Copyright statement

Plant tissue culture and artificial seed production techniques for cauliflower and their use to study molecular analysis of abiotic stress tolerance

by

Hail Zuhir Rihan

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

School of Biological Sciences
Faculty of Science and Environment

2014

Plant tissue culture and artificial seed production techniques for cauliflower and their use to study molecular analysis of abiotic stress tolerance

Hail Zuhir Rihan

A protocol for cauliflower micro-propagule production was developed and optimised for both micropropagation and artificial seed production techniques using meristematic tissues from cauliflower curd. All steps in the protocol were empirically optimised including: blending, sieving, culture methods, liquid culture media composition and plant growth regulator combinations and concentrations. The cost of the micro-propagules could be reduced by as much as 50% on the initial costings reported previously since treatments doubled the number of microshoots produced per culture unit.

The research confirmed the suitability of cauliflower microshoots to be encapsulated as artificial seeds and an effective protocol for microshoot encapsulation was designed through the optimization of 1) the production of cauliflower microshoots suitable for encapsulation, 2) encapsulation procedures, 3) artificial seed artificial endosperm structure, 4) conversion materials. The possibility of culturing cauliflower artificial seeds in commercial substrates such as perlite, sand, vermiculite and compost was confirmed. The use of plant preservative mixture (PPM) for the control of contamination in cauliflower culture media and artificial seeds was optimised and the effect of this material on the development of plant material was assessed. It was confirmed that cauliflower artificial seed could be stored in a domestic refrigerator for up to 6 months which could have a great impact in cauliflower breeding programmes.

The huge number of cauliflower microshoots that could be produced using this protocol and the homogeneity of the culture system, provided a tool for the molecular analysis of cauliflower microshoots (and artificial seed) abiotic stress tolerance analysis. Various treatments were conducted to improve microshoot cold tolerance and the up-regulation of the *CBF/DREB1* transcription factor including low temperature acclimation, mannitol, ABA (abscisic acid) and Mo (molybdenum). Microshoots were confirmed to acclimate successfully using low temperature. Mo was shown to improve the cold tolerance of cauliflower microshoots and to up-regulate *CBF/DREB1* in the absence of low temperature acclimation. Acclimation did not increase the accumulation of dehydrin proteins and it is concluded that dehydrins do not play a significant role in the cold tolerance of cauliflower microshoots.

Since cauliflower breeding and seed multiplication protocols make extensive use of micropropagation, the studies reported in this research could make a significant impact by decreasing the cost of micropropagation and increasing its reliability. It also opens new perspectives for further research for cauliflower artificial seed production and the possibility of sowing these seeds directly in the field. Furthermore, this research helps to facilitate cauliflower breeding programmes by improving the understanding of abiotic stress tolerance mechanisms and the relationship between different types of abiotic stresses such as cold and drought.

Contents

Chapter 1: General Introduction and Literature Review

1.1. Introduction 1

1.2. Cauliflower curd structure and formation 2

1.3. Stages of cauliflower growth 3

- Juvenile stage 4
- Curd induction stage 4
- Curd growth stage 5

1.4. The breeding of cauliflower 5

a) Open pollinated varieties 5

b) F1 hybrid varieties 5

1. Genetic (nuclear) male sterility 6
2. Cytoplasmic male sterility 7

c) Cauliflower micropropagation 7

1. Phytohormones 9
2. Culture system contamination 11

1.5. Artificial seeds 13

1.6. Artificial seeds definition 13

1.7. The importance, uses and advantages of artificial seeds 14

1.8. Artificial seed concept 16

1.9. Ex-plant material 17

1.10. Artificial seed gelling agents and adjuvant materials 20

1.11. Artificial endosperm structure and their effects in the synthetic seed 22

1.12. Artificial seeds storage ability: 23

1.13. Brassica species artificial seeds 27

1.14. Abiotic stress 28

1.15. The role of Ca^{2+} and ROS (Reactive Oxygen Species) in the abiotic stress signaling 30

1.16. Low temperature stress (Frost damage) 31

1.17. Cold acclimation and frost stress tolerance 33

1.18. Gene expression induction in response to low temperature 34
1.19. CBF cold response pathway 35
1.20. Drought stress 39
1.21. Abscisic acid and abiotic stress tolerance 43
1.22. The role of Molybdenum (Mo) in abiotic stress tolerance 45
1.23. Thesis aims 48
1.24. Thesis objectives 48
Chapter 2: Cauliflower Micropropagation 50
2.1. Introduction 50
2.2. Plant material 54
2.3. Culture system (Standard Operating Procedure for Culture System) ($S.O.P_{CS}$) 54
2.4. Experiments 56
2.4.1. Experiment 1 56
Optimization of the blending duration 56
2.4.1.1. Aims and objectives 56
2.4.1.2. Materials and methods 56
2.4.1.3. Results 58
2.4.1.4. The amount of produced explants: 58
2.4.1.5. The proportion between produced explants which have meristematic domes and debris 58
2.4.1.6. Explant growth ability during subsequent culture 59
2.4.2. Experiment 2 64
The effect of sieving size class on explants and microshoots 64
2.4.2.1. Aims and objectives 64
2.4.2.2. Materials and methods 64
2.4.2.3. Results 65
2.4.3. Experiment 3 67
The determination of explant developmental stage 67
2.4.3.1. Aims and objectives 67
2.4.3.2. Materials and methods 67

2.4.3.3. Results 67
2.4.4. Experiment 4 70
The determination of the optimal agitation methods suitable for explants growth 70
2.4.4.1. Aims and objectives 70
2.4.4.2. Materials and methods 70
2.4.4.3. Results 71
2.4.5. Experiment 5 73
The effect of plant growth regulators (PGR) added to the liquid media on the number and the viability of cauliflower microshoots 73
2.4.5.1. Aims and objectives 73
2.4.5.2. Materials and methods 73
2.4.5.3. Results 74
2.4.5.4. Stage 1 74
2.4.5.5. Stage 2 76
2.4.6. Experiment 6 78
The effect of sugar type, source and concentration cauliflower microproshoot production 78
2.4.6.1. Aims and objectives 78
2.4.6.2. Materials and methods 78
2.4.6.3. Results 79
2.4.7. Experiment 7 85
The effect of the PPM (plant preservative mixture) added to the culture media and blending media on the number and viability of produced microshoots 85
2.4.7.1. Aims and objectives 85
2.4.7.2. Materials and methods 85
2.4.7.3. Results 86
2.5. Discussion 91
2.6. Conclusion 101

Chapter 3: Cauliflower Artificial Seed Production 103
3.1. Introduction 103
3.2. Experiments 105
3.2.1. Experiment 1 105
Optimization of micro-shoot encapsulation 105
3.2.1.1. Aims and objectives 105
3.2.1.2. Materials and methods 105
3.2.1.3. Results 106
3.2.1.4. Standard Operating Procedure for artificial seed production (SOP_{ASP}) 109
3.2.2. Experiment 2 110
The effect of plant growth regulator combinations used in the liquid culture medium for the production of microshoots on their suitability for artificial seed production 110
3.2.2.1. Aims and objectives 110
3.2.2.2. Materials and methods 110
3.2.2.3. Results 111
3.2.3. Experiment 3 118
The determination of the optimal cauliflower microshoots age suitable for encapsulation 118
3.2.3.1. Aims and objectives 118
3.2.3.2. Material and methods 118
3.2.3.3. Results 118
3.2.4. Experiment 4 120
The effect of the PGRs used with the artificial seed matrix 120
3.2.4.1. Aims and objectives 120
3.2.4.2. Materials and methods 120
3.2.4.3. Results 121
3.2.5. Experiment 5 124
The effect of air bubbles involved in artificial seed matrices 124
3.2.5.1. Aims and objectives 124
3.2.5.2. Materials and methods 124

3.2.5.3. Results 124
3.2.6. Experiment 6 126
The effect of sucrose concentration used with cauliflower artificial seed matrixes in their growth and development 126
3.2.6.1. Aims and objectives 126
3.2.6.2. Materials and methods 126
3.2.6.3. Results 126
3.2.7. Experiment 7 128
The optimization of artificial seed conversion in semi-solid media 128
3.2.7.1. Aims and objectives 128
3.2.7.2. Materials and methods 128
3.2.7.3. Results 128
3.2.8. Experiment 8 132
The optimization of artificial seed conversion in commercial substrates 132
3.2.8.1. Aims and objectives 132
3.2.8.2. Materials and methods 132
3.2.8.3. Results 133
3.2.9. Experiment 9 137
The effect of using PPM (plant preservative mixture) on the conversion of cauliflower artificial seed produced 137
3.2.9.1. Aims and objectives 137
3.2.9.2. Materials and methods 137
3.2.9.3. Results 137
3.2.10. Experiment 10 142
The effect of the amount of culture medium used with perlite on the conversion and viability of artificial seed produced 142
3.2.10.1. Aims and objectives 142
3.2.10.2. Materials and methods 142
3.2.10.3. Results 142
3.2.11. Experiment 11 144
Cauliflower artificial seed dehydration tolerance 144

3.2.11.1. Aims and objectives 144
3.2.11.2. Materials and methods 144
3.2.11.3. Results 145
3.2.12. Experiment 12 147
Cauliflower artificial seed cold storage 147
3.2.12.1. Aims and objectives 147
3.2.12.2. Materials and methods 147
3.2.12.3. Result 147
3.2.13. Experiment 13 149
Artificial seeds cryopreservation 149
3.2.13.1. Aims and objectives 149
3.2.13.2. Materials and methods 149
3.2.13.3. Results 150
3.2.14. Experiment 14 152
Cryopreservation of dehydrated artificial seeds 152
3.2.12.1. Aims and objectives 152
3.2.12.2. Materials and methods 152
3.2.12.3. Results 153
3.3. Discussion 154
3.3.1. Microshoot production 155
3.3.2. Microshoot encapsulation 157
3.3.3. Artificial seed conversion (germination) 161
3.3.4. Artificial seed preservation 164
3.4. Conclusion 166
Chapter 4: Cauliflower Artificial Cold Tolerance and The Up-regulation of The *CBF/DREB1* Gene 168
4.1. Introduction 168
Objectives 173
4.2. General Material and Methods 173

4.2.1. Standard Operating Procedure for frost damage evaluation using relative electrical conductivity technique (REC %)($SOP_{EC\%}$) 173
4.2.1.1. $SOP_{EC\%}$ protocol used with cauliflower mature leaves ($SOP_{ECL\%}$) 173
4.2.1.2. $SOP_{EC\%}$ protocol with cauliflower microshoots ($SOP_{ECM\%}$) 175
4.2.2. Standard Operating Procedure for RNA extraction and purification (SOP_{RNAext}) 175
4.2.3. Standard Operating Procedure for *BoCBF/DREB1* regulatory gene identification ($SOP_{CBF/DREB1}$) 177
4.2.3.1. mRNA reverse transcription 177
4.2.3.2. Standard Operating Procedure for cDNA amplification using ordinary PCR (SOP_{OPCR}) 178
4.2.3.3. Standard Operating Procedure for cDNA amplification using quantitative PCR (SOP_{QPCR}) 180
4.2.4. Standard operating procedure for protein extraction and purification SOP_{PEX} 181
4.2.5. Standard Operating Procedure for protein estimation SOP_{PES} 181
4.2.6. Standard Operating Procedure SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) SOP_{SDS} 182
4.2.7. Standard operating procedure for Western blotting SOP_{WB} 184
4.2.8. Standard Operating Procedure for statistical analysis (SOP_{SA}) 186
4.3. Experiments 187
4.3.1. Experiment 1: 187
The effect of cold acclimation on the cold tolerance of cauliflower microshoots and up-regulation of *CBF/DREB1* 187
4.3.1.1. Aims and Objectives 187
4.3.1.2. Materials and Methods 187
4.3.1.3. Results 188
4.3.2. Experiment 2: 196
The effect of drought simulation on the artificial seeds (microshoots) cold tolerance and on the induction of *CBF/DREB*1 expression in the cauliflower microshoots 196
4.3.2.1. Aims and objectives 196
4.3.2.2. Material and Methods 196

4.3.2.3. Results 198
4.3.3. Experiment 3: 202
The effect of cold acclimation on the cold tolerance and the induction of *CBF/DREB1* gene in mature cauliflower plants 202
4.3.3.1. Aims and Objectives 202
4.3.3.2. Material and Methods 202
4.3.3.3. Results 203
4.3.4. Experiment 4 205
The effect of drought on mature cauliflower plant cold tolerance and on the induction of *CBF/DREB1* gene expression in mature cauliflower plants 205
4.3.4.1. Aims and Objectives 205
4.3.4.2. Materials and Methods 205
4.3.4.3. Results 207
4.3.5. Experiment 5 210
The effect of Abscisic acid (ABA) on the development of, cold tolerance and *CBF/DREB1* expression in cauliflower microshoots 210
4.3.5.1. Objectives 210
4.3.5.2. Materials and methods 210
4.3.5.3. Results 213
4.4. Discussion 235
4.5. Conclusion 245
Chapter 5: The Effect of Molybdenum on the Cauliflower Artificial Seed Production System and the Molecular Mechanism of Cold Tolerance 247
5.1. Introduction 248
Objectives 249
5.2. Materials and Methods 250
5.2.1. The effect of Mo on the development of microshoots 250
5.2.2. The effect of Mo used with artificial seed capsules and artificial seed conversion media 250
5.2.3. The effect of Mo on the cold tolerance of cauliflower artificial seeds 251

5.2.4. The effect of Mo on the induction of *CBF/DREB1* expression and on the cold tolerance of acclimated cauliflower microshoots 252
5.3. Results 253
5.3.1. The effect of Mo used with microshoots culture media on the development of microshoots 253
5.3.2. The effect of Mo applied to artificial seed matrix 255
5.3.3. The effect of molymbenum applied with artificial seed conversion media ... 256
5.3.4. The effect of Molybdenum on the cold toleranceof articial seeds of cauliflower 257
5.3.5. The effect of low but non damaging temperature on the viability of artificial seeds derived from both MO treated and non-treated microshoots 258
5.3.6. The effect of molybdenum treatment on the cold tolerance of acclimated cauliflower microshoots 259
5.3.7. The effect of molybdenum treatment on the induction of *CBF/DREB1* gene expression 260
5.3.8. Detection of COR15 protein in molybdenum treated cauliflower microshoots 263
5.4. Discussion 265
5.5. Conclusion 269
Chapter 6: An Analysis of the Development of Cauliflower Seed as A Model to Improve Artificial Seed 270
6.1. Introduction 270
Objectives 274
6.2. Material and methods 274
6.1.1. Plant materials 274
6.1.2. Determination of moisture content 277
6.1.3. Determination of lipid content (rapid soxhlet extraction) 277
6.1.4. Determination of nitrogen content (Kjeldahl method) 278
6.1.5. Determination of ash content 278
6.1.6. Determination of carbohydrate level 278
6.1.7. The accumulation of dehydrin proteins during the development of cauliflower seeds (standard operating procedure for western blotting SOP_{WBD}) 278

6.1.8. The effect of acclimation on the accumulation of dehydrin proteins in cauliflower microshoots 279
6.1.9. The effect of mannitol used with the culture media on the accumulation of dehydrin proteins in cauliflower microshoots 280
6.1.10. The effects of ABA on the accumulation of dehydrin proteins in acclimated cauliflower microshoots 280
6.1.11. The effect of molybdenum on the accumulation of dehydrin proteins in acclimated cauliflower microshoots 280
6.3. Results 281
6.3.1. The development of cauliflower seed structure (moisture, lipids, carbohydrate and ash)..... 281
6.3.2. The accumulation of dehydrin proteins during the development of cauliflower seeds 283
6.3.3. The effect of mannitol on the accumulation of dehydrin proteins in cauliflower microshoots 287
6.3.4. The effect of cold acclimation on the accumulation of dehydrin protein in cauliflower microshoots 289
6.3.5. The effect of ABA on the accumulation of dehydrin protein in acclimated cauliflower microshoots 291
6.3.6. The effect of molybdenum on the accumulation of dehydrin proteins in acclimated cauliflower microshoots 293
6.4. Discussion 295
6.5. Conclusion..... 303
Chapter 7: General Discussion 304
Cauliflower micropropagation 304
Cauliflower artificial seed production 307
Molecular analysis of cauliflower abiotic stress tolerance 311
Future work 317
Appendixes 320
Appendixe 1: Determination of ash content in cauliflower seeds 321
Appendixe 2: Determination of lipid content in cauliflower seeds using rapid Soxhelt extraction..... 322
Appendixe 3: Determination of nitrogen and protein content by Kjeldahl methods 324

Principle 324
1. Digestion stage 324
a) Apparatus 324
2. Vapodest distillation stage 325
3. Calculation 325
References 327

List of figures

Figure 1. Artificial seed uses and benefits (modified from (Mohamed et al., 2009)). 15

Figure 2. Artificial seed concept. 16

Figure 3. Artificial seed storage approaches (modified from (Mohamed et al., 2009). .. 25

Figure 4. Cauliflower artificial seeds (Kieffer, 1996) 28

Figure 5. Cold acclimation induces changes in cellular processes. Different responses are observed while exposing plants to low non-freezing temperatures. Modified from (Xin and Browse, 2000). 34

Figure 6. The effect of the blending duration treatments on the A: explant weights (LSD= 1.171 for 212-300 µm, LSD= 0.167 for 300-600 µm size class). B: explant volumes (LSD=756.74 for 212-300 µm, LSD=1626.43 for 300-600 µm size class)..... 61

Figure 7. The effect of the blending treatments on the proportion of explants carrying meristimatic domes and debris at two size classes 212-300 µm and 300-600 µm (LSD= 0.257 for 212-300 µm size class, LSD= 1.047 for 300-600 µm size class. 62

Figure 8. The effect of the blending duration treatments on the number of growing microshoots at two size classes 212-300 µm and 300-600 µm (LSD= 19.58 for 212-300 µm size class, LSD= 70.52 for 300-600 µm size class). 62

Figure 9. The effect of the blending duration treatments on the microshoots average weights at two size classes, 212-300 µm and 300-600 µm (LSD= 0.022 for 212-300 µm size class, LSD= 0.0045 for 300-600 µm size class). 63

Figure 10. The explants which have meristematic domes and debris (three days explants age- 30 second blending duration treatment- 300-600 µm sieving size class) (100 times magnification). 63

Figure 11. The affect of sieving size class on the average number of meristamatic domes carried per explant and produce microshoots (LSD= 1.335 for meristematic domes, LSD= 1.707 for the number of produced microshoots). 65

Figure 12. (A and B) the meristimatic domes carried per explant at size class 212-300 µm and 300-600 µm respectively, (C and D) the microshoots growing per explant at size classes 210-300 µm and 300-600 µm respectively. 66

Figure 13. Cauliflower explants growth stages assessed by measuring their length changes over time. 68

Figure 14. Cauliflower produced explants at different developmental stage. 69

Figure 15. Explants production and agitation methods: A) orbital stirrer. B) Air bubbles. C) Air bubbles and magnetic stirrer. 71

Figure 16. The microshoots hyperhydricity produced when the current of air bubbles was used as an agitation method. 72

Figure 17. The effect of the agitation methods on the average numbers and weights of microshoots. 72

Figure 18. The effect of the various combination of Kinetin (1. 2) mg L^{-1} incorporated with IBA (1, 2) mg L^{-1} or NAA (1, 2) mg L^{-1} added to the lequid media on the number of developing microshoots (LSD=28.85). 75

Figure 19. The effect of various combination of Kinetin (1. 2) mg L^{-1} incorporated with IBA (1, 2) mg L^{-1} or NAA (1, 2) mg L^{-1} added to the liquid media on the average weights of developing microshoots (LSD=0.0083). 75

Figure 20. The effect of the various combination of Kinetin (1. 2 mg L^{-1}) incorporated with IAA (1, 2 mg L^{-1}) added to the liquid media on the number of developing microshoots (LSD=50.76)..... 77

Figure 21. The effect of various combination of Kinetin (1. 2 mg L^{-1}) incorporated with IAA (1, 2 mg L^{-1}) added to the liquid media on the average weight of developing microshoots (LSD=0.0247)..... 77

Figure 22. The effect of sucrose concentration used with the culture media on the number and average weight of cauliflower microshoots (LSD=35.43 for microshoot number, LSD=0.0051 for the micoshoots average weight). 80

Figure 23. Microshoots produced using 4.5% sucrose concentration in the culture media. B) Microshoots produced using 1.5 % sucrose in the culture media. 80

Figure 24. The effect of sugar types and concentrations on the number of microshoots produced (LSD= 8.18 for both sugar types and concentrations). 82

Figure 25. The effect of sugar types and concentrations on the average weights of microshoots produced (LSD=0.216 for the sugar concentration and LSD=0.376 for the interaction between the sugar type and concentration). 82

Figure 26. The effect of sugar (sucrose) source used with culture media on the number of growing microshoots (LSD=14.83). 83

Figure 27. The effect of sugar cane concentration used with culture media compared with the use of 3% of sugar beet (standard) on the average weights of microshoot produced (LSD=0.028). 84

Figure 28. A) The microshoots produced when low concentration of PPM was used with culture media (0.25) ml L^{-1}. B) The contamination obtained when free PPM culture medium was used. 89

Figure 29. A) The produced microshoots using (0.5) ml L^{-1} of PPM added to the culture medium. B) The microshoots produced using (1) ml L^{-1} with the culture media. 89

Figure 30. The effect of the PPM (plant preserve mixture) used with culture medium and blending medium on the number of developing microshoots after 21 culture days (LSD=7.601)..... 90

Figure 31. The effect of the PPM (plant preserve mixture) used with culture medium and blending medium on the average weight of microshoots after 21 culture days (LSD= 0.0066)..... 90

Figure 32. The effect of the $CaCl_2$, 2H2O concentrations on artificial seeds produced viability. 108

Figure 33. Artificial seeds produced using 2% concentration of sodium alginate and 15 g L^{-1} of $CaCl_2$, $2H_2O$. 108

Figure 34. The effect of the interaction between the kinetin concentrations and the type of auxins used on the artificial seed conversion rate (LSD=10.986)..... 113

Figure 35. The effect of the PGR combinations on the artificial seeds conversion rate (LSD= 7.498)..... 114

Figure 36. The effect of the interaction of the kinetin concentration and auxin types on the viability of artificial seeds produced (LSD=0.6985)..... 114

Figure 37. The effect of microshoot culture PGRs on the viability of artificial seeds (LSD=0.075)..... 115

Figure 38. The effect of the PGR combinations on the artificial seeds conversion rate (LSD= 4.862)..... 116

Figure 39. The effect of PGR combinations used with microshoots liquid culture media on the viability of produced microshoots. 117

Figure 40. The effect of the optimal microshoot age suitable for encapsulation (LSD= 0.687 for the conversion rate and LSD= 0.0675 for artificial seeds viability). 119
Figure 41. The interaction effect between kinetin concentrations and auxin types on the conversion rate of artificial seeds. 122

Figure 42. The effect of the artificial seed PGR matrix combinations on the artificial seed conversion rate..... 123

Figure 43. The effect of the PGR combinations used with artificial seeds matrix on the produced artificial seed viability..... 123

Figure 44. The average weights of plantlets obtained using two types of artificial seeds, one with air bubbles in its capsules and another without it. 125

Figure 45. The effect of sucrose used with artificial seed matrixes on their conversion rate and viability (LSD= 11.65 for the conversion rate and LSD= 0.741 for the viability) 127

Figure 46. The effect of sucrose used with artificial seed matrixes on their development and growth. 127

Figure 47. The effect of artificial seeds culture media PGRs on their conversion rate. 130

Figure 48. The effect of artificial seeds culture PGRs on their viability (LSD = 0.364). 130

Figure 49. A) Callus produced when 0.5 mg L^{-1} NAA was used with the culture media. B) Plantlets obtained when 2 mg L^{-1} IBA was used with the culture media. 131

Figure 50. The effect of the irrigation PGR combinations on the conversion rate of artificial seeds. 134

Figure 51. The effect of culture substances on the artificial seed conversion rate (LSD = 7.018). 135

Figure 52. The effect of artificial seeds culture substances on their viability (LSD= 0.022). 136

Figure 53. The effect of using PPM in the media used for irrigation on the artificial seeds viability and conversion rate (LSD = 8.333 for conversion rate and LSD = 0.0377 for plantlet fresh weights). 139

Figure 54. The interaction effect between the culture substances and PPM on the artificial seeds conversion rate. 139

Figure 55. Plantlets obtained using Compost and S23 free PGRs after 50 days of culture. 140

Figure 56. Plantlets obtained using Sand and S23 free PGRs after 50 days of culture. 140

Figure 57. The effect of irrigation media sizes on the artificial seeds conversion rate (LSD = 32.92). 143

Figure 58. Artificial seed moisture levels during subsequent drying steps. 145

Figure 59. The effect of the artificial seed moisture level on its conversion rate (LSD = 18.695). 146

Figure 60. The effect of the artificial seed moisture level on its viability (LSD = 0.0928). 146

Figure 61. The effect of cold storage duration on the conversion rate of artificial seeds (LSD=17.1). 148

Figure 62. The effect of cold storage duration on the viability of cauliflower artificial seeds (LSD=0.61). 148

Figure 63. Artificial seeds cryopreservation experiment design. 151

Figure 64, general view of the set-up for measuring electrical conductivity..... 175

Figure 65. Standard curve of protein concentrations and absorbance at 562 nm 182

Figure 66. The effect of cold acclimation on artificial seeds cold tolerance assessed by their conversion rate at different low temperatures (LSD=10.11) 189

Figure 67. The effects of temperature treatments on the average weights of cauliflower plantlets produced from both acclimated and non-acclimated artificial seeds (LSD=0.0885)..... 190

Figure 68. The effect of annealing temperature on the expression of *CBF/DREB1* gene (N: sample tested to have negative *CBF/DREB1* expression and P: sample tested to have a positive *CBF/DREB1* expression)..... 191

Figure 69. The effect of using three annealing temperature (54, 57, 60 ºC) on the expression of *CBF/DREB1* gene (band intensity proportions) in the samples which have positive gene expression (LSD=884.635). 192

Figure 70. The effect of cold acclimation on the up-regulation of *CBF/DREB1* in cauliflower microshoots. 193

Figure 71. Plantlets converted from acclimated artificial seeds treated at -10°C..... 193

Figure 72. The effect of low temperature (4°C) at various exposure times on the induction of *CBF/DREB1* expression in cauliflower microshoots (LSD=0.285). 194

Figure 73. The SDS-PAGE analysis for protein samples derived from both acclimated and non-acclimated microshoots. 195

Figure 74. Western blot analysis for the detection of COR protein (23 kDa) under cold acclimation. 195

Figure 75. Standard curves for culture media osmotic potential obtained using different concentrations of mannitol. 198

Figure 76. The effect of culture osmotic potential (mannitol concentration) on the average weight of cauliflower microshoots (LSD=0.016). 199

Figure 77. The negative effect of using high culture osmotic potential (mannitol concentration) on the development of cauliflower microshoots. 199

Figure 78. The effect of using a high osmotic potential culture media (-2.95) on the development of cauliflower microoshots. 200

Figure 79. The effect of the culture osmotic potential (mannitol concentration) on the conversion rate of artificial seed treated with various low temperatures (LSD= 1.181). 201

Figure 80. The effect of culture osmotic potential (mannitol concentration) on the conversion rate of artificial seeds treated with -10 °C. 201

Figure 81. The effect of temperature treatment on the relative electrical conductivity (REC %) of both acclimated and non-acclimated leave disks derived from cauliflower mature plants (LSD=14.4). 203

Figure 82. The effect of cold treatment at (4°C) treatments for different periods on the induction of CBF/DREB1 in mature cauliflower plants (LSD=0.098). 204

Figure 83. Standard curve of the relation between the Theta Probe and soil moisture content. 207

Figure 84. The effect of drought on the mature cauliflower frost damage under irrigation and drought (LSD=8.99 at -6°C)..... 207
Figure 85. The effect of soil moisture level on the induction of *CBF/DREB1* expression in the cauliflower mature plants (LSD= 0.240)..... 208

Figure 86. The effect of moisture level on the stomatal conductance of cauliflower full extended leaves. 209

Figure 87. The effect of ABA on the development of microshoots (LSD=0.029 for the average weights of microshoots)..... 213

Figure 88. The effect of temperature treatments on the relative REC% (frost damage) in cauliflower microshoots (LSD=4.53)..... 214

Figure 89. The effect of ABA on the REC % of microshoots treated with 20, -4, -8, and -12 º C (LSD for REC% at -8 º C= 6.7). 215

Figure 90. The effect of ABA on the REC % of microshoots treated with 20, -4, -8, and -12 º C (LSD for the -8 treatment = 0.056)..... 216

Figure 91. The effect of ABA on the concentration of mRNA derived from cauliflower microshoots treated with low temperature (4°C) for several durations (LSD=26.72)… 217

Figure 92. The effect ABA concentrations on the induction of CBF/DREB1 gene (CBF/Actin ratio) expression after 6 hours of low temperature treatment at 4°C (LSD=0.08)..... 218

Figure 93. The effect of using 4 mg L^{-1} concentration of ABA on the expression of CBF/DREB1 in cauliflower microshoots exposed to several duration of low temperature at 4°C (LSD= 0.186)..... 219

Figure 94. The effect of using 4 mg L^{-1} of ABA on the expression of *CBF/DREB1* (using P2 primers) in cauliflower microshoots treated to several duration of low temperature at 4°C compared to that in no-treated microshoots. 221

Figure 95. The effect of using 4 mg L^{-1} of ABA on the expression of *CBF/DREB1* using the quantitative (Real time) PCR in cauliflower microshoots exposed to several duration of low temperature at 4°C (LSD=9.87). 222

Figure 96. (*1CBF/DREB1*) sequence obtained using primers (P1) (BLAST (NCBI)) Fasta sequences :(F premix 52..433 of sequence) (R premix 25..385 of sequence). 223

Figure 97. (*2CBF/DREB1*) sequence obtained using primers (P2) (BLAST (NCBI)) Fasta sequences: (F premix 16...159 of sequence) (R premix 14...155 of sequence). 223

Figure 98. Nucleotide sequences (cDNA) alignment. 230

Figure 99. Phylogenic relation of the BoCBF/DREB1 cDNA. 230

Figure 100. Multiple alignment of the BoCBF/DREB1 deduced amino acids sequence with members of Brassicacea family and other plant families. 233

Figure 101. Phylogenic relation of the BoCBF/DREB1 proteins. 233

Figure 102. The SDS-PAGE analysis for protein samples derived from both ABA treated and non-treated samples..... 234

Figure 103. Western blot analysis for the detection of COR protein (23 kDa) from ABA treated and non-treated microshoot samples (LSD=0.29). 235

Figure 104. The change of media colour with the change of molybdenum concentration in the culture media. 254

Figure 106. The effect of using Mo used with the culture media on the number and average weight of microshoots produced (LSD=15.53 for the number of microshoots, LSD=0.029 for the microshoots average weights)..... 254

Figure 107. The effect of Mo concentration used with the culture liquid media on the colour of microshoots produced (A and B were derived from 30ppm of MO treatment, C microshoots were derived from Mo free culture media)..... 255

Figure 108. The effect of Mo used with artificial seed matrix on their conversion rate and viability (LSD=13.26 for artificial seed conversion rate)..... 255

Figure 109. The effect of Mo used with artificial seed conversion media on their conversion rate and viability (LSD= 0.20 for artificial seed viability). 256

Figure 110. The effect of Mo used with artificial seed conversion media on the growth of plantlets..... 257

Figure 111. The effect of using 15 PPM of Mo with microshoots culture media on the artificial seeds cold tolerance (LSD= 25.78). 258

Figure 112. The effect of temperature treatments (°C) on the average weight of plantlets derived from the conversion of encapsulated microshoots produced in free MO liquid media (LSD= 0.22) and liquid media supplemented with 15 ppm Mo (LSD= 0.15). 259

Figure 113. The effect of Mo treatment on the cold tolerance assessed as EC% of acclimated cauliflower microshoots (LSD = 4.30 at -10°C). 260

Figure 114. The effect of different concentrations of Mo and acclimation treatment on the expression of *CBF/DREB1* gene observed using ordinary PCR (LSD = 0.175 for Mo treatment)..... 262

Figure 115. The effect of different concentrations of Mo (PPM) and acclimation treatmens on the expression of CBF/DREB1 observed using quantitative PCR (LSD = 30.31 for Mo treatment, LSD = 36.98 for the temperature effect)..... 262

Figure 116, the SDS-PAGE analysis for protein samples derived from both Mo treated and non-treated samples..... 263

Figure 117. Western blot analysis for the detection of COR15 protein (23 kDa) from Mo treated and non-treated microshoot samples (LSD = 0.054)..... 264

Figure 118. The growth stages of cauliflower plants used for seed production. 275

Figure 119. Cauliflower seed samples at different developmental stages..... 276

Figure 120. Moisture, protein and ash level changes during the development of cauliflower seeds..... 282

Figure 121. Carbohydrate and lipid level changes during the development of cauliflower seeds..... 282

Figure 122. The SDS-PAGE analysis for protein samples derived from cauliflower seeds at different developmental stages. 284

Figure 123. Western blot analysis for the detection of dehydrin proteins from cauliflower artificial seeds at different developmental stages using a blocked dehydrin k-segment antibody..... 284

Figure 124. Western blot analysis for the detection of dehydrin proteins from cauliflower artificial seeds at different developmental stages using a k-segment dehydrin antibody. 285

Figure 125. The accumulation of dehydrin proteins during the development of cauliflower seeds..... 286

Figure 126. SDS-PAGE analysis for protein samples derived mannitol treated microshoots..... 287

Figure 127, Western blot analysis for the detection of dehydrin proteins from cauliflower mannitol treated microshoots using a k-segment dehydrin antibody. 288

Figure 128, The accumulation of dehydrin proteins in mannitol treated microshoots. . 288

Figure 129. The SDS-PAGE analysis for protein samples derived from acclimated and non-acclimated microshoots. 289

Figure 130. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated and non-acclimated microshoots using a k-segment dehydrin antibody. .. 289

Figure 131. The effect of cold acclimation on the accumulation of dehydrin proteins in cauliflower microshoots. 290

Figure 132. SDS-PAGE analysis for protein samples derived acclimated cauliflower microshoots treated with ABA. 291

Figure 133. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated microshoots treated with ABA using a k-segment dehydrin antibody. 292

Figure 134. the effect of ABA used with cauliflower microshoots liquid culture media on the accumulation of dehydrin proteins in the acclimated cauliflower microshoots. 292

Figure 135. SDS-PAGE analysis for protein samples derived acclimated cauliflower microshoots treated with Mo. 293

Figure 136. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated microshoots treated with Mo using blocked k-segment dehydrin antibody. 294

Figure 137. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated microshoots treated with Mo using a k-segment dehydrin antibody. 294

Figure 138. The effect of Mo used with cauliflower microshoots liquid culture media on the accumulation of dehydrin proteins in the acclimated cauliflower microshoots. 295

List of tables

Table 1. The recent applications of artificial seeds on different types of plants. 27

Table 2. The effect of microshoot culture PGRs on the conversion rate of artificial seeds produced. 112

Table 3. The effect of microshoot culture PGRs on the viability of artificial seeds produced. 113

Table 4. Artificial seed matrix PGRs effect on their conversion rate statistical analysis results..... 121

Table 5. Result analysis of the effect of culture substances and PGRs used with irrigating media on the artificial seeds conversion rate. 133

Table 6 The effect of irrigation composition and culture substrates on artificial seed conversion rate (%) S23 was supplemented with the described PGRs. (LSD=10.528 for PGR combinations, LSD = 5.84 for Culture substrates and LSD = 2.105 for PGR combinations x Culture substrates (interaction)). 134

Table 7. Least significant differences between PGR combinations and between culture substances and for the interaction between them. 134

Table 8. The statistical analysis results showed the effect of liquid culture media structure and culture substances on the artificial seeds viability. 135

Table 9. The effect of irrigation composition and culture substrate on the plantlet fresh weight (g) S23 was supplemented with the described PGRs. (LSD = 0.022 for culture substrates and LSD = 0.080 for the interaction between the PGR combinations and culture substrates). 136

Table 10. Least significant differences between culture substances and for the interaction between the PGR combinations and culture substances..... 136

Dedication

I would like to dedicate my thesis to my beloved parents (May Almighty God bless them).

I also would like to dedicate my thesis to my brother and sisters.

Acknowledgements

It is my pleasure to express sincere thanks to my supervisors, Professor Mick Fuller and Dr. Stephen Burchett for their assistance, encouragement, guidance, criticism and support throughout the present research project.

I greatly appreciate the assistance and support from Angela Harrop, Dr. Michele Kiernan, Dr. Wondwossen Abate Woldie and Dr. Raid Nisr for their support in the laboratory, without whom the experiments could not have been completed. I also offer my gratitude to Sarah, Andy (technicians in the laboratory) and Mr Jack Shannon for their help during my project work. I also would like to thanks my dear friends Momammed Al-Issawi, Faiz Fadhel, Magda Al Shamari, Anas Satar, Fadil Al-Swedi and Dr.Mohammed Elsayed Elmahrouk for their help and support throught my study.

I also offer my sincere gratitude to all the members of staff in Graduate school and the school of Biomedical and Biological Sciences University of Plymouth and especially to my colleagues on third and eigth floor Davy Building and on the fourth floor Portland Square Building.

I am very thankful to Dr. Michael F Thomashow and Dr. Sarah Gilmour of the Michigan State University (USA) for providing the antibodies for the COR15a protein. I am also thankful to Dr. Michael Wisniewski‘for providing the antibodies for the dehydrins protein.

I would like to acknowledge The Ministry of Higher Education (Syria) for the award of scholarship that provided the financial support for this research project.

I am very grateful for the encouragement and support of my Mother, Father, Sisters and Brother who patiently waiting for me.

Finally, my entire deep thanks to Almighty God, Who blessed me with the strength, confidence and determination needed for the completion of my research project.

Author's Declaration

At no time during the registration for the degree of Doctor of philosophy has the author been registered for any other University award. I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance where acknowledged.

This study was sponsored by the Syrian Ministry of Higher Education-Damascus University

----

Candidate

Word account of main body of thesis: 74785 words, total 89897 words

Modules and Courses Attended:

1. Techniques in Botanical Conservation (10 credits).
2. Plant Taxonomy and Identification (10 credits).
3. Postgraduate Research Skills and Methods (20 credits).
4. General Teaching Associate (GTA).
5. LTHE300 (20 credits).
6. Practical Techniques in Molecular Biology workshop, 16th-19th July, 2012. Plymouth University.
7. Plymouth consortium Student Associate Scheme (SAS) 2010/11.
8. An internship for three days at Elsoms Seeds Ltd, Spalding, Lincolnshire, PE11 1QG, United Kingdom January 24th -26th. 2012, PE11 1QG, United Kingdom January 24th -26th. 2012. The workshop included training about the practical skills in seed production techniques.

Postgraduate Research Skills attended (Plymouth University):

1- Ms Word introduction, 26 / 11 / 2009.

2- MS Excel introduction to essential features, 15 / 01 / 2010.

3- Plagiarism-your words or other people, 20 / 01 / 2010.

4- Endnote clinic, 21 / 01 / 2010.

5- Transfer process Mphil/PhD, 27 / 12 / 2009.

6- Microsoft PowerPoint 2007 (Creating a Presentation), 10/02/2010.

7- Essential tools for the Viva: Debating Skills, 26/01/2010.

8- MS project, 14/02/2010.

9- Presenting to an audience –Part 1, 03/03/2010.

10- Simulated assessment centre, 10/03/2010.

11- Viva la viva, 29/03/2010.

12- Plant physiology-dormancy-1, October 2011.

13- Plant physiology-dormancy-2, October 2011.

Publications (achieved in last 3 years):

1. **Rihan, H. Z.** Al Shamari, M & Fuller, M. P. 2012. The production of cauliflower microshoots using curd meristematic tissues and hypocotyl-derived callus. *Acta Horticulturae. (ISHS),* 961**,** 427-434.

2. **Rihan, H. Z.** Al-Issawi, M. Burchett, S & Fuller, M. P. 2012. Artificial seed production from encapsulated microshoots of cauliflower (*Brassica oleraceae* var botrytis). *Acta Horticulturae (ISHS),* 961**,** 419-425.

3. **Rihan, H. Z.** Al-Issawi, M. Burchett, S & Fuller, M. P. 2011. Encapsulation of cauliflower (Brassica oleracea var botrytis) microshoots as artificial seeds and their conversion and growth in commercial substrates. *Plant Cell, Tissue and Organ Culture (PCTOC),* 107**,** 243-250. **(This article has been reviewed and featured in Agricell Reports Vol 57 no 5. This international review newsletter selects the most noteworthy publications in the field of plant biotechnology each month).**

4. **Rihan, H. Z.** Al-Issawi, M. Al-Swedi, F & Fuller, M. P. 2012. The effect of using PPM (plant preservative mixture) on the development of cauliflower microshoots and the quality of artificial seed produced. *Scientia Horticulturae*, 141, 47-52. **(Article reviewed and featured in Agricell Reports). (Article selected and featured on Earth Emphasis website)** (http://earthemphasis.com/key-research-articles/the-effect-of-using-ppm-plant-preservative-mixture-on-the-development-of-cauliflower-microshoots-and-the-quality-of-artificial-seed-produced/)

5. Al-Issawi, M. **Rihan, H. Z.** El-Sarkassy, N & Fuller, M. P. 2013. Frost Hardiness Expression and Characterisation in Wheat at Ear Emergence. *Journal of Agronomy and Crop Science,* 199**,** 66-74.

6. Al-Issawi, M. **Rihan, H. Z**. Woldie, W. A., Burchett, S & Fuller, M. P. 2013. Exogenous application of molybdenum affects the expression of CBF14 and the development of frost tolerance in wheat. *Plant Physiology and Biochemistry*, 63, 77-81.

7. Fuller, M. P. Hamza, J. H. **Rihan, H. Z** & Al-Issawi, M. 2012. Germination of Primed Seed under NaCl Stress in Wheat. *ISRN Botany,* 2012**,** 5.

8. **Rihan, H. Z**. Al-Shamari, M. Al-Swedi, F. Burchett, S & Fuller, M. P. 2013. The effect of sugar type, source and concentration on Brassica oleraceae var botrytis microproshoot production. *Acta Horticulturae*, under review.

9. Al-Swedi, F. **Rihan, H. Z**. Al Shamar, M. Lane, S & Fuller, M. P. 2013. Genetic transformation of cauliflower with Ascorbate Peroxidase (APX) gene. *Acta horticulturae*, under review.

10. Al Shamar, M. **Rihan, H. Z.** Al-Swedi, F & Fuller, M. P. 2013. The use of somatic embryogenesis in artificial seed production in cauliflower (*Brassica oleracea* var. *botrytis*). *Acta Horticulturae*, under review.

11. Al Shamar, M. **Rihan, H. Z.** Al-Swedi, F & Fuller, M. P. 2013. Secondary somatic embryo production in cauliflower (*Brassica oleraceae* var. botrytis) and the effect of activated charcoal. *Scientia Horticulturae*. Under review.

12. Al Shamar, M. **Rihan, H. Z.** Al-Swedi, F & Fuller, M. P. 2013.The effect explant size of root –derived embryogenic callus tissue and kinetin concentrations on somatic embryo formation in cauliflower (Brassica oleraceae var. botrytis). *In Vitro Cellular and Developmental Biology -Plant*. Under review.

13. Three papers are in progress for 2013-2014.

Conference papers (delivered in last 3 years):

1. **Rihan, H. Z.** Al-Issawi, M. Al Shamari, M. Burchett, S and Fuller M.P. 2012. Cold tolerance and CBF/DREB1 up-regulation both cauliflower mature plants and microshoots. Centre for Agriculture and Rural Sustainability Postgraduate Symposium 2012. The New Continental Hotel, Plymouth, UK.

2. Al Shamari, M. **Rihan, H. Z.** Al-Swedi, F and Fuller, M. P. 2012. Primary and secondary somatic embryos in cauliflower (*Brassica oleraceae* var. *botrytis*). Centre for Agriculture and Rurale Sustainability Postgraduate Symposium 2012. The New Continental Hotel, Plymouth, UK.

3. Al-swedi, F. Al Shamari, M. **Rihan, H. Z**. Lane, S and Fuller, M. P. 2012. Micropropagation and genetic transformation in *Brassica olereacea* var *botrytis*. 12th congress of the European Society for Agronomy, Helsinki, Finland, 20-24 August 2012. P: 252-253.

4. Al Shamari, M. **Rihan, H. Z**. Al-swedi, F. Lane, S and Fuller, M. P. 2012.Regeneration of cauliflower (*Brassica olereacea* var *botrytis*) via somatic embryogenesis. 12th congress of the European Society for Agronomy, Helsinki, Finland, 20-24 August. P: 482-483.

5. Al-Issawi, M. **Rihan, H. Z**. Fadhel, F. Burchett, S and Fuller, M. P. The effect of Mollybdenum on frost tolerance to wheat genotypes. The postgraduate society annual conference. Plymouth University, June 26, 2012.1.4, 15.

6. Al-swedi, F. **Rihan, H. Z**. Al Shamari, M. S. Lane and Fuller, M. P. Cauliflower (*Brassica oleracea*) transformed by using *Agrobacteruim tumefaciens* as a vector. The postgraduate society annual conference. Plymouth University, June 26, 2012. 2.6, 24.

7. **Rihan, H. Z**. Al-Issawi, M. Al Shamari, M. Burchett, S and Fuller, M. P. Cold tolerance and *CBF/DREB1* up-regulation in *Brassica oleracea* var *botrytis.* Plant and microbe adaptation to cold 2012. Hokkaido University, Japan, June 24-28, 2012. P-7, 60.

8. Al-Issawi, M. **Rihan, H. Z**. Burchett, S and Fuller, M. P.The expression of *CBF* and physiological effect of frost to wheat during ear emergence. Plant and microbe adaptation to cold 2012. Hokkaido University, Japan, June 24-28, 2012.O19, 31.

9. **Rihan, H. Z** and Al-swedi, F. 2012. The teaching of biology in Syria. (Researches-Teachers-Learners, We are all in this together) international symposium. London, 27th – 29th March. EPA1, 22. **(I was successful in obtaining a travel grant from Plymouth award scheme to present my research in this meeting).**

10. Al-swedi, F and **Rihan, H. Z**. 2012. Formative assessment in higher education towards theory and enhancement of practice. (Researchers-Teachers-Learners, We are all in this together) international symposium. London, 27th – 29th March. EPA1, 30.

11. **Rihan, H. Z**. Al-Issawi, M. Al-swedi, F and Fuller, M. P. 2011. The improvement of cauliflower artificial seed cold tolerance. Plant Environmental Physiology Group – First Annual Mini-Symposium 20th December, Darwin House, London **(I was successful in obtaining a travel grant to present my research in this meeting).**

12. Al-Issawi, M. **Rihan, H. Z**. Al-Swedi, F, and Fuller, M. P. 2011. *CBF* expression in wheat at ear emergence stages Plant Environmental Physiology Group – First Annual Mini-Symposium 20th December, Darwin House, London.

13. Al-swedi, F. **Rihan, H. Z**. Al-Issawi, M. Al-Shamari, M and Fuller, M. P. 2011. Agrobacterium-mediated transformation of cauliflower (*Brassica oleracea* var. *botrytis*): optimizationof protocol and development of transgenic cauliflower. Plant Environmental Physiology Group – First Annual Mini-Symposium 20th December, Darwin House, London (poster presentation).

14. **Rihan, H. Z**. Al-Issawi, M. Al-swedi, F and Fuller, M. P. 2011. An easy way to understand the mechanism of cold tolerance gene function in plants. University of Plymouth postgraduate conference 23th November, house stone theatre, Portland square building.

15. Al-swedi, F. **Rihan, H. Z**. Al-Issawi, M. and Fuller, M. P. 2011. A micro propagation technique for cauliflower (Brassica oleracea var.botrytis) to facilitate Agrobacterium transformation. University of Plymouth postgraduate conference, 23th November, House stone theatre, Portland square building.

16. Al-Issawi, M. **Rihan, H. Z.** Fuller, M. P and Al-Shamari, M. 2011. Cross-protection of wheat to abiotic stress. BioResources young researcher 2011 meeting at the University of Reading (8th July).

17. Al-Shamari, M. **Rihan, H. Z** and Fuller, M., P. 2011. The control of hyperhydration on cauliflower shoots produced in liquid media. BioResources Young Researchers 2011 meeting at the University of Reading (8th July).

18. **Rihan, H. Z**. Fuller, M. P. Al-Issawi, M and Al-Shamari, M. 2011. The mass production of cauliflower microshoots for the production of artificial seeds and the optimization of artificial seed capsule structure. BioResources Young Researchers 2011 meeting at the University of Reading (8th July).

19. **Rihan, H. Z.** Al-Issawi, M. Al Shamari, M and Fuller, M. P. 2011.Cauliflower artificial seeds cold storage. University of Plymouth postgraduate conference 29th June, House stone theatre, Portland square building.

20. **Rihan, H. Z.** Al-Issawi, M. Fuller, M. P and Burchett, S. 2011. The determination of the optimal cauliflower microshoot development stage suitable for capsulation as artificial seeds and the optimization of conversion using semi solid media and commercial substrates. Society of experimental biology annual main meeting, Glasgow, UK, P1.25, 187. (**I was successful in obtaining a travel grant from the socity of experimental biology to present my research in this meeting).**

21. **Rihan, H. Z**. Fuller, M. P. Al-Issawi, M and Burchett, S. 2011. Cloning of cauliflower (Brassica oleraceae var. botrytis) and the production of artificial seeds as a promising cost-effective alternative to conventional methods of seed production. University of Plymouth postgraduate conference. **I was rewarded as the winner of the best oral presentation in the conference.**

22. **Rihan, H. Z**. 2010. The production the preservation of cauliflower artificial seeds The University of Plymouth- Plant physiology symposium 9th March.

23. **Rihan, Z, H**., Fuller, M. P and Burchett, S. 2010. Cauliflower micropropagation and artificial seed production. Society of experimental biology annual main meeting, Czech Republic, Prague, (poster presentation), C 5.18, 247.

24. The annual meeting and conference of the UK CEUG (controlled environmental user‘s group) (2009) (observer).

Conferences organised:

1. The postgraduate society annual conference. Plymouth University, June 26, 2012.
2. Practical Techniques in Molecular Biology workshop, 16th-19th July, 2012. Plymouth University, United Kingdom.

Grants and Awards:

1. The winner of the Early Career Researcher Award in the Expert category in the Vice-Chancellor’s Enterprise Awards 2012 at Plymouth University.

2. The winner of the best oral presentation in the postgraduate conference at the Plymouth University 2011.

3. Travel grant from Plymouth award scheme to present my research in the (Researchs-Teachers-Learners, We are all in this together) international symposium.

4. Travel grant from the Society of Experimental Biology to present my research in the society annual meeting in Glasgow, UK.

Professional Membership:

1. Higher Education Academy (**HEA**) /UK (from 2010).
2. Society for Experimental Biology (**SEB**) (from 2009).
3. International Society for Horticulture Science (**ISHS**) (from 2011).
4. Society of Chemical Industry (**SCI**) (from 2010).
5. Postgraduate society/University of Plymouth/ (from 2010)/*acted Chairman of the Society for the year 2012.*
6. Plymouth University graduate committee (2012).
7. School of Biomedical and Biological Sciences Research Committee.
8. School of Biomedical and Biological Sciences Safety Committee.

List of Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

ABA: Abscisic Acid

ANOVA: Analysis of variances

APS: Amonium Persulfate

BLAST: Basic Local Alignment Search Tool

BoCBF: Brassica oleracea C-repeat binding factor

BSA: Bovin Serum Albumin

CAMTA: Calmodulin Binding Transcription Activator

CAT: Chloramphenicol acetyltransferase

CBF: C-repeat binding factor

cDNA: Complementary deoxyribonucleic acid

COR: Cold regulated

CRT: C-repeat

dATP: Deoxyadenosine triphosphate

dCTP: Deoxycytidine triphosphate

dH_2O: distilled-sterilized water

DNA: Deoxyribonucleic acid

dNTPs: Deoxynucleoside Triphosphates

DRE: Dehydration Responsive Element

DREB: Dehydration Responsive Element Binding Factor

DTT: Dithiothreitol

dTTP: Deoxythymidine triphosphate

EC: Electrical Conductivity

EDTA: Ethylenediaminetetraacetic acid

ERD: Early Responsive Dehydration Gene

gDNA: Genomic deoxyribonucleic acid

H_2O_2: Hydrogen peroxide

HCl: Hydrogen chloride

HRP: Horseradish Peroxidase xx

IBA: Indole-3-butyric acid

ICE: Inducer of CBF Expression

$K_2HP\ O_4$: Dipotassium Hydrogen Phosphate

kDa: Kilo Dalton

KH_2PO_4: Potassium Dihydrogen Phosphate

KPO_4: Potassium Phosphate

LEA: Late Embryogenesis Abundant (LEA) proteins

LSD: Least Significant Difference

MS: Murashige and Skoog

MSU: Michigan State University

Na_2HPO_4 : Sodium Hydrogen Phosphate

NaCl: Sodium chloride

NaH_2PO_4 : Sodium Dihydrogen Phosphate

NaOH: Sodium Hydroxide

NCBI: National Center for Biotechnology Information

OH: Hydroxyl radicals

PAGE: Polyacrylamide Gel Electrophoresis

PBS: Phosphate Buffer Saline

PBST: Phosphate Buffer Saline Tween

PCR: Polymerase Chain Reaction

POX: Peroxisomal enzyme

RNA: Ribonucleic acid

ROS: Reactive Oxygen Species

RWC: Relative Water Content

SDS: Sodium Dodecyl Sulfate xxi

SOD: Superoxide dismutase

TEMED: Tetramethylethylenediamine

UDS: Unscheduled DNA synthesis

UV: Ultra Violet

Chapter 1: General Introduction and Literature Review

1.1. Introduction

Cauliflower (Brassica oleraceae var. botrytis L.group) is an important vegetable grown worldwide with production assessed to be 20,876,817 tonnes worldwide (cauliflower and broccoli). The biggest crop is produced in Asia (The production in Asia has been evaluated at 17,000,754 tonnes of cauliflower and broccoli)(FAO., 2011).

Cauliflower contains high level of vitamins K, A, C and folic acid, flavonoids, and fibres. Therefore, cauliflower has a high important role as an antioxidant and anti-inflammatory, and it is considered as an important crop for human and animal feed. Cauliflower has a positive influence in decreasing the symptoms of asthma in children and it also helps in in lessening the risk of multiple sclerosis (MS) since this type of vegetable has adequate amount of vitamin C. Pattison et al (2004) indicated that cauliflower could also be useful to protect against artherosclerosis and to decrease bruising. Cauliflower and cruciferous vegetable generally play a role in lowering the risk of cancer, especially bowel, breast and other female cancers. Moreover, the flavonoids contained in cauliflower help to reduce the risk of cancer formation (Metwali Gharieb, 2006).

Cauliflower is a cool season vegetable which requires a well-drained and rich fertile soil, good moisture level and sunny location for the production of good quality of curds (the human edible part of cauliflower). While summer cauliflower are grown all over UK, winter cauliflower cultivation is limited to the mild coastal areas (Cornwall) where the probability of frost damage is low because cauliflower is a frost sensitive crop. Therefore, cauliflower cannot be considered as a profitable crop between the month of

November and April in most areas of UK a part from the south and south west of the country. Therefore, large quantities of winter cauliflower needs are imported from Spain, France and sometimes Italy.

Cauliflower was first mentioned rather late compared with other cultivated *Brassica oleraceae* L. Cauliflower was mentioned in the 12th century by Ibn-al-awam in his Spanish-arabic study who reported three cauliflower types called Syrian and Mosul cabbages. The origin of cauliflower and broccoli seem to be from eastern Mediterranean and it was suggested by Song et al (1990) that that wild *Brassica oleraceae* L. derived kale is the progenitor of cauliflower. The authors also reported that there is a genetic affinity between cauliflower and broccoli suggesting that they could be derived from the same gene poll by careful selection.

The distinctive feature of this crop is that the part fit for human consumption is a large 'preinflorescence, called a curd. The curd is a large part of the cauliflower plant produced at the top of the stem after a period of vegetative growth (Hand and Atherton, 1987). It is produced from elongated and branched shoots carrying on its surface a great number of meristems which have the ability to grow into flowers (Sadik, 1962). This large number of meristems in this species offers exceptional possibilities for micropropagation manipulation (Kieffer, 1996).

1.2. Cauliflower curd structure and formation

Cauliflower curd is a much organised structure that has a main monopodial steam carrying numerous branches (Sadik, 1962). Booij and Struik (1990) indicated that an

acceleration of the widening rate of the apex is the first step of the curd induction in cauliflower. However, Sadik and Ozbun (1968) mentioned that the rising number of cell layers of the meristem mantle is the most remarkable modification in the curd formation. It was reported that curd formation begins when the apex reaches the break point (about 0.6 mm in diameter) (Wurr et al., 1990). The apex then starts to form bract like leaves which will develop the curd covering leaves in later stage, and the part of the stem below the bracts extends letting the inflorescence to appear as a small tower on the end of the stem (Kieffer, 1996). Salter (1960) reported that the meristems in the bract exiles start emerging to form tips of branches of first order and later a second order branch primordial is started from the apices of the first order branches. This process is continues to form a huge number of shoot apices the cover the curd (Torres et al., 1980).

Cauliflower curd shapes are different depending on the variety. However, the vast majority of the cultivated genotypes have a relatively smooth surface despite of some Italian varieties 'Jesi' shows a very angular surface since each branch forms a small pyramidal arrangement with a specific conserved angle. This structure could be caused by a highly specific stem development (Kieffer, 1996).

1.3. Stages of cauliflower growth

Cauliflower has three main stages of growth during its life cycle from sowing to curd initiation to curd harvest (Luckhurst, 2007):

- **Juvenile stage**

This is a vegetative stage when the plant is in-sensitive to cold stimulation and grows in biomass without developing to a floral productive phase (Thomas, 1999). The period of this stage is variable depending on the genotype. It can be easily estimated using the number of leaves produced to arrive at the point when the plant become responsive to cold induction (Kieffer, 1996).

- **Curd induction stage**

In this stage the apex is induced to change from vegetative to floral prior to producing a curd. For winter varieties this requires an exposure to low temperatures and is called vernalization. The duration of this stage is essentially dependent on the genotype (Kalloo and Bergh, 1993). When the plant reaches its inductive phase, the apex will be impelled to change from vegetative to generative followed by the evolution of the curd and then the flower. In the induction stage, the plant apexes can be affected by low temperature degrees resulting in increase to their diameters, expansion in their width and having a “vaulted” structure (Hadley and Pearson, 1998).

The basic changes were observed in the shoot tip during the induction stage. While Burn et al (1993) mentioned that cold treatment induces a modification of the level of DNA methylation, decreasing the time needed for flowering, Thomas and Lester (1972) reported that the cold treatment increased the activity of gibberellin which was at its highest level in the cauliflower apex just prior to curd initiation. They mentioned that other phytohormones such as auxin and cytokinin could have a part to play in curd induction. An essential role of carbohydrate in the curd induction was also observed by (Hand and Atherton, 1987, Williams and Atherton, 1990) cited in (Kieffer, 1996).

However, Bernier (1988) mentioned that there are several factors affecting in cauliflower induction in interaction with the physiology and genetic stage.

- **Curd growth stage**

In this stage the curd development can be affected by three main factors: A. genetic structure. B. climatic conditions. C. culture components (Kieffer, 1996). The vegetative apex becomes generative and grows into the curd. However, the curd is a prefloral formation which shares some attributes of the reproductive and vegetative apices. If not harvested, it is able to grow into flower buds, which can lengthen up to 50 cm, in suitable environmental conditions (Sadik, 1962, Wiebe, 1975). The edible portion of the cauliflower is this prefloral structure, the curd.

1.4. The breeding of cauliflower

a) Open pollinated varieties

These varieties logically depend on out-pollination because of cauliflower ‘s sporophytic self–incompatibility system producing high level of heterozygote (Trick and Heizmann 1992) cited in (Kieffer, 1996). A consequence of open pollinated varieties is that they exhibit heteroogeneity reflecting high cost of agricultural treatment especially at harvest because of asynchronous ripening. These varieties usually need 10-12 cuts for crop harvesting (Kieffer, 1996, Luckhurst, 2007).

b) F1 hybrid varieties

F1 hybrid cauliflowers have valuable features such as: uniform maturity (1-3 harvesting cuts instead of 10-12 in open pollinated varieties), high yield and better quality (Kučera et al., 2006) and in general are preferred by growers. The production of cauliflower F1

hybrids varieties has faced several difficulties such as, the laboratory/glasshouse inbreeding work required in order to produce valuable parental inbred lines. The production of purebred inbred lines is hard to achieve given the outbreeding nature of cauliflower (Bhalla and de Weerd, 1999). It takes up to 15 years in winter heading types using the technique of hand pollination of the immature flower (Kieffer, 1996) and the continuance of breeding lines is labour intensive (Bhalla and de Weerd, 1999). Moreover, although the most cauliflower hybrid varieties have been produced by means of self incompatibility depending on the ability to generate hybrid seed using two self incompatibility lines homozygous for different S alleles as parental constituent (Kučera et al., 2006), self pollination is not easily avoided in unfavorable seed production conditions and there can be up to 5% of off –type seeds (Kieffer, 1996).

Considering the difficulties of F1 hybrid production depending on SI character, new seed production strategies have been studied relying on male sterility features in the cauliflower. Male sterility, which seems to be more dependable than SI in providing no self pollination, includes two types:

1. Genetic (nuclear) male sterility

The gene is initiated by back cross breeding into deliberated female lines. Mariani et al (1990) mentioned that the male sterility was formed by expression of a chimeric gene which demolishes tapetal cell layers spoiling pollen production. However, the applicable use of genetic male sterility is restricted because of its common instability (Kučera et al., 2006).

2. Cytoplasmic male sterility

The most extensive cytoplasmic male sterility system used in cauliflower hybrid breeding is the development of the 'Ogura' (radish) CMS (Pelletier et al., 1983) cited in (Kučera et al., 2006). However, the uses of cytoplasmic male sterility were not viable because of flower abnormality (ineffective nectar production, reduced female fertility) and lack of chlorophyll at low temperature. These problems were avoided by somatic mixture with protoplast derived from 'normal' *Brassica*. Therefore, the cytoplasmic male sterility has been utilized for hybrid seed production since 1992 (Kieffer, 1996).

c) Cauliflower micropropagation

Considering the disadvantages of open pollinated cauliflower varieties and the difficulties of production male sterility and incompatibility purebred lines for producing F1 hybrid cauliflower varieties, *in vitro* propagation systems have an important role in cauliflower breeding. It can be used for clonal reproduction of selected genotypes such as self incompatible and male sterile lines (Kieffer, 1996). In this way, Fuller et al (1990) could produce di-haploid plants with similar properties of inbred lines using cauliflower anther culture. Thus, the production of purebred lines for producing F1 varieties of cauliflower is much easier using tissue culture technique saving an intensive work and requiring less time.

In vitro shoot propagation from Brassica seedling explants is interesting because of a plentiful supply of the explants from many plant structures including cotyledons, seedling roots, hypocotyls, stem sections, petals and leaf tissue (Bhalla, 1998, Cao, 2003). Many studies on *in vitro* cauliflower propagation have been reported using

seedling explants (Arora et al., 1997, Vandemoortele et al., 1993), anther culture (Fuller et al., 1990, Yang et al., 1992), protoplast culture (Delpierre and Boccongibod, 1992, Yang et al., 1994), roots (Horeau et al., 1988, Narasimhulu and Chopra, 1988) and peduncles (Christey and Earle, 1991). However, *in vitro* propagation favors the culture of pre-existing meristems because of their superior genetic stability (Kieffer, 1996). Hence, the cauliflower curd has a great advantage, since it contains millions of meristemes forming an ideal basic material for tissue culture applications (Kieffer et al., 2001). Cauliflower micropropagation using curd meristimatic tissue has been mentioned by (Crisp and Gray, 1979, David and Margara, 1979). Kieffer et al (1995) created a new simple effective protocol for in 'mass' cauliflower micopropagation using propagules from fractionated and graded curd. More than 10,000 microshoots were produced within two weeks and over 80% of them were rooted in the rooting medium. Furthermore, Kieffer et al (2001) were able to produce 2000 cauliflower plants from one mother curd using the modified protocol of (Kieffer et al., 1995). They mentioned that the plants produced had greatly homogenous curd in the field with a short cutting phase opening wide vistas to use this technology in commercial production.

Plant micropropagation via tissue culture is essentially produced either through organogenesis or somatic embryogenesis (Deng et al., 2009). Somatic embryogenesis is the *in vitro* generation of somatic embryos which are a bipolar formation with both apical and basal meristematic districts and capable of producing shoots and roots, respectively (Saiprasad, 2001). Straight somatic embryogenesis is a more desirable route for plant regeneration avoiding the dedifferentiated callus stage and producing

genetically stable plants (Deng et al., 2009). However, a crucial appliance of somatic embryos is their employment in production of synthetic seed providing great ease of handling, storage, transportation and planting in comparison with conventional tissue culture techniques (Ara et al., 1999) cited in (Rai et al., 2008a) . This study aims to determine an efficient protocol to produce cauliflower artificial seeds.

However, the optimization of cauliflower micropropagation needs to optimize both of use phytohormones combinations with culture media and to overcome the contamination problem observed with tissue culture protocols.

1. Phytohormones

Auxins and cytokinin

The use of phytohormones, and especially the balance of auxin and cytokinin in the media, was reported as a key factor for the control of organogensis in tissue culture (Skoog and Miller, 1957). Induction of caulogenesis is normally caused by a high ratio of cytokinin:auxin. However, it was reported that the phytohormone concentrations were also essential even at constant proportion (Hachey et al., 1991). The presence of cytokinin whether alone or in combination with a low concentration of auxin was found to be very important for inducing the best level of shoots regeneration of *Brassica spp*. This point was proved working with different types of explants used for microshoot production such as curd stem segments (Margara, 1969), immature flower buds (Anderson and Carstens, 1977), root segments (Lazzeri and Dunwell, 1984), stem thin cell layer (Lazzeri and Dunwell, 1986), leaf discs (Lazzeri and Dunwell, 1986, Glendening and Sjolund, 1988), inflorescence axis segments (Christey and Earle,

1988), stem segments (Pua et al., 1989), primary leaves (Ovesna et al., 1993), hypocotyl segments and cotyledons (Narasimhulu and Chopra, 1987, Narasimhulu and Chopra, 1988, Sharma et al., 1990, Hachey et al., 1991). The type and concentration both of cytokinin and auxin vary according to the plant species and the part of the plant used since exogenous growth regulators of the explants must be taken in to account (Sharma et al., 1990, Sharma et al., 1991). These authors mentioned that the presence of the lamina was crucial for the initiation of shoot regeneration of the cotyledonary explants of petiole cut end in *B. juncea* (L.) Czern. It was found later that the lamina produced an auxin-like structure that was capable of regenerating shoots the end of cutting.

Although several types of cytokinins have been investigated with *Brassica spp*, N^6-benzyladenin (BA) was reported to be the best in terms of shoot regeneration induction (Pua et al., 1989, Sharma et al., 1991). It is important to note here that the use of a high concentration of (BA) affects the quality of shoots, increasing the level of hyperhydricity. The use of a combination of cytokinin and auxin has positive effects, increasing the frequency of regeneration and the number of shoots obtained per explants. However, the ratio of cytokinin: auxin should be adjusted accurately since this ratio varies depending on the plant species (Julliard et al., 1992), the type of explants, even the clones derived from the same explants required different ratios during the stage of shoot proliferation (Deng et al., 1991).

Although several types of auxins were used with *Brassica spp* micropropagation protocols, the use of NAA and IBA was reported to be the most commonly auxins used

although with noticeably different concentrations (Zarske, 1988, Kumar et al., 1992, Kumar et al., 1993)

Gibberellin

Although the synergic activity of gibberellic acid on cauliflower stem explants shoot regeneration was demonstrated by Margara (1969), Sharma et al (1991) reported no beneficial results in relation to cotyledonary explants. However, inhibition of growth and shoot regeneration in broccoli explants was observed by Lazzeri and Dunwell (1986).

Ethylene

Although the positive effects of using silver nitrate and silverthiosulphate was demonstrated in research on the shoot regeneration *of B.campestris* L (Mukhopadhyay et al., 1992), continuous was found to result in symptoms of hyperhydricity. However, Block et al and Hachey et al (1989, 1991) found no significant effect of silver on shoot regeneration, and there was even some inhibitory activity.

2. Culture system contamination

Despite the strict sterilized conditions applied with tissue culture techniques, the contamination of plant culture remains a persistent problem. Contamination can cause culture losses which could be ranged from small number of cultures to the whole batches culture medium. Contamination by bacteria and fungi is an inside process that frequently threatens plant tissue cultures during the duration of the culture phase. Therefore, it would be valuable to supply a chemical agent which decreases or prevents

the microbial contamination of plant tissue culture media maintaining the sterility of the culture system.

However, although different antimicrobial chemical agents have been tested in the plant tissue culture system, the uses of antibiotics have been widely evaluated for their ability to stop the development of bacteria in the plant cultures. Phillips *et al* (1981) investigated the effect of six antibiotics, i.e benzyl penicillin, phosphomosin, choramphenicol, nalidixic, streptomycin and rifampicin on preventing bacterial infection in Jerusalem artickoke (*Helianthus tuberosus*) cultures. They found that only rifampicin had given significant results controlling bacterial contamination without the rate of cell division. Gilbert *et al* (1991) investigated the use of antibiotics to control latent bacterial contamination in potato cell culture. They tested the use of two combinations of antibiotics, penicillin, streptomycin and amphotericin or erythromycin, streptomycin and carbenicillin. The authors reported that the both combination reduced the plant growth when they used with the media of culture. Moreover, these combinations induced chlorosis when they were used at high concentration.

Antibiotics are expensive and only effective against bacteria and not fungi. Moreover, antibiotics range of efficiency against type of bacteria is usually narrow. They are generally heat-labile. They are usually either phototoxic or competent of altering the behavior of cultured plant tissues. However, considering these disadvantages for using antibiotics, the use of PPM was recommended for controlling the contamination of plant culture (more details in section 2.1(page 52)) since:

1. It is efficient against fungi and bacteria (Lunghusen, 1998a).
2. PPM is less expensive compared with antibiotics.
3. The creation of resistant mutants toward PPM is very unlikely since it inhibits multiple enzymes.
4. When it is used in the suitable concentrations, PPM does not affect the viability of plant materials (Digonzelli *et al*., 2005).

Niedz (1998) mentioned that the use of 0.5 to 2 ml l^{-1} concentrations of PPM can be efficient controlling fungi and bacterial contaminations because PPM affects necessary enzymes on the cycle of citric acid and on the transport chain of electrons.

1.5. Artificial seeds

The improvement of synthetic seed production techniques is considered a valuable alternate technology of propagation in many commercially important crops and a significant method for mass propagation of elite plant species (Saiprasad, 2001). However, the production of cauliflower clones multiplied by tissue culture and distributed as artificial seeds could be useful alternative to the costly F1 hybrids for homogenous harvest production (Kieffer, 1996).

1.6. Artificial seeds definition

These are synthetically encapsulated somatic embryos (usually) or other vegetative parts such as shoot buds, cell aggregates, auxiliary buds, or any other micropropagules which can be sown as a seed, converted into a plant under *in vitro* or *ex vitro* conditions,

keeping this ability for an extended period (storage ability) (Ara *et al*., 2000, Daud *et al*., 2008, Saiprasad, 2001).

1.7. The importance, uses and advantages of artificial seeds

Artificial seed is an essential technique for proliferation of plant species which are not able to produce seed such as some varieties of grapes and water melon or have difficulties in seed propagation (for instance, hetrozygosity of seeds, endosperm lacking seeds, tiny seed size and the necessity of seed with mycorrhiza fungi connection for development and growth) (Cartes *et al*., 2009, Saiprasad, 2001). Synthetic seeds can be employed for production of polyploids with elite trails, avoiding the genetic recombination when these plants are propagated using conventional plant breeding systems, thus saving time and costs (Figure 1). Synthetic seeds can be also used in the proliferation of male or female sterile plants for hybrid seed production (Saiprasad, 2001). Artificial seeds produced through somatic embryos are an important technique for transgenic plants where a single gene can be placed in a somatic cell and then this gene will be located in all the plants which are produced from this cell. Therefore, artificial seeds could be an efficient technology used for reproduction of transgenic plants (Daud *et al*., 2008). Moreover, artificial seeds, which are produced using tissue culture techniques which are aseptic, are free of pathogens, giving great advantages to these materials for transport across frontiers and for avoiding the spread of plant diseases (Daud *et al*., 2008, Nyende *et al.*, 2005). Artificial seeds are also valuable in terms of their role in providing protective coating, increasing the level of micropropagules success in the field. However, these micropropagules need a

protective coating to increase successful establishment in the field situation because of the sensitivity of uncovered micropropagules to drought and pathogens under nature environmental conditions (Ara *et al*., 2000). Furthermore, artificial seeds are more durable for handling, transporting and storage. Artificial seeds are also a useful technique as clonal propagation systems in terms of preservation of genetic uniformity of plants, straight delivery to the field, low cost and fast reproduction of plants (Figure 1). Germplasm preservation applying artificial seed cryopreservation technology could be useful especially for recalcitrant species (such as the species which their seeds are sensitive to storage treatment as dehydration, for instance, mango and coconut) (Saiprasad, 2001).

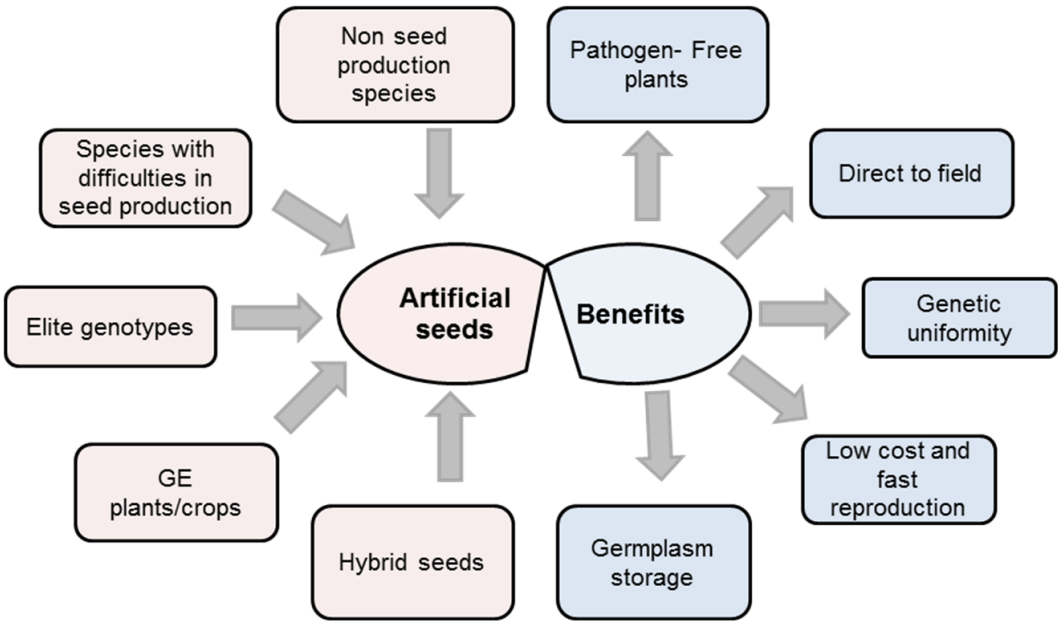


Figure 1. Artificial seed uses and benefits (modified from (Mohamed *et al*., 2009)).

1.8. Artificial seed concept

Artificial seed structure mimics conventional seed. It consists of A- explants material which imitate the zygote embryos in the conventional seed (the essential difference between explants (usually somatic embryos) and zygote embryos is that the somatic embryos develop from somatic cells instead of zygotes in zygote embryos) B- The capsulation (gel agents and additional materials such as: nutrients, growth regulators, anti-pathogens, bio-controllers and bio fertilizers) which emulate the endosperm in conventional seed (Cartes *et al*., 2009) (Figure 2).

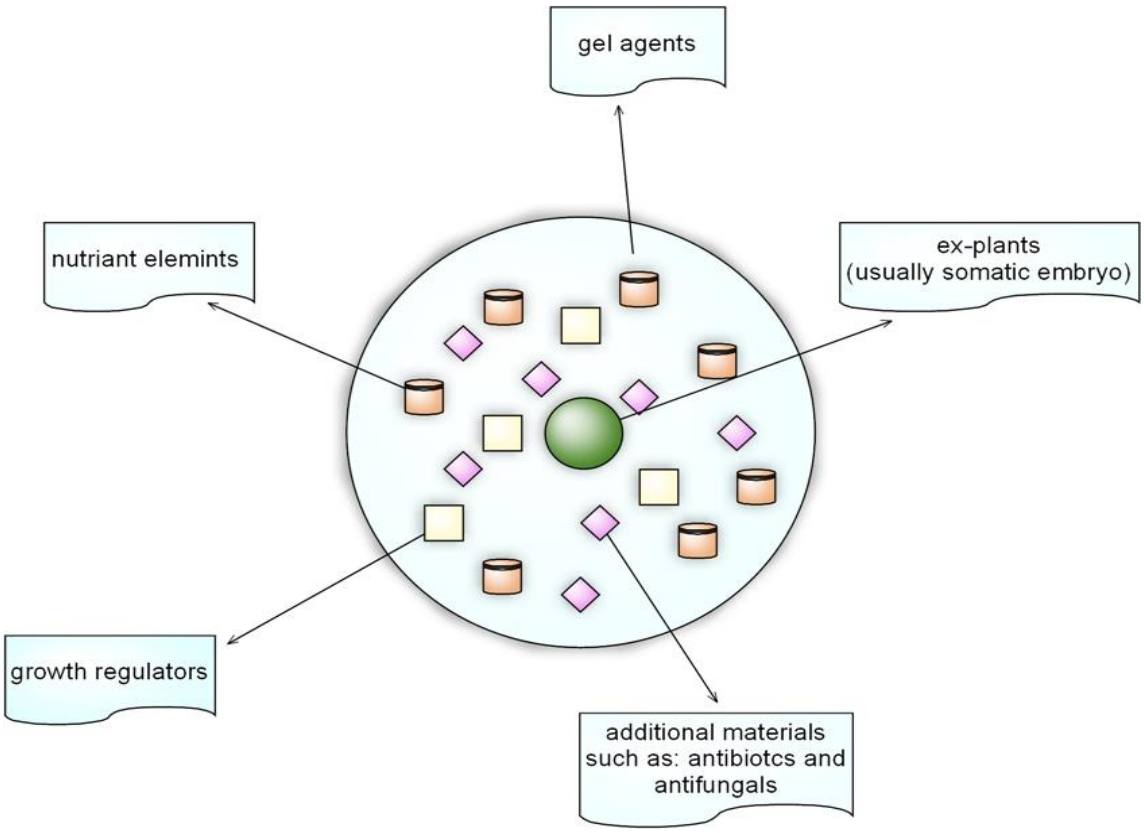


Figure 2. Artificial seed concept.

Essential requirements for the production of artificial seeds:

1.9. Ex-plant material

Explant materials is the basic generative component of artificial seed. It could be:

I. Somatic embryos

Somatic embryos are the most common micropropagule used for artificial seed production because these structures are able to produce the radical and plumule which have the capability to progress into root and shoot in one step (Ara *et al*., 2000). Synthetic seeds through somatic embryos can also provide high level of reproduction. Somatic embryos have a great feature in that the structure of an external bipolar configuration from plant tissue without correlation to the plant vascular system. However, the plant lines, which are produced via somatic embryos, are capable of keeping their regenerating capacity for a long time producing uniform plant production (Leroy *et al*., 2000) because the technique of artificial seed production using somatic embryos avoids the dedifferentiation callus stage, resulting in constant genetic structure production (Deng *et al*., 2009).

The use of somatic embryos for artificial seed production has become wide spread with time and the number of species which seem to have propagation ability using this technique is increasing. The production of artificial seeds via somatic embryos have been investigated in several plant species such as carrot (*Daucus carota*) (Kitto and Janick, 1982), alfalfa (*Medicago sativa*), norway spruce (*Picea abies*) (Gupta and Durzan, 1987), Pistachio (*Pistacia vera*)(Onay *et al*., 1996), sandalwood (*Solanium*

album) (Ghosh and Sen, 1994) and in mango (*Mangifera indica L*.) (Ara *et al*., 1999) and many other species (Table 1). However, while Attree *et al* (1994) indicated that the somatic embryos of white spruce (*Picea glauca*) survived desiccation and grew to plantlets more strongly than removed zygotic embryos cultured *in vitro*, Lulsdorf *et al* (1993) mentioned that the germination level of encapsulated white spruce (*Picea glauca*) and black spruce (*Picea mariana*) of reduced frequency in comparison with the correspondent zygotic embryos. Cartes *et al* (2009) reported that the encapsulated somatic and zygotic Rauli-Beech (*Nothofagus alpine*) embryos had the same germination habits which depended on the type of encapsulation applied and they mentioned that the germination level of zygotic embryos was higher in comparison with somatic embryos. An automatic production and encapsulation artificial seeds protocol was established by (Onishi *et al*., 1994). These authors mentioned that the optimal sowing state such as nursery bed in the field or in the greenhouse provides a high and homogeneous conversion of artificial seeds. They demonstrated that the conversion level of celery and carrot embryos can be raised from (0%) to (53-80 %) applying three essential treatments: 1. Culturing the embryos in medium culture with high osmolarity for 7 days. The embryos size was increased from (1-3 mm) to (8 mm) and the chlorophyll was also increased during this treatment. 2. The embryos water content was reduced from (95-99 %) to (80-90 %). 3. Post-dehydration culture on SH medium containing 0.01 mg l^{-1} $GA_{3,}$ 0.01 mg l^{-1} BAP and 2 % sorbitol. The bead quality was also modified by adding 3% sucrose and a mixture of fungicides.

II. *Apical shoot tips and axillary shoot buds*

Although unipolar axillary shoot buds and apical shoot tips contain no root meristem, they have been encapsulated to produce synthetic seeds in several plant species. However, although these explants required some special treatment for induction of the reformation of roots before the encapsulation stage, various studies have reported the conversion of encapsulated buds of banana and mulberry into plantlets without specific induction treatment (Ganapathi *et al*., 1992) and (Bapat and Rao, 1990) respectively. Ganapathi *et al* (1992) mentioned that 100 % conversion of encapsulated banana shoot tips into plantlets was obtained using white's culture medium and these plantlets were effectively based in soil. Piccioni and Standardi (1995) reported that encapsulated micropropagated buds of six woody species, apple (*Malus spp*), blackberry (*Rubus spp*), birch (*Betula pendula*), kiwifruit (*Actinidia deliciosa*), raspberry (*Rubus idaeus* L.) and hawthorn (*Crataegus oxyacantha*) were successfully regrown after encapsulation and cultivation on enriched media. Working in M.26 apple rootstock, encapsulated apical buds (synthetic seed) showed higher level of conversion in comparison with artificial seeds from axillary buds (the maximum conversion rates for encapsulated apical and axillary buds were 85% and 25%, respectively) (Capuano *et al*., 1998). Lata *et al* (2009) mentioned that 100% conversion of encapsulated axillary buds was produced in the suitable capsulation matrix and the plantlets produced were effectively passed to the soil. These consequences prove the ability of such explants for encapsulation and artificial seed production.

III. Other explants materials

Several other explant materials such as embryogenic masses and protocorm-like bodies (small swollen tuber consisting mostly from undifferentiated cells which has potential to produce shoots *in vitro*) have been investigated to test their ability to produce artificial seeds. However, although the supporting of embryogenic masses in culture tubs is expensive and labour intensive and although mechanically provoked bio-reactors require regular transfer of tissue to new media. Nonetheless, Onay *et al* (1996) were able to successfully produce artificial seeds via an embryogenenic mass. They reported that the encapsulated embryogenic mass fractions regained their primary reproductive ability after two month storage. The production of synthetic seeds through encapsulated protocrom-like bodies of orchid (*Geodorum densiflorium*) was investigated by (Datta *et al* (1999). These authors mentioned that the encapsulated protocorm-like bodies retained their viability after three month storage at 4^{o}C while non encapsulated protocorm-like bodies appeared non-viable after one month storage at 4^{o}C. However, there is a variety of explants which could be used for artificial seed production and this depends essentially on the plant species.

1.10. Artificial seed gelling agents and adjuvant materials

The basic limitation for using somatic embryos as micropropagules for plant propagation is that the somatic embryos are delicate structures without a quiescent resting stage. Therefore, they require essential supplementary tissues which should provide the nutrient elements and a protective layer making them easier to handle and store. Thus, the main objective of artificial seed research is the production of a artificial seed

structure that stimulates the conventional seed in their characters (such as, handle, storage, viability and germination level) (Ara *et al*., 2000).

At the beginning of artificial seed studies, polyoxyethylene was selected as a suitable capsulation material to encapsulate celery embryos due to their positive properties such as sustained embryo growth, non toxicity to explants and solubility in water (Kitto and Janick, 1982) . Later and applied to alfalfa embryos, Redenbaugh *et al* (1984) reported a new technology using hydrogel encapsulation. Since then the hydrogel materials have been the main structure for somatic embryos encapsulation.

However, Although many gel materials such as agar, alginate, carrageenan, guar gum, and sodium pectate were investigated for artificial seed production, alginate matrix was discovered to be the optimal encapsulation for artificial seed production because of its sensible thickness, weak spinnability of solution, low toxicity of micro-organism, low expense, bio-suitability characteristics and fast gellation. This material improves capsule structure and bead rigidity, supplying better protection to covered explants against mechanical hurt (Ara *et al*., 2000, Saiprasad, 2001).The major principle for alginate encapsulation formation depends on the exchange ions between Na+ in sodium alginate with Ca+ in the $CaCl_2.2H_2O$ which happens when sodium alginate droplets involving the synthetic embryos dropped into the $Ca_2.2H_2O$ solution producing stable explant beds. The solidity and rigidity of the capsule (explant beads) depend upon the two gelling agents (sodium alginate and $CaCl_2.2H_2O$) concentrations and their mixing duration. On other hand, the addition of growth regulators and nutrients to the capsule is an essential factor to successful synthetic seed production technique, increasing the

competence of germination and viability of these seeds. These materials are considered as artificial endosperm and they also play an important role in the synthetic seed storage capability (Saiprasad, 2001). However, there are many other materials such as pesticide, antibiotics, fungicide, which have positive effects in the capsule features.

1.11. Artificial endosperm structure and their effects in the synthetic seed characters

While Saiprasad (2001) mentioned that usually 3% sodium with complexing solution containing 75 mM $Ca_2Cl_2.2H_2O$ for half an hour mixing duration provides the optimal structure for synthetic seed bead formation, Ara *et al* (2000) indicated that generally 2% sodium alginate gel upon complexation with 100 mM $CaCl_2.2H_2O$ is the best. However, not just the concentrations of gel agents but also mixing duration have important effects on the rigidity and hardness of artificial seeds which in turn greatly affect their characters (such as germination, storage ability). Daud *et al* (2008) mentioned that the germination level of African violet species (Saintpaulia ionantha Wendi) was higher (72-80%) for (30) min sodium alginate including micro-organism exposure duration in the 100 mM $CaCl_2.2H_2O$ solution than (52%) for (10) min exposure duration. However, these factors depend on others such as plant species, explants type and the aim of study such as short storage, long storage, obtain high germination level. However, lots of studies working in different plant species have investigated the optimal capsule structure which provides the top germination rate, best seed viability and their effects in the synthetic seed storage duration (Table 1).

Artificial endosperm has a great effect on the germination level. In this way, Ara *et al*. (1999) mentioned that the percentage of encapsulated somatic embryos germination of (*Mangifera indica* L.) was higher than non-encapsulated somatic embryos of the similar size in the same medium. Many studies also have demonstrated the important role of artificial endosperm structure (capsulation structure) in the synthetic seed storage capability (Table 1). Lakshmana Rao and Singh (1991) indicated that the reduction in germination level of encapsulated somatic embryos of hybrid *Solanium-melongenal* was much lower than in naked somatic embryos after (60) days storage at 4°C. Furthermore, while the encapsulated pieces of embryos mass got back their basic proliferative ability after (60) days storage, naked fragments failed in that (Onay *et al.*, 1996).

1.12. Artificial seeds storage ability:

Several studies have investigated the synthetic seed storage capability (Table 1). Rai *et al* (2008a) reported that high concentration of sucrose or ABA could be useful for short term conservation of guava (*Psidium guajava* L.) because of their temporarily inhibition in encapsulated somatic embryos germination (Table 1). Working in *Rauvolvia serpentine* and applying three different temperature degrees (20°C, 12°C and 4°C) Ray and Bhattacharya (2008) indicated that 4°C, where storage achieved up to 14 weeks with high regrowth percentage (Table 1), was the optimal degree for short storage duration. However, while short artificial seed storage can be obtained applying several procedures such as using suitable temperature degree (usually 4°C), using suitable capsulation materials and optimal storage conditions (reduce heat, light, oxygen …etc)

(Figure 3), long storage can be achieved using dehydration or/and cryopreservation techniques (Figure 3).

Fabre and Dereuddre (1990) working on Solanum shoot tips and aiming to increase the tolerance of plant tissue to dehydration-cryopreservation storage conditions, reported a full protocol for encapsulation-dehydration and storage. This protocol consists of three procedures: a. preculturing encapsulated explants in a medium containing high concentration of sucrose. B. drying of encapsulated micro-organism. C. direct plunging into liquid nitrogen. Unfortunately, few research projects have investigated in depth the artificial seed preservation (dehydration-cropreservation) and this technique still needs more studies in view of the great value of synthetic seeds as an easy and cost- efficient method of germplasm preservation (Danso and Ford-Lloyd, 2003). Furthermore, artificial seed conservation facilitates the exchange and distribution of trait plant germplasm decreasing the requirement for transferring and subculturing, out of season (West *et al*., 2006, Naik and Chand, 2006).

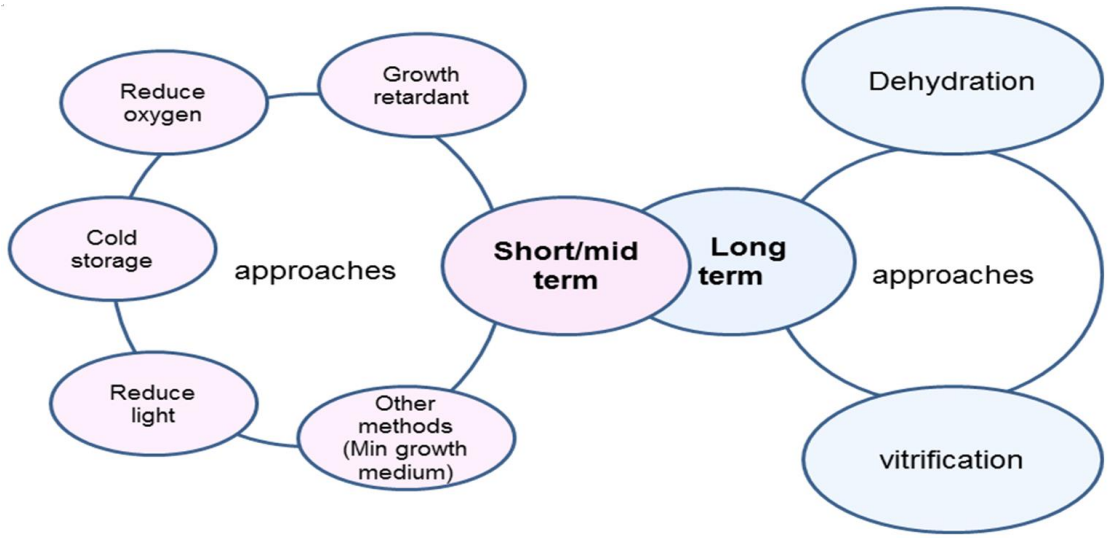


Figure 3. Artificial seed storage approaches modified from (Mohamed *et al*., 2009).

Reference	Species and explant used	Pre-encapsulation culture	Capsule structure	Additional materials	Storage conditions	Post storage treatment or culture	Storage duration	Experiment assessment
(Tsvetkov and Hausman, 2005)	Apical segments of Turkey oak (*Quercus cerris* L.)	Modified GD medium + 0.88 BAP + 7.5 $g.l^{-1}$ agar	4% (w/v) SA immersed in 1.4%(w/v) $CaCl_2$ for 10 min	GD modified medium+0.88mM BAP	4º C	-	6 weeks	85% regeneration and 95% viability
(Verleysen *et al*., 2005)	Shoot tips of azalea (*Rhododendorn simsii* Planch.)	Modified (WPM) medium+(0.3, 0.45, 0.6 M) sucrose (24)h	drops SA immersed in $CaCl_2$ for 15 min	1.62 M glycerol +0.32 M sucrose (cryoprotectants)	Dehydration to WC (38.6)% + transfer to LN	Detoxification in (1) M sucrose for (2)h	24 hours	40% survived after thawing
(Naik and Chand, 2006)	Nodal segments of promegranate (*Punica granatum* L.)	-	3% Sa + 100 mM $CaCl_2$ for 30 min	MS medium +4.44 mM (BA) + 0.54 mM (NAA)	4° C	(MS) medium + 4.44 mM (BA) + 0.54 mM (NAA)	30 days	The nodals were capable of sprouting.
(Faisal *et al*., 2006)	Nodal segments of *Rauvolfia tetraphylla* L.	MS medium + 10mM BA + 0.5 mM NAA + 3%(w/v) sucrose + 0.8%(w/v) agar	3% SA + 100 mM for 30 min	MS medium	4°C	MS medium +10mM BA+0.5NAA + 3% sucrose + 0.8% agar	4 weeks	Shoot proliferation 86.7 %
(Wang *et al.,* 2007)	Shoot buds were derived from lam (*Progonatherum paniceum*) calli	MS medium supplemented with 2.0 mg L_1 BAP and 0.2 mg L^{-1} NAA.	3.0% SA +2% $CaCl_2$ for 20 min	1% activate carbon(w/v) + 0.3% bavistin (w/v) + MS medium containing 3% sucrose	-	substrata containing 8 g L_{-1} agar with half-strength MS medium and 2% sucrose	-	61.58% conversion rate
(Sujatha and Kumari, 2008)	Node explants of mugwort (Artemisia vulgaris)	Nodal segments were excised from poliferating microshoots	2% SA + 50 mM $CaCl_2$ for 15-25 min	-	5°C	MS + 3%(w/v) sucrose	60 weeks	85% of encapsulated nodal segments survived on poliferation mendium
(Ray and Bhattacharya, 2008)	Shoot tips of *Rauvolfia serpentina*	MS medium + 3% sucrose+ 0.8% agar	3% SA + 100mM $CaCl_2$ for 45 min	Encapsulated shoot tips kept in vessels containing MS + 3% sucrose during storage period	4°C	MS + 3% scrose	14 weeks	(68.5-100%) regrowth rate during storage period (several tests in the normal culture conditions)
(Rai *et al*., 2008a)	Somatic embryos of guava(*Psidium guajava* L.)	-	2% SA + 100 mM $CaCl_2$ for 20-30 min	Encapsulated somatic embryos + MS + 9% sucrose or + MS+ 1 $mg.l^{-1}$ ABA	25 ± 2°	MS + 3% sucrose	60 days	28.8% germination level and 37.5% germination level For (sucrose and ABA respectively)
(Cartes *et al*., 2009)	Somatic embryos from mature seeds of rauli-beech (*Nothofagus alpine*)	-	3% SA + 5.5 $g.l^{-1}$ $CaCl_2$ for 30 min	MS + 0.5 $mg.l^{-1}$ IAA + 0.5 $mg.l^{-1}$ NAA + 2 $mg.l^{-1}$ BAP + 30 sucrose	-	culture MS + 30g.l-1 sucrose + 7 g.l-1 agar	-	The germination rate was 45% after 4 weeks
(Lata *et al*., 2009)	Axillary buds of *Cannabis sativa* L.	MS medium + 3% (w/v) sucrose + 0.8% agar+ 0.5 mM (TDZ)	5% SA + $CaCl_2$ for 30 min	MS + 0.5 mM (TDZ) + 2.5 mM (IBA). A + fungicide + 5% (PPM)	-	MS medium + 0.5 TDZ + 0.075% PPM	-	100% artificial seed conversion rate

Table 1. The recent applications of artificial seeds on different types of plants.

Abbreviations:

SA: sodium alginate.

WPM: woody plant medium.

BAP: N^6–benzylaminopurine.

BA: 6-Benzylaminopurine.

MS: Murashige and Skoog medium.

NAA: naphathaleneacetic acid

GD: Gresshoff and Doy medium (Gresshof.Pm and Doy, 1972) cited in (Tsvetkov and Hausman, 2005)

IAA: indolacetic acid

1.13. Brassica species artificial seeds

There are not many studies that have investigated the ability and effectiveness of synthetic seed production within Brassica species. However, working in broccoli (Takahata *et al*., 1993) and in the Chinese cabbage (Wakui *et al*., 1994) cited in (Kieffer, 1996) have reported the production of artificial seeds via microspore derived embryos. These embryos were haploid because they were derived from microspore produced after meiosis. Therefore, they did not replicate parental phenotype.

In the cauliflower, several protocols have been investigated to produce synthetic seeds through encapsulated somatic embryos (Fransz *et al*., 1993, Redenbaugh *et al*., 1986) . However, although all these authors emphasized the difficulty faced in producing artificial seeds ‘en masse’. Kieffer (1996) created a new protocol for producing artificial cauliflower seeds using micro-explants derived from fractionated and graded curd of cauliflower. He mentioned that the 2% sodium alginate was the optimal concentration producing suitable synthetic seed bead formation (5-7 mm micro-explants capsulations diameters were produced) (Figure 4). Moreover, he indicated that micro-explants were successfully able to keep their regeneration ability after six months storage duration at 4°C. More recently, (Bubu and Fuller, 2005) reported that more than 80% conversion rate of encapsulated cauliflower micro-

shoot into plantlets was obtained. They mentioned that although the storage at 4°C for 28 days was achieved without a reduction in viability, cryopreservation at (-20, -80, -196 ºC) killed the micro-shoots. On other hand and after applying different dehydration levels, these authors mentioned that moisture content had no damaging result in the cauliflower synthetic seed conversion level which was assessed to be 68-78%.

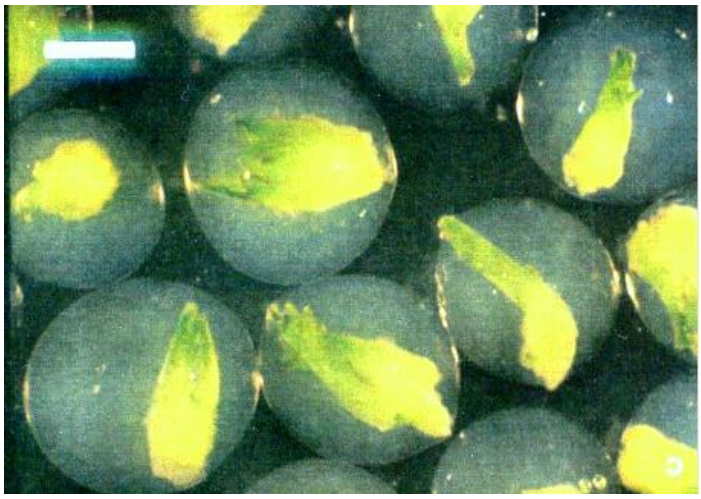


Figure 4. Cauliflower artificial seeds (Kieffer, 1996) (Bar = 0.2cm).

1.14. Abiotic stress

Plant growth and productivity are limited by both biotic and abiotic factors (Seki *et al*., 2003). It was reported that abiotic stresses can cause a decrease in yield of crops up to 50 % resulting in very high economic losses (Vij and Tyagi, 2007). The world population is increasing rapidly and at the same time food production is decreasing due to the effect of various abiotic stresses (Mahajan and Tuteja, 2005). Therefore, the minimization of these losses is one of the main objectives for plant and crop specialists and since it is very difficult to control abiotic stress resulting from climate change and human activities, the development of stress tolerant crop genotypes is necessary (Mahajan and Tuteja, 2005). Salinization of arable land is predicted in 30

% land loss within the next 25 years, and up to 50 % by the year 2050 (Wang *et al*., 2003).

Different scientists have defined stresses differently depending on their mode of studies. Lichtenthaler (2006) defined stress as any substance or condition which stops a plant's metabolism, development or growth. Stress was also defined by Mahmood (2002) as any element that decreases plant reproduction and development below the genotype's potential. However, plant response to stress is very complex including morphological, physiological, biochemical and molecular changes. Some plants have morphological adaptations in order to avoid undesirable conditions and others can alter their physiology metabolism, gene expression and various developmental activities to tolerate the effect of specific stresses.

Abiotic stresses affect different plant organ structures and functions in various ways with the main target area considered to be the cellular membrane. Membrane systems play essential roles in the regular cell functions such as, electron transport, ATP generation, signal perception, ion pumps and channels. All these functions can easily be affected by abiotic stresses (Shanker and Venkateswarlu, 2011). However, other plant functions such as photosynthesis, nitrogen assimilation, respiration and many other processes can also be affected by abiotic stresses.

Plant abiotic stress is a very complex trait involving different biochemical and physiological mechanisms that are coded by a high number of genes. However, the understanding of abiotic stress tolerance mechanisms in plants developed dramatically with the growing power of genomic and proteomic tools (Gao *et al*., 2007). Plants have some common mechanisms for withstanding abiotic stresses and it usually starts by stress recognition followed by up-regulation of specific genes that

are involved in cell protection and damage fixation. The signal transduction pathways that sense stress have a fundamental role in the induction of abiotic stress tolerance in plants (Smalle and Vierstra, 2004). The cellular perception of abiotic stress induces signaling cascades that activates ion channels, the production of Reactive oxygen Species (ROS), Kinase cascades and the accumulation of some plant growth regulators such as ABA. Eventually, the activation of such pathways induces the expression of defence genes and their products play important roles in the tolerance of abiotic stress (Sauter *et al*., 2001).

1.15. The role of Ca^{2+} and ROS (Reactive Oxygen Species) in the abiotic stress signaling

Ca^{+2} and ROS play essential roles as common signaling molecules in very early stages of the plant abiotic stress response. The level of these increases significantly in cells and local tissues under the effect of abiotic stresses. Ca^{+2} is considered to be the main signal transducer in signaling cascades motivated in responses to plant abiotic stress types. Ca^{2+} also seems to be the cross talk between abiotic stress pathways since the level of this cation increases under the effects of different types of abiotic stresses. Ca^{+2} binding proteins (Ca^{+2} sensor) mediate the signal activated by Ca^{2+} influx under the effect of abiotic stresses. Jenks and Wood (2010) reported three main Ca^{2+} proteins in plants: Ca^{2+} dependent proteins kinases (CDPK), Calmodulin (CaM) and calcineurin B-like (CBL) proteins. Rudd and Franklin-Tong (2001) reported that CaM, which a highly conserved protein, is the first sensor to the Ca^{2+} cytosolic level in all Eukaryotic cells. Therefore, calmodulin genes, which control calcium fluxes, are considered to be the main sensors of environmental triggers such as extreme temperatures. Marc *et al* (2010) reported clear evidence

that physical changes in membrane fluidity initiated by the stoichiometry of lipids could be the main trigger for calcium influx into the cell.

Reactive oxygen species (ROS) also play an important role as a second messengers responding to various abiotic stresses. Rao *et al* (2006) reported that abiotic stresses cause an oxidative burst and that the low level of ROS induce an increase in Ca^{2+} influx into the cytoplasm. The high level of Ca^{2+} activates NADPH oxidase in order to produce ROS through yielding O_2^- which is then converted to H_2O_2 (ROS) under the effect of super oxidase dismutase (SOD). Therefore, the production of ROS is Ca^{2+} dependent and the concentration of Ca^{2+} is also regulated by the concentration of ROS by the activation of Ca^{2+} channels in the plasma membrane (Kwak *et al*., 2003). Therefore, a cross talk between Ca^{2+} and ROS modulates the activity of specific proteins that control the expression specific definitive defense genes in the nucleus. However, several other genes have been demonstrated to have roles in the abiotic stress responses such as, Kinases and transcription factors which in turn have roles in the crosstalk between signaling cascades involved in responses against two or more kind of stresses (Rao *et al*., 2006).

1.16. Low temperature stress (Frost damage)

Low temperature is one of the main abiotic stresses that affects plant growth and production globally. It limits the geographical distribution of agronomic species and significantly decreases the yield of several crops around the world (Pearce and Fuller, 2001). It is very important to study the frost damage mechanism and to breed for cold tolerant varieties since the average minimum temperature is below 0 ºC for about 64% of the earth's land area and it is below -10 ºC for about 48% (Deane, 1994).

Frost technically means the formation of ice crystals and this includes a phase change from vapor or liquid to solid. Ice formation (crystallization) consists of two main steps: a) nucleation. b) crystal growth. Nucleation is the process when the molecules start to gather forming clusters, at the nanometer scale. However, these clusters are required to grow to a specific size in order to form stable nuclei and at this point the atoms arrange in a defined manner to form the crystal structure. Subsequently, the nuclei keep growing to form ice and cause damage effects on plant cells (Sakai and Larcher, 1987).

Ice formation may take place either in the intracellular or extracellular spaces depending on the cooling conditions (Guy, 1990a). Intercellular freezing can happen when cooling rates are high or after a significant supercooling. Supercooling is defined as when liquid water cooled below the melting temperature (0 ºC) without any ice formation. The supercooling point is the minimum low temperature reached before ice formation. Extracellular freezing takes place in the spaces between cells in water transporting elements (xylem and phloem) or on the external surfaces of the plant. Ice will grow and spread from the initial formation point (nucleation site) through the extracellular spaces in contiguous water. Moreover, as long as the plasma membrane is unharmed and the cooling rate is low, the ice will stay in the extracellular spaces and it will draw the water from the cells since water has a lower osmotic potential in the ice (solid) phase than that in the liquid phase. This process will continue until equilibrium of water potential is achieved resulting in a dehydration effects on the plant cells (Wisniewski and Fuller, 1999). Several forms of membrane damage can result as a consequence of dehydration caused by freezing effects. This could include expression induced lysis, lamellar-to-hexagonal-phase transitions, and additional jump lesions (Steponkus *et al*., 1993). McKersie and Bowley (1997)

reported that freezing contributes to membrane damage through the induction of reactive oxygen species production. It has been demonstrated that protein denaturation caused by the effect of low temperature could cause cellular damage (Guy *et al*., 1998).

1.17. Cold acclimation and frost stress tolerance

Cold acclimation is defined as the exposure of plants to low non-freezing temperatures leading to significant positive effect on the cold tolerance of plant (Thomashow, 1999, Smallwood and Bowles, 2002). Extensive researches have been conducted to improve the understanding of the biochemical and molecular basis of the cold acclimation response and the changes that take place throughout this process (Thomashow, 1990). However, the increase in cold tolerance obtained by acclimation is not static. Moreover, it depends on the season and it is lost quickly (deacclimation) when plants are exposed to warm temperatures. Wanner and Junttila (1999) also reported that cold acclimation is a photosynthesis activity demanding process. The authors reported that light plays a role in cold acclimation and moderate to high light intensity is required for a sufficient cold acclimation process.

Extensive physiological and biological changes occur during cold acclimation (Figure, 5) starting by a reduction in the growth rate and water content of various plant tissues (Levitt, 1980). Cold acclimation also increases the level of ABA (Chen *et al*., 1983). Low temperature (cold acclimation) changes lipid membrane composition (Uemura and Steponkus, 1994). Denesik (2007) reported that cold treatment affects membrane fluidity resulting in an increase in the membrane rigidity. This more fluid state of cell membranes help to protect cells under the effect of low

temperature stress through maintaining the cell shape and preventing cellular component from water loss. Moreover, the rigidity of membrane could reduce cells collapsing during extracellular freezing by creating a negative pressure in the cells (Heidarvand and Maali Amiri, 2010, Rajashekar and Lafta, 1996). It has also been reported that cold acclimation increases the accumulation of compatible osmolytes such as, proline, betaine, polyols and soluble sugars, and it also cause an increase in antioxidants levels (Kishitani *et al.*, 1994, Murelli *et al*., 1995, Nomura *et al*., 1995, Dörffling *et al*., 1997, Tao *et al*., 1998).

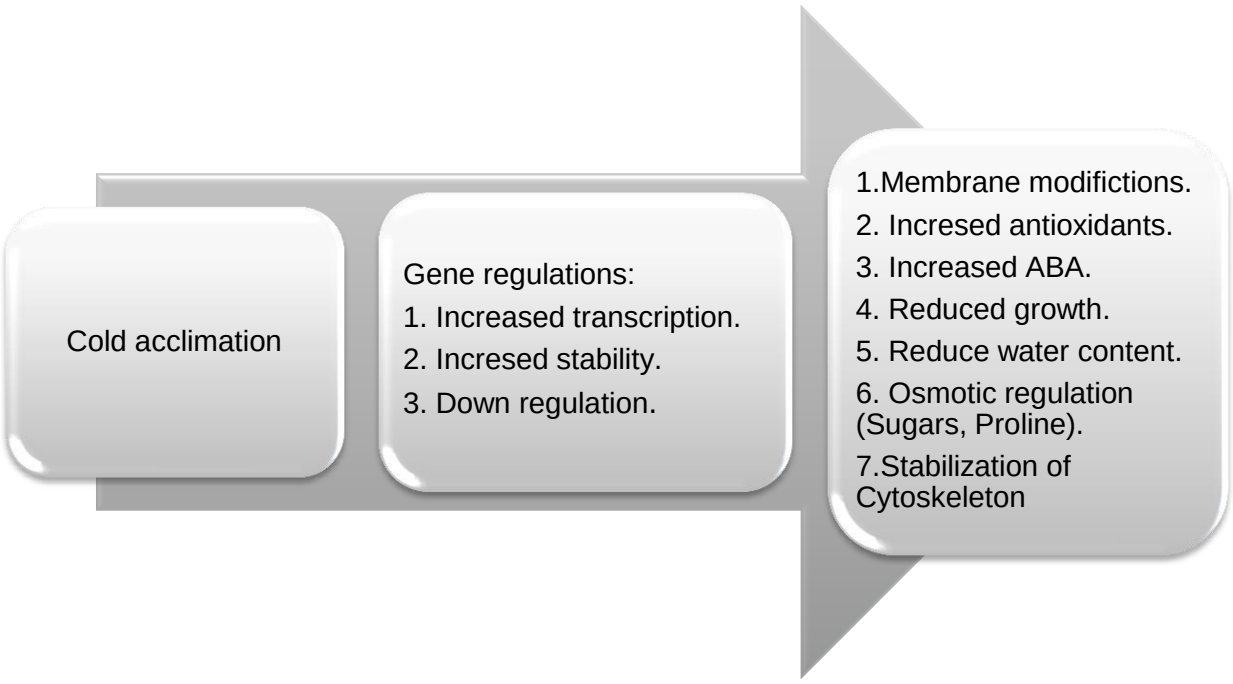


Figure 5. Cold acclimation induces changes in cellular processes. Different responses are observed while exposing plants to low non-freezing temperatures. Modified from (Xin and Browse, 2000).

1.18. Gene expression induction in response to low temperature

Cold acclimation affects the expression of a huge number of genes by either up or down regulations (Seki *et al*., 2002, Xiong *et al*., 2002). Furthermore, there is cross talk between the response of different abiotic stresses and some of the genes up-

regulated by drought or salt stress (Thomashow, 1999, Seki *et al*., 2003). Fowler and Thomashow (2001) reported that cold acclimation activates different gene expression pathways which in turn mean that cold induced genes could be members of more than one cold regulon. However, it should be mentioned that plant response to abiotic stresses is mediated by a common group of reactions, cooperatively known as signal transduction (Heidarvand and Maali Amiri, 2010). Therefore, plant response to low temperature goes through specific process starting by cell recognition of low temperature (sensing) followed by the signaling process in order to induce the cold responsive genes resulting in an increase in plant cold tolerance (Denesik, 2007).

As discussed ealier, Ca^{+2} is thought to be important for stress signaling. Free Ca^{+2} has been observed to increase rapidly in the cytoplasms of plant cells during the acclimation process and therefore, it is most likely to be involved in this process (Eckardt, 2009). Knight and Knight (2000) indicated that the free cytoplasm Ca^{+2} observed under low temperature effects comes from extra cellular and intracellular calcium stores. Significant evidence has confirmed the essential role of Ca^{+2} signaling on cold induction of CBF pathways (Knight and Knight, 2000). The role of Ca^{+2} was approved by the discovery of calmodulin binding transcription activator (CAMTA) since calmodulin is a calcium binding protein. CAMTA binds to a regulatory element in the *CBF*2 gene promoter which plays a role in controlling the CBF regulon and freezing tolerance (Doherty *et al*., 2009). Eckardt (2009) reported that CAMTA play a significant part in transduction of low temperature induced cytosolic Ca^{+2} signals into downstream regulation of gene expression.

1.19. CBF cold response pathway

Intensive studies in expression of cold-response gene expression in Arabidopsis resulted in the identification of the *CBF/DREB1* transcription factors family which have an essential role in cold acclimation and freezing stress tolerance in plants (Gilmour *et al*., 1998). CBF/DREB1 is a family of transcription factors that has A/GCCGAS as a conserved DNA-binding motif which binds to the DRE/CRT element and belongs to the ERF/AP2 transcription family (Yamaguchi-Shinozaki and Shinozaki, 2005). Thomashow (2001) has characterized three different cold inducible *CBF/DREB1* genes in Arabidopsis described as: *CBF/DREB1B, CBF2/DREB1C* and *CBF3/DREB1A*. The expression of these genes in transgenic plants activated downstream cold responsive genes (*COR* genes) even at warm temperature improving the cold tolerance of these plants (Liu *et al*., 1998, Kasuga *et al*., 1999).

The CBF/DREB1 pathway has been identified and described is several plant species indicating that the CBF transcriptional cascade under the cold stress is highly conserved in the plant kingdom (Jaglo *et al*., 2001). A significant relation between the expression of *CBFs/DREBs* and changes in the temperature has been demonstrated indicating that the lower the temperature the higher the expression of these genes. However, the expression can desensitize at a given low temperature (Zarka *et al*., 2003).

The increase in transcription levels of three *CBF*s (CBF 1, 2, 3) has been observed within 15 min exposure to low temperatures. The rapid response to the cold effect strongly suggests that the ‘thermometer’ and signal ‘transducer’ of low temperature and which regulates the expressions of *CBF*s is present at warm temperatures. In this context, Gilmour *et al* (1998) demonstrated that a transcription factor, tentatively

designed inducer of CBF expression (ICE), is present at warm temperature. They also reported that ICE recognizes DNA regulatory elements in the CBF promoter and called these 'ICE boxes'. It was also reported that either 'ICE' or proteins with which it interacts is activated under the effect of low temperature and as a consequence the promoter of *CBF* genes was suggested to be activated and response to low temperature (Zarka *et al*., 2003). Chinnusamy *et al* (2003) reported that *ICE1,* which is a nuclear gene encoding a MYC-like bHLH protein, has a crucial role in regulating some *CBF* genes. However, it should be mentioned that apart from ICE proteins, there are several proteins such as, LOS4, HOS1 and LOS1 proteins that have been reported to have positive effects of the induction of *CBFs* expression (Matthew, 2005).

CBF proteins, which are DNA-binding proteins, recognise the DNA regulatory element CRT (C repeat)/DRE (dehydration responsive element) that are located in the promoter of *COR* genes. Therefore the induction of 20-30% of COR gene expression is controlled by CBF proteins (Baker *et al*., 1994, Yamaguchi-Shinozaki and Shinozaki, 1994). These proteins (CBF1, 2 and 3) are encoded by three *CBF* genes located in tandem in chromosome 4 and they are considered as transcription factors having masses of about 24 kDa. These proteins are about 90% identical in amino acid sequence and they contain a conserved DNA-binding motif of about 60 amino acids designated the AP2/ERF domain (Riechmann and Meyerowitz, 1998). However, 144 AP2/ERF genes were found and described in Arabidopsis (Riechmann *et al*., 2000) and based on the comparisons of their DNA binding domain, Sakuma *et al*.(2002) has classified them into five subgroups: AP2 subfamily (14 genes), RAV subfamily (6 genes), DREB/CBF subfamily (55) genes, ERF

subfamily (65 genes) and the others which were considered as fifth group consists of 4 genes.

The up-regulation of COR (cold regulated) genes through the activation of CRT/DRE elements in their promoters by CBF proteins is considered to be a small part of the CBF regulon since intensive studies, which have been conducted to determine the expression profiling of CBF, reported that a total of 109 genes were being assigned to CBF regulon (Fowler and Thomashow, 2002, Vogel *et al*., 2005). However, according to the nature of proteins that are encoded, Matthew (2005) classified CBF regulon assigned genes into four categories, the first group is the largest containing more than 50% of the proteins and these proteins have unkown function, the second group is the cryoprotective proteins which involves the COR proteins, the third group is the regulatory proteins and the last group if the biosynthetic proteins.

COR (cold regulated) genes encode extremely hydrophilic proteins that are either novel or members of the dehydrins or LEA (Late Emberyogensis Abaundant) proteins (Close, 2006a). However, most COR protein functional activities are still hypothetical. Artus *et al*.(1996) reported some evidence that CORa protein, which is one of the most well studied COR proteins, supports in stabilizing cell membranes against freezing damage. COR15a that encodes a 15 kDa polypeptide is targeted to the chloroplast where it is processed to 9.4 kDa mature polypeptide (Lin and Thomashow, 1992). Steponkus *et al* (1998) reported that COR15a polypeptide could act directly as a cryoprotective agent improving the cold tolerance of plants. Moreover, it has been demonstrated that COR proteins reduce the tendency of membranes forming hexagonal-II structures which is harmful non-bilayer construction that take place due to cellular dehydration associated with freezing (Thomashow, 1999). Bravo *et al* (2003) and Hara *et al* (2003) have reported that

COR proteins also have a positive role in protecting other proteins against freeze-thaw inactivation *in vitro.* Moreover, it has been suggested that CORs could play a role as a hydration buffer that isolate ions and help to re-nature unfolded proteins (Bray, 1993). The essential role of CORs in the cold tolerance of plants was confirmed by Artus *et al* (1996) who reported that over expression of *COR15* a in transgenic Arabidopsis improved chloroplast freezing tolerance by about 2 ºC in non-acclimated plants.

1.20. Drought stress

Drought is one of the main environmental stresses that affects the development and yield of plants. The effect of drought is predicted to increase because of climate change and a growing water scarcity (Harb *et al*., 2010). The availability of water is decreasing in the view of the current and future human population growth and societal needs emphasising the necessity of sustainable water use policies (Rosegrant and Cline, 2003). Therefore, an understanding of drought stress and the relation between water use and plant growth is an important issue for sustainable agricultural production.

Drought is generally defined as a period without significant rainfall. Drought stress happens when the available water in the soil decreases and the conditions of the atmosphere cause constant loss of water through evaporation or transpiration (Jaleel *et al*., 2009). However, plants have developed complex acclimation and adaptation mechanisms to survive drought stresses. Plants have two main strategies in response to water soil deficit, drought escape and drought resistance mechanisms (Price *et al.*, 2002). Drought escape occurs when plants can complete their life cycle before severe stress happens. On the other hand, drought tolerance is the

maintenance of high tissue water potential despite a soil water shortage and this could be through the improvement of water uptake under stress or the ability of plants to hold onto their acquired water. Plants have evolved several mechanisms to withstand soil water deficit such as improved root traits, decreasing water loss through reduced epidermal (stomatal and cuticular) conductance, reduced leaf area (evaporative surface) and reduced radiation absorption (Harb *et al*., 2010).

Plants respond to drought by the induction of both regulatory and functional sets of genes (Bartels and Sunkar, 2005). It is supposed that the LEA protein family is involved, among other functions, in the process of cellular response to abiotic stresses. LEA proteins are linked with tolerance to water stress in both plants and animals. However, although significant numbers of studies have proposed the crucial effects of these proteins in abiotic stress tolerance, their precise functions has not been determined (Goyal *et al*., 2005). *LEA* proteins are induced during embryo maturation in both orthodox and recalcitrant angiosperm seeds (Bartels and Sunkar, 2005). Galau *et al* (1986) reported a significant positive correlation between the accumulation of LEA proteins during embryogenesis and the concentrations of ABA and attainment of desiccation tolerance. Bartels *et al*. (1988) also found that early developmental stage embryos treated with ABA showed a precocious accumulation of LEA proteins suggestion a connection between these two parameters. However, although LEA proteins are not typically produced in vegetative tissues, they are expressed by exogenous application of ABA or osmotic stress (Bartels and Sunkar, 2005). The role of LEA proteins in protecting molecules to survive protoplasmic water depletion have been strongly supported by evidence derived from gene expression profiles (Ingram and Bartels, 1996).

Although there is some debate about their classification, LEA proteins have been divided into five different groups depending on the conserved structural characteristics (Dure *et al*., 1989). Group 1 of LEA proteins is featured by a high level of glycine content (ca. 20%), amino acid with charged R-groups (ca. 40%) and the existence of a section of 20 hydrophilic amino acids. The high hydrophilicity of these proteins causes them to remain them soluble after boiling indicating that they are greatly hydrated and do not have a globular tertiary construction. This group of proteins could have a role in binding or replacement of water. Group 2 of LEA proteins are known as dehydrins and are produced in a wide range of plant species in response to stress stimuli with a dehydrative component, involving drought, extreme temperature, salinity and developmental stages of plant tissue such as seed and pollen maturation (Campbell and Close, 1997). Dehydrins are characterised by a territory of serine residues, a conserved motif having the amino acid consensus sequence DEYGNP close the N-terminus and a lysine-rich 15 amino acid sequence motif in most cases at or near the carboxy terminus and this composition is predicted to form an amphipathic α-helix (Bartels and Sunkar, 2005).The distinctive characteristic of dehydrins is the existence of a supposed amphipathic a-helix-forming domain, the K-segment, which is conserved in higher and lower plants and which defined by the consensus of fifteen amino acid sequence for angiosperms the K-segment is EKKGIMDKIKEKLPG. Because of this consensus, antibodies designed against this sequence have been used to detect dehydrin proteins in a wide range of plants (Campbell and Close, 1997).

Several studies using immunolocalization and sub cellular fractionation techniques have demonstrated that dehydrins can be found in the nucleus or cytoplasm. However, the existence of these proteins in the nucleus is seemingly affected by the

cell type (Campbell and Close, 1997). Asghar *et al* (1994) reported the presence of dehydrins in both nucleus and cytoplasm of the scutellar parenchyma, aleurone, scutellar epithelium, scutellar provascular strands and embryonic leaves of mature maize seeds imbibed in 0.1 mM ABA. However, the authors reported the existence of dehydrins only in the cytoplasm of shoot and root apices. These results were supported by immunolocalization of maize dehydrin RAB17 in embryos and wheat dehydrins (WCS120 family) reported by Goday *et al* (1994) and Houde *et al* (2003) respectively. Dehydrins were also identified using immune-electron microscopy techniques in the cytosol of dried leaf cells of *Craterostigma plantagineum* Hochst (Campbell and Close, 1997) and in the euchromatin, cytosol and nucleolus of different cells in tomato seedling and mature plant tissues (Goday *et al*., 1994).

High similarity between the putative amphipathic alpha-helices formed by dehydrin K-segments and class A amphipathic alpha -helices occur in apolipoproteins, which is considered the major protein components of blood plasma lipoproteins, has been observed. Apolipoproteins ease the transport of water-insoluble lipids in plasma signifying that dehydrins might play a role at the interface between membrane phospholipids and the cytosol in plant cells. Another analogy between dehydrin K-segments and chaperone machines was observed. Chaperone machines interact with partly unfolded polypeptides to help their proper folding via hydrophobic interactions suggesting that dehydrins may work at the surface of exposed hydrophobic spots on polypeptides to inhibit protein-protein accretion when protoplasmic water activity drops (Campbell and Close, 1997).

Group 3 LEA proteins are characterised by the presence of several copies of an 11-amino acid peptide expected to create an amphipathic α-helix with potentials for intra-and intermolecular interactions (Dure, 1993). A conserved N-terminus expected

to make an α-helix and a varied C-terminal portion with a random coil construction is the main characteristic of group 4 LEA proteins. Group 5 LEA proteins are characterized by a higher content of hydrophobic residues than the other groups of LEA proteins and possibly assume a globular conformation (Dure *et al*., 1989).

1.21. Abscisic acid and abiotic stress tolerance

The plant hormone ABA plays an essential role as a chemical signal in response to biotic and abiotic stresses such as salt, cold, drought and wounding. ABA stimulates several changes in plant physiological, molecular and developmental progressions resulting in plant adaptation to the stress environment (Ton *et al*., 2009). Abiotic stresses such as drought induce the synthesis of ABA which in turn activates the expression of stress related genes and stomatal closure (Lee and Luan, 2012). Wilkinson and Davies (2010) reported the accumulation of ABA under drought conditions and the role of ABA on stomatal closure help plants to reserve water under stress conditions. Several ion channels at the plasma membrane controlling the stomatal closure are induced by ABA (Schroeder *et al*., 2001). While ABA activates the S-type anion channels and outward K-channels, inward K^+ channels are deactivated, resulting in a net ionic efflux leading to a decrease in guard cell turgor (Lee and Luan, 2012). Water efflux and reduced guard cell volume caused by the reduction of ionic concentration results in stomatal closure which helps plants to tolerate drought conditions (Wasilewska *et al*., 2008). At the molecular level drought induces the expression of the *AtNCED3* gene which encodes key enzymes for ABA biosynthesis and the overexpression of the *NCED* gene resulted in higher rate of ABA accumulation and a subsequent decrease in transpiration (Schwartz *et al*., 2003). ABA is now considered as endogenous messenger regulating the plants

water status. ABA is also produced the signal to control seed germination and developmental processes (Tuteja, 2007).

ABA has an essential role in the induction of various stress signals controlling downstream stress responses. Therefore, it is necessary that plants adjust their ABA content constantly in response to changes in physiological and environmental conditions. Plants respond to stresses by two main mechanisms described as ABA-dependent and ABA-independent pathways. The expression of ABA-responsive genes could be induced by several transcription factors such as DREB2A/2B, AREB1, RD22BP1 and MYC/MYB through interacting with their corrosponding cis-acting elements such as DRE/CRT, ABRE and MYCRS/MYBRS, respectively (Tuteja, 2007). Koornneef *et al* (1998) have reported ABA deficient mutants namely aba1, aba2 and aba3 in *Arabidopsis.* However, while no differences were observed between the growth of these mutants and wild type plants under no stress conditions, ABA deficient mutants readily wilt and die under drought stress and showed poor growth under salt stress. ABA can also be essential for freezing tolerance through the induction of dehydration tolerance genes (Xiong *et al*., 2001).

Several ABA induced genes such as *Arabidopsis* cold-regulated (*COR*) genes *RAB18, LTI78*, and *KIN2*, are up-regulated under the effect of various abiotic stresses such as cold, salt and drought (Knight *et al*., 2004). However, although Shinozaki and Yamaguchi-Shinozaki (2000) described several signalling pathways including both ABA-dependent and ABA-independent pathways resulting in *COR* gene (cold responsive genes) expression up-regulation, Thomashow (1999) and Shinozaki and Yamaguchi-Shinozaki (2000) reported cold effects on the induction of gene expression was mainly through an ABA-independent pathway. The existence

of ABRE cis-acting element (ABA-responsive element) is an essential requirement for the up-regulation of ABA-induced gene expression (Shinozaki and Yamaguchi-Shinozaki, 2000). It has been demonstrated using genetic analysis that there is no strong connection between ABA dependent and ABA independent pathways, however, the mechanism involved could usually cross talk or sometimes converge in the signaling pathway (Uno *et al.*, 2000). In the view of this information, calcium was suggested to be a strong candidate that can mediate such cross talks since calcium concentration was observed to rapidly increase in the plant cells under the effect of ABA and various abiotic stresses such as drought, cold and salinity (Mahajan and Tuteja, 2005). Moreover, various changes in plant molecular and biochemical such as, the transcript accumulation of RD29A gene and the accumulation of proline, were observed and mediated by both ABA dependent and ABA independent pathways (Tuteja, 2007).

Finkelstein *et al* (2002) reported an important role of ABA in the induction of *LEA* gene expression. Bies-Etheve *et al* (2008) also reported that the application of exogenous ABA induced the expression of specific *LEA* genes in plant vegetative tissues. The expression of *LEA* genes during the development of seed and under abiotic stress were up-regulated by ABA (Dalal *et al*., 2009). It has also been reported that ABA induces the expression of *LEA* genes resulting in an increase in the desiccation tolerance of cultured embryos (Leung and Giraudat, 1998). The role of ABA on the up-regulation of *LEA* genes is considered to be one of the main mechanisms that ABA has to increase plant drought tolerance.

1.22. The role of Molybdenum (Mo) in abiotic stress tolerance

Molybdenum is an essential trace element present in the soil and is required by both plants and animals. Many oxidation statuses of molybdenum ranging from zero to VI have been reported in agricultural soils. However, VI is considered to be the most existed state (Sun *et al*., 2009). Molybdenum plays an essential role in plant reduction and oxidation reactions. Molybdenum is not biologically active but it forms a part of organic complex called the molybdenum co-factor (Moco). Moco is very important for the molybdenum-requiring enzymes (molybdoenzymes) that have been reported in most biological systems (Williams and Frausto da Silva, 2002).

Li *et a*l (2001) proposed that molybdenum is involved in the amelioration of wheat frost tolerance. Several mechanisms, of which molybdenum can improve the frost tolerance, have been suggested. However, the mechanism for this enhancement has not been definitively determined. Molybdenum increases the activity of the anti-oxidative enzymes SOD (Superoxide dismutase), CAT (Chloramphenicol acetyltransferase) and POX (Peroxisomal enzyme) resulting in an increase in plant anti-oxidative defenses under low temperature stress (Yu *et al*., 1999) since frost stress leads to the production of Reactive Oxygen Species (ROS) that has cellular damage effects on plant tissues (Sattler *et al*., 2000). Sun *et al* (2006b) demonstrated that molybdenum application increases the capacity of scavenging the ROS resulting in the reduction in membrane damage of winter wheat under low temperature.

Another hypothesis proposed for the role of molybdenum in plant cold tolerance was that molybdenum increases the activity of AO (Aldehyde Oxidase) which has an important role in the production of ABA. ABA triggers bZIP and the up-regulation of

the ABA dependent COR gene expression pathways (Sun *et al*., 2009) and thereby protects against frost damage. However, the role of molybdenum in the biosynthesis of ABA suggests a significant role of this element in plant drought and salinity tolerance. A third mechanism is suggested to be through nitrogen reductase (NR) and enhancing nitrate assimilation since molybdenum has significantly increased NR activity in wheat as it has been found that NR activity significantly increased in Mo-treated wheat and was associated with protein accumulation (Hamdia et al., 2005, Hale *et al*., 2001, Yu *et al*., 1999). Al-Issawi *et al* (2013) recently reported a fourth mechanism of molybdenum effects on the plant cold tolerance through the up-regulation of the CBF pathway which in turns lead to COR up-regulation and improvements in frost tolerance.

1.23. Thesis aims

Cauliflower is an open pollinated species and there are technical challenges to producing in-bred lines with reliable self-incompatability or male sterility for producing F1 hybrids particularly amongst the winter-heading maturity sub-group. The production of cauliflower clones multiplied by tissue culture and distributed as artificial seeds could be useful alternative to F1 hybrids varieties and could also be useful in the context of the maintenance of elite clonal germplasm in cauliflower breeding programmes. Therefore, this study seeks to develop full protocols for producing and storage of cauliflower artificial seeds as a cost effective technique and good alternative to other cauliflower propagation methods. It also aimed to utilize the cauliflower micropropagation system designed for the molecular analysis of cauliflower microshoots abiotic stress tolerance aiming to improve the quality of the artificial seed produced.

1.24. Thesis objectives

The objectives of this study are

1. To further optimize cauliflower micropropagation systems designed by Kieffer (1996) through conducting several experiments to reduce the cost per culture unit and to decrease the risk of contamination.
2. To investigate the capacity of cauliflower microshoot encapsulation as artificial seeds and to design a full encapsulation and conversion protocol.
3. To assess the capacity of using cauliflower artificial seeds in commercial substrates.

4. To make use of designed cauliflower micropropagation protocol for the molecular analysis of plant abiotic stresses (cold- drought) generally and cauliflower microshoots especially.
5. To investigate the existence of the CBF regulon in cauliflower and its relation to abiotic stress resistance in microshoots.
6. To assess the effect of some chemical materials on cold tolerance and the induction of *CBF* genes in the cauliflower microshoots.
7. To study the development of cauliflower seeds (nutrient accumulation) as a module for artificial seed improvement.
8. To investigate the accumulation of dehydrin proteins during the development of cauliflower seed and the possibility of inducing the expression of these proteins in the cauliflower microshoots.

Chapter 2: Cauliflower Micropropagation

2.1. Introduction

Cauliflower microprogation is essential for cloning of important germplasm such as the inbred lines or dihaploid lines in F1 hybrid programmes. However, although a lot of studies have been applied to cauliflower micropropagation using various plant tissue types such as, leaf veins (BuiattiI *et al*., 1974), inflorescence axis segments (Singh, 1988, Christey and Earle, 1988), cotyledons (Murata and Orton, 1987, Horeau *et al*., 1988, Narasimhulu and Chopra, 1988, Ovesná *et al*., 1993, Dash *et al*., 1995), shoot tips (Yanmaz *et al*., 1986), inflorescence stem segments (Zarske, 1988), immature petals (Turton and Fuller, 1989), hypocotyls segments and curd meristems (Crisp and Walkey, 1974, Yanmaz *et al*., 1986, Kumar *et al*., 1992), the use of these protocols have been found to be labour intensive (Pow 1969; Kumar *et al* 1993). Kieffer (1996) designed an effective protocol for the production of cauliflower propagules from fractionated and graded curd.

The outermost layer of cauliflower curd is made of several million meristems (Kieffer, 1996), of which the majority are able to produce shoots via *in-vitro* culture. The use of curd meristematic tissue for *in-vitro* culture has been investigated for micropropagation and the production of virus free plants (Grout and Crisp, 1977, David and Margara, 1979, Kumar *et al*., 1993). The use of the meristematic layer of cauliflower curd is considered to be a superior compared with the use of conventional protocols which have been found to be labour intensive (Pow, 1969, Kumar *et al*., 1993). However, as the meristematic tissue derived from cauliflower curd has no chlorophyll, all the required elements necessary for growth should be provided by the culture medium especially in

the early stages of explant development. Carbohydrates (sugars) are essential as a source of carbon for energy for biosynthesis (Amiri and Kazemitabar, 2011) and also have an stabilizing osmotic effect *in-vitro*. Sugars can have a large effect on the development of plant cultures (Gibson, 2001). The type and the concentration of sugar used with the culture medium should always be considered to obtain optimal results in micropropagation systems (Mendoza and Kaeppler, 2002). Sucrose has frequently been used as an essential carbohydrate source in plant culture media. Chen (1978) reported that the use of a high level of sucrose induced the growth of callus. However, they also demonstrated that the callus derived from 9 % sucrose containing medium differentiated into more albino plants than those from low concentrations. The use of 2-5% sucrose was suggested to be suitable for anther culture in rice. Usually the growth and development of cultures increases with sugar concentration until it reaches an optimum level and then decreases when higher concentrations are used (Thapa *et al*., 2007).

Although sucrose has been chosen for use with the vast majority of plant micropropagation culture media in the past, recent work suggests that it is not always the most effective sugar to be used (Amiri and Kazemitabar, 2011). Michel *et al* (2008) reported that the percentage of induction and dry weight of callus in cotton are more efficient with glucose followed by fructose and sucrose. Maltose was also indicated to be a superior source of carbohydrate than sucrose in several other plant species (Pande and Bhojwani, 1999, Last and Brettell, 1990, Shahnewaz and Bari, 2004). Furthermore, t has been demonstrated that morphogenesis was attributable to the concentration and type of carbohydrate used with the culture media (Romano *et al*.,

1995). Glucose also has been used as the preferred source of carbon in culture media used for the micropropagation of several plant species (Michel *et al*., 2008, Salvi *et al*., 2002).

Kieffer‘s protocol involves the use of a commercial blender and sieves for the production of explants emphasizing the importance of using a fully sterilized conditions. There is however, a high risk of contamination during the protocol despite strict sterilized conditions which are imposed. Contamination by bacteria, fungi and yeasts is an constant challenge that frequently threatens plant tissue cultures during the culture phase (Leifert and Cassells, 2001). Several studies have investigated the elimination of bacterial and fungal contamination in culture systems using various materials including the use of fungicidal (Haldeman *et al*., 1987) and antibiotic treatments (Kneifel and Leonhardt, 1992, Leifert *et al*., 1992). However, although the use of these materials can be effective for controlling or restraining contamination (Reed and Tanprasert, 1995), two essential limitations, have been encountered in their use firstly, the reduction of a microorganisms sensitivity to these substances i.e. building up of resistance (Seckinger, 1995) and secondly, their inactivation caused by heat during autoclaving (Barrett and Cassells, 1994).

Plant Preservative Mixture (PPM) is a combination of two broad-spectrum industrial isothiazolone biocides, chloromethylisothiazolone and methylisothiazolone. PPM is heat stable and is thus able to be autoclaved with the culture medium (Lunghusen, 1998b). Resistant mutants towards PPM are unlikely to be formed since PPM targets specific multiple enzyme sites in the Krebs cycle and the electron transport chain of micro-organisms (Chapman and Diehl, 1995). As with any anti-microbial compound there is a

risk that it is phyto-toxic and checks need to be made to ensure that they do not inhibit or alter plant growth during *in-vitro* culture or in subsequent *in-vivo* establishment.

Niedz (1998) reported that PPM has tested PPM with many types of citrus tissue culture and he demonstrated that PPM could be used with the culture media to control bacterial and fungal contamination without impairing the plant material. Fuller and Pizzey (2001) indicated the important role of PPM to control the contamination in brassicas culture media. The effects of PPM on several species have been studied for example, non-embryogenic callus of sweet orange (*Citrus sinensis* (L.) Osbeck `Valencia'), shoot regeneration of trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and rough lemon (*Citrus jambhiri* Lush.) (Niedz, 1998), adventitious melon, petunia and tobacco (Compton and Koch, 2001), and pepper (*Capsicum annuum* L.), eggplant (*Solanum melongena* L.) (Guri and Patel, 1998). However, all these studies demonstrated the positive effect of PPM to control the contamination and they highlighted the importance of using PPM at a proper concentration which was indicated to be different depending on the plant species and since the use of PPM at relatively high concentration could have negative effects on the development of plant tissue.

The current study aimed to further optimize the production of cauliflower propagules through optimizing explant production stages and culture composition (sugar types and concentrations). It also aimed to investigate the capacity of PPM for reducing the risk of contamination in the cauliflower micropropagation system designed.

2.2. Plant material

Plants of known provenance were used in this work. Plants were obtained from the field in Cornwall, courtesy of Simmonds & Sons Ltd, via Mr Ellis Luckhurst and replanted in raised beds in Portland Villas South at the University of Plymouth. Several varieties were obtained: Mascaret Mar, Jan Alpen, Thumphant Dec, Optimist Nov, Reclon table Feb, Feb Clemen, Jan Dionis, Dec Cendis, Cho829 Apric, Chfo474 Afr. The plants were transplanted on October 21st 2009 and were grown according to good commercial practice.

Unfortunately the exceptionally cold winter of 2009/10 caused a large degree of frost damage to the plants of Jan Alpen, Thumphant Dec, Optimist Nov, Reclon table Feb, Dec Cendis, Cho829 Apric and Chfo474 Afr cauliflower varieties. Plants from the remaining varieties (Clemen, Dionis, Mascaret MAR) were raised to maturity when the curds were harvested and stored at 2–4 ºC until required. The use of 3 varieties gave a continuous supply of cauliflower heads over the experimental period. The produced curds were used and additional Broden cauliflower curds variety were derived directly from Cornwall via Mr Ellis Luckhurst.

2.3. Culture system (Standard Operating Procedure for Culture System) (S.O.P$_{CS}$)

Explants were produced using the system described as follows;

A. Cauliflower curd was divided using scalpel and knife to small pieces (1-5 cm) and sterilized by immersing cauliflower pieces in 10% by volume un-thickened domestic bleach (0.06% sodium hypochlorite) for 15 min followed by a double wash with sterile distilled water.

B. Upper meristematic layer (<1mm) was shaved off using sterilized scalpel in order to remove the mass of non-responsive tissues (stem tissues).

C. Meristem clusters were disrupted and homogenized using commercial blender ((Waring Model 800) at approximately 17 000 rev min^{-1}). Blending step was made in maintenance culture media supplemented with 1 ml L^{-1} PPM (plant preservative mixture) (Sigma Chemical Company Ltd.). 30 s blending was used as a standard.

D. The produced explants were classified into two size classes (212-300 µm and 300-600 µm) using commercial sieves (Endecotts Ltd., London).

E. After the sieving process the resulting product was placed in liquid culture media supplemented with (2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA + 1 ml L^{-1} of PPM) as a standard recommended by previous studies. 74 µm explants per culture pot was used as a standard.

F. Liquid media containing the produced explants were placed in the agitation phase for 13-20 days depending on the applied experiments. An orbiter shaker was used as a standard method for agitation.

G. The produced microshoots then were transferred to maintenance semi solid media (4.4 g L^{-1} + 7 g L^{-1} agar + 30 g L^{-1} sucrose) to continue their growth.

H. Results (Data) represented as means ± standard error (S.E.). All data was subjected to analysis of variance (ANOVA) using minitab software (version 15) and comparison of means were made with least significant difference test at 5 % level of probability.

2.4. Experiments

2.4.1. Experiment 1

Optimization of the blending duration

2.4.1.1. Aims and objectives

The aim of this experiment was the investigation of the impact of the blending duration treatment on the explants damage and the determination of the optimal blending duration providing the highest amount of the best quality of explants at desirable size classes.

2.4.1.2. Materials and methods

Eight blending durations (15, 30, 45, 60, 75, 90, 105, 120 s) were applied using commercial blender ((Waring Model 800) at approximately 17 000 rev min^{-1}). For each treatment, 100 g of predisrupted material was derived from the Clemen cauliflower variety curds. The explants were then classified into two size classes (212-300 µm and 300-600 µm) using commercial sieves (Endecotts Ltd., London).

Blending and sieving steps were made in maintenance culture liquid media supplemented with 1 ml L^{-1} PPM (plant preserve mixture) (Sigma Ltd.). The produced explants were cultured in liquid culture media derived from Murashige and Skoog (1962), according to Kiffer (1996) and supplemented with 2 mg L^{-1} Kinetin + 1 mg L^{-1} of IBA (Indol-3-Butyric acid) + 1 ml L^{-1} PPM. Light in the lab was supplied by cool white fluorescent tubes (spectral photon fluence 34 µmol.m^{-2}.s^{-2}) with a photo period of 16 hours. The optimal blending duration was determined by considering three aspects:

1. **The total amount of explant material produced in a size class**

For every blending duration treatment, the amount of explants produced in terms of volume and weight within every size class was assessed using precise volumetric measures and 5 decimal point balance.

2. **The proportion of explants which have meristematic domes and the proportion of debris**

Samples each consisting of 4 ml of agitated culture media containing eight day old explants was derived from every pot. The relative proportions were assessed under zoom stereo binocular microscope (Nikon SMZ-2T) for each blending duration treatment.

3. **Explant growth ability during subsequent culture**

The number of growing explants which had been cultivated for 20 days was assessed for the two size class 212-300 µm and 300-600 µm and, the average weight of the microshoots produced was evaluated.

For each blending duration, four pots (30 ml culture medium per pot) were set in culture with a constant volume of produced explants (74 ± 2 µl) at two size classes, 212-300 µm and 300-600 µm.

2.4.1.3. Results

2.4.1.4. The amount of produced explants:

The explants were assessed in terms of volume and weight because it was observed that the foam level was frequently increasing with blending duration. Therefore, it was necessary to make sure that the assessed volume was related to the explants rather than foam. The 60 s blending duration was found to be optimal producing the highest amount of explants in terms of volume and weight at size class (300) µm at high significant difference (P< 0.001). At 212-300 µm explants however increased with the blending duration in term of volume and weight up to 120 s (Figure 6).

It was found that the amount of explants decreased when the blending treatments were longer than 60 s at size class 300-600 µm. Therefore, the majority of explants were smaller than at 300 µm. It was noticed also that there was no significant difference between 15 and 30 s blending duration treatment in terms of the amount of explants at both two size classes (Figure 6).

2.4.1.5. The proportion between produced explants which have meristematic domes and debris

This proportion decreased frequently with the blending duration treatments with a highly significant deference (P< 0.001) between the blending treatments at two size classes 212-300 µm and 300-600 µm (Figure 7). However, it is was found that there was no significant difference between 15 s and 30 s blending duration treatments for the size classes and there were highly significant differences for these two treatments in comparison with other blending duration treatments (Figure 7). It is clear that after 30 s

blending the damage to meristematic domes started to increase very quickly (the proportion was less than 0.5) (Figure 7).

2.4.1.6. Explant growth ability during subsequent culture

The maximum number of growing explants at size class 212-300 µm was produced using 30 s blending duration treatment at highly significant deference ($P< 0.001$) in comparison with other blending durations. 15 s blending duration was found to be the optimal giving the highest number of developing explants at size class 300-600 µm at highly significant deference ($P< 0.001$) (Figure 8) (there was no significant deference between 15 s and 30 s blending duration treatments in this size class).

Although the maximum explant weight average was produced using 60 s blending duration treatment at size class 212-300 µm at highly significant difference, the number of growing explants was very low. The maximum average weight of produced explants (fast growing) was obtained using 15 s blending duration treatment at highly significant difference ($P< 0.001$) at size class 300-600 µm (There was no significant difference between 15 and 30 sec blending durations in terms of the average weight of produced explants at size class 300-600 µm) (Figure 9).

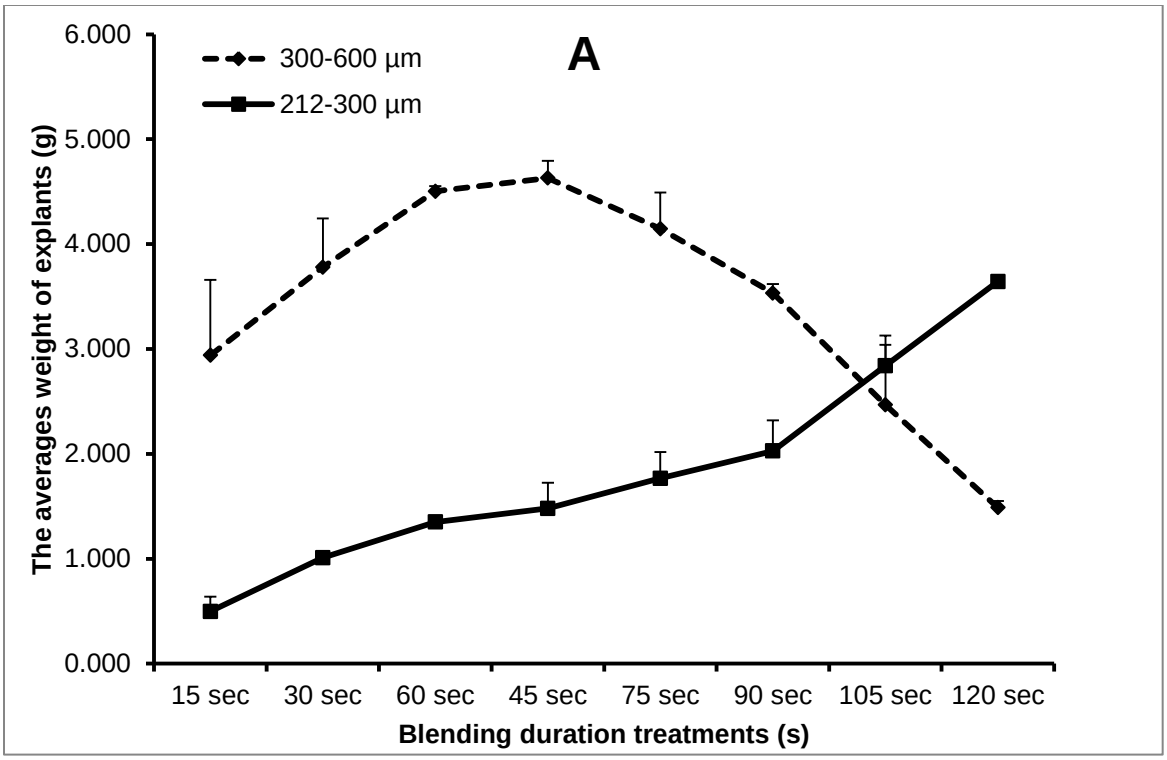
It was clear that the blending treatments started to damage meristematic domes after a limited period of blending treatment 30 s at both size classes reducing the explants viability very quickly (Figure 9). Therefore, the best options were found to be 15 s and 30 s blending duration treatments. However, the following points should be considered:

1. It was found that there was no significant difference between 15 s and 30 s blending duration treatments in terms of the proportion of explants which have

meristematic domes and debris at two size classes 212-300 µm and 300-600 µm (Figure 7).

2. There was no significant difference between the two treatments in terms of the explants number and viability at the two size classes (Figures 8 and 9).
3. There was a highly significant difference between15 s and 30 s blending duration treatments in terms of the explants volume and weight at size classes 212-300 µm and 300-600 µm and the amount produced was much higher using 30 (sec) blending duration treatment (Figure 6).

Therefore, 30 (sec) blending duration treatment was found to be the optimal, giving highest number and best viability of explants at both size classes 212-300 µm and 300-600 µm.



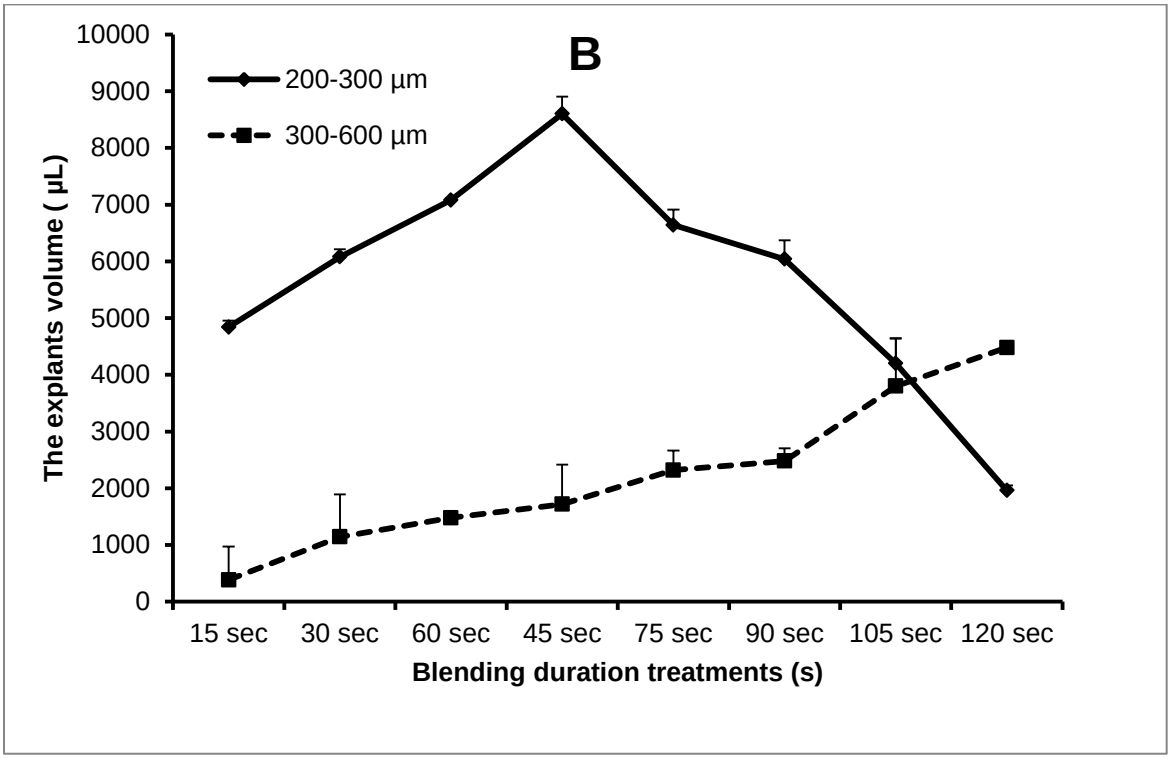


Figure 6. The effect of the blending duration treatments on the A: explant weights (LSD= 1.171 for 212-300 µm, LSD= 0.167 for 300-600 µm size class). B: explant volumes (LSD=756.74 for 212-300 µm, LSD=1626.43 for 300-600 µm size class).

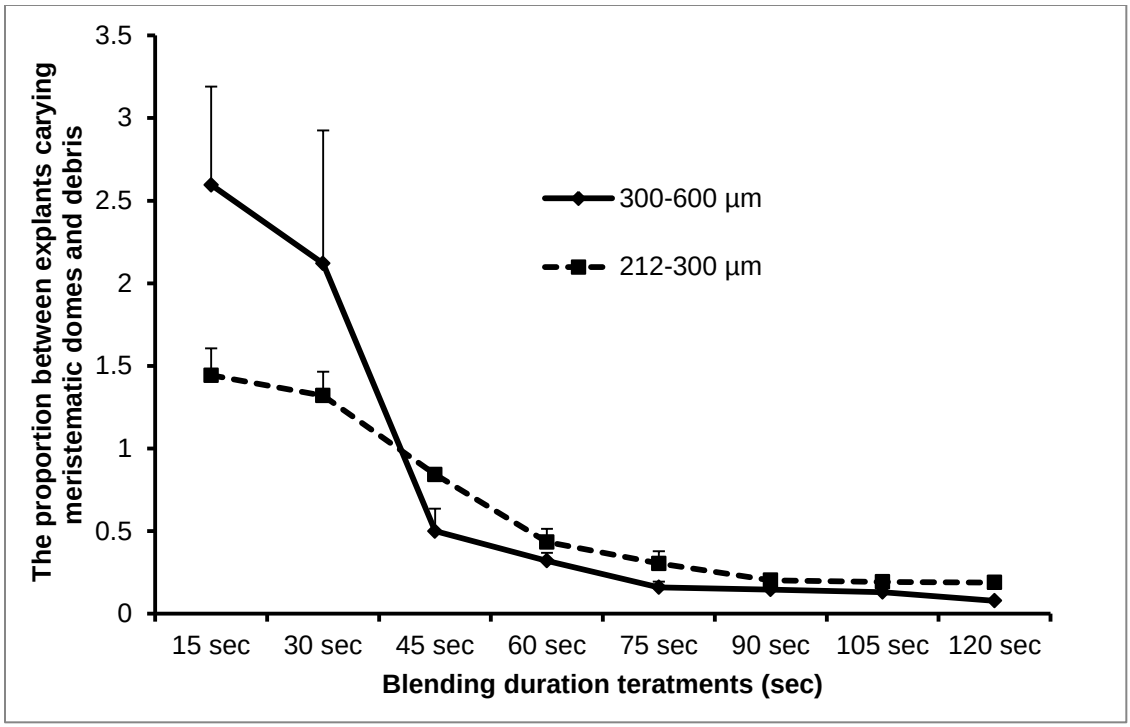


Figure 7. The effect of the blending treatments on the proportion of explants carrying meristimatic domes and debris at two size classes 212-300 µm and 300-600 µm (LSD= 0.257 for 212-300 µm size class, LSD= 1.047 for 300-600 µm size class.

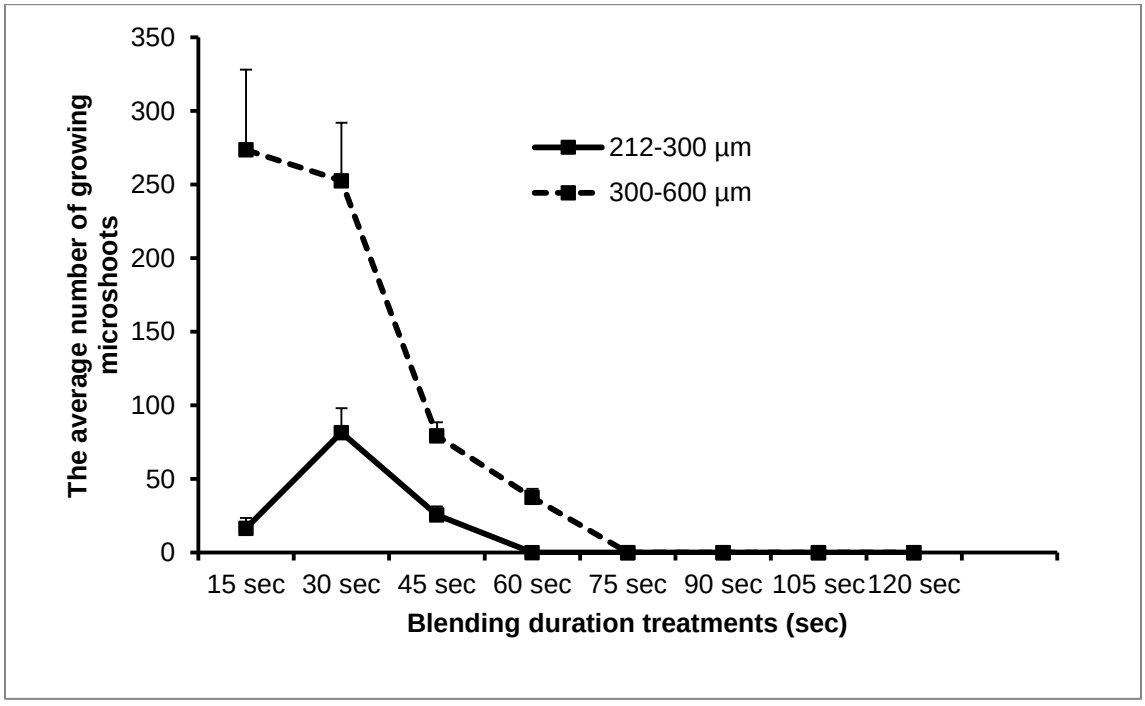


Figure 8. The effect of the blending duration treatments on the number of growing microshoots at two size classes 212-300 µm and 300-600 µm (LSD= 19.58 for 212-300 µm size class, LSD= 70.52 for 300-600 µm size class).

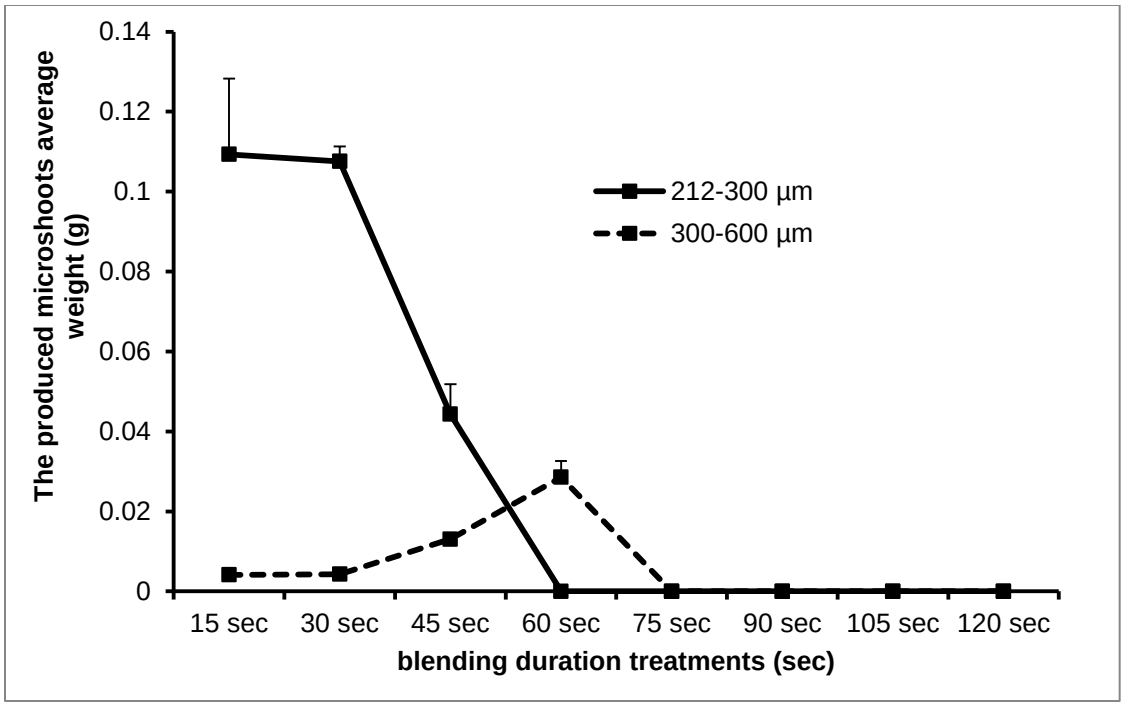


Figure 9. The effect of the blending duration treatments on the microshoots average weights at two size classes, 212-300 µm and 300-600 µm (LSD= 0.022 for 212-300 µm size class, LSD= 0.0045 for 300-600 µm size class).

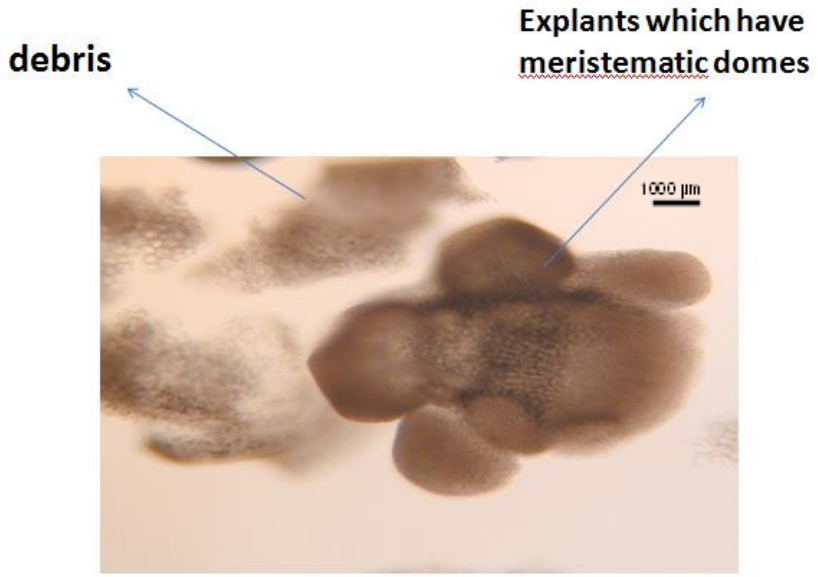


Figure 10. The explants which have meristematic domes and debris (three days explants age- 30 second blending duration treatment- 300-600 µm sieving size class) (100 times magnification).

2.4.2. Experiment 2

The effect of sieving size class on explants and microshoots

2.4.2.1. Aims and objectives

The investigation of the effect of sieving size class on the mean number of meristematic domes carried per explant and the average number of microshoots produced per explant. Moreover, the determination of the optimal size class producing one microshoot per explant was one of the main objectives to this experiment.

2.4.2.2. Materials and methods

The SOP_{CS} was used for the production of explants and later microshoots. 30 s blending was used for the production of explants. The culture pots containing the culture media were cultured in a constant volume of cauliflower explants (cv Clemen) (74 ± 2 µl). The effect of two sieve sizes (212-300 µm and 300-600 µm) was investigated. For each sieving size class, four pots (30 ml cultured media) were cultured in a constant volume of explants (74 ± 2 µl).

While 3 ml agitated liquid media solution containing 7 days old explants was taken from each pot in order to investigate the average number of meristimatic domes carried per explant, The average number of micro-shoots produced per explant at each size class was calculated at each container after 18 days of culture. The number of microshoots were investigated by eye (explants were big enough to be countable).

2.4.2.3. Results

The number of meristematic domes carried per explant and the number of microshoots produced per explant were observed to be significantly higher using 300-600 µm than those obtained using 212-300 µm sieve size class. While the average meristemtic domes and microshoots were found to be 1.8 and 1.56 respectively at 212-300 µm sieve size class, they were observed to be 3.15 and 3.625 at 300-600 µm sieve size class (Figures 11 and 12).

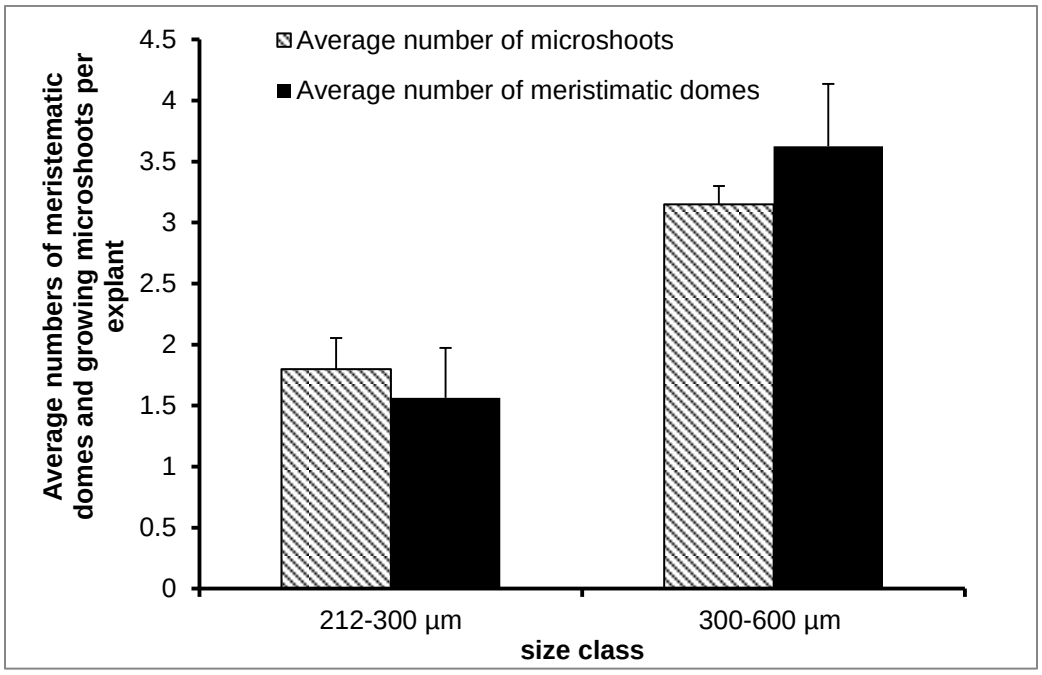


Figure 11. The affect of sieving size class on the average number of meristematic domes carried per explant and produce microshoots (LSD= 1.335 for meristematic domes, LSD= 1.707 for the number of produced microshoots).

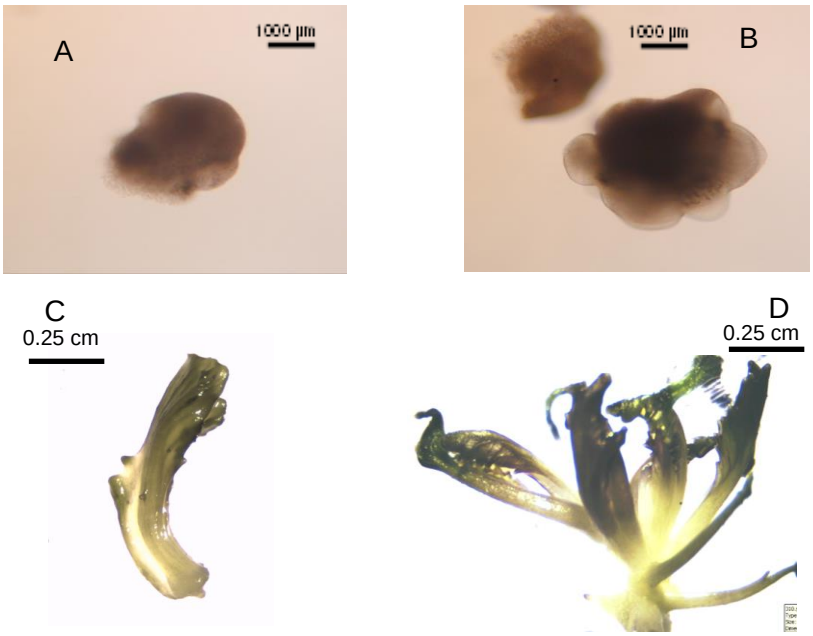


Figure 12. (A and B) the meristimatic domes carried per explant at size class 212-300 µm and 300-600 µm respectively, (C and D) the microshoots growing per explant at size classes 210-300 µm and 300-600 µm respectively.

2.4.3. Experiment 3

The determination of explant developmental stage

2.4.3.1. Aims and objectives

The aim was the determination of the explant developmental stages during culture. Microshoots were used for the production of artificial seeds and the optimal microshoot age suitable for encapsulation was determined (chapter 3). Therefore, the investigation of microshoot development mechanism could be useful for the understanding of which stage microshoots can be used for encapsulation.

2.4.3.2. Materials and methods

Explants were produced using the protocol previously described ($S.O.P_{CS}$) (cv Broden). Five containers were used at each size class (212-300 µm and 300-600 µm). Random microshoot samples (n = 15) were taken from each culture every 3 days and their length measured under a zoom binocular microscope. Measurements commenced when the microshoots were 5 days old and continued until 20 days old.

2.4.3.3. Results

Explant growth was determined for produced explants 212-300 µm and 300-600 µm size classes. The explants started growing very slowly for the first 11 days at the two both size classes. After that the growth rate increased exponentially with the time (Figures 13 and 14). At the age of 20 days, the explants were too big to be maintained in pots and it was necessary to transfer them to semi solid media to continue their growth.

The better growth rate at size class 212-300 µm than 300-600 µm size class might be caused by better nutrient supply for produced explants at size class 212-300 µm taking into account the fact that the majority of produced explants at this size class contains only 1 meristematic domes giving one microshoot while the explants at size class 300-600 µm include 2 to 3 meristiimatic domes giving 2 to 3 microshoots and more localized competition for nutrients.

Microshoots developmental can be divided into three main stages

1. 0-11 day stage when the growth rate was very low.
2. 11-15 day stage of culture when an acceleration of microshoot growth was observed.
3. A stage after 15 days when the growth rate was rapid (Figures 13 and 14).

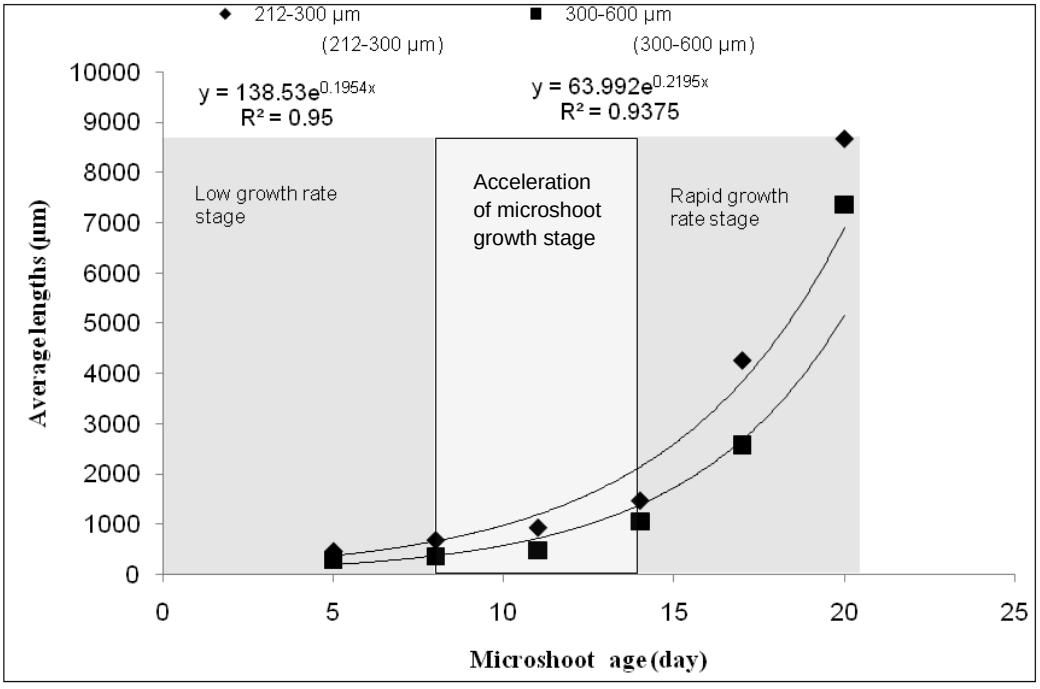


Figure 13. Cauliflower explants growth stages assessed by measuring their length changes over time.

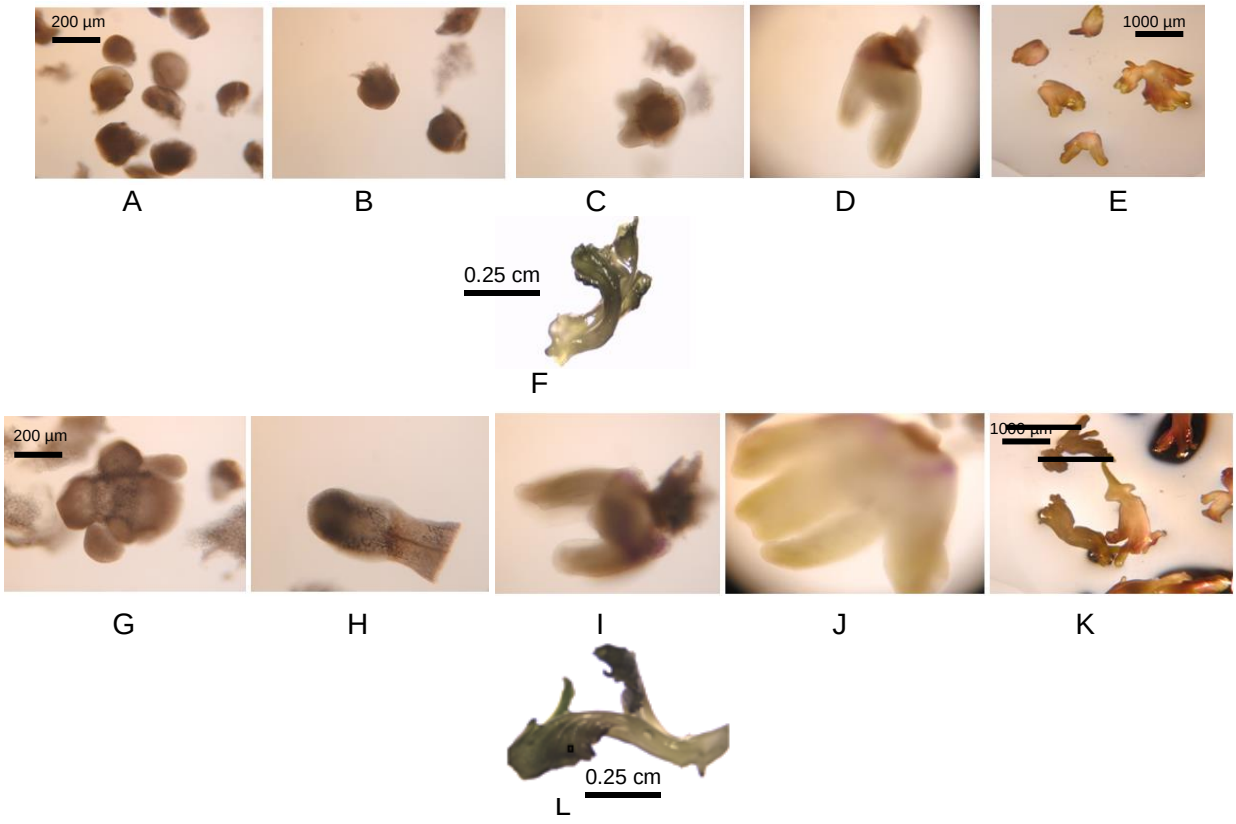


Figure 14. Cauliflower produced explants at different developmental stage.

(A, B, C, D, E and F) and (G, H, I, J, K, L) photos were taken at size classes 212-300 µm and 300-600 µm respectively. These photos were taken at 3, 6, 9, 12, 15, 17 and 20 days microshoots old respectively. (A, B, C, D, G, H, I, and J) pictures were taken at 100 time magnifications (E and K) were taken at 40 times magnification and (F and L) without any magnification.

2.4.4. Experiment 4

The determination of the optimal agitation methods suitable for explants growth

2.4.4.1. Aims and objectives

The aim was to investigate the effect of different agitation methods on the growth of explants and to determine the optimal method for microshoot mass production.

2.4.4.2. Materials and methods

Three methods of agitation were tested in terms of their effect on explant growth and viability. S.O.P_{CS} was used for the production of the cauliflower explants. 300,600 µm in diameter explants (cv Mascaret MAR) were set in S23 liquid media supplemented with 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA + 1 ml L^{-1} PPM. The assessed methods were evaluated as follows,

1. Four culture containers each with 74 µl explants set in constant volume of liquid media (30 ml) were set in the orbital shaker at (150) rev min^{-1} (Figure 15). The number of microshoots and their viability assessed as average fresh weight were evaluated after 22 days culture.
2. Four containers each containing 30 ml of liquid media were cultured in a constant volume of explants (74 µl) supplied with a current of air bubbles produced by an air pump (Hozelock cyprio, model 1380-0000). The air was purified using air filter (Millex HA, 0.22 µm) (Figure 15). The number of microshoots and their viability as average fresh weight were evaluated after 29 culture days.
3. 296 ml of explants, which adjusted to be equal to the amount of explants used in the each of the first two agitation methods, were cultured in 120 ml of liquid

media contained in 1 liter size flask. The cultivated explants were agitated both by a magnetic stirrer and by releasing a current of air bubbles (the amount of air bubbles released were adjusted to mimic that used for four containers which were used in the previous method.

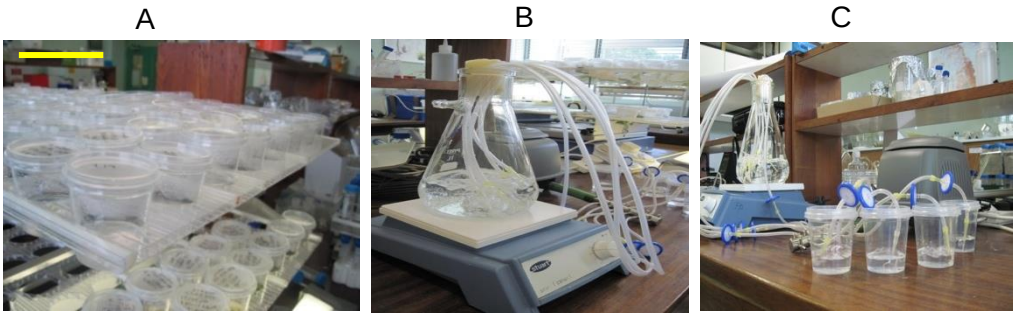


Figure 15. Explants production and agitation methods: A) orbital stirrer. B) Air bubbles. C) Air bubbles and magnetic stirrer (Bar = 30 cm).

2.4.4.3. Results

The main reason for agitation is to maintain oxygen in solution in the liquid media providing a suitable condition for explant growth. It was clear that the use of the orbital shaker at 150 rev min^{-1} gave better results in terms of the number of developing microshoots and their viability measured as fresh weight (Figure 17). Moreover, the level of microshoot hyperhydricity was quite high using the current of air bubbles as an agitation method (Figure 16).

It was observed that the use of current of air bubbles delayed the growth of explants. Thus, the number and the viability of microshoots was evaluated after 28 days of culture rather than 13 culture days. Moreover, the amount of culture media used with this agitation method was much higher than that used when the orbital shaker was employed as an agitation mean. It was necessary adding some liquid media every while to keep the culture media on the required level (the amount of culture media used was four times the amount used with orbital shaker).

None of the cultivated explants developed using a current of air bubbles with the magnetic stirrer. This suggested that explants might be damaged by the magnetic stirrer. Therefore, the use of orbital shaker as an agitation method was found to a suitable methodology for mass microshoot production.

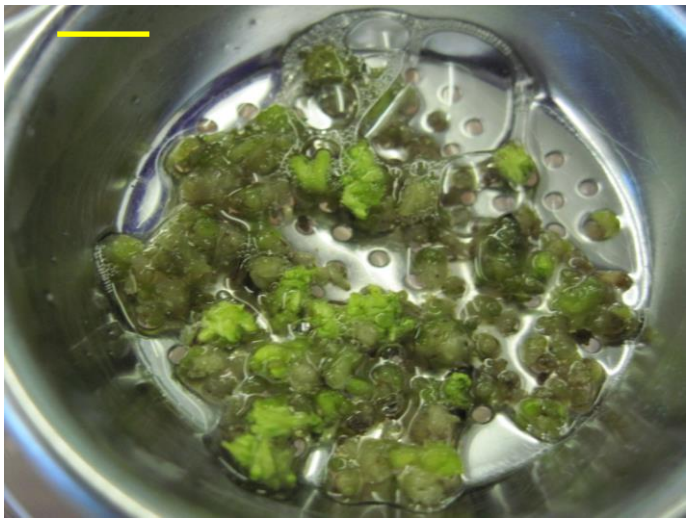


Figure 16. The microshoots hyperhydricity produced when the current of air bubbles was used as an agitation method (Bar = 1 cm).

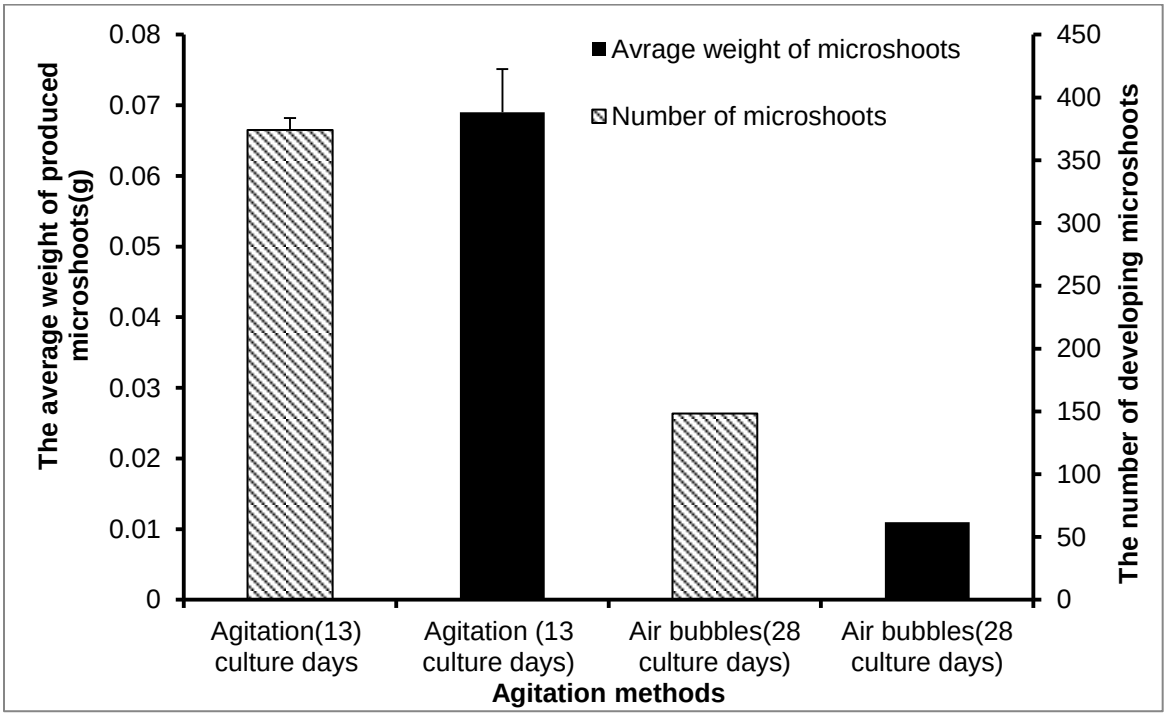


Figure 17. The effect of the agitation methods on the average numbers and weights of microshoots.

2.4.5. Experiment 5

The effect of plant growth regulators (PGR) added to the liquid media on the number and the viability of cauliflower microshoots

2.4.5.1. Aims and objectives

The effect of the PGR on the growth of microshoots and the determination of the optimal combination suitable for the use with culture media providing the highest number of the best quality of microshoots were the main aims of this experiment.

2.4.5.2. Materials and methods

S.O.P$_{CS}$ was used for the production of the cauliflower microshoots. The effects of several PGR combinations were evaluated in terms of their ability to induce micro-explant development. Nine PGR combinations consisting of various combinations of the cytokinin (Kinetin) (1 and 2 mg L^{-1}) with the auxins IBA (1 and 2 mg L^{-1}) or NAA (1 and 2 mg L^{-1}) were evaluated in the first stage and another 4 combinations consisting of various combinations of Kinetin (1 and 2 mg L^{-1}) with IAA (1 and 2 mg L^{-1}) compared with the use of Kinetin (2 mg L^{-1}) and IBA (1 mg L^{-1}) were evaluated in the second stage. Four containers, each with 30 mL of culture medium, were cultivated with a constant volume of explants (74 µL) of the 300–425 µm explant size class and used with every treatment. In order to preserve culture sterility the culture media was supplemented with 1 mL L^{-1} PPM^{TM} (Plant Preservative Mixture) and used with all the treatments. The effects of PGRs ware evaluated after 21 days of culture.

2.4.5.3. Results

2.4.5.4. Stage 1

The use of 1 mg L^{-1} Kinetin + 1 mg L^{-1} of IBA as PGR combination added to the culture liquid media was found to be the optimal combination producing the highest number of microshoots. It was observed that there was no significant difference between this treatment and the use of 2 mg L^{-1} Kinetin + 1 IBA mg L^{-1} as a PGR mixture in terms of the number of developing microshoots (Figure 18).

In terms of the produced microshoots average weight, 1 mg L^{-1} Kinetin + 2 mg L^{-1} IBA was found to be the best, with high significant difference in comparison to the other combinations ($P< 0.001$) (Figure 19) but no significant differences were found this treatment and the use of the 2 mg L^{-1} Kinetin + 2 IBA mg L^{-1} and 2 mg L^{-1} Kinetin + 1 IBA mg L^{-1} treatments.

Although the use of 1 mg L^{-1} Kinetin + 2 mg L^{-1} IBA was found to be the optimal in terms of the microshoots average weight, the number of growing microshoots using this treatment was significantly lower in comparison with the use of 1 mg L^{-1} Kinetin +1 mg L^{-1} IBA and 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA. Therefore, overall the best PGR combinations were observed to be 1 mg L^{-1} Kinetin + 1 mg L^{-1} IBA and 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA in terms of the number of developing microshoots and their viability measured as average fresh weight. However, the use of 2 mg L^{-1} Kinetin + 1 mg L^{-1} PGR mixture was found to be better than the use of 1 mg L^{-1} Kinetin + 1 mg L^{-1} IBA in terms of the microshoots average weights. Thus, 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA PGR combination treatment was chosen as the best given priority of the microshoots quality (viability) in the first step and considering the high number of produced microshoots

using both of the 1 mg L^{-1} Kinetin + 1 mg L^{-1} IBA and 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA treatments.

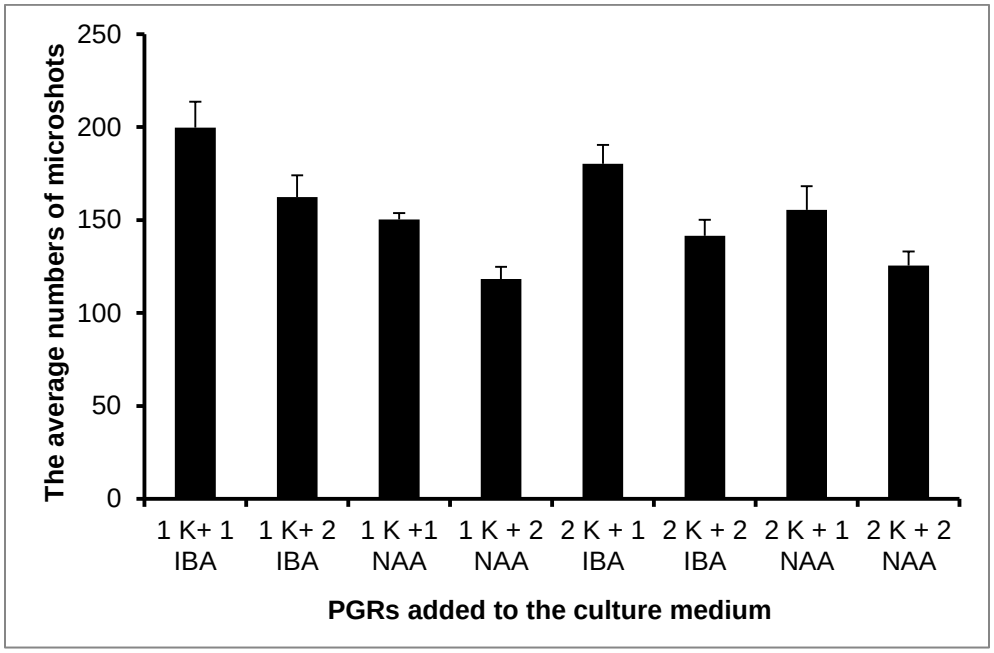


Figure 18. The effect of the various combination of Kinetin (1. 2) mg L^{-1} incorporated with IBA (1, 2) mg L^{-1} or NAA (1, 2) mg L^{-1} added to the lequid media on the number of developing microshoots (LSD=28.85).

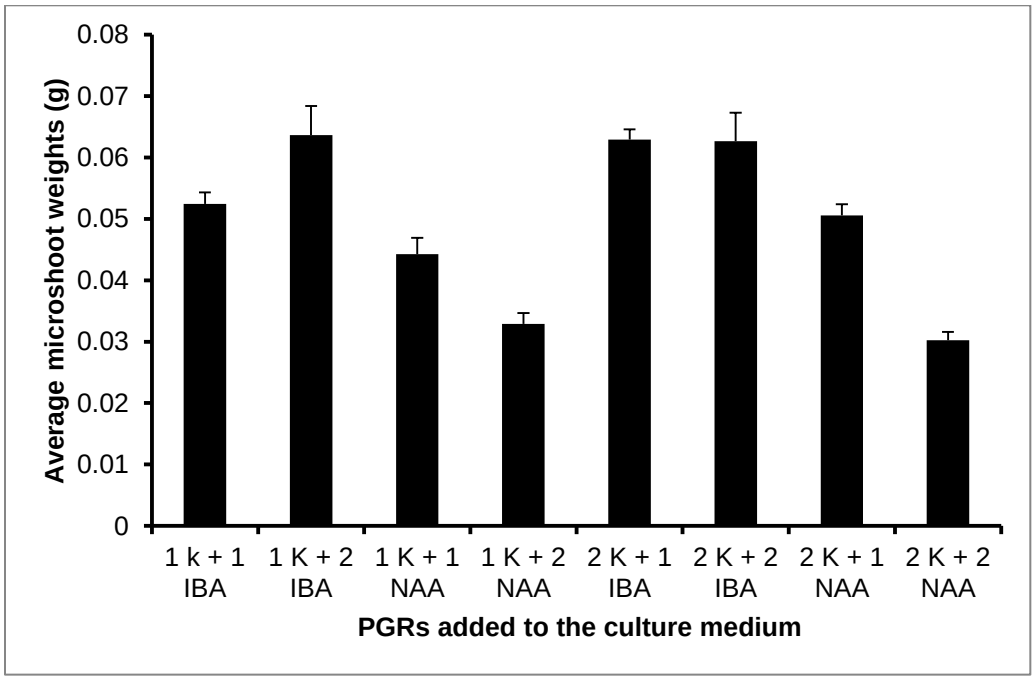


Figure 19. The effect of various combination of Kinetin (1. 2) mg L^{-1} incorporated with IBA (1, 2) mg L^{-1} or NAA (1, 2) mg L^{-1} added to the liquid media on the average weights of developing microshoots (LSD=0.0083).

2.4.5.5. Stage 2

In terms of the number of growing microshoots in the culture liquid media, 2 mg L^{-1} Kinetin + 1 mg L^{-1} ABA treatment was found to be the optimal combination giving the highest number of developing microshoots at high significant difference in comparison with other PGR combinations ($P< 0.001$) (Figure 20). While the average number of developing microshoots passed 374 microshoots per container using this treatment, the number of developing microshoots was less than 100 microshoots per pot using other PGR combinations.

In terms of the average weights of microshoots, it was found that 1mg L^{-1} Kinetin + 1 mg L^{-1} IAA (Indol Acetic Acid) PGR mixture to be the optimal combination. The average microshoot weights produced using 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA PGR combination was lower than using other PGR combinations at significant difference ($P= 0.002$) (Figure 21). However, this big difference could be caused by the better explant nutrient supply when other PGR combinations used in comparison with the use of 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA PGR combination considering the big difference in terms of the number of developing microshoots produced between using this treatment and the use of other PGR combinations. Thus, the use of 2 mg L^{-1} of Kinetin + with 1 mg L^{-1} of IBA PGR combination was considered the optimal.

It was clear from these two experiments that the best PGR combination suitable for explants induction was found to be 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA producing the highest number of the best quality of cauliflower microshoots.

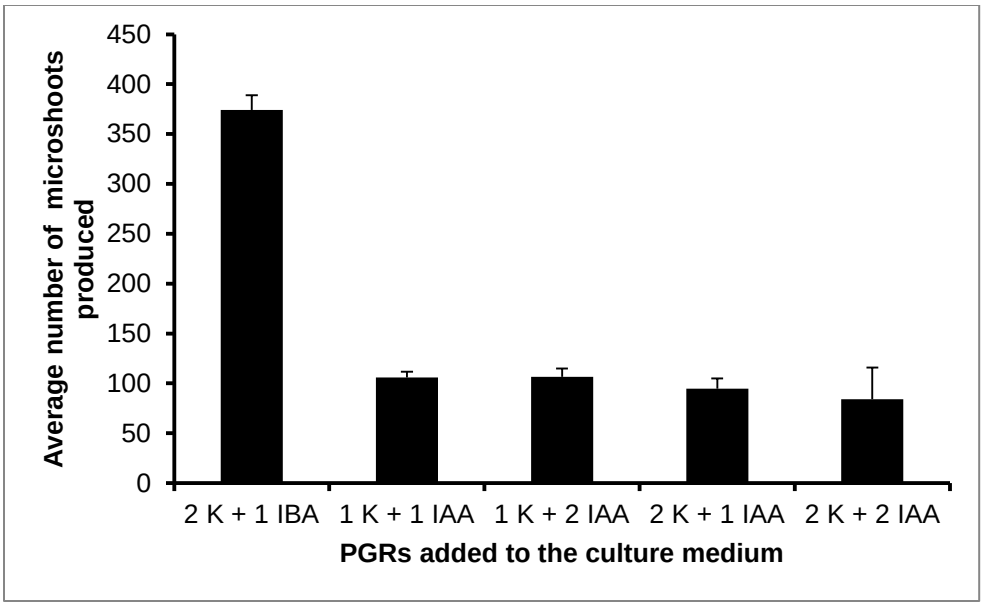


Figure 20. The effect of the various combinations of Kinetin (1. 2 mg L^{-1}) incorporated with IAA (1, 2 mg L^{-1}) added to the liquid media on the number of developing microshoots (LSD=50.76).

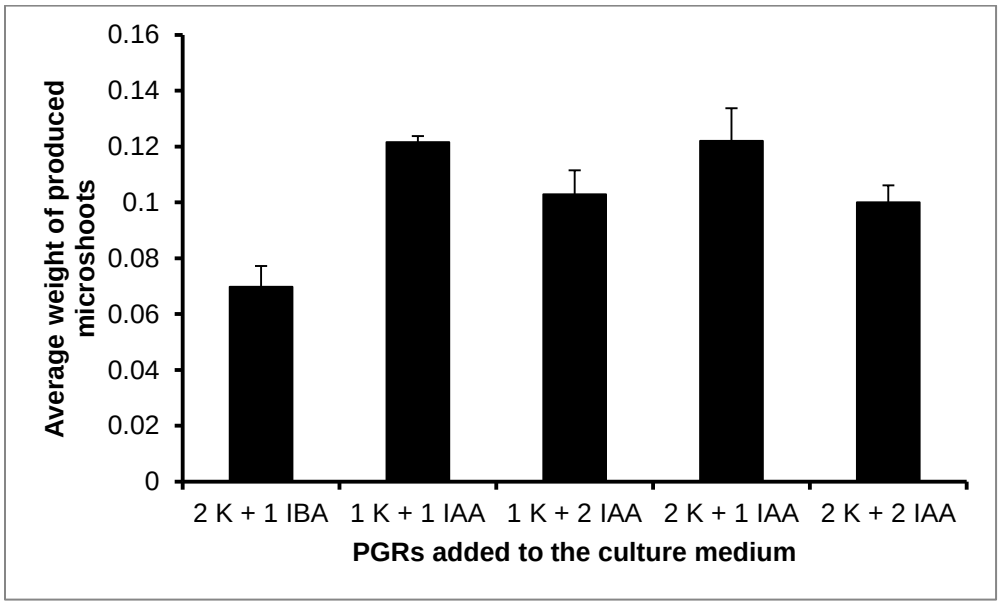


Figure 21. The effect of various combinations of Kinetin (1. 2 mg L^{-1}) incorporated with IAA (1, 2 mg L^{-1}) added to the liquid media on the average weight of developing microshoots (LSD=0.0247).

2.4.6. Experiment 6

The effect of sugar type, source and concentration on cauliflower microproshoot production

2.4.6.1. Aims and objectives

This study aimed to investigate the effect of several types and concentrations of sugars used with culture media on the development and growth of cauliflower microshoots. Moreover, the effect of the sucrose source (sugar beet and sugar cane) on the cauliflower micropropagation system used was tested.

2.4.6.2. Materials and methods

Curds of three varieties of cauliflower, Diwan, Cendis and Galicia, were obtained from Sainsbury supermarket (variety names were obtained from courtesy of Simmonds & Sons Ltd (Cornwall)). The use of 3 varieties gave a continuous supply of cauliflower heads over the experimental period. S.O.P_{CS} was used for the production of the cauliflower microshoots.

- **The effect of sucrose concentration**

Six concentrations of sucrose derived from sugar beet (Silver Spoon™, Tesco) (0, 1.5% (43 mM) , 3% (86 mM), 4.5% (131 mM) , 6% (172 mM) and 7.5% (215 mM) were used with the culture media. 10 culture pots (replicates) were used with each treatment. The number and average weights of microshoots were recorded after 25 days of the culture.

- **The effect of sugar type and concentration**

The culture media were supplemented with three types of sugars glucose (1.5% (83.26 mM), 3% (166.52 mM) and 4.5 % (249.78 mM)), fructose (1.5 % (83.26 mM), 3% (166.52 mM) and 4.5% (249.78 mM)) and maltose (1.5% (22.81mM), 3% (45.63 mM) and 4.5% (68.45 mM). The effects of these sugars were investigated and compared with effect of using 3% sucrose (sugar beet) which was considered as a standard. Five culture pots (replicates) were used with each treatment. The number and average weights of microshoots were recorded after 25 days of the culture.

- **The effect of sucrose source**

Five concentrations of sucrose derived from sugar cane (Tate & Lyle^{TM}, Tesco) (0, 1.5, 3, 4.5 and 6%) were used with culture media and their effects were compared with use of 3% of sucrose derived from sugar beet (silver spoon, Tesco).). Seven culture pots (replicates) were used with each treatment. The number and average weights of microshoots were recorded after 25 days of the culture.

2.4.6.3. Results

- **The effect of sucrose concentration**

The use of sugar with culture media was shown to be an essential requirement for cauliflower explants growth since none of them grew using sugar free culture media (Figure 22). The optimal microshoot number was obtained using 3% sucrose at high significant difference compared with other treatments (P< 0.001). The highest microshoot average weight was obtained using 4.5% of sucrose at high significant differences compared with the other treatments (P< 0.001) (Figure 22). The use of

relatively high concentration of sucrose affected the colour of microshoots and led to an increase in the level of anthocyanin (Figure 23). Therefore, the use of 3% of sucrose was recommended to produce highest number of good quality (colour) of cauliflower microshoots.

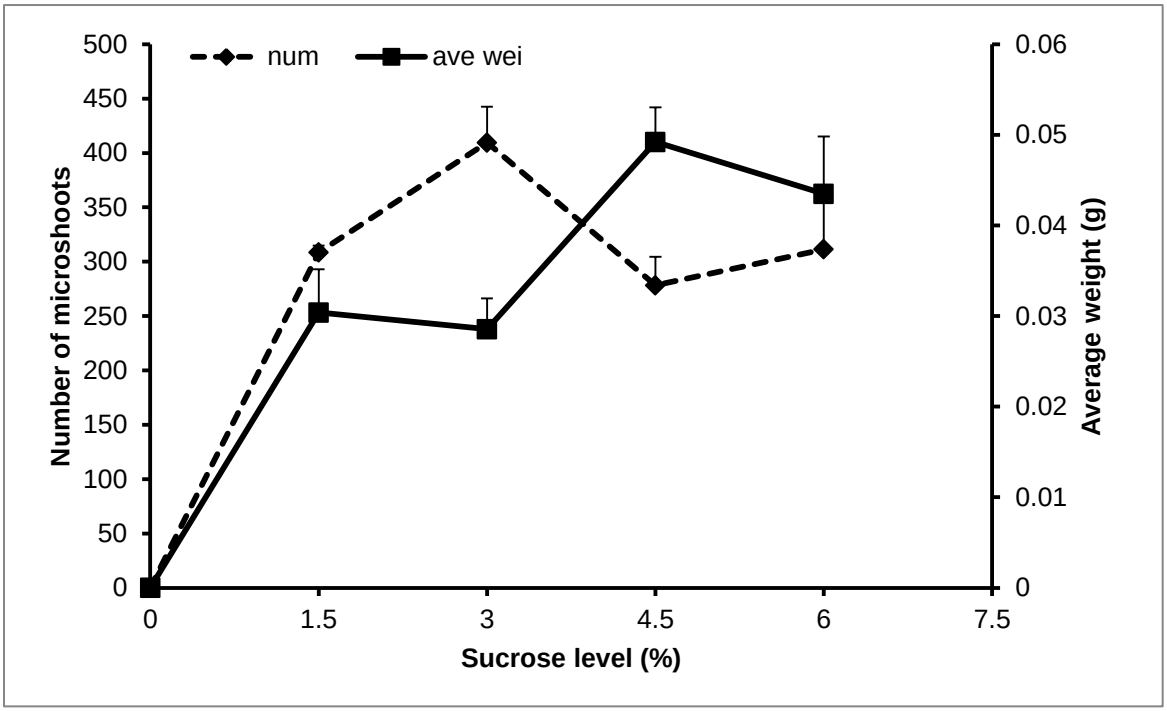


Figure 22. The effect of sucrose concentration used with the culture media on the number and average weight of cauliflower microshoots (LSD=35.43 for microshoot number, LSD=0.0051 for the micoshoots average weight).

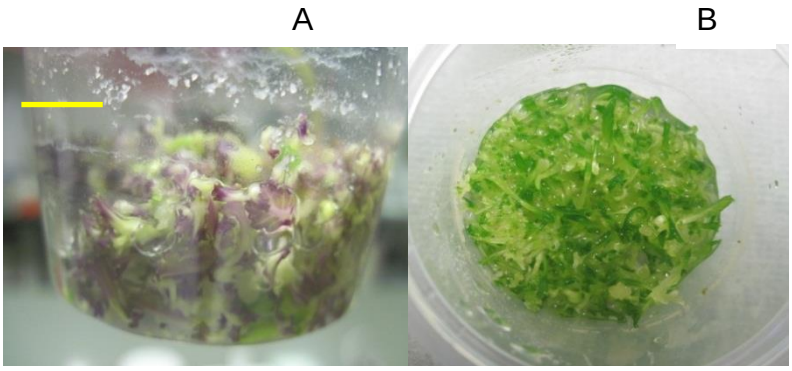


Figure 23. Microshoots produced using 4.5% sucrose concentration in the culture media. B) Microshoots produced using 1.5 % sucrose in the culture media (Bar = 2 cm).

- **The effect of sugar types in concentrations**

Sugar type had a highly significant effect on the number of microshoots produced ($P< 0.001$). The use of maltose was found to be the best comparing with other types of sugars used. However, no significant difference was observed between the use of maltose and sucrose in terms of the number of microshoots. A significant effect of the sugar concentration was observed ($P< 0.01$) and the use of 1.5 % was recommended. No significant interaction was found between sugar type and concentration ($P=0.099$) (Figure 24).

Concerning the effect of sugar type and concentration on the average weights of microshoots produced, while no significant effect of the sugar type was observed ($P=0.09$), a highly significant effect of sugar concentration was found ($P<0.01$) and the optimal concentration was found to be 1.5%. A highly significant effect of the interaction between sugar type and concentration ($P<0.001$) was observed and the optimal treatment was found to be 1.5% fructose (Figure 25). However, this treatment could not be recommended considering the very low number of microshoots that developed using this treatment (Figure 24).

Overall in this experiment, the use of 1.5% maltose gave optimal number of microshoots but without a significant difference compared with the use of 3% of sucrose (control). The low average microshoot weight observed using these treatments could be caused by the big number of growing microshoots and the competition for limiting nutrients in the culture media. The use of 3% sucrose was recommended because the cheaper cost of this sugar and the positive influence on the economics of the protocol efficiency.

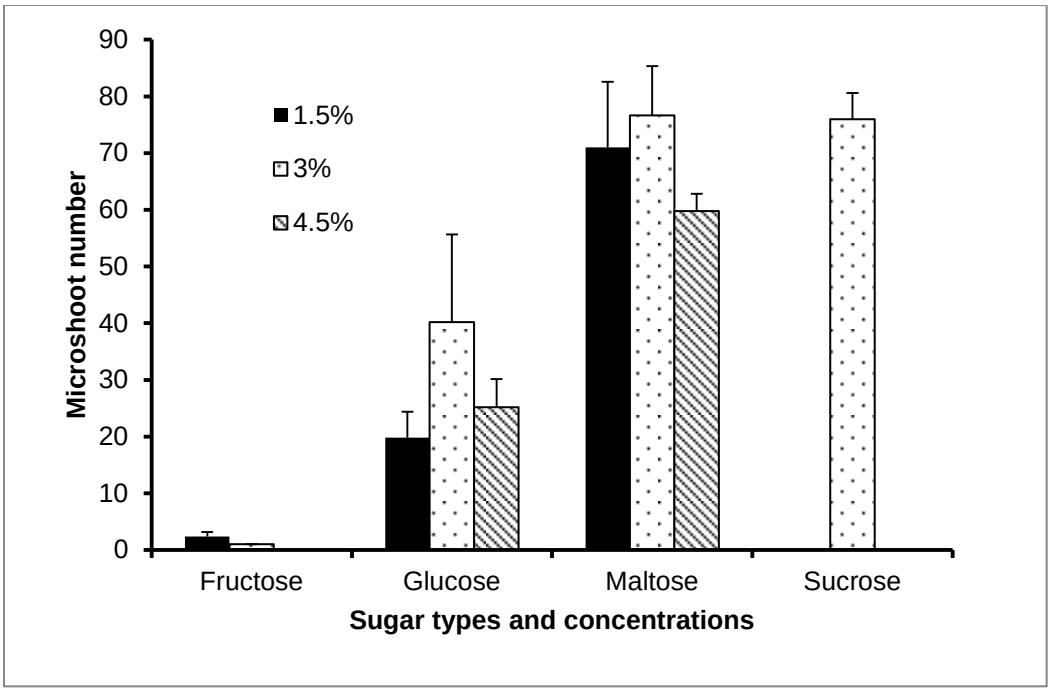


Figure 24. The effect of sugar types and concentrations on the number of microshoots produced (LSD= 8.18 for both sugar types and concentrations).

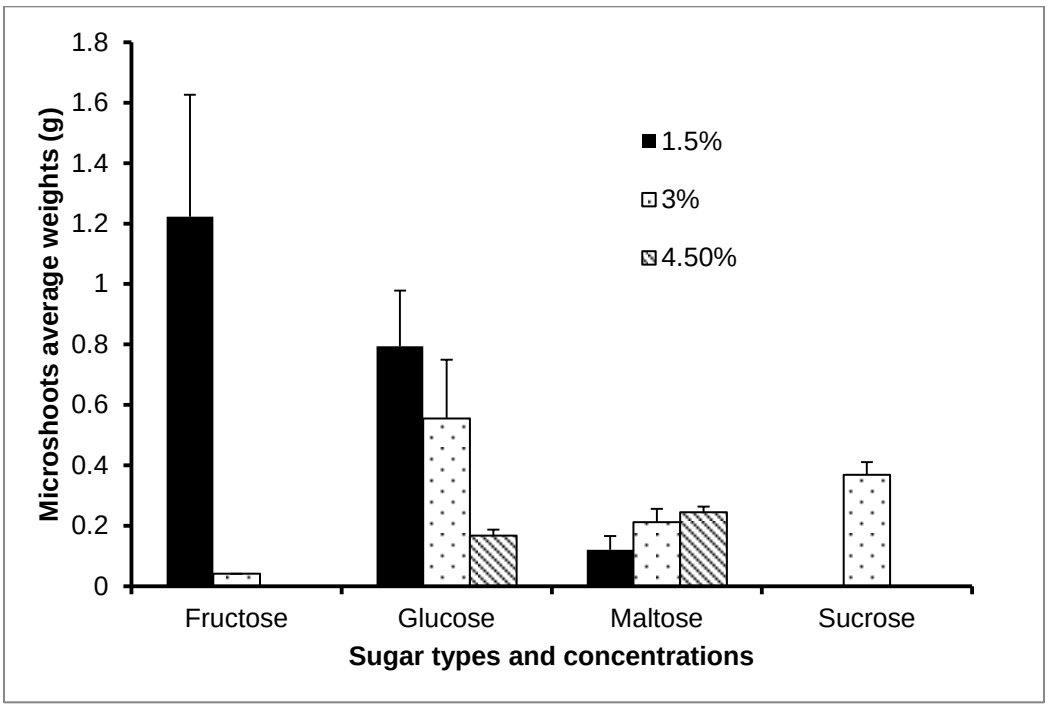


Figure 25. The effect of sugar types and concentrations on the average weights of microshoots produced (LSD=0.216 for the sugar concentration and LSD=0.376 for the interaction between the sugar type and concentration).

- **The effect of sucrose source**

A highly significant effect of the sucrose source was observed and the highest microshoot number was obtained using 4.5% sugar cane (P< 0.001) (Figure 26). However, although the use of sugar cane significantly reduced the average weight of microshoots (P<0.001) (Figure 27), this could be caused by the big number of growing microshoots and the competition for the availability of other nutrient elements from the culture media. The use of 4.5% of sugar cane was recommended to be used with cauliflower culture media

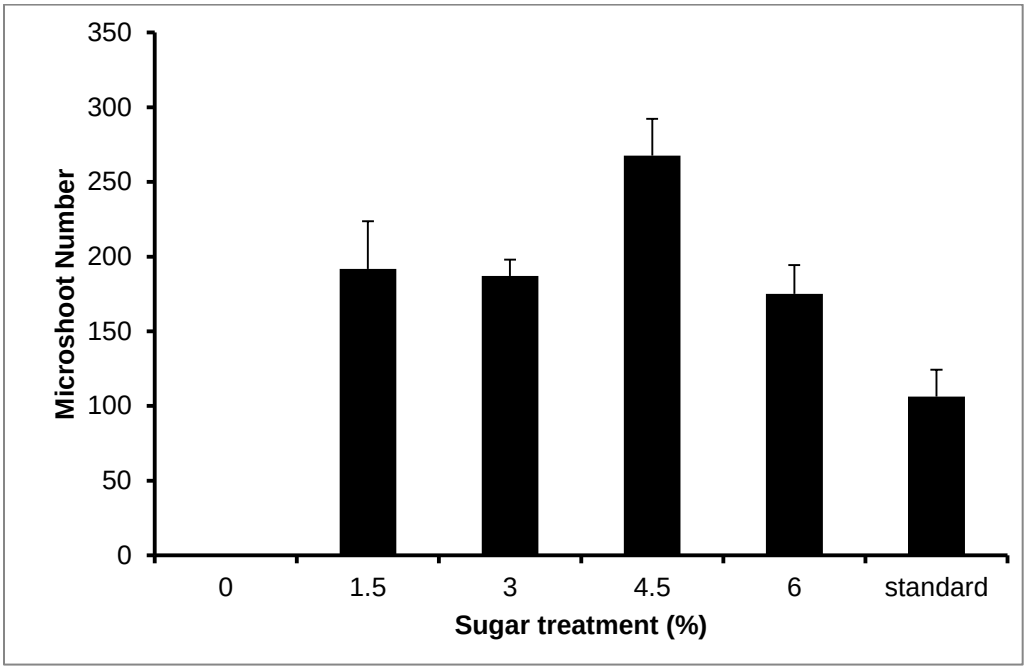


Figure 26. The effect of sugar (sucrose) source used with culture media on the number of growing microshoots (LSD=14.83).

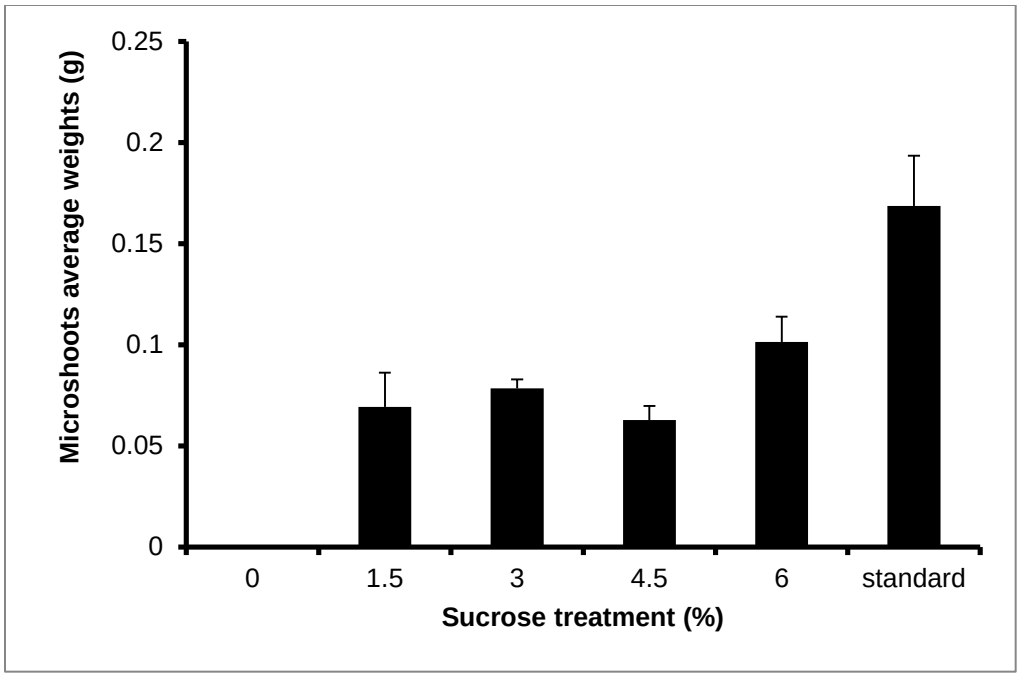


Figure 27. The effect of sugar cane concentration used with culture media compared with the use of 3% of sugar beet (standard) on the average weights of microshoot produced (LSD=0.028).

2.4.7. Experiment 7

The effect of the PPM (plant preservative mixture) added to the culture media and blending media on the number and viability of produced microshoots

2.4.7.1. Aims and objectives

This current study aimed to determine the best concentration of PPM for limiting the contamination of cauliflower microshoot production without affecting explant growth ability, and also the impact of the presence of PPM at particular growth stages, i.e. whether the presence of the PPM in the blending medium (S23) (the medium used in the blending stage for the homogenizing of curd clusters) is enough or whether it is necessary for the PPM to be in the culture medium in order to control contamination. However, only visible contamination screening was completed and the culture which visually appeared “sterile” could be contaminated because the PPM is considered to be a bacteriostatic agent.

2.4.7.2. Materials and methods

S.O.P$_{CS}$ was used for the production of cauliflower microshoots (cv Dionis).The experiment was divided into three stages,

1. Three concentrations of PPM (1, 2, 3 mL L^{-1}) (Apollo Scientific Limited, UK) were used with both plant growth regulator (PGR) free S23 liquid medium and S23 supplemented with 2 mg L^{-1} kinetin and 1 mg L^{-1} IBA. Five culture vessels were used with each treatment. The aim of this experiment was to investigate the effect of using relatively high concentrations of PPM on the growth of

microshoots and to determine if the PPM has any effect on the induction of micro-explants or their subsequent development

2. Various concentrations of PPM (0, 0.25, 0.5, 1 ml L^{-1}) were added to liquid media used for blending treatments in interaction with the same concentrations used for culture media and their effects on the growth of cauliflower microshoots were investigated. Five culture vessels were used with each treatment.
3. 1 ml L^{-1} PPM was used of culture media free PGRs and its effect on the development of cauliflower microshoots was evaluated. Five culture vessels were used with this treatment.

The number of developing microshoots and their viability assessed as microshoot fresh weights were evaluated after 21 days of culture.

2.4.7.3. Results

None of the explants developed when I ml L^{-1} of PPM was used with PGRs free culture media. There was no interaction between PPM material and PGRs used with culture media and that the PPM material had only an antibiotic role in controlling the contamination in the culture system.

It was found to be that the use of higher concentration than 1 ml L^{-1} of PPM had a damaging effect on the plant material because none of the explants grew using these concentrations. The average number of growing microshoots using 1 ml L^{-1} PPM was found to be (36.166) microshoots per pot with average weight of microshoot assessed to be (0.313) g.

The use of PPM material added to the blending media was found to have a minor effect on the number and viability of developing microshoots (Figures 30 and 31). The addition of PPM to the blending media did not control the contamination even when it was used at high relatively concentration (1 ml L^{-1}) and the presence of PPM in the culture medium was found to be an essential requirement for controlling the contamination because the level of contamination was found to be quite high when free PPM culture medium was used whatever the used concentration of PPM with blending medium was (Figure 28).

However, it was observed that the higher concentrations of PPM were used with culture medium, the lower number of explants grew. Nevertheless, the presence of PPM material was essential because of the high level of contamination obtained using PPM free culture media (Figure 28). The use of relatively low concentration of PPM (0.25) ml L^{-1} was found to be the optimal in terms of the number of developing microshoots but unfortunately their viability assessed as average weights was found to be quite low in comparison with other treatments with a high significant difference ($P< 0.001$) (Figure 30 and 31) and this might be caused by high level of darkness and browning of culture media which is known to be caused by the accumulation of phenol compounds (Figure 28). Phenol like compounds that were observed using these treatments could be the causing microshoots to stop growing.

The best results in terms of microshoots quality assessed as average microshoot fresh weights (Figure 31) were obtained using the following three treatments:

1. 0.5 ml L^{-1} PPM used with culture medium + 0.5 ml L^{-1} PPM used with Blending medium.
2. 0.5 ml L^{-1} PPM used with culture medium + 1 ml L^{-1} PPM used with Blending medium.
3. 1 ml L^{-1} PPM used with culture medium + 0.5 ml L^{-1} PPM used with Blending medium.

However, although it was found that there were no significant differences between these treatments in terms of the microshoot fresh weight (Figure 31), the use of (0.5) ml L^{-1} PPM added to the culture medium (the first two treatments) was considered to be better than the use of 1 ml L^{-1} PPM (third treatment) because of the high significant difference between these treatment in terms of the number of microshoots (Figure 30).

It was observed that there was difference in the colour of microshoots using different concentrations of PPM material added to the culture. The colour of microshoots produced using 1 ml L^{-1} PPM treatments were lighter than the color produce using 0.5 ml L^{-1} PPM after 21 days of culture (Figure 29).

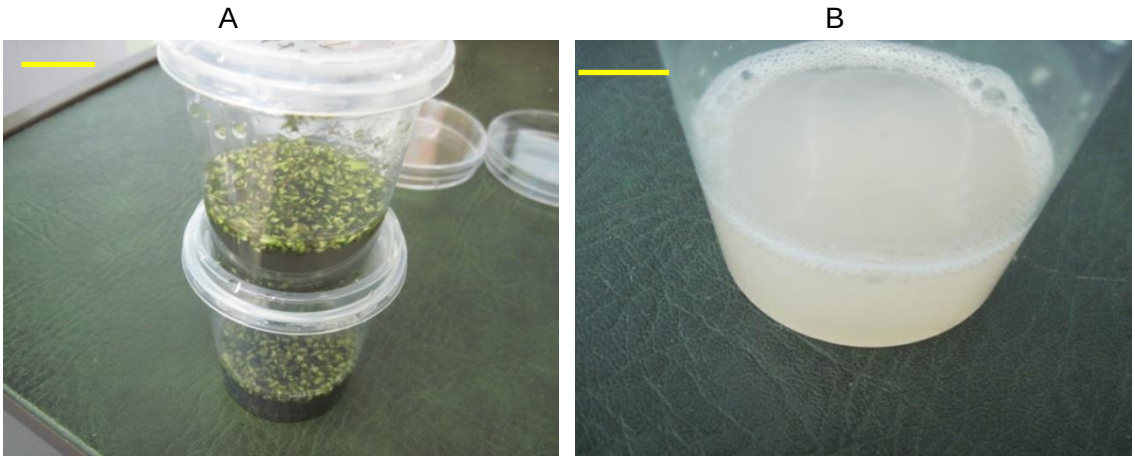


Figure 28, A) The microshoots produced when low concentration of PPM was used with culture media (0.25) ml L^{-1}. B) The contamination obtained when free PPM culture medium was used (Bar = 2 cm)**.**

Figure 29, A) The produced microshoots using (0.5) ml L^{-1} of PPM added to the culture medium. B) The microshoots produced using (1) ml L^{-1} with the culture media (Bar = 2 cm)**.**

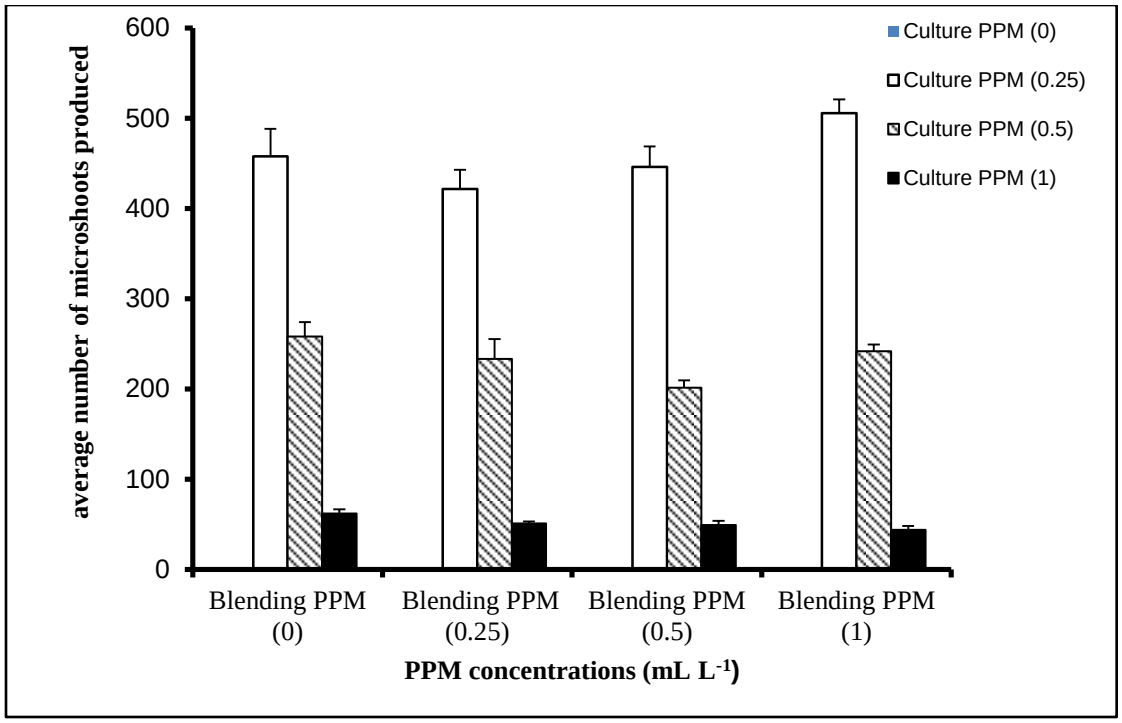


Figure 30. The effect of the PPM (plant preserve mixture) used with culture medium and blending medium on the number of developing microshoots after 21 culture days (LSD=7.601).

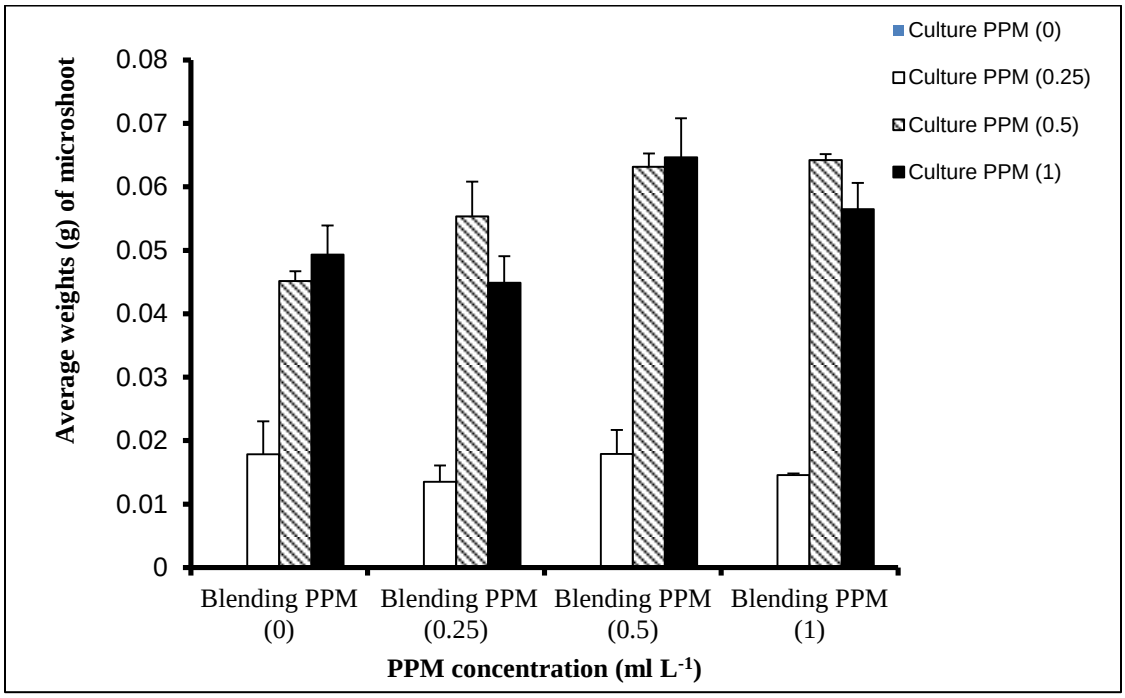


Figure 31. The effect of the PPM (plant preserve mixture) used with culture medium and blending medium on the average weight of microshoots after 21 culture days (LSD= 0.0066).

2.5. Discussion

The use of a blender for the mass production of cauliflower explants was found to be a reasonably effective methodology providing a huge amount of uniform explants which are impossible to produce using other ways. The use of the blender helped to maximize the amount of explants produced per curd taking advantage of the huge number of merstematic domes carried per cauliflower curd which seemed to be particularly suitable for mechanical explants production by homogenizing treatment. It was observed that the optimization of the blending durations was very important because it was clear that the explants started losing their viability after 30 s blending because of mechanical damage to the meristematic domes. The use of blenders has also been described for mass production of initial explants of fern (Cooke, 1979, Janssens and Sepelie, 1989, Knauss, 1976, Teng and Teng, 1997) and for separating meristemoid aggregates of several species (Levin *et al*., 1988, Standardi and Piccioni, 1998, Teng and Ngai, 1999, Ziv and Ariel, 1991, Ziv *et al*., 1998). The use of blender for cauliflower explants production was also previously reported by Bubu and Fuller (2005), Kieffer *et a*l (1995) and Kieffer *et al* (2001).

The sieving step worked efficiently and it helped to classify the explants produced into desirable size classes and remove the big clusters of meristems. The use of sieves enables the production of uniform very small sizes of explants (212-300 µm and 300-600 µm) impossible to produce manually. Moreover, classification of the explants into accurate small sizes facilitates the determination of the number of meristematic domes carried per explants and therefore the number of potential microshoots. The similarity between the estimate of the number of meristematic domes carried per explant and the eventual number of microshoots produced

showed that the microshoots had developed from existing meristematic domes. The use of meristem tissue in tissue culture techniques is very common because of their high rates of cell division and their genetic stability for subsequent type cloning.

The determination of the optimal sieve size class producing explants carrying one meristematic dome per each and later producing one microshoot per explant was one of the essential aims of this study. However, the choice of the most suitable sieving size class was a controversial issue. The selection of the (300-600 µm) size class produced explants carrying several meristematic domes and this can be a negative point since these explants will give more than one microshoot each and yet this can considered as a positive point because having more than one meristematic dome per explant provides insurance against the risk of losing any of the explants to damage. However, the results showed that 212-300 µm was the best sieving size class producing one meristematic dome per explant and later one microshoot, with viability of explants providing the disruption step was only 30 s.

Volumetric measures were used for explant cultivation to control a precise density for culturing. A huge number of explants were cultivated in each culture pot making contamination a significant risk. Nonetheless, the impact of culture infectivity was reduced by strict application of proactive rules, such as the use of cauliflower curds in the early stage of development when the curds are surrounded by leaves which semi protect the curds from environmental conditions and other sources of contamination, and proactive rules, such as absolute aseptic experimentation, use of PPM (plant preservative mixture) and culture of the explants in many culture vessels.

The use of liquid media suited cauliflower micropropagation and the use of liquid media for plant micropropagation has been proposed as an optimal solution for

decreasing plantlet production costs and for the automation of the production (Debergh, 1988). The use of liquid media has been successfully used for micropropagation of a large number of species and it was found to be more effective in comparison with semi solid media. For example, a higher number of shoots were obtained in *Prunus persica* L. Batseh (Hammerschlag, 1982) , more somatic embryos were created in soft winter wheat (Jones and Petolino, 1988), and *Gossypium hirsutum* (Gawel and Robacker, 1990). However, although the use of liquid media for micropropagation has great advantages such as offering much more regular culturing conditions, more of the container volume is able to be used (in comparison with semi solid media) and the relocate times can be decreased since explants are no longer positioned, These advantages, however, are often counterbalanced by technical problems such as hyperhydricity. Hyperhydricity is a physiological abnormality of water-soaked large cell appearance disturbing clonally propagated plants in tissue culture conditions (Debergh *et al*., 1992, Kevers *et al*., 1984). Hyperhydricity badly obstructs the regeneration of mature, standard plants (Sato *et al*., 1993, Böttcher, 1988). Hyperhydricity also negatively influences the growth of shoots and is recognized as a serious limitation on shoot development at the proliferation phase (Gaspar and Bajaj, 1991) resulting in a irreparable loss of regeneration capability of the tissue (Gaspar *et al*., 2000). Several sorts of abnormal formation have been observed in the hyperhydric tissue such as, abnormal stomata (Lessey *et a*l., 2005, Leshem *et al*., 1988, Ivanova and van Staden, 2008, Gaspar *et al*., 2000), a decreased number of palisade cells layers, erratic epidermal tissue, huge intracellular spaces in the mesophyll cell layer, thin cell wall, and the existence of a thin cuticle or no cuticle at all (Ivanova and van Staden, 2008). However, several means have been tested to reduce hyperhydricity in tissue culture, including raising

the concentration of carbohydrate in the media (Zimmerman and Cobb, 1989), changing the light intensity (Sutter and Langhans, 1979) and deceasing humidity (in semi-solid culture) (Bottcher *et al*., 1988).

In terms of the current research, hyperhydricity was only observed when the release of air bubbles was used as a means of agitation. The use of a current of air bubbles is supposed to reduce the level of hyperhydricity because it decreases the level of accumulated ethylene which is considered as one of the main reasons behind this physiological phenomenon (Akhter Zobayed *et al*., 1999). In contrast our results indicated that the level of hyperhydricity was higher using a current of air bubbles released in the liquid media when compared to the use of orbital shaker for agitation. Thus, an alternative hypothesis is needed. The high level of hyperhydricity might be caused by the presence of a high level of cytokinins (kinetin) for the long period considering, that when the current of air bubbles was used, the growth of explants was delayed significantly and the amount of the liquid media used was much higher because of the loss of the liquid media caused by the release of air current and the necessity of adding some extra media every week (the amount of liquid media used was four times in comparison with the agitated liquid media used a shaker). Therefore, there could have been a concentration effect of PGRs over the period of the experiment. High levels of cytokinins are known for their effect of raising the level of hyperhydricity (Gaspar *et al*., 1995, Ivanova *et al*., 2006, Ivanova and van Staden, 2008). Although several studies have investigated the effect of the cytokinins on the hyperhydricity phenomenon (Bornman and Vogelmann, 1984, Paˆques and Boxus, 1987, Thomas *et al*., 2000), the function of plant PGRs in hyperhydricity has yet to be fully determined and evidence exists for their effects on regular or hyperhydritic morphogenesis is still controversial (Picoli *et al*., 2001).

Despite this, the use of an agitated liquid culture media appeared to allow an optimal shoot regeneration frequency and growth rate in accordance with previous reports (Walkey and Woolfitt, 1970, Grout and Crisp, 1977).

The development of microshoots was observed to be very slow during the initial growth stage and this might be the result of shock caused by blending treatments and the required time for the transport of cytokinins and its accumulation in the active plant tissue.

The results showed that the supplementation with PGRs was an essential requirement for the induction of the cultivated explants. Cytokinins (i.e. kinetin) have a crucial position in the organization of sink activity and nutrient partitioning (Kuiper, 1988, Kuiper *et al*., 1989). Cytokinins are essentially made in the root apex (Komor *et al*., 1993) and because the cauliflower explants have no roots, the main cytokinins source is provided by the culture media. The propagation of cauliflower is accomplished by changing the morphogenetic pathway that generates floral buds to an alternative which gives rise to vegetative buds and this depends on the level of endogenous cytokinins. The addition of kinetin has been shown to increase the level of endogenous cytokinin (Feito *et al*., 1994, Kataeva *et al*., 1991, Vankova *et al*., 1991). It seems to be that kinetin was involved in the production of vegetative shoots from cauliflower curd pieces and has an essential role in cauliflower micropropagation.

In terms of the role of added auxins in the development of cauliflower explants, the results showed that the type of auxin is important, because there was found to be a large difference between the number and the viability of produced microshoots depending on the type of auxin used. IBA (indol butyric acid) was found to be best

for the development of explants. However, the differences obtained between the auxins types used gave a clear idea that the auxins have a role with the induction of cauliflower explants in contrast to what was mentioned by Vandemoortele *et al* (2001) that it is difficult to associate endogenous auxins with a function in the induction of cauliflower curd explants.

It was observed that the development of explants roots was quite limited. Even the use of a high concentration of NAA (naphthalene acetic acid), which is considered to be one of the strongest in the auxin phytohormone group, had negative results in terms of the rooting of cauliflower microshoots. However, the limitation of microshoot rooting might be caused by one or a combination of the following reasons:

- An interaction with kinetin since transferred microshoots to semi solid medium containing 2 mg L^{-1} ABA displayed roots within a few days.
- The existence of PPM (plant preservative mixture) in the liquid medium might prevent the development of the microshoots root.

The observations contrast with that reported by Kieffer (1996) who reported that the capability of NAA at low concentration to encourage early rooting of microshoots even in the presence of kinetin in the culture medium. It was observed that there was variability of microshoot vigour and this seems to be caused by a combination of many factors, such as initial meristem physiological size and status, culture situation and mechanical damage caused by the blending treatment.

The carbohydrate content in the liquid culture media was optimized to increase the efficiency of the designed protocol since plant, cell and tissue culture usually requires a carbohydrate source in order to satisfy energy demands (Amiri and Kazemitabar, 2011). Sucrose has been used widely as a main carbohydrate source

in plant tissue culture (Shahnewaz and Bari, 2004). The use of sucrose significantly increased the growth of micropropagated potatoes (Mohamed and Alsadon, 2010, Pruski *et al*., 2002). Mohamed and Alsadon (2010) reported that the use of higher concentration than 30 g L^{-1} resulted in a significant lower content of chlorophyll in the plantlet of micropropagated potato. However, the use of a higher than specific concentration of carbon source with the culture media could result in negative effects on the plant materials caused by an excessive osmotic contribution or by the toxicity of the carbon source (Ślesak *et al*., 2004) and this could explain the colour change observed in this study when higher than 3% sucrose was used in the culture media. The optimal concentration of sucrose in a medium should be enable to satisfy the energy requirements for cell division and differentiation without having any negative osmotic effect on shoot formation (Javed and Ikram, 2008). In agreement with the current results, the use of 2 to 3% sucrose in micropropagation media has been widely reported (Hazarika *et al*., 2004).

Although, sucrose has been used in the most of the work done in the field of *in vitro* micropropagation (Ahmad *et al*., 2007), it has not been always the best option. Michel *et al* (2008) indicated that glucose was the sugar that induced the best response of cotton callogensis. Salvi *et al* (2002) demonstrated that the shoot length and number and length of roots was significantly increased when glucose was used with the medium. Maltose has been also reported as a superior sugar compared with sucrose in many species such as cereals (Pande and Bhojwani, 1999, Last and Brettell, 1990). Ren *et al* (2010) reported that the use of maltose significantly increased the frequency of callus formation in wheat. However, in agreement with our results, Baskaran and Jayabalan (2005) demonstrated that sucrose was a better option than glucose and fructose for the regeneration of Eclipta alba shoot. The use

of sucrose was reported to be the optimal compared with fructose and glucose for the regeneration of *Vigna radiate* (Amutha *et al*., 2003). Nowak *et al* (2004) demonstrated that the use of sucrose was better than glucose for organogenesis of *Prunus domestica*. Indeed, it seems to be that the type and concentration of carbon source used with plant tissue culture depends on the species, type and age of growth material (Sul and Korban, 1998).

Sugar cane has been identified as a plant which contains a high amount of sucrose and sugar cane juice has been used as a source of carbon in the micropropagation of different plant species. Sul and Korban (1998) reported that the use of sugar cane juice gave a better results than graded granular sucrose in *Pinus sylvestris*. The use of sugar cane juice was also found to be suitable for Grand Naine banana micropropagation (Kodym and Zapata-Arias, 2001). However, according to our knowledge, there is no study that has reported a comparison between the sugar types, sugar cane and sugar beet, in terms of their suitability for plant tissue culture. The current study is the first report of the superior effects of sugar cane to be used for cauliflower micropropagation. However, further analysis is still required to find out the component differences between these two sugars and to determine the constituent that has the reported positive effect of sugar cane.

The level of contamination observed was very high when the PPM free culture medium was used. This high level of contamination observed showed that the use of 10 % of bleach for 15 minutes for plant material (curd fractions) sterilization was an ineffective methodology. However, since the cauliflower curd fractions are sensitive to harder sterilization treatments, it could be difficult to surface sterilize them completely. Thus, some antibiotic and fungicide should always be used with the culture media for preventing the contamination.

The use of PPM was found to be quite effective in controlling the contamination of the culture system. Our results showed the necessity of using this material since the use of PPM free culture media displayed a high level of contamination. Nevertheless, the determination of the optimal concentration of this material controlling the contamination without reducing the explants viability was found to be essential. It was observed that a high level of PPM has negative effects on the explant viability. It was observed that the lower the concentration of PPM used the higher the number of explants developed this accords with (Compton and Koch, 2001) who found that the percentage of melon cotyledon explants which formed callus and somatic embryos and furthermore the number of embryos per explants were decreased when incubated in embryo initiation and embryo development media containing more than 5 ml L^{-1} PPM, and Niedz (1998) who mentioned that PPM does not affect the viability of sweet orange (*Citrus sinensis* L.) explants and does not reduce their growth ability when it was used in concentration lower than 1.0 ml L^{-1}. Kraj and Dolnicki (2003) indicated that there was no negative effect of PPM upon the growth and development of callus in European beech (*Fagus Sylvatica* L.) when it was used at a suitable concentration.

It seems that the determination of the optimal PPM concentration to control the contamination and not affect the growth ability in the culture media depends on the plant species in the first instance. In terms of cauliflower, the relatively low concentration of PPM (0.25 ml L^{-1}) was found to control the contamination effectively and led to the production of the highest number of developing explants. Nevertheless, the viability of these developing explants was limited by high level of phenol-like components produced. The numerous numbers of growing explants affected by the blending treatment could lead to this high level of phenols. However,

the darkening of culture media caused by phenolic compounds has been considered to be a serious problem to tissue culture systems (Titov *et al*., 2006, Ozyigit, 2008). The process starts by browning of the outside of plant tissue due to the oxidation of phenolic compounds resulting in the production of quinines that are very reactive and toxic to plant tissue. The production and composition of phenolic in the plant tissue might be determined by genetic and environmental conditions like oxidative reaction during processing, culturing and storage (Lux-Endrich *et al*., 2000). In case of the current research, it seems to be that there is a relation between chemical composites of medium and phenolic exudation and this accords with (Chamandoosti, 2010). There were no signs of the presence of these compounds when concentrations higher than 0.25 ml L^{-1} PPM were used. However, the large number of developing explants and the fast rate of growth using low concentration of PPM led to an accumulation of released phenolic components since PPM might work as growth retardant in this case, limiting the number and the speed of explant growth.

Several methods have been investigated in terms of their effectively on stopping the production of phenolic compounds. Titov *et al* (2006) found that phenolic compound excretion could be stopped by pre soaking *Musa spp*. cv. Kanthali floral bud explants in an antioxidant solution of 0.125 % potassium citrate. Mante and Tepper (1983) reported that the browning caused by phenolic compounds in the culture medium can be prevented using a mixture of ascorbic acid, citric acid and cysteine. However, while Gupta *et al* (2000), Kumria *et al* (2003) and Ozyigit *et al* (2008) suggested that the ages of the genotype and explants are the most efficient factors for controlling the total amount of phenolic compound in media, Chamandoosti (2010) also mentioned that the composition of media and type of explants are the most important factors for decreasing the phenolic compounds in the culture media. It might worth

trying some of these methods with cauliflower in order to reduce the amount of phenolic compounds produced using relatively low concentration of PPM taking advantage from the huge number of developing explants observed when low concentration of PPM was applied.

2.6. Conclusion

The described protocol was found to be effective for cauliflower mass production. All the protocol steps were optimized in order to produce the highest level of best quality cauliflower microshoots. The use of a commercial blender was found to be a useful methodology for plant material distribution and 30 s blending duration treatments was found to be the best in terms of the number and viability of produced microshoots. The use of sieves helped to classify the produced explants in certain size classes and affected the number of meristematic domes carried per explant. The method was useful since it was possible to produce one microshoot per explant using the 212-300 µm sieve size.

The use of PGRs was essential for microshoot development and the best PGR combination was found to be 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA. The use of an orbital shaker for agitation was observed to be superior in comparison with other methods. The optimal sugar type and concentration suitable for the use with cauliflower micropropagation system was also determined. Although the use of maltose and sucrose gave the optimal effect on the cauliflower microshoot development, the use of sucrose was recommended as a cheaper option. Moreover, this study highlighted the effect of the source of sucrose used with the culture media on the effectiveness of the cauliflower micropropagtion. This study was the first to demonstrate the superior effect of sugar cane compared with sugar beet for the use with this culture

system. The results reported in this study will play a role reducing the cost of cauliflower micro-propagule production.

The contamination was a real problem and the use of bleach for curd piece sterilization did not fully control the contamination. Therefore, PPM was used and the use of 0.5 ml L^{-1} with culture medium was found to be optimal for controlling the contamination and keeping the explant viability at an acceptable level.

The use of the described protocol is considered to be a cost effect methodology for cauliflower micropropagation to produce huge numbers of microshoots per curd. The microshoots produced can be transferred to the semi-solid media and then to the field for commercial production and can also be used for cauliflower artificial seed production (see chapter three).

Chapter 3: Cauliflower Artificial Seed Production

3.1. Introduction

Artificial seed is defined as the production of somatic embryos or any other vegetative tissue (shoot buds, cell aggregates, micro-shoots) produced using tissue culture techniques and encapsulated in a protective coating (Ara *et al*., 2000, Saiprasad, 2001). Artificial seed may offer a tool suitable to the extensive scale-up required for multi-clone commercial production (Lata *et al*., 2009). Moreover, the use of this technique economizes upon space, medium and time requested by the traditional tissue culture methods (Mathur *et al*., 1989). The encapsulation of vegetative propagules to produce artificial seeds has been used recently as a proper alternative to the employment of somatic embryos (Sarkar and Naik, 1998). Artificial seeds have great advantages in comparison with traditional tissue culture methods. They are reasonably inexpensive to produce, and easy to handle, plant and transport. They can also be stored for a long period using dehydration and cryopreservation techniques (Pennycooke and Towill, 2001, Wang *et al*., 2002).

Several studies have investigated the production of artificial seeds working with different plant species including vegetables, fruits, medical plants, ornamentals, forest trees, orchids and cereals (Bapat *et al*., 1987, Bapat and Rao, 1988, Mathur *et al*., 1989, Ganapathi *et al*., 1992, Corrie and Tandon, 1993, Sharma et al., 1994, Sarkar and Naik, 1998, Mandal *et al*., 2000, Nyende *et al*., 2003, Chand and Singh, 2004, Naik and Chand, 2006, Singh *et al*., 2006, Faisal and Anis, 2007, Micheli *et al*., 2007, Rai and Jaiswa, 2008, Rai *et al*., 2008b, Rai *et al*., 2009, Singh *et al*., 2009) cited in (Rai *et al*., 2009).

In terms of Brassica species, artificial seeds have been produced using somatic embryos derived from broccoli microspores (Takahata *et al*., 1993) and Chinese cabbage microspores (Wakui *et al*., 1994). However, these embryos are normally haploid because they are derived from microspores formed after meiosis and were not clones and therefore produced a form different to the parental phenotype.

With regard to cauliflower, although Redenbaugh *et al* (1986) was able to produce artificial seed using somatic embryos, they emphasize the difficulty encountered in the production of SE' in mass. However, several protocols like those designed by (Pareek and Chandra, 1978) and (Fransz *et al*., 1993) found negative results when it was attempted to produce cauliflower somatic embryos in mass.

Since the production of cauliflower microshoots was optimized (chapter two) and because of the difficulty encountered in producing cauliflower somatic embryos, this study aimed to produce cauliflower artificial seeds using encapsulated microshoots. However, a basic study, which has investigated the ability of cauliflower artificial seed production using cauliflower microshoots, was designed by Kieffer (1996) and the work here sought to optimize a full protocol for mass production and preservation of cauliflower artificial seed.

3.2. Experiments

3.2.1. Experiment 1

Optimization of micro-shoot encapsulation

3.2.1.1. Aims and objectives

1. Determination of optimal steps for cauliflower microshoots encapsulation.
2. Investigation of the effect of sodium alginate and $CaCl_2,2H_2O$ solution concentrations on the artificial seed bead formation, plant toxicity, conversion rate and viability of artificial seeds assessed as fresh weights of plantlets produced.

3.2.1.2. Materials and methods

Micro-shoots were encapsulated in sodium alginate hydrogel following a protocol derived from Kieffer (1996). However, because of the marked decrease in the setting ability of alginic acid solution caused by high temperature when it is sterilized by autoclaving (1 bar, 121°C for 15 min) (Larkin *et al*., 1988, Kieffer, 1996), the sodium alginate solution was sterilized following a tyndallisation protocol described as follows:

i. Heat at 80°C for 15 minutes (Kills most micro-organisms, but not spores).
ii. Rest at room temperature for five hours (allows spores to germinate).
iii. Heat at 90°C for 15 minutes (kills germinated spores).
iv. Rest overnight and heat at 90°C for 15 minutes (Insurance).

Microshoots were mixed with a sterile solution of alginic acid containing S23 (4.4 g L^{-1} MS + 30 g L^{-1} sucrose) and supplemented with 1 ml L^{-1} PPM. Different concentrations of

alginic acid (1, 2, 3, 4, 5 % (w/v)) (Sigma Ltd) were investigated. Thirteen day old micro-shoots (300-600 micrometer explants size class, 240 micro liter explants per pot containing 30 ml liquid medium supplemented with 2 mg L^{-1} kinetin and 1 mg L^{-1} IBA, cv Clemen) were encapsulated in sodium alginate gel (1 to 5 % concentrations). The microshoots contained in sodium alginate solution dropped individually in 10 g L^{-1} (68 mM) concentration of sterile $CaCl_2,2H_2O$ solution using a sterile pipette ($CaCl_2,2H_2O$ solution was sterilized by autoclave (1 bar, 121°C for 15 minutes). The artificial seeds produced were left for 30 minutes mixing duration and then transferred to maintenance liquid culture media for 30 min followed by a quick wash with sterile water. The artificial seeds produced were dispatched on a maintenance semi-solid media (4.4 g L^{-1} MS + 3% sucrose + 7 g L^{-1} agar). The cultivated artificial seeds were kept in a growth cabinet at 23º C and 16 hours light period.

Applying the same described system but using 2 % sodium alginate solution, the effect of three concentrations of $CaCl2,2H_2O$ (5, 10 and 15 g L^{-1}) (34, 68 and100 Mm) was evaluated. For each treatment, three plastic pots (10*10*8 cm), each containing 100 ml of maintenance semi solid media supplemented with 1 ml L^{-1} PPM were used. 12 artificial seeds were cultivated in each container (3 lines each containing 4 seeds) and each container was considered as a replication. The effects of the sodium alginate and $CaCl_2,2H_2O$ concentrations on the conversion rate and viability of artificial seeds assessed as average plantlet fresh weights were evaluated after two weeks of culture.

3.2.1.3. Results

Sterilization by tyndallisation was found to be an efficient method for keeping sodium alginate capable of setting. 2% sodium alginate concentration was found to be the

optimal, producing spherical beads of 5-7 mm diameter (Figure 33).However, while the viscosity of sodium alginate solution was increased with higher concentration producing drop-shaped bead, concentration lower than 2% gave poor quality small beads. The optimal concentration of 2 % was therefore used throughout all subsequent experiments. Encapsulated micro-shoots placed in a growth chamber germinated within few days whatever the initial sodium alginate concentration was (1-5%). Thus, there was no toxic effect of sodium alginate found on the plant tissues. There was also no effect of sodium alginate concentration on the viability of produced artificial seeds since there was no significant difference between the average weights of plantlets obtained after two weeks of culture (P = 0.568).

In terms of the effect of the $CaCl_2$, $2H_2O$ concentration on the produced artificial seeds, while all encapsulated micro-shoots showed the ability to convert regardless of the initial concentration of $CaCl_2$,$2H_2O$ used, a significant difference was observed in terms of artificial seed viability assessed by measuring the fresh weights of plantlets obtained after two weeks of culture. The concentration of 15 g L^{-1} $CaCl_2$, $2H_2O$ was found to be the optimal producing the highest plantlets average weight (P = 0.022) in comparison with the use of other $CaCl_2$,$2H_2O$ concentrations (Figure 32).

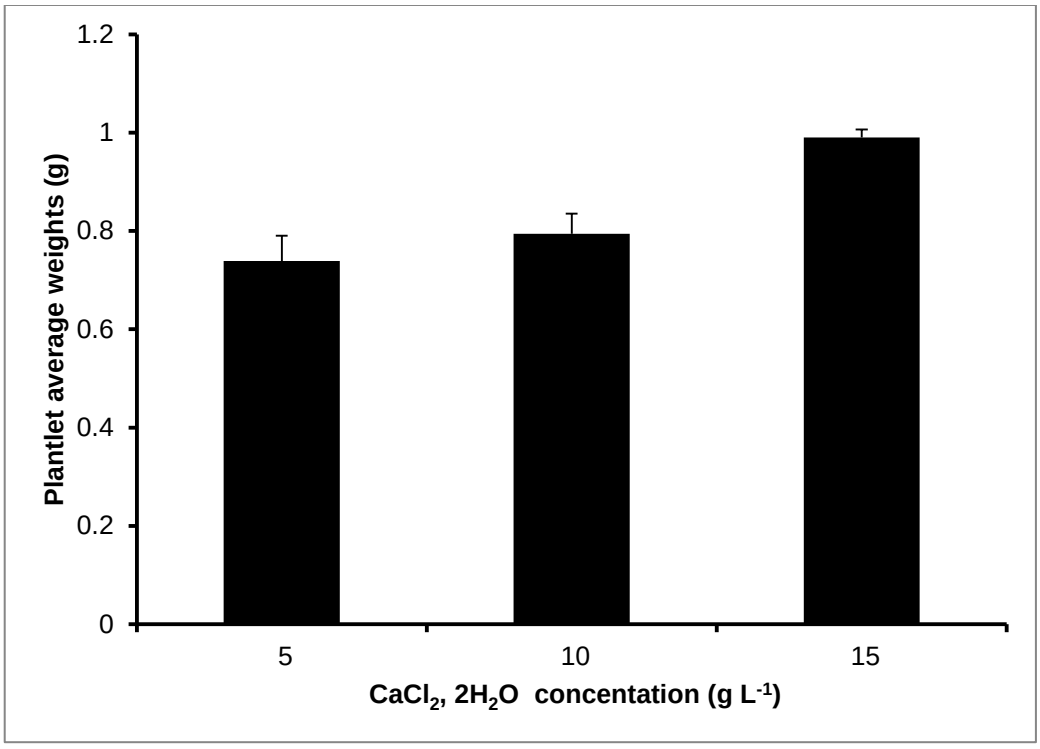


Figure 32. The effect of the CaCl2, 2H2O concentrations on artificial seeds produced viability.

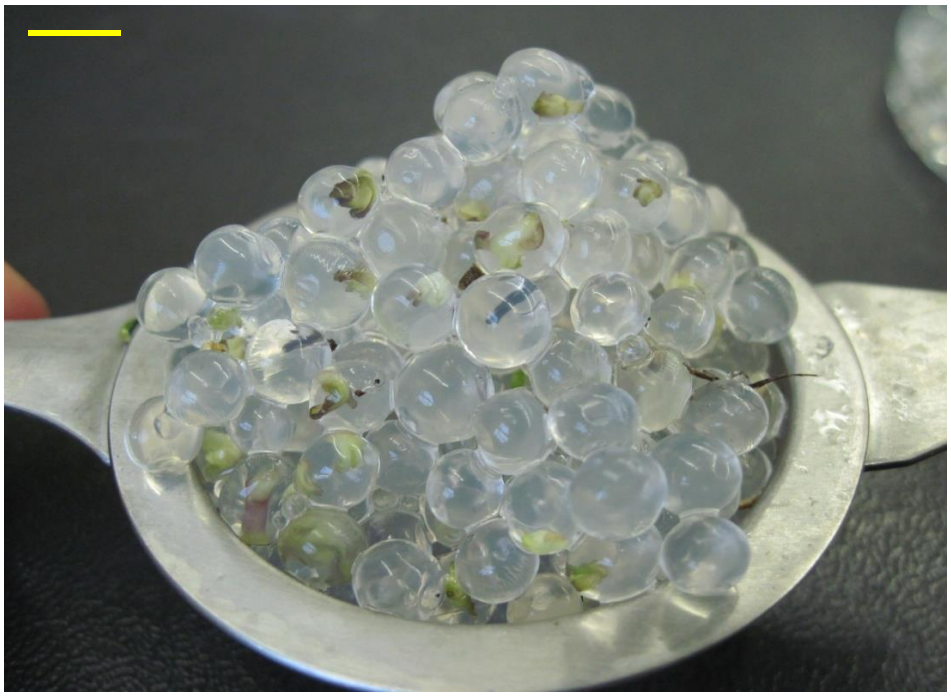


Figure 33. Artificial seeds produced using 2% concentration of sodium alginate and 15 g L^{-1} of $CaCl_2$, $2H_2O$ (Bar = 1 cm).

3.2.1.4. Standard Operating Procedure for artificial seed production (SOP_{ASP})

In general, the same procedures were subsequently followed in the production of cauliflower artificial seeds enabling a SOP to be produced; any variation in the procedure will be noted in the course of describing the experiment. The procedures are as follows,

1. SOP_{CS} was used for cauliflower microshoots production.
2. 2 % w/v of sodium alginate solution supplemented with (4.4 g L^{-1} MS + 30 g L^{-1} sucrose + 1 ml L^{-1} PPM) was sterilized by tyndallisation.
3. Microshoots were mixed with the sterilized sodium alginate solution and dropped using sterile pipette in 15 g L^{-1} $CaCl_2$, $2H_2O$ for 30 min mixing duration for full complexing. The produced artificial seeds were transferred to maintenance liquid media supplemented with 1 ml L^{-1} PPM for 30 min followed by a quick wash with sterile water
4. Artificial seeds were then cultivated in maintenance semi solid media (7 g L^{-1} agar + 4.4 g L^{-1} MS + 3 % sucrose + 1 ml L^{-1} PPM) contained in culture pot. Each pot contained 100 ml of the mixture described. The cultivated artificial seeds were kept in the growth cabinet at 23ºC and 16 hours photoperiod until the experiment results were estimated.

All the work was done under strict sterilized conditions in a laminar flow hood.

3.2.2. Experiment 2

The effect of plant growth regulator combinations used in the liquid culture medium for the production of microshoots on their suitability for artificial seed production

3.2.2.1. Aims and objectives

The aim of this experiment was the investigation of the effect of PGR combinations used with explant culture media on the suitability of microshoot for the production of artificial seeds. The PGRs in the liquid culture medium were manipulated in an attempt to promote the production of optimal microshoot quality suitable for encapsulation in sodium alginate matrix and giving the highest level of the best quality artificial seed conversion rate.

3.2.2.2. Materials and methods

SOP_{ASP} was used for the production of cauliflower artificial seeds (cv Mascaret MAR). Different PGR combinations were used with microshoot culture liquid medium. 300-425 µm explants size class were used for microshoot production and 14 day old microshoots were used for the production of artificial seeds.

This experiment was divided in two steps. Firstly, maintenance S23 was supplemented with nine combinations of Kinetin (1 and 2 mg L^{-1}) and IBA or NAA (1 and 2 mg L^{-1}). Secondly, S23 was supplemented with four combinations of Kinetin (1 and 2 mg L^{-1}) and IAA (1 and 2 mg L^{-1}) and was compared with the use of S23 supplemented with 2 mg L^{-1} of Kinetin and 1 mg L^{-1} of IBA. Artificial seed conversion rate and their viability was assessed as the number and fresh weight of plantlets evaluated after 20 days of culture in semisolid medium supplemented with 2 mg L^{-1} IBA. Three replications, each consisting of 10 artificial seeds, were used for each treatment. Each ten artificial seeds

were cultivated in plastic culture vessels. The treatments were distributed randomly within the vessels.

3.2.2.3. Results

- **Stage 1**

1. The effect of phytohormone combinations on the artificial seed conversion rate

The conversion rate of artificial seeds was assessed as the number of converted artificial seeds. It was observed that the kinetin concentration had a significant effect on the conversion rate of artificial seeds (Table 2) and the use of relatively lower concentration 1 mg L^{-1} gave the optimal conversion results at that was assessed to be 96.7%.

It was also observed that the concentrations of auxin had a significant effect (Table 2) and the use of 1 mg L^{-1} of auxin was found to be better than the use of 2 mg L^{-1}. The conversion rate was found 97.5 % and 89.17% at 1 mg L^{-1} and 2 mg L^{-1} auxin concentrations respectively.

A very high interaction between the kinetin concentrations and auxin types was found ($P < 0.001$). The use of 1 mg L^{-1} kinetin with IBA as an auxin type was considered to be the best given conversion rate assessed to be at 100% (Figure 34).

However, very high interaction was also observed between kinetin concentration, auxin type and auxin concentration (Table 2). It was found that the use of (1 mg L^{-1} Kinetin + 1 mg L^{-1} IBA), (2 mg L^{-1} Kinetin + 2 mg L^{-1} NAA), (1 mg L^{-1} Kinetin + 1 mg L^{-1} NAA) and (1 mg L^{-1} Kinetin + 2 mg L^{-1} IBA), gave the best results (100% conversion rate) (Figure 35) and it was observed that there was no significant difference between these

treatments. The use of 2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA was considered as a standard in the next experiment.

Table 2. The effect of microshoot culture PGRs on the conversion rate of artificial seeds produced.

Source	*P*	Sig
Kinetin concentration	0.019	*
Auxin type	0.075	-
Auxin concentration	0.004	**
Kinetin concentration * Auxin type	0.00	***
Kinetin concentration * Auxin concentration	0.546	-
Auxin type * Auxin concentration	0.231	-
Kinetin concentration * Auxin type * Auxin concentration	0.001	***

2. The effect of phytohormone combinations on the artificial seed viability

Auxin concentration had a significant effect on the viability of artificial seeds (Table 3) with 1 mg L^{-1} of auxin concentration giving the optimal effect 1.111 g than 2 mg L^{-1} giving plantlets average weights assessed to be 0.490 g.

A highly significant interaction was found between the kinetin concentration and auxin type used with microshoot culture media on the viability of artificial seeds ($P < 0.001$) (Figure 36). It was found that optimal results were produced using either low concentration kinetin with IBA or high concentration of kinetin with NAA.

A highly significant interaction between kinetin concentration, auxin types and auxin concentrations was also observed (Table 3). However, the best PGR combination was obtained using 1 mg L^{-1} Kinetin + 1 mg L^{-1} IBA and 2 mg L^{-1} Kinetin + 1 mg L^{-1} NAA treatments (Figure 37). It was found that there was a highly significant difference

between these treatments and the use of (2 mg L^{-1} Kinetin + 1 mg L^{-1} IBA) which was used as a standard in the next experiment.

Table 3. The effect of microshoot culture PGRs on the viability of artificial seeds produced.

Source	P	Sig
Kinetin concentration	0.468	-
Auxin type	0.513	-
Auxin concentration	0.000	***
Kinetin concentration*Auxin type	0.000	***
Kinetin concentration*Auxin concentration	0.831	-
Auxin type*Auxin concentration	0.599	-
Kinetine concentration*Auxin type*Auxin concentration	0.000	***

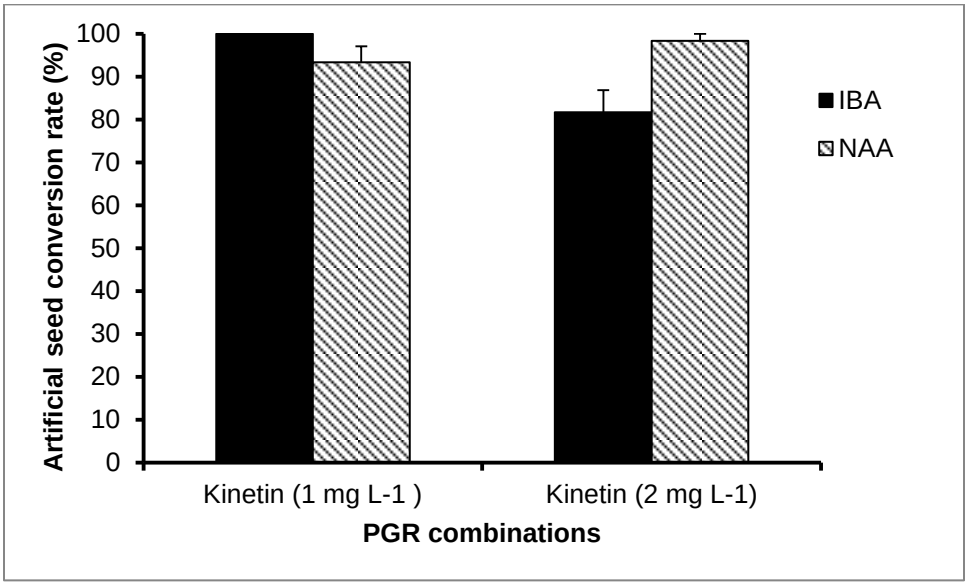


Figure 34. The effect of the interaction between the kinetin concentrations and the type of auxins used no the artificial seed conversion rate (LSD=10.986).

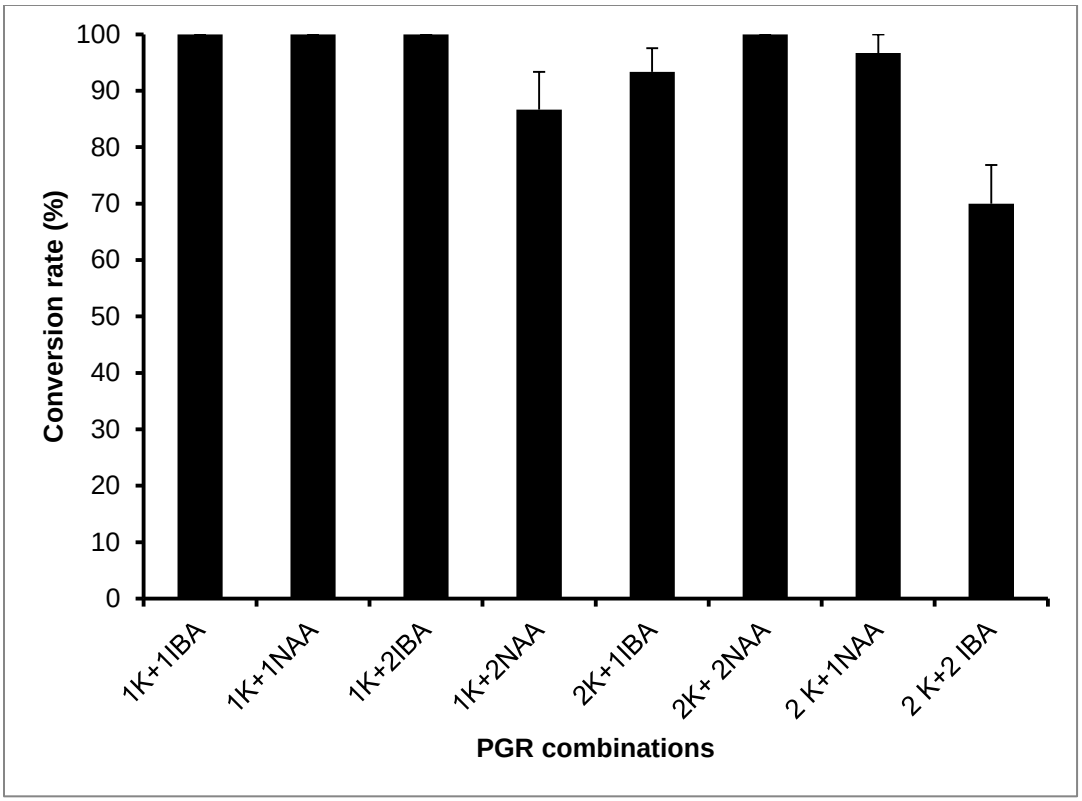


Figure 35. The effect of the PGR combinations on the artificial seeds conversion rate (LSD= 7.498).

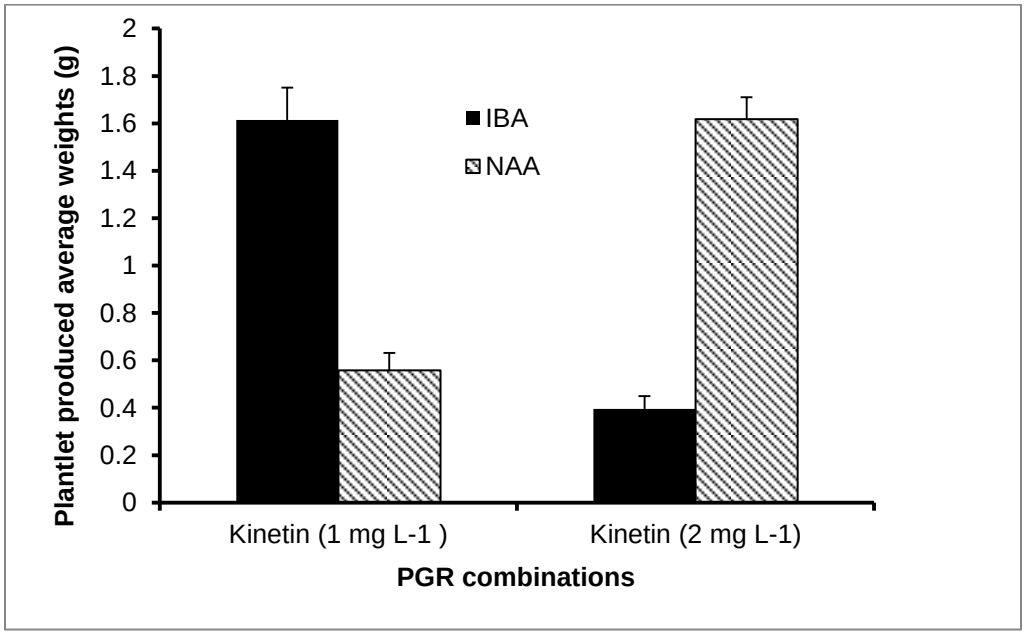


Figure 36. The effect of the interaction of the kinetin concentration and auxin types on the viability of artificial seeds produced (LSD=0.6985).

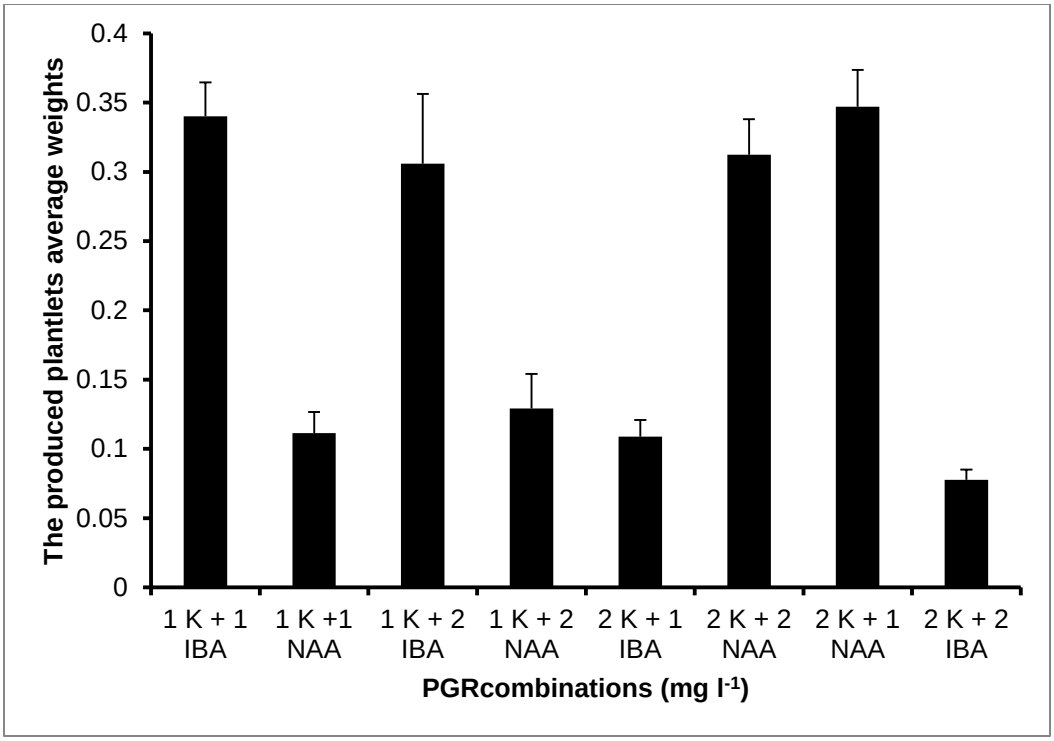


Figure 37. The effect of microshoot culture PGRs on the viability of artificial seeds (LSD=0.075).

Over all in this experiment the use of 1 mg L^{-1} of kinetin incorporated with 1 mg L^{-1} of IBA as a PGR combination was found to be the optimal in terms of artificial seed conversion rate and viability.

- **Stage 2**

It was observed in this experiment that the use of 2 mg L^{-1} Kinetin +1 mg L^{-1} IBA was the optimal in terms of the artificial seeds conversion rate (76.25 %) with a high significant difference assessed in comparison with other PGR combinations used with microshoots culture media ($P < 0.001$) (Figure 38). In terms of the viability of artificial seeds, however, no significant difference was found between 2 mg L^{-1} Kinetin +1 mg L^{-1} IBA and other treatment ($P = 0.200$) (Figure 39).

It was clear from these two experiments that the PGR combinations applied with liquid culture media used for microshoot production had great effects on the artificial seed conversion rate and viability. However, optimal artificial seeds were produced using 1 mg L^{-1} kinetin + 1 mg L^{-1} IBA applied with the microshoot culture media.

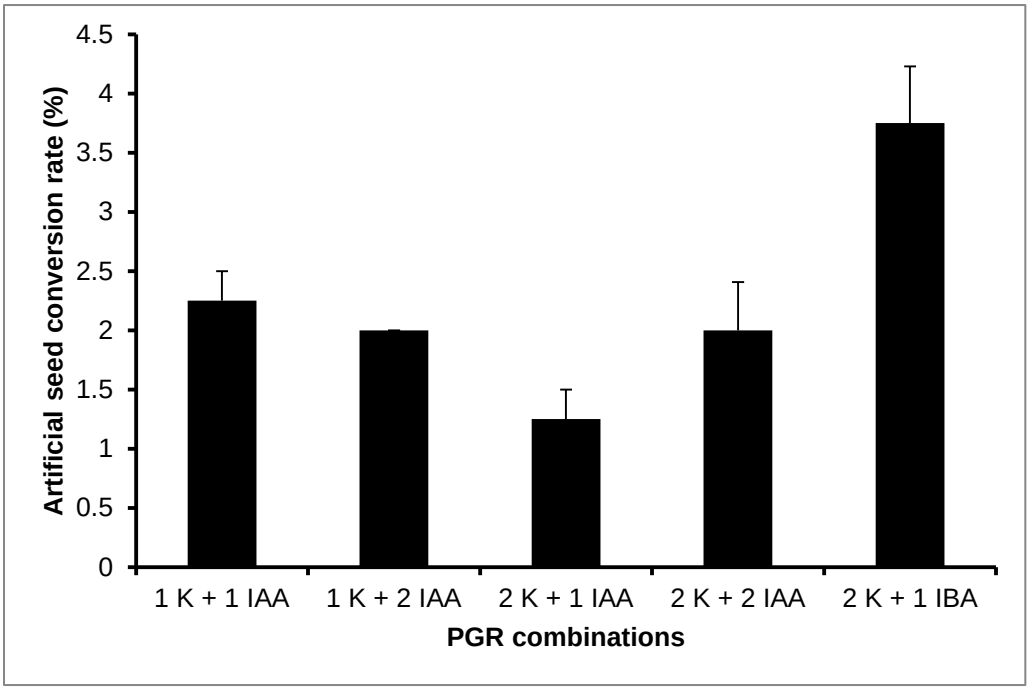


Figure 38. The effect of the PGR combinations on the artificial seeds conversion rate (LSD= 4.862).

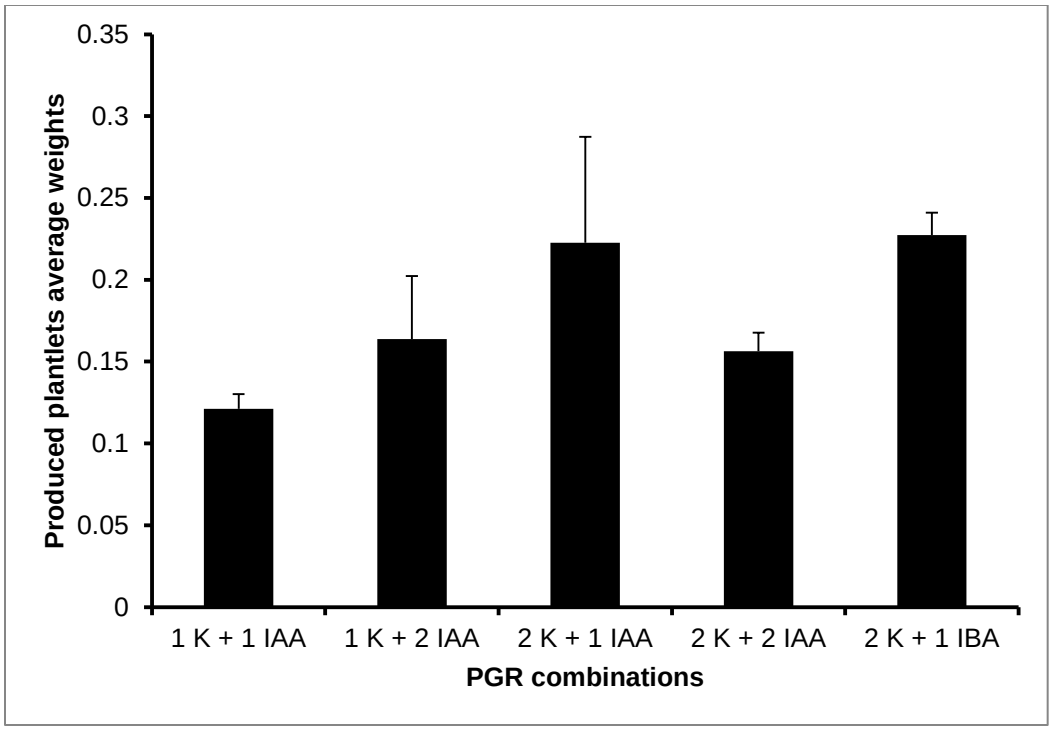


Figure 39. The effect of PGR combinations used with microshoots liquid culture media on the viability of produced microshoots.

3.2.3. Experiment 3

The determination of the optimal cauliflower microshoots age suitable for encapsulation

3.2.3.1. Aims and objectives

The investigation of the effect of microshoot age on the conversion rate and viability of artificial seeds produced and the determination of the optimal microshoot age suitable for encapsulation as artificial seeds.

3.2.3.2. Material and methods

SOP_{ASP} was used for the cauliflower artificial seed production. 212-300 µm explants size class (cv Mascaret) were used for microshoots production. The production of artificial seeds started when the microshoots were 9 days old and stopped when they were 15 days old since after that the microshoots were too big to be encapsulated. The effect of microshoot age was assessed in terms of the artificial seed conversion rate and viability which was evaluated as fresh weight of plantlets obtained after 24 days of culture. 7 replications each consisting of 6 artificial seeds were cultivated in plastic pots (10*10*8). Four lines were cultivated per pot and each one consisted of 6 seeds. The treatments were distributed randomly within the pots.

3.2.3.3. Results

The optimal age for microshoot encapsulation was observed to be 13–14 days (Figure 40). The encapsulation of both younger and older microshoots had a significant negative effect on the subsequent artificial seed conversion rate ($P < 0.05$) and fresh weights of plantlets produced ($P < 0.001$). The growth of microshoots younger than 13 day-old was observed to be very slow and encapsulation seemed to be an inhibitor of

microshoot growth at this sensitive stage. The growth of microshoots older than 14 days old was observed to be rapid in culture but this fast growth brought about metabolic activity which seemed to negatively affect the subsequent of microshoot encapsulation. It was concluded that microshoots derived in an accelerating stage of growth (chapter 2) were optimal for encapsulation.

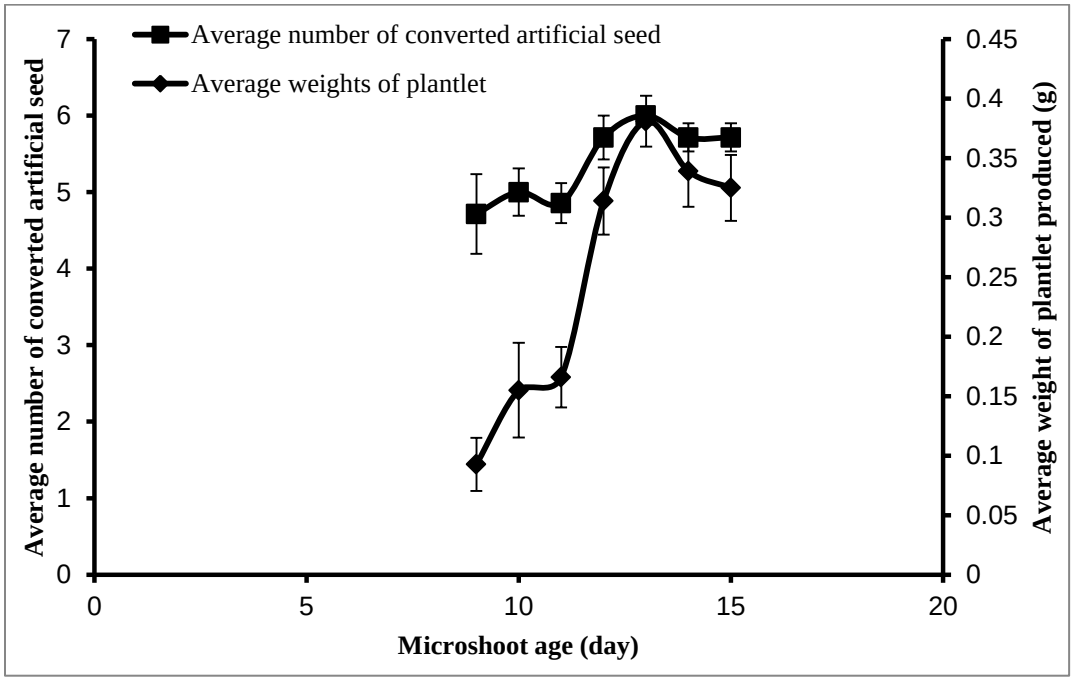


Figure 40. The effect of the optimal microshoot age suitable for encapsulation (LSD= 0.687 for the conversion rate and LSD= 0.0675 for artificial seeds viability).

3.2.4. Experiment 4

The effect of the PGRs used with the artificial seed matrix

3.2.4.1. Aims and objectives

The aim of this experiment was the investigation of the effect of the PGR combinations incorporated with the artificial seed matrix on the artificial seeds conversion rate and viability. The determination of the optimal PGR structure was the main objective of this experiment.

3.2.4.2. Materials and methods

SOP_{ASP} was used for the production of Mascaret MAR cauliflower (cv Mascaret MAR) artificial seeds, with the exception that 148 µl of 212-300 µm explants size class was used per container (30 ml culture media). 13 day-old microshoots were used for the production of artificial seed. Various combinations of PGRs were incorporated with the sodium alginate solution before it was steralized. 27 PGR combinations were incorporated in artificial seed matrixes. Three concentrations of kinetin (0.5, 1 and 2 mg L^{-1}) were used with three types of auxins (IBA, NAA, and IAA). Three concentrations (0.5, 1 and 2 mg L^{-1}) of each auxin were investigated.

The effect of PGRs on the conversion rate of artificial seed was investigated and their viability assessed as fresh weight of plantlets, was evaluated after 34 days of culture. Five replications of each treatment were employed, each consisting of 5 artificial seeds cultivated in plastic pots (10*10*8). Four lines each containing 5 artificial seeds were cultivated within each pot. The treatments were distributed randomly within the pots (every treatment was replicated within each of 5 pots).

3.2.4.3. Results

1. The effect of PGR combinations on the conversion rate of artificial seeds

A significant effect of the kinetin concentration was observed (Table 4) and the best kinetin concentration was found to be 1 mg L^{-1}. While the conversion rate was 94.22 % using 1 mg L^{-1} of kinetin, it was evaluated to be 88.44 % and 87.56 % using 0.5 mg L^{-1} and 2 mg L^{-1} concentrations of kinetin respectively.

A very highly significant interaction was observed between kinetin concentration and auxin types ($P < 0.001$) (Figure 41). However, no significant effect was found to be for the interaction between the kinetin type, auxin type and auxin concentrations and 100% conversion rate was obtained using several PGR combinations described as follows, (0.5 mg L^{-1} kinetin + 0.5 mg L^{-1} IBA), (1 mg L^{-1} kinetin + 0.5 mg L^{-1} NAA), (1 mg L^{-1} kinetin + 1 mg L^{-1} IAA), (1 mg L^{-1} kinetin + 1 mg L^{-1} NAA), (1 mg L^{-1} kinetin + + 2 mg L^{-1} IAA), (2 mg L^{-1} kinetin + 2 mg L^{-1} IBA), (2 mg L^{-1} kinetin + 0.5 mg L^{-1} IBA) and (2 mg L^{-1} kinetin + 1 mg L^{-1} IBA) (Figure 42).

Table 4. Artificial seed matrix PGRs effect on their conversion rate statistical analysis results.

Source	*P*	Sig
Kintine concentration	0.035	*
Auxin type	0.616	-
Auxin concentration	0.285	-
Auxin type*Auxin concentration	0.108	-
Auxin type*Kinetin concentration	0	***
Auxin concentration*Kinetin concentration	0.267	-
Auxin type*Auxin concentration*Kinetin concentration	0.142	-

2. The effect of PGR combinations on the viability of artificial seeds

No significant effects were observed of the PGR combinations used with artificial seed matrix on its viability (Figure 43).

However, since there were several combinations of PGRs giving 100% conversion rate and no effect of the PGR combination on the conversion rate of artificial seeds was found to be, the economic factor could be decisive in choosing the cheapest combination considering the fact that PGRs are expensive materials.

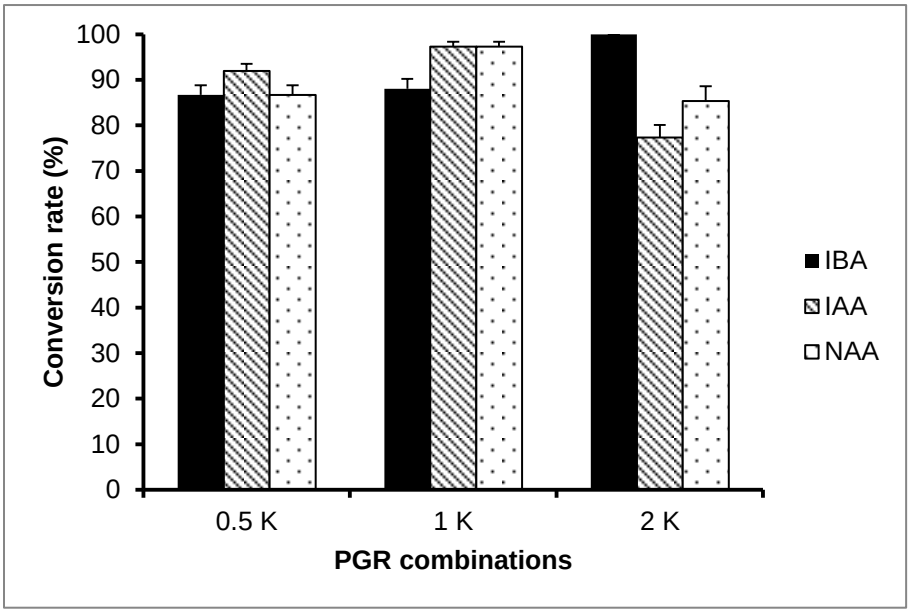


Figure 41. The interaction effect between kinetin concentrations and auxin types on the conversion rate of artificial seeds.

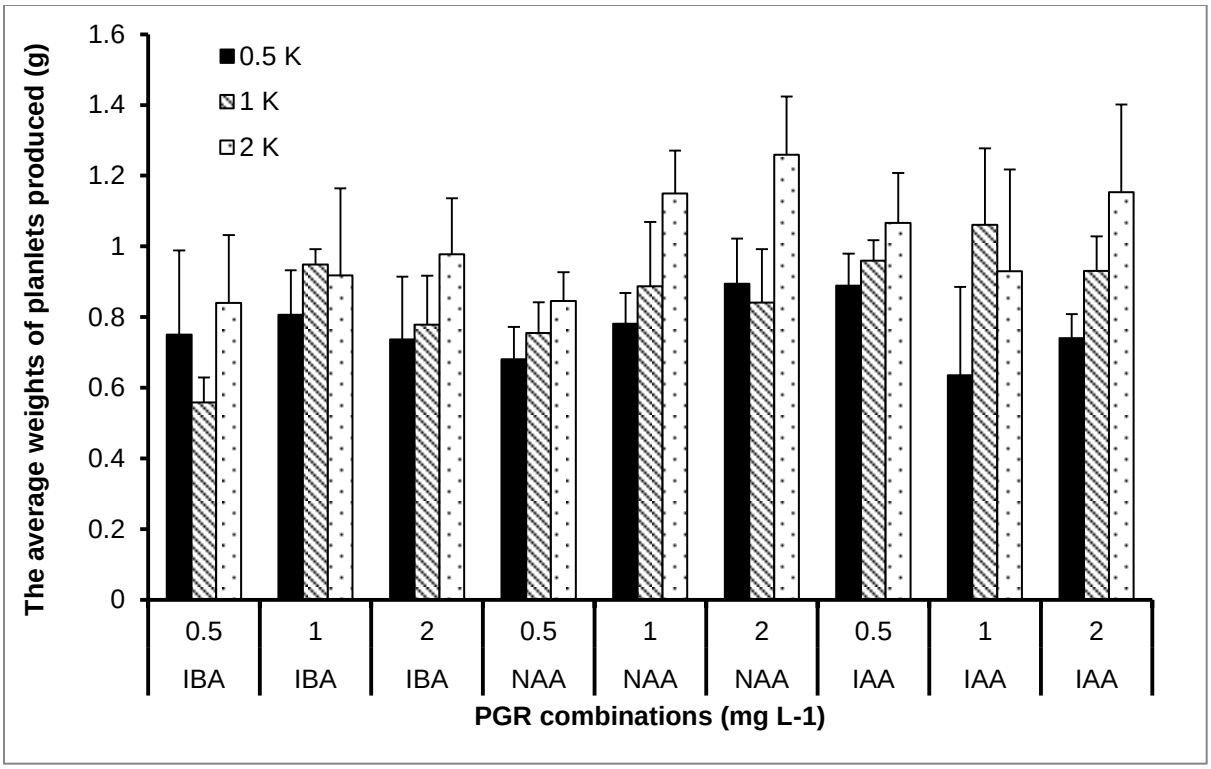


Figure 42. The effect of the artificial seed PGR matrix combinations on the artificial seed conversion rate.

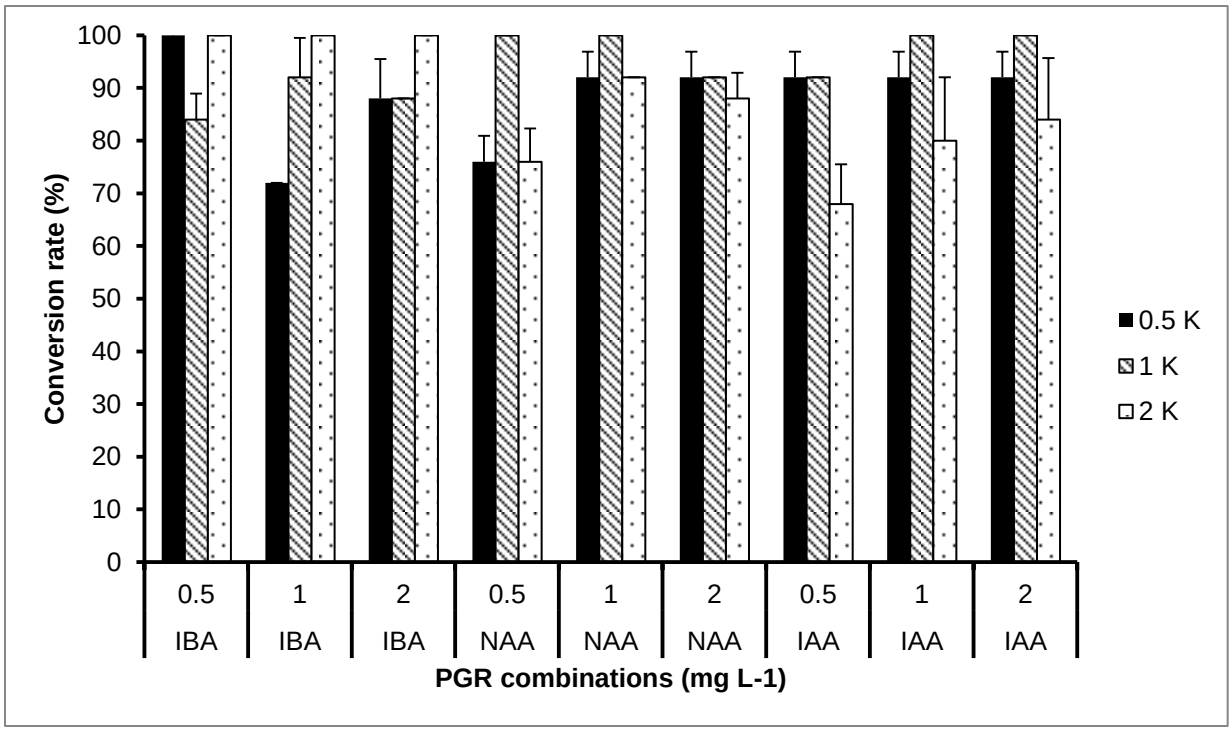


Figure 43. The effect of the PGR combinations used with artificial seeds matrix on the produced artificial seed viability.

3.2.5. Experiment 5

The effect of air bubbles involved in artificial seed matrices

3.2.5.1. Aims and objectives

The aim was to investigate whether the insertion of air bubbles in the artificial seed capsules would improve its conversion rate and viability.

3.2.5.2. Materials and methods

SOP_{ASP} was used for the production of cauliflower artificial seeds (cv Borden). 300-600 µm explants size class were used for microshoot production and 13 day-old microshoots were used for artificial seed production. Small air bubbles were contained in the artificial seed coats. A sterile air current was released within a sodium alginate solution using air pump (Hozelock cyprio, model 1380-0000) and filter (Millex HA, 0.22 µm). Because of the high rigidity of 2% sodium alginate small bubbles of air were trapped in the solution and these bubbles were involved then inside the artificial seed coat. The conversion rates of artificial seeds and fresh weights of produced plantlets were evaluated after 15 days of culture in maintenance semi solid medium. Three replications, each consisting of 10 artificial seeds were used for each treatment. Artificial seeds were cultivated in plastic pots (10*10*8 cm). Four lines, each containing 5 seeds were cultivated within each pot. The treatments were distributed randomly within the pots.

3.2.5.3. Results

It was found that there was no significant difference between the two types of artificial seeds tested in terms of the conversion rate and viability (Figure 44). The conversion rate was evaluated at 100% using both types of artificial seeds.

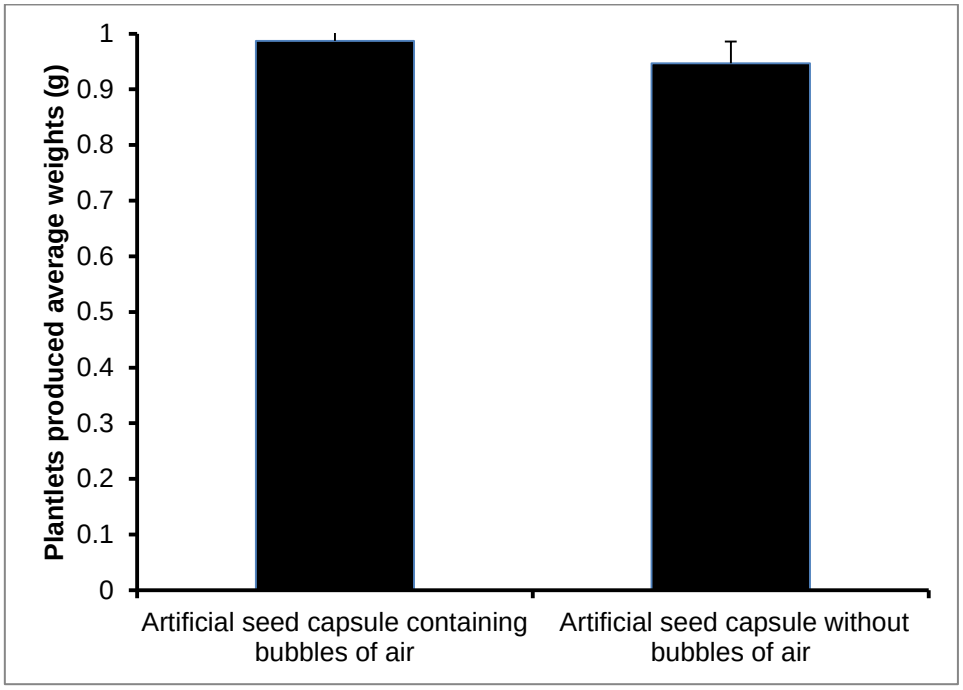


Figure 44. The average weights of plantlets obtained using two types of artificial seeds, one with air bubbles in its capsules and another without it.

3.2.6. Experiment 6

The effect of sucrose concentration used with cauliflower artificial seed matrixes in their growth and development

3.2.6.1. Aims and objectives

This experiment aimed to investigate the effect of sucrose sugar concentration used with artificial seed matrixes on both artificial seed conversion rate and viability.

3.2.6.2. Materials and methods

SOP_{ASP} was used for the production of cauliflower artificial seeds (cv Dionis). 300-600 µm explants size class were used for microshoot production and 13 day-old microshoots were used for artificial seed production. Six concentrations of sucrose were used with artificial seed matrixes: 0, 1.5, 3, 4.5, 6 and 7.5 %. Artificial seeds were cultured in semi-solid media supplemented with 2 mg L^{-1} IBA. 10 replicates, each consisting of 4 artificial seeds were cultivated in culture pots (four seeds per pot) and used for each treatment. The cultures were kept in a growth cabinet at 23 ºC for 25 days when artificial seeds conversion rate and viability (plantlets fresh weight) were assessed.

3.2.6.3. Results

The use of sucrose with artificial seed matrixes significantly reduced both artificial seed conversion rate and viability (P<0.001). The higher the sucrose concentration used, the lower than conversion rate and viability. Moreover, none of cultivated artificial seeds was able to convert when sucrose concentration higher than 6 % was used with artificial seed capsules (Figures 45 and 46).

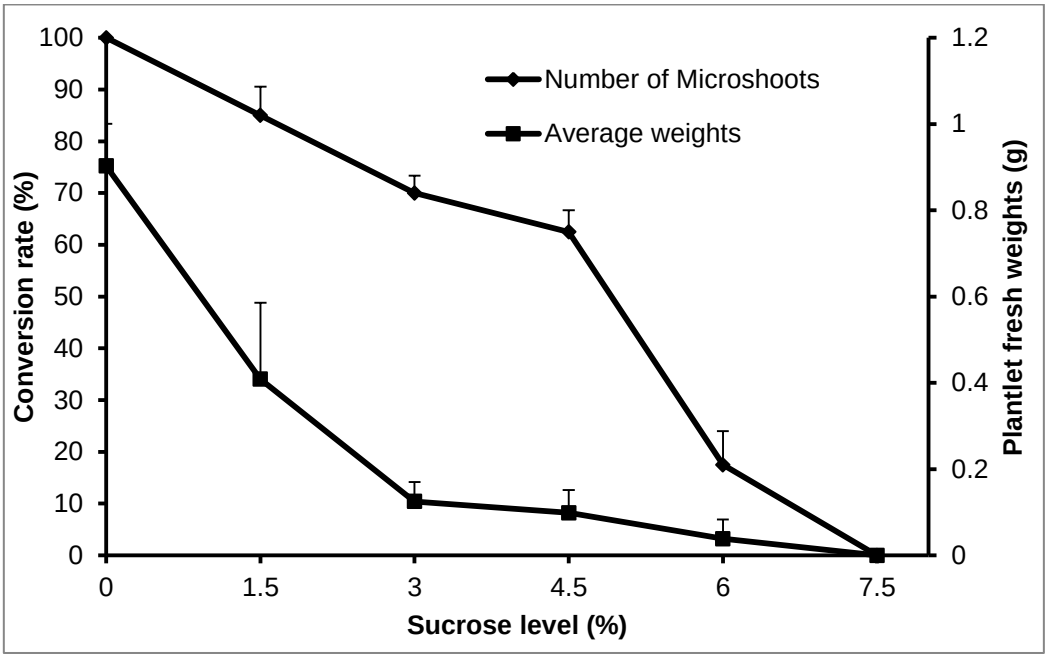


Figure 45. The effect of sucrose used with artificial seed matrixes on their conversion rate and viability (LSD= 11.65 for the conversion rate and LSD= 0.741 for the viability)

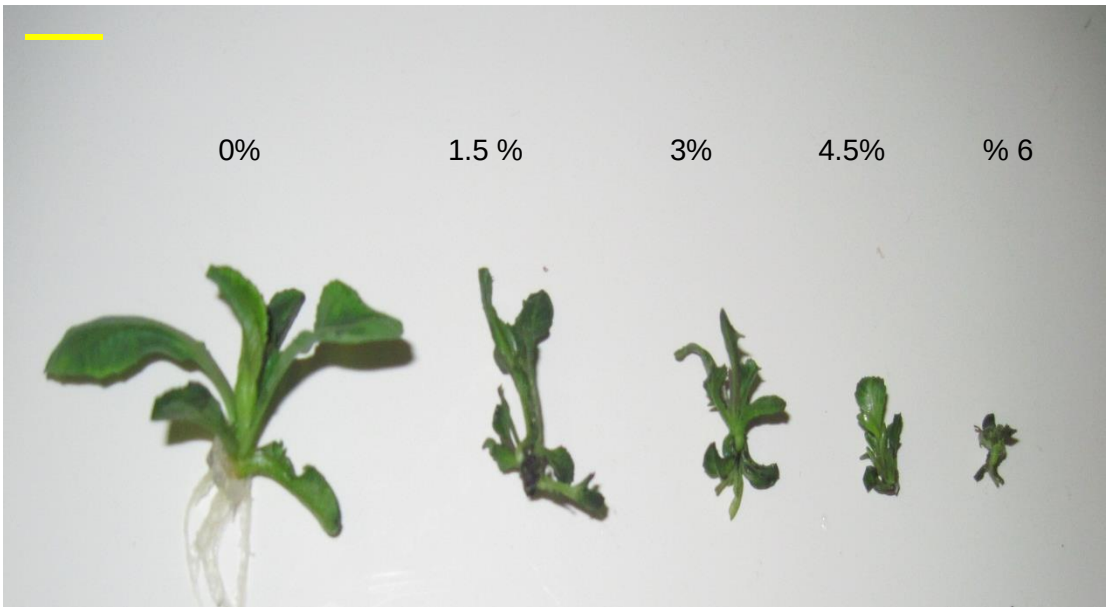


Figure 46. The effect of sucrose used with artificial seed matrixes on their development and growth (Bar = 1 cm).

3.2.7. Experiment 7
The optimization of artificial seed conversion in semi-solid media

3.2.7.1. Aims and objectives

The aim was the investigation of the effect of PGRs used with artificial seed semi solid culture media on their conversion rate and viability assessed as plantlets produced average fresh weights. The determination of the optimal PGR type and concentration used with artificial seed culture media was also one of the main objectives of this experiment.

3.2.7.2. Materials and methods

SOP_{ASP} was used for the production of cauliflower artificial seeds (cv Clemen). 212-300 µm explants were used for microshoots production.13-day-old microshoots were encapsulated. Various auxins types and concentrations were added to the semi solid media used for artificial seed cultivation. 9 PGR treatments were used. Three types of auxins (IBA, IAA, NAA) each at three concentration (0.5 mg L^{-1}, 1 mg L^{-1} and 2 mg L^{-1}) from each were tested. For each treatment, 5 replications each consisting of 3 artificial seeds were cultivated in culture pots. Three seeds were cultivated in each pot. The effect of PGRs on the artificial seed conversion rate and viability assessed as plantlets fresh weights were evaluated after 21 culture days.

3.2.7.3. Results

No significant effect of auxin on the artificial seed conversion rate was found (Figure 47), irrespective of the type and concentration used with semi-solid culture medium (P = 0.871). However, the conversion rate was found to be quite high and exceeded 90 % in most cases.

In terms of artificial seed viability assessed as plantlets fresh weights, auxin concentrations had a significant effect (P < 0.05). The presence of auxin at low

concentration (0.5 mg L^{-1}) in the culture media gave the optimal results at average plantlets fresh weight assessed to be 0.973 g whereas it was 0.660 g and 0.764 g using 1 mg L^{-1} and 2 mg L^{-1} auxin concentrations respectively.

A highly significant interaction was also observed between the concentrations and types of auxins ($P < 0.05$) (Figure 48). However, although the use of 0.5 mg L^{-1} of NAA gave the best results (Figure 48) in terms of artificial seeds viability, the level of callus observed was very high (Figure 49).

Callus was observed using different treatments such as (1 mg L^{-1} and 2 mg L^{-1}) IAA, (0.5 mg L^{-1}, 1 mg L^{-1}, 2 mg L^{-1}) NAA. It seemed to be that the type and concentrations of auxins had a significant effect on the callus formation. The stronger the auxin and the higher its concentration, the more callus was observed.

The use of IBA improved the viability of artificial seeds (in comparison with the use of maintenance S23 (Figure 46) and did not lead to callus formation (Figure 49). The use of 2 mg L^{-1} of IBA gave the best results in terms of artificial seeds viability and considering the point that the conversion rate obtained using this treatment was found to be 100%, this treatment is recommended to be used with subsequent semi-solid culture media.

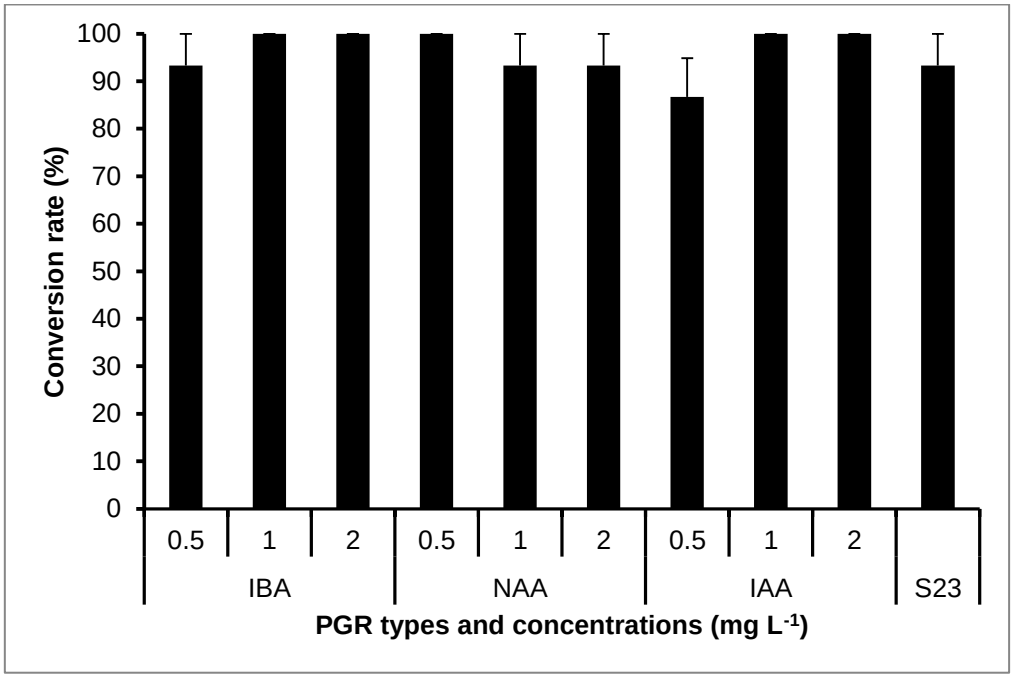


Figure 47. The effect of artificial seeds culture media PGRs on their conversion rate.

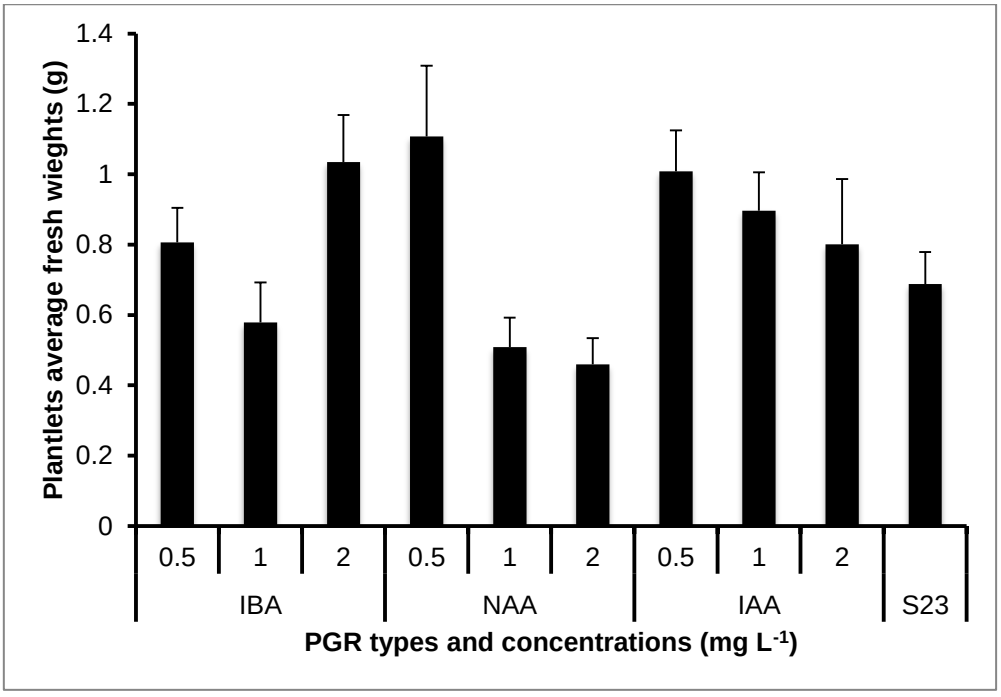


Figure 48. The effect of artificial seeds culture PGRs on their viability (LSD = 0.364).

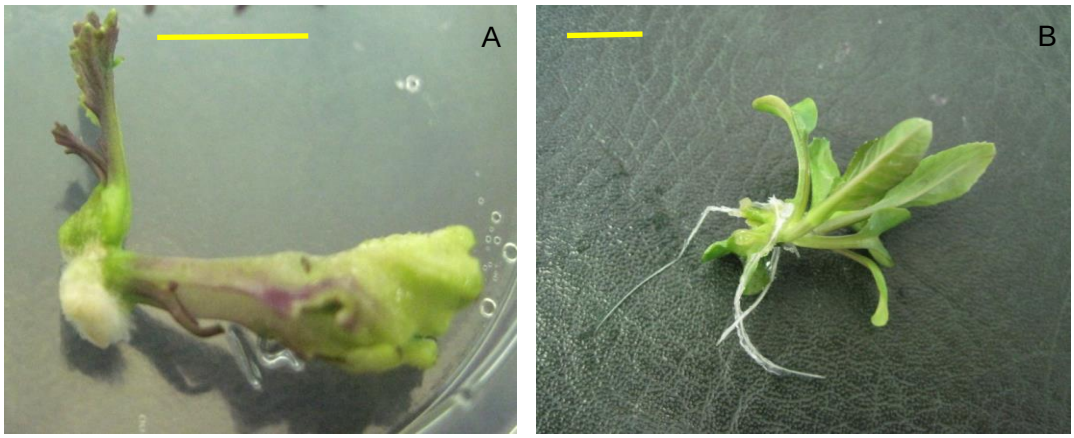


Figure 49. A) Callus produced when 0.5 mg L^{-1} NAA was used with the culture media. B) Plantlets obtained when 2 mg L^{-1} IBA was used with the culture media (Bar = 1cm).

3.2.8. Experiment 8

The optimization of artificial seed conversion in commercial substrates

3.2.8.1. Aims and objectives

This experiment was designed to achieve several aims described as follows,

1. To investigate the suitability of sterilized commercial substances (compost, perlite, sand and vermiculite) (Plymouth Garden Centre, Crownhill, Plymouth) for artificial seed conversion as a cheaper alternative to using semi solid media and as a first step for testing the feasibility of using artificial seeds in unsterilized conditions (culture in the field).
2. To explore whether the use of sterilized water can be sufficient for artificial seeds conversion or whether the use of S23 solution is to be considered as essential for artificial seed conversion.
3. To investigate the effect of adding PGRs to the liquid media used for the moistening of culture substances.
4. To investigate the effect of using PPM used with maintenance liquid media used for substrate irrigation.

3.2.8.2. Materials and methods

SOP_{ASP} was used for cauliflower artificial seed production (cv Mascaret MAR). Five replications of 6 artificial seeds were placed onto different sterilized substrates (compost, vermiculite, perlite and sand) to assess their suitability for conversion and establishment using 11 day old microshoots produced from 212 to 300 µm explants and cultivated in S23 liquid medium supplemented with 2 mg L^{-1} Kinetin and 1 mg L^{-1} IBA. Ten different irrigation solutions were assessed with each substrate as follows, (1) SDW. (2) S23 PGRs free. (3) Eight combinations of S23 supplemented with

various combinations of Kinetin (1 and 2 mg L^{-1}) with IBA or NAA (1 and 2 mg L^{-1}). Each pot was irrigated with 75 mL of the irrigation solution. Artificial seed conversion rate and the fresh weight of plantlets produced were evaluated after 50 days of culture.

3.2.8.3. Results

The use of sterile distilled water did not support the growth of artificial seeds since the conversion rate of artificial seed was low using this treatment whatever culture substance was used. Thus, it was concluded that it was necessary to provide the artificial seeds with nutrient elements required for growth during conversion.

Highly significant differences were caused by PGR treatments used with irrigating culture media on the conversion rate of artificial seeds (Table 5). However, the use of PGR did not improve the conversion rate and the use of S23 and S23 supplemented with 2 mg L^{-1} Kinetin + 2 mg L^{-1} NAA treatments gave the optimal results (Figure 50).

A highly significant effect of the culture substrates was observed (Table 4). The use of perlite was found to be the optimal at conversion rate assessed to be 90.74 % (Figure 51).

A very high interaction was found between culture substrates and liquid irrigation structure (Table 5, 6 and 7). The best combinations were found to be the use of S23 PGR free and S23 supplemented with 2 mg L^{-1} Kinetin + 2 mg L^{-1} NAA treatments with perlite with a conversion rate assessed at 96.7 % for both of them.

Table 5. Result analysis of the effect of culture substances and PGRs used with irrigating media on the artificial seeds conversion rate.

Source	*P*	Significant
PGR combinations	<0.001	***
Culture substances	<0.001	***
PGR combinations*Culture substances	<0.001	***

Table 6 The effect of irrigation composition and culture substrates on artificial seed conversion rate (%) S23 was supplemented with the described PGRs. (LSD=10.528 for PGR combinations, LSD = 5.84 for Culture substrates and LSD = 2.105 for PGR combinations x Culture substrates (interaction)).

PGRs (mg L^{-1})			Culture substrates				
Kin	**IBA**	**NAA**	**Perlite**	**Sand**	**Compost**	**Vermiculite**	**Treatment averages**
1	1	0	90^{c}	67^{g}	36^{l}	63^{h}	**64^{b}**
1	2	0	82^{e}	93^{b}	34^{lm}	33^{m}	**60^{bc}**
1	0	1	93^{b}	43^{k}	33^{m}	56^{i}	**56^{bc}**
1	0	2	83^{e}	63^{h}	26^{o}	30^{n}	**50^{c}**
2	1	0	93^{b}	97^{a}	73^{f}	30^{n}	**73^{ab}**
2	2	0	87^{d}	43^{k}	55^{i}	26^{o}	**52^{c}**
2	0	1	93^{b}	90^{c}	43^{k}	30^{n}	**64^{b}**
2	0	2	97^{a}	87^{d}	67^{g}	46^{j}	**74^{a}**
0	0	0	97^{a}	83^{e}	90^{c}	46^{j}	**79^{a}**
average			**91^{a}**	**74^{b}**	**50^{c}**	**40^{d}**	

Table 7. Least significant differences between PGR combinations and between culture substances and for the interaction between them.

Source	LSD
PGR combinations	10.528
Culture substances	5.84
PGR combinations*Culture substances	2.105

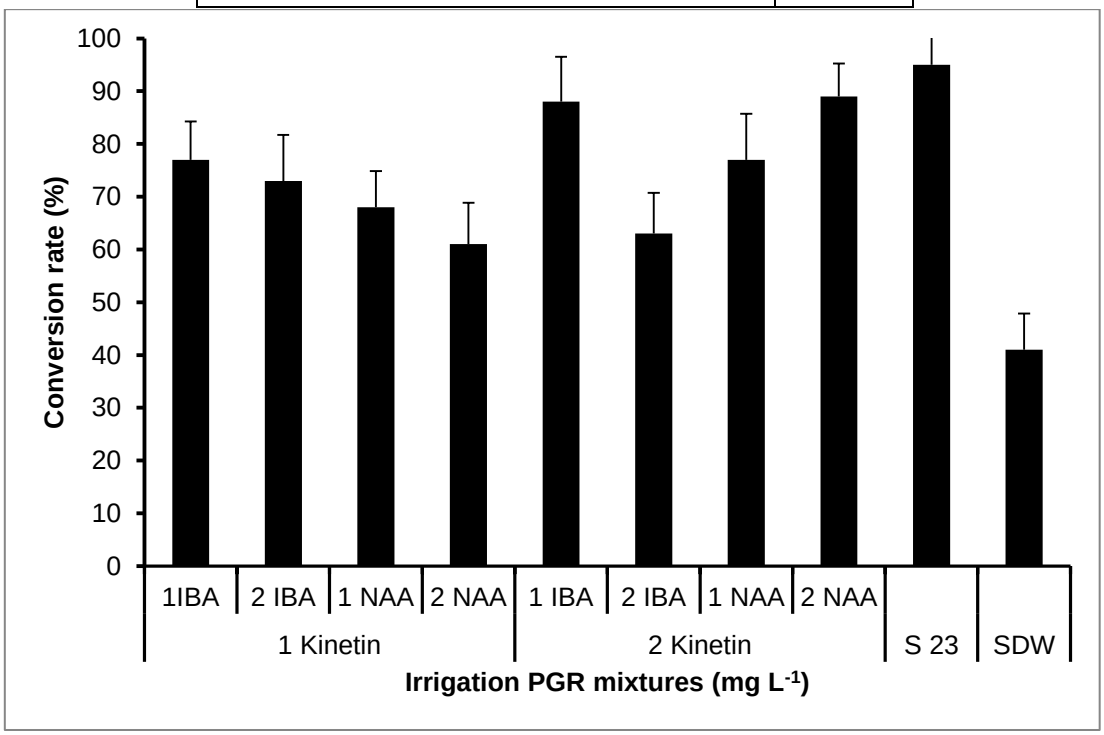


Figure 50. The effect of the irrigation PGR combinations on the conversion rate of artificial seeds.

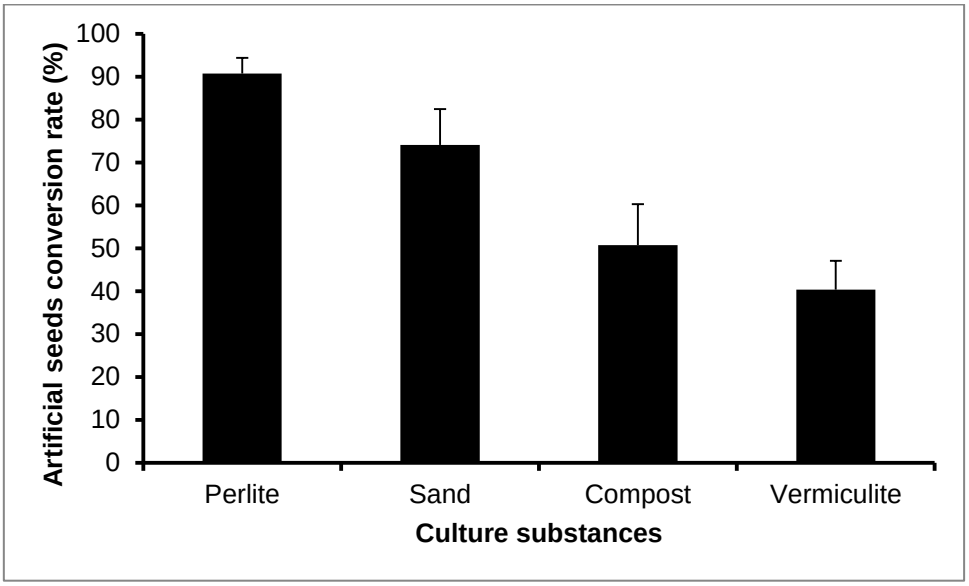


Figure 51. The effect of culture substances on the artificial seed conversion rate (LSD = 7.018).

No significant effect on artificial seed viability was found when PGRs were used with liquid media (Table 8).

A very highly significant effect on the artificial seeds viability was observed for the case of culture substances (Table 8) (Figure 52). However, the use of compost was found to be optimal with an average plantlet fresh weight assessed to be 0.127 g per plantlet (Figure 52).

A very high interaction between the culture substances and PGR combinations was also found (Table 8). The best combination observed was when compost was used as a substrate and moistened with culture media supplemented with 2 mg L^{-1} Kinetin + 2 mg L^{-1} NAA or 2 mg L^{-1} Kinetin + 2 mg L^{-1} IBA PGR combinations (Tables 8, 9 and 10).

Table 8. The statistical analysis results showed the effect of liquid culture media structure and culture substances on the artificial seeds viability.

Source	P	Significances
Phytohormone combinations	0.237	-
Culture substances	<0,001	***
Phytohormone combinations*Culture substances	0.011	**

Table 9. The effect of irrigation composition and culture substrate on the plantlet fresh weight (g) S23 was supplemented with the described PGRs. (LSD = 0.022 for culture substrates and LSD = 0.080 for the interaction between the PGR combinations and culture substrates).

PGRs (mg L^{-1})			Culture substrates				
Kin	**IBA**	**NAA**	**Perlite**	**Sand**	**Compost**	**Vermiculite**	**Average**
1	1	0	0.068^{efghij}	0.064^{fghij}	$0.109^{bcdefghi}$	0.050^{ghij}	**0.072**
1	2	0	0.051^{ghij}	0.075^{efghij}	0.178^{ab}	0.030^{ij}	**0.083**
1	0	1	0.071^{efghij}	0.025^{j}	0.234^{a}	0.050^{ghij}	**0.095**
1	0	2	0.073^{efghij}	$0.12^{bcdefgh}$	0.044^{hij}	0.033^{ij}	**0.067**
2	1	0	0.061^{ghij}	0.073^{efghij}	$0.092^{cdefghij}$	0.034^{ij}	**0.065**
2	2	0	$0.087^{defghij}$	0.054^{ghij}	0.167^{abc}	0.033^{ij}	**0.085**
2	0	1	0.061^{ghij}	0.141^{bcdef}	0.124^{bcdefg}	0.031^{ij}	**0.089**
2	0	2	0.072^{efghij}	0.160^{abcd}	0.144^{bcde}	0.044^{hij}	**0.105**
0	0	0	0.052^{ghij}	0.072^{efghij}	0.059^{ghij}	0.027^{ij}	**0.052**
Average			**0.066^{b}**	**0.087^{b}**	**0.128^{a}**	**0.037^{c}**	

Table 10. Least significant differences between culture substances and for the interaction between the PGR combinations and culture substances.

Source	LSD
culture substances	0.022
PGR combinations*culture substances	0.0803

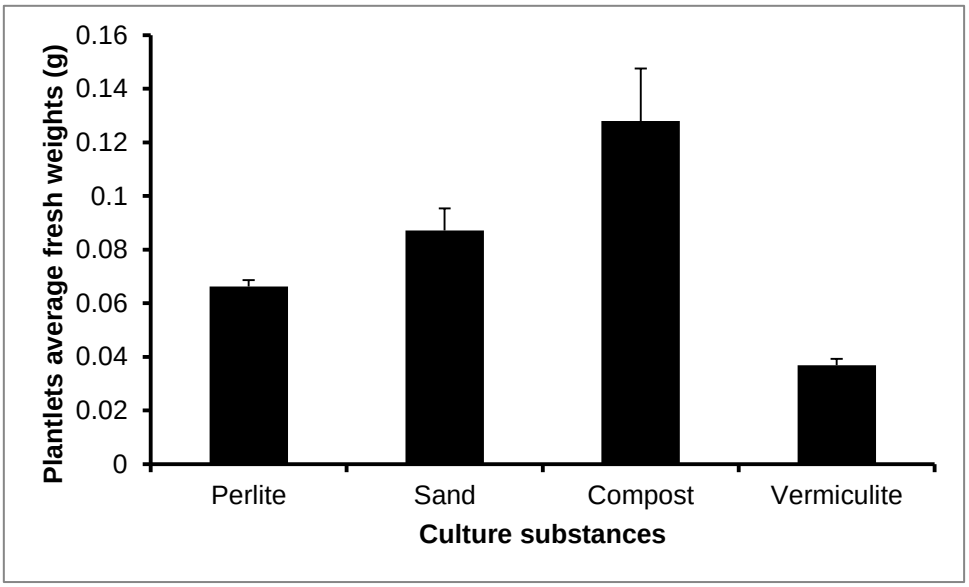


Figure 52. The effect of artificial seeds culture substances on their viability (LSD= 0.022).

3.2.9. Experiment 9

The effect of using PPM (plant preservative mixture) on the conversion of cauliflower artificial seed produced

3.2.9.1. Aims and objectives

This study aimed to investigate the effect of using PPM with liquid medium applied as fertigation during artificial seed conversion in various commercial growing substrates (compost, perlite, sand and vermiculite).

3.2.9.2. Materials and methods

SOP_{ASP} was used for cauliflower artificial seed production (cv Mascaret MAR). Eleven day-old microshoots produced using the 212–300 µm size class explants were used for artificial seed production. Artificial seeds were then cultivated on four different sterilized substrates (compost, vermiculite, perlite and sand) contained in plastic cuboid culture vessels (10*10*8 cm). Each vessel used contained 200 ml of the substrate described. Four different irrigation solutions were assessed with each culture substrate as follows: (1) S23 supplemented with 1 ml L^{-1} PPM; (2) S23 supplemented with 5 ml L^{-1} PPM; (3) S23 supplemented with 10 ml L^{-1} PPM; (4) S23 with no PPM. Each vessel was irrigated with 75 mL of the irrigation solution and the vessels were covered and kept in a growth cabinet (Sanyo, Leicestershire, UK) at 23 ◦C. Artificial seeds were cultivated in 5 lines of 6 seeds and each line was considered as a replicate. Artificial seed conversion rate and viability was assessed as fresh weights of plantlets produced after 50 days of culture.

3.2.9.3. Results

Very highly significant effects of the culture substrates were observed in terms of artificial seed conversion rate ($P < 0.001$) and viability ($P = 0.001$). However, the use

of sand was found to be the optimal with a conversion rate assessed at 91.667% and fresh plantlets average weights evaluated at 0.1263 g (Figure 56).

Significant differences were also observed when PPM was used with artificial seed irrigation liquid media in terms of both the conversion rate ($P < 0.001$) and viability ($P = 0.037$). While the presence of the PPM at low concentrations (1 and 5 ml L^{-1}) had negative effects on the conversion rate, the use of it at relatively higher concentration (10 ml L^{-1}) did not have this effect and while positive effects were observed of using PPM at relatively low concentration on the artificial seeds viability, the relatively high concentration did not have this effect. However, the higher the PPM concentration that was used, the lower the artificial seed viability was observed (Figure 53).

A very high interaction between culture substrates and PPM was observed ($P < 0.001$) in terms of artificial seed conversion rate. However, high conversion rates were obtained using different treatments such as the use of relatively high concentration of PPM (10 ml L^{-1}) with compost or the use of relatively low concentrations of PPM with sand (Figure 54). However, considering the point that the viability of artificial seeds was much better using sand as a culture substance rather than perlite, the use of 1 ml L^{-1} or 5 ml L^{-1} of PPM was recommended to be the optimal

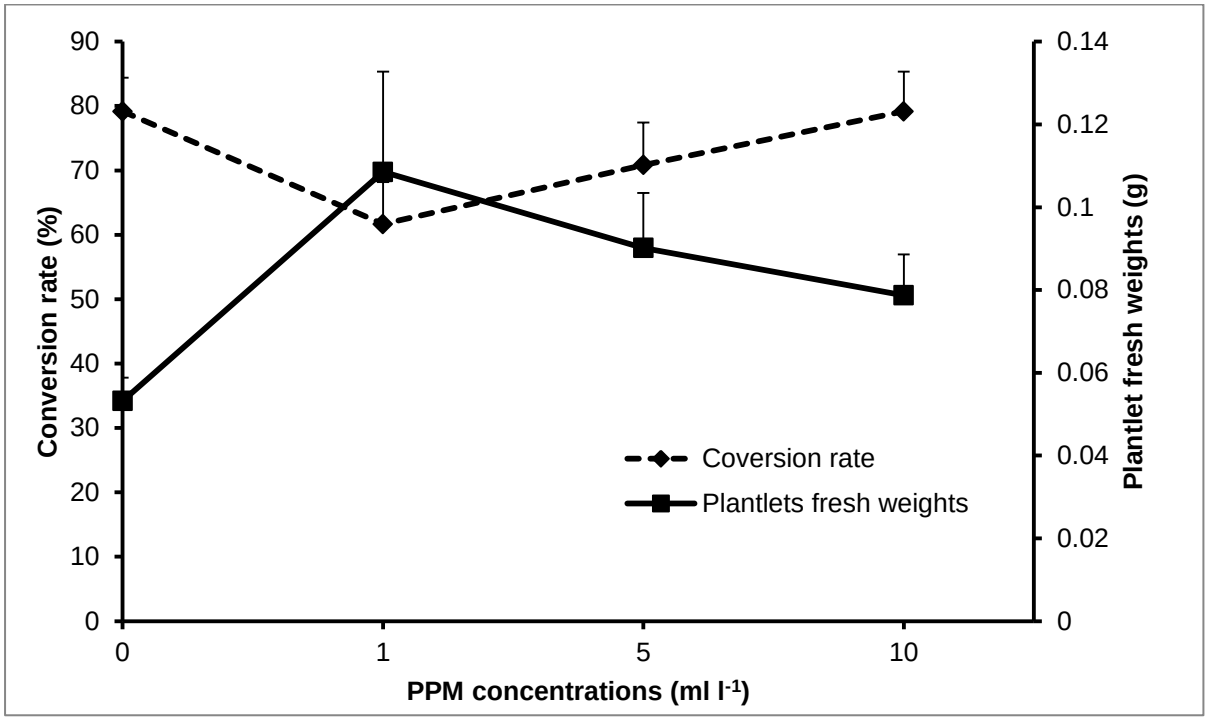


Figure 53. The effect of using PPM in the media used for irrigation on the artificial seeds viability and conversion rate (LSD = 8.333 for conversion rate and LSD = 0.0377 for plantlet fresh weights).

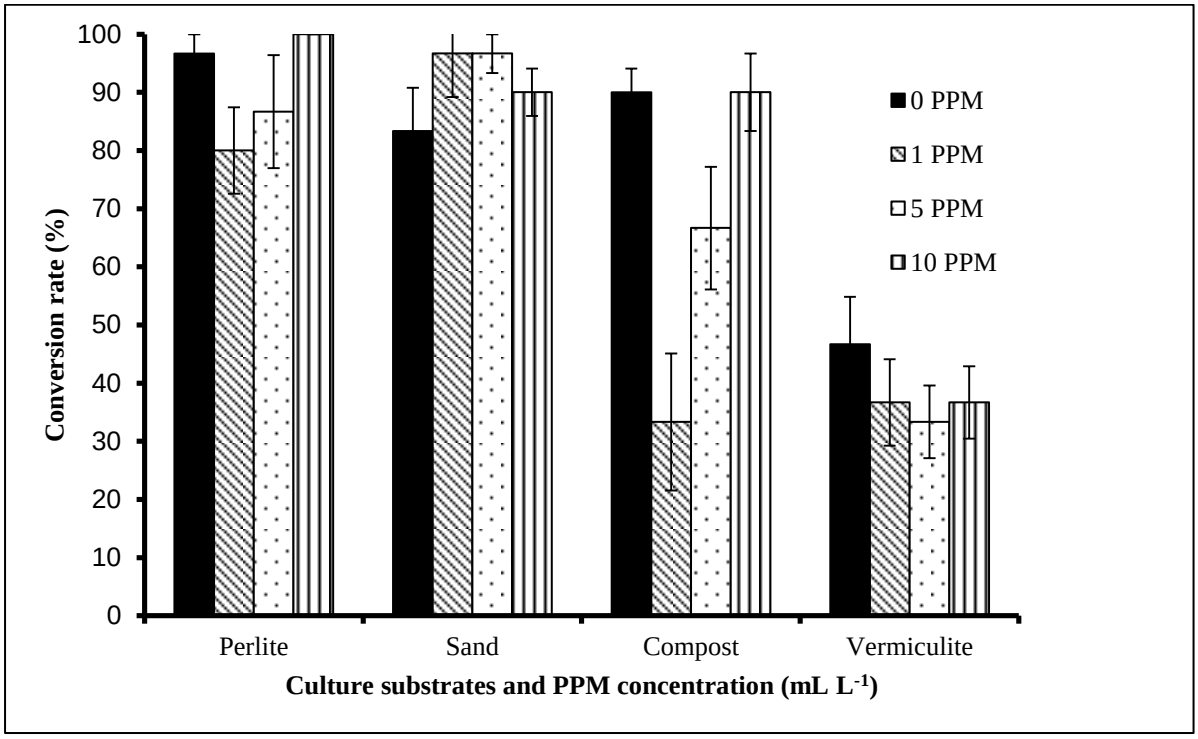


Figure 54. The interaction effect between the culture substances and PPM on the artificial seeds conversion rate.

Figure 55. Plantlets obtained using Compost and S23 free PGRs after 50 days of culture (Bar = 1 cm).

Figure 56. Plantlets obtained using Sand and S23 free PGRs after 50 days of culture (Bar = 1 cm).

Important points emerged from experiments 6, 7 and 8 as a whole; these can be summarized as follows,

1. The irrigation culture structure played an essential role in the artificial seeds conversion rate and viability. It seemed that the presence of sucrose could be

the key factor since the use on S23 free PGRs was able to support artificial seed conversion.

2. The use of PGR combinations did not have positive effects on the artificial seed conversion rate and viability when it was used with culture semisolid media.
3. A high interaction was observed between PGR combinations with culture substances used and high effects of these materials were observed on the artificial seed conversion rate and viability.
4. Regardless the interaction with PGR combinations used with irrigation media, the use of perlite gave the optimal conversion rate and the use of compost gave the optimal artificial seeds viability but, interestingly, the use of sand gave the optimal artificial seeds viability and conversion rate when PPM was used with the irrigation culture media.
5. It was observed that the use of culture substrates delayed the growth of artificial seeds (when the results were evaluated after 50 days of culture).

It was noticed that the perlite was quite dry when the results were evaluated. However, considering the difference between the physical structures of culture materials used and the fact that the same amount of irrigating structure was used with all substrates (75 ml), and since the use of perlite had given the optimal results in terms of artificial seeds conversion rate in the first stage of this experiment, different amounts of liquid media were used in an attempt to improve the viability of artificial seed in the next experiment.

3.2.10. Experiment 10

The effect of the amount of culture medium used with perlite on the conversion and viability of artificial seed produced

3.2.10.1. Aims and objectives

Since the optimal conversion rate was obtained using perlite as a culture substance and irrigated with S23 and since it was noticed that the perlite was dry when the results were assessed in the previous experiment, the aim of this experiment was the assessment of the possibility of raising artificial seeds viability using higher amount of S23 liquid media for irrigation.

3.2.10.2. Materials and methods

SOP_{ASP} was used for cauliflower (cv CHF 0474-APR) artificial seed production.212-300 µm explants were used for microshoots production and 14-day-old microshoots were used for artificial seed production. Four different amounts of S23 liquid media (75ml, 100 ml, 125 ml and 150 ml) were applied. Artificial seeds were cultivated in plastic pots, each containing 225 ml of perlite. The effects of the amount of liquid media used for artificial seeds irrigation on the artificial seeds conversion rate and viability assessed as plantlets average fresh weights were evaluated after 54 days of culture. Five plastic pots each containing 225 ml of perlite and cultivated with 16 artificial seeds (4 lines and 4 seeds per line) were used for every treatment and each line was considered as a replication. Treatments were distributed randomly in the culture pots.

3.2.10.3. Results

While the use of 100 ml of S23 media gave the optimal conversion rate and was significantly different compared with other treatments used (P = 0.026) (Figure 57),

the amount of irrigation media was found to have no significant effect on the viability of artificial seeds (*P* = 0.254).

It was observed that the plantlets started growing to a certain level and then stopped. The use of this material does not support the growth of plantlets in the long term (i.e. until they could be transferred to the field).

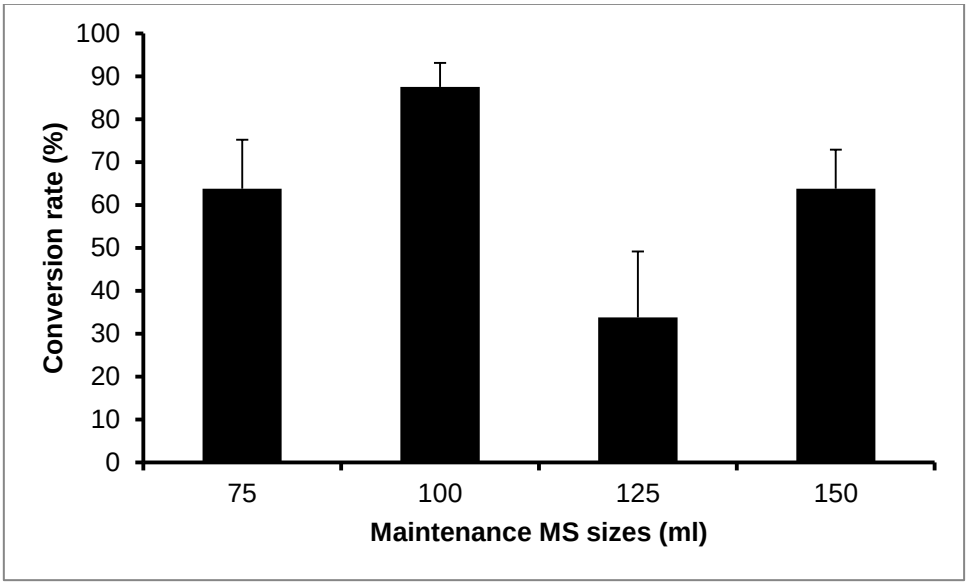


Figure 57. The effect of irrigation media sizes on the artificial seeds conversion rate (LSD = 32.92).

3.2.11. Experiment 11

Cauliflower artificial seed dehydration tolerance

3.2.11.1. Aims and objectives

The aim of this experiment was to determine the artificial seed capability for dehydration (the effect of dehydration on the artificial seeds viability and conversion rate) and later, in the following steps the feasibility of using this to improve cauliflower artificial seeds cryopreservation ability.

3.2.11.2. Materials and methods

SOP_{ASP} was used for cauliflower artificial seed production (cv Mascaret MAR). 148 µl explants (212-300 µm size class) was cultivated per pot for microshoots production.15 day-old microshoots were used for artificial seed production. The artificial seeds produced were left in a laminar flow hood for 19 hours. The weight of 30 artificial seeds were taken after 0, 2.5, 5, 7.5, 10, and 19 hours using 5 decimal point balance (Oxford- Model A 2204) and then the dry weight of these was measured after drying at 105ºC for 48 hours (Figure 58). Artificial seed moisture levels were calculated using the following equation,

$$\text{Moisture level} = \frac{\text{artificial seed measured weight} - \text{artificial seed dry weight}}{\text{Artificial seed fresh weight}} * 100$$

Samples of artificial seeds were cultivated after 0, 2.5, 5, 7.5, 10, and 19 hours of dehydration in plastic pots each containing 100 ml of maintenance liquid media supplemented with 2 mg L^{-1} of IBA. The effect of the moisture level of artificial seeds on their conversion rate and viability evaluated as fresh plantlets weights was assessed after 28 days of culture.

5 replications each consisting of 6 artificial seeds were cultivated in a plastic pot for every assessment period (0, 2.5, 5, 7.5, 10, and 19 hours of dehydration). 5 lines each consisting of 5 seeds were cultivated in each pot (10*10*8 cm). The treatments were distributed randomly within the pots.

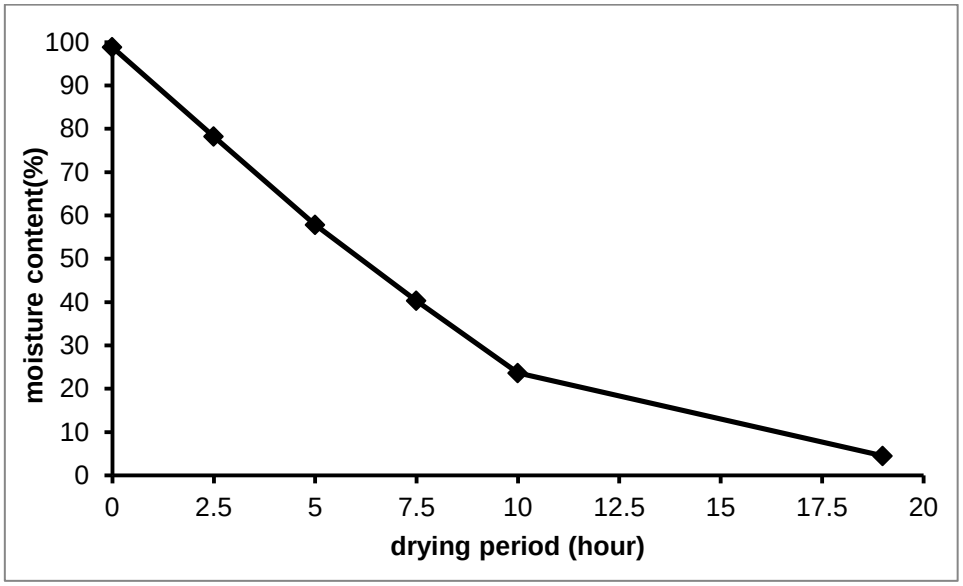


Figure 58. Artificial seed moisture levels during subsequent drying steps.

3.2.11.3. Results

No significant effects of the artificial seeds moisture level on its conversion rate (*P* = 0.284) (Figure 59) and viability (*P* = 0.537) were observed (Figure 60). The conversion rate was found to be high (73.33 %) at very low moisture level (4.478 %) (Figure 60) and the viability of plantlets produced assessed to be relatively good with average weight assessed to be 0.106 g.

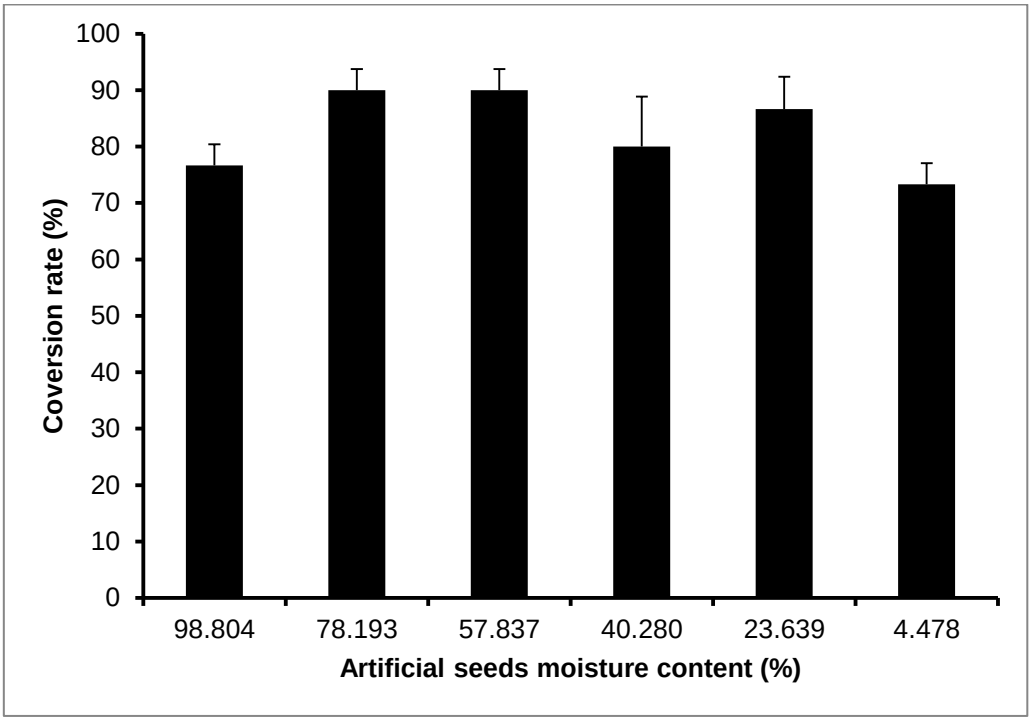


Figure 59. The effect of the artificial seed moisture level on its conversion rate (LSD = 18.695).

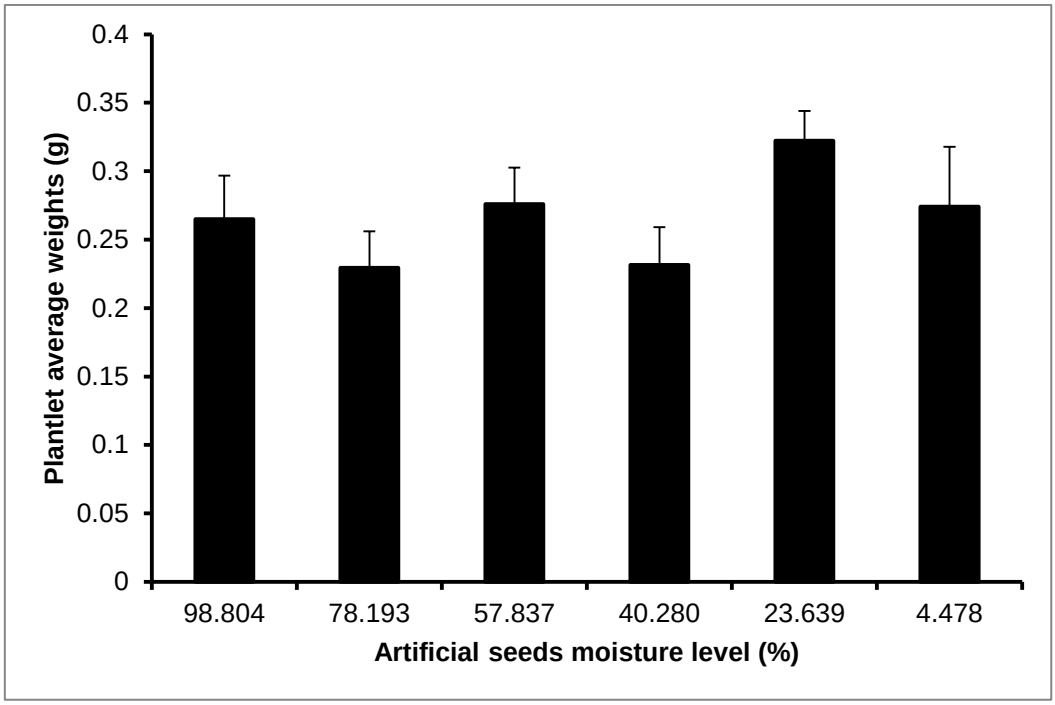


Figure 60. The effect of the artificial seed moisture level on its viability (LSD = 0.0928).

3.2.12. Experiment 12

Cauliflower artificial seed cold storage

3.2.12.1. Aims and objectives

The aim of this experiment was to investigate the suitability of cold storage at 4 ºC for cauliflower artificial seed short term storage. The effect of cold storage on both artificial seed conversion rate and viability was investigated.

3.2.12.2. Materials and methods

SOP_{ASP} was used for cauliflower artificial seed production (cv Dionis). 74 µl explants (300-600 µm size class) was cultivated per culture pot and15 day-old microshoots were used for artificial seed production. Artificial seeds were cultured in culture pots which each contained 30 ml of semisolid S23 media supplemented with 2 mg L^{-1} of IBA. 5 artificial seeds were cultured per pot. Artificial seeds were then kept in a cold room at 4 ºC Plymouth University. Artificial seed samples were collected 16 times at 15 day intervals and transferred to growth cabinet at 23ºC. 5 culture pots were used per treatment (cold storage duration). Artificial seed conversion rate and viability were assessed after one month of treatment samplings.

3.2.12.3. Result

Cauliflower artificial seeds showed high capacity for cold storage and the conversion rate was assessed to be over 80% for the first 7 months. However, the conversion rate decreased significantly with longer storage durations (P<0.001) (Figure 61).

In terms of the artificial seed viability, artificial seeds kept their viability for 6 months and 15 days, however, the viability significantly decreased with a longer storage period (P<0.001) (Figure 62). It was also observed that artificial seeds were growing slowly during the storage at 4 ºC indicating that a temperature lower than that is

required to stop the growth of artificial seeds. No artificial seed was able to germinate after 8 months of cold storage.

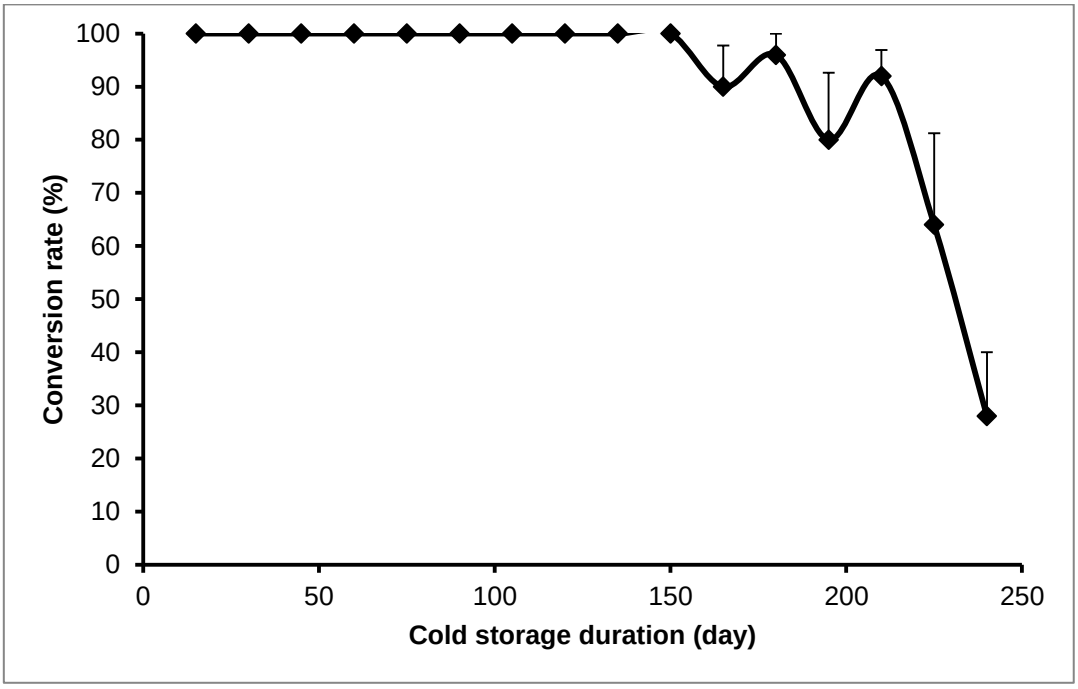


Figure 61. The effect of cold storage duration on the conversion rate of artificial seeds (LSD=17.1).

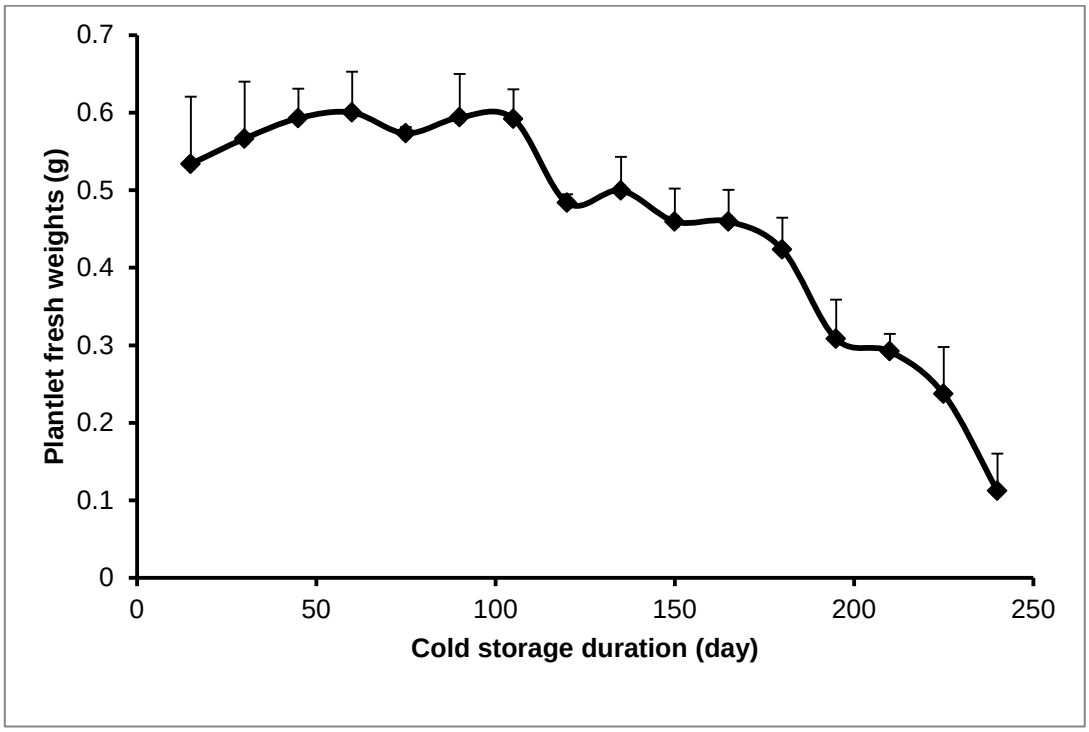


Figure 62. The effect of cold storage duration on the viability of cauliflower artificial seeds (LSD=0.61).

3.2.13. Experiment 13

Artificial seeds cryopreservation

3.2.13.1. Aims and objectives

The aim of this experiment was the investigation of cauliflower artificial seed cryopreservation capacity as a major option for long term storage. Different protocols were examined in order to determine the optimal procedure suitable for artificial seeds storage in LN (liquid nitrogen) at -196ºC as a main aim and -80 ºC and -20 ºC as alternatives approaches.

3.2.13.2. Materials and methods

Mascaret MAR cauliflower variety microshoots were used for artificial seed production using SOP_{ASP}. 212-300 µm size class explants were employed for microshoot production. The microshoots were classified using accurate mesh size made for this purpose and 2-4 mm size class of 18-day-old microshoots were used for artificial seed production. Four cryoprotectant materials were incorporated with sodium alginate solution before tyndallisation process, DMSO (Dimethyl sulfoxide), Glycerol, Mannitol and Sucrose. DMSO, Glycerol and Mannitol were used at 6% and 12% concentrations, whilst 15 and 30 % sucrose concentration were used (Figure 63). Three cryofreezing programmes were applied to each cryoprotectant material, using the cryo-freezer (KRYO10 series cryo-freezer). The cryofreezing programmes were described as follows,

1. **Programme 1**: straight immersion in liquid nitrogen.
2. **Programme 2**:
    1. Frozen to -15º C at – 4º C per minute
    2. Frozen to -20º C at -1º C per minute
    3. Held at -20 º C for 1 hour

4. Frozen to -60 º C at -25 º C per minute
5. Transferred to liquid nitrogen

3. Programme 3:

1. Frozen to -15 º C at 4 º C per minute
2. Frozen to -20 º C at -1 º C per minute
3. Transferred to liquid nitrogen

The treated artificial seeds were kept in liquid nitrogen for one hour and then transferred to warm maintenance liquid media (30º C) for 15 min before being cultivated in semi-solid medium supplemented with 2 mg L^{-1} of IBA.

5 replications each consisting of 6 seeds were cultivated in plastic pots (10*10*8 cm) for every treatment. Four lines each containing 6 seeds were cultivated within each pot and the treatments were distributed randomly within the pots. The conversion rate of artificial seeds cultivated was assessed after 10 days of culture.

3.2.13.3. Results

None of the artificial seeds cultivated tolerated the storage in liquid nitrogen since none of them converted.

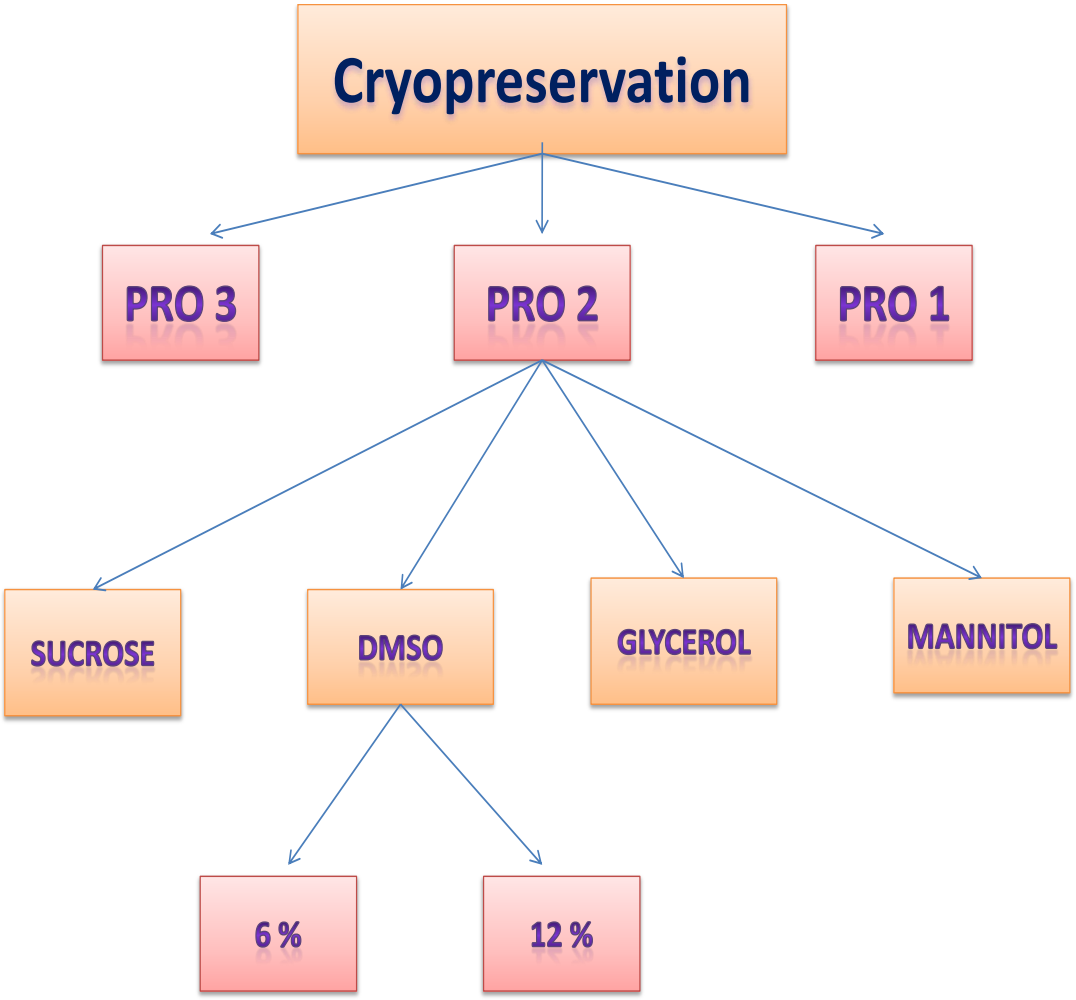


Figure 63. Artificial seeds crypreservation experiment design.

3.2.14. Experiment 14

Cryopreservation of dehydrated artificial seeds

3.2.12.1. Aims and objectives

The aim of this experiment was to investigate whether artificial seed dehydration could improve cryopreservation ability and whether the use of different low temperatures degrees (-20 and -80) could be a good alternative to using liquid nitrogen.

3.2.12.2. Materials and methods

Tozer 3031 cauliflower variety microshoots were encapsulated as artificial seeds using SOP_{ASP}. 300-425 µm explants size class was used for microshoot production. 18-day-old microshoots were classified using accurate mesh and 2-4 mm microshoots size class was used for artificial seed production.

Artificial seeds were dehydrated and cryopreserved before being cultivated in maintenance semi solid media supplemented with 2 mg L^{-1} IBA. Artificial seeds were left in the laminar flow hood for 19 hours (moisture level about 4 %) for dehydration and then cryopreserved using cryo-freezer (KRYO10 series cryo-freezer). All the cryofreezing programmes started at +20º C and the temperature was reduced at -1º C per min to +10º C after which different cryofreezing protocols were applied and described as follows,

- ✓ The temperature was lowered at -4º C per min to -15º C and then reduced at -1º C to -20º C. The artificial seeds were stored at -20º C.
- ✓ The temperature was reduced at -4º C per min to -15º C and then at -1º per min C to -20º C. After that, the temperature was reduced at -25 per min to -80º C. The artificial seeds were stored at -80º C.

- ✓ The temperature was reduced very quickly at -40º C per min to -20º C at which point a quantity of artificial seeds was taken out of the cryofreezer and stored at this temperature, while the reminder were kept at temperature reducing at -40º C per min to -80º c and then stored at that temperature.

Artificial seeds were kept at storage temperature for one week before being transferred to warm liquid culture media for 30 min. The artificial seeds were then cultivated in semi-solid media. Artificial seeds conversion rate was assessed after one week of culture. For each treatment, 5 replications, each consisting of 6 seeds were cultivated in plastic pots. Four lines each containing 6 seeds were cultivated in each pot and the treatments were distributed randomly within the pots.

3.2.12.3. Results

None of artificial seeds cultivated tolerated the storage at described low temperature (-20, -80 and -196 (LN)) since none of them was able to converted.

3.3. Discussion

The described protocol enables production of full plantlets of cauliflower from encapsulated microshoots (artificial seeds). However, the success of cauliflower artificial seed production requires to be optimized at four steps,

1. The production of viable microshoots, which have the capacity to be encapsulated as artificial seed without significant loss of viability.
2. The optimization of encapsulation procedures which includes two important elements:
    a) The optimization of gelling agent types and concentrations in order to produce the optimal quality of artificial seed beads. The artificial seed capsules should protect the micropropagules against mechanical injuries without affecting its viability.
    b) The optimization of the artificial endosperm which is defined as a mixture of additional material such as nutrient elements, sugars, antidesease and growth regulators. The artificial endosperm should be optimized in order to provide the microshoots with all elements necessary for growth.
3. The conversion stage of artificial seeds should be optimized in order to provide the best conditions for artificial seed germination. However, the germination of artificial seed could be affected using semi solid media or commercial substances like compost, vermiculite, sand and perlite.
4. The optimization of artificial seed preservation (short and long storage) applying different approaches such as cold storage, dehydration and cryopreservation.

3.3.1. Microshoot production

This study showed that the organization of cauliflower microshoots seemed highly suitable for encapsulation as artificial seeds. Interestingly, the types and concentrations of plant growth regulators used with microshoots culture media had a great effect on the artificial seed conversion rate and viability. The optimal combination of PGR used for the production of microshoots suitable for artificial seed production (1 mg L^{-1} kinetin + 1 mg L^{-1} IBA) was found to be different from that used for the production of maximum number with highest average weight microshoots (2 mg L^{-1} kinetin + 1 mg L^{-1} IBA)(Chapter 2). Therefore, PGRs used had major effects not only on the number or the fresh weight of developing microshoots but also on the physiological structure of the microshoots produced. It is known that kinetin induces cell division provided that auxin is present in the medium. However, while the relatively high concentration of kinetin (2 mg L^{-1}) induced the growth of explants and the growth of microshoots, it affected their quality and suitability for artificial seed production. The use of a relatively high concentration of kinetin led to the production of poor microshoots which were affected by the encapsulation procedures, reducing the conversion rate and the viability of artificial seed. The use of relatively low concentration (1 mg L^{-1}) was found to be optimal.

The type and the concentration of auxins also affected the conversion rate and the viability of artificial seeds. However, although several studies have demonstrated the positive effect of auxins on the root formation and development (Reed *et al*., 1998, Celenza *et al*., 1995, Boerjan *et al*., 1995, Kares *et al*., 1990, Blakely *et al*., 1988), in the current research, none of the auxin types and concentrations used induced root formation. This could be attributed to the presence of kinetin or/and PPM in the liquid media or it might be caused by the physical structure (liquid) of the medium itself.

Since the artificial seeds were able to germinate and the majority of them established roots in the semi-solid media in the later stage and since the semi-solid media contained 1 ml L^{-1} of PPM, the rooting problem could be caused by the presence of kinetin or the use of liquid media for microshoots production. However, it has been reported by others that the implementation of cytokinin decreases the number of lateral roots (Hinchee and Rost, 1986, Goodwin and Morris, 1979, Böttgor, 1974). Eriksen (1974) working in peas mentioned that the presence of high concentrations of cytokinin could have negative effects on the initial step of rooting by deterring the activity of auxin. Rani Debi *et al* (2005) indicated that cytokinin inhibits lateral root initiation in rice (Oryza sativa). Nakashimada *et al* (1995) also observed inhibition effects of kinetin presence in the culture media on the root elongation of horseradish hairy roots (*Armoracia rusticana*) plantlets. However, Hinchee and Rost (1986) reported that the auxin:cytokinin ratio has an essential role in co-ordinating lateral root growth in pea seedlings.

The problem in the root formation was considered to be a critical point in the current study since it affected other steps of the protocol. The delay observed in microshoot root formation until artificial seed cultivation in the semi-solid media led to a negative phenomenon that the leaves, which continued their growth, raised the base of microshoot above the semi-solid culture media where the root formation was supposed to occur. This type of growing plantlets died after a short period of growth. Therefore, there an obvious necessity for more research to be conducted with the aim of improving the rooting of microshoots, possibly by reducing the concentration of kinetin in the culture medium. The examination of the possibility of using other PGR combination and / or the manipulation of explants and later microshoots culture

medium are important in order to promote their development. This could be done by changing the culture medium in accordance with the stage of micoshoots growth.

The optimal age of microshoot encapsulation was observed to be 13 and 14-days old. The encapsulation of younger and older microshoots had negative effects on the artificial seed conversion rate and viability. However, the acceleration of microshoot growth was also observed at this age (Chapter 2). The growth of microshoots younger than 13 day-old was observed to be very slow and they could not withstand the encapsulation in this stage. Encapsulation seemed to be an inhibitor of microshoot growth at this sensitive stage. The growth of microshoots older than 14 days old was observed to be relatively rapid. However, the fast growth brought about metabolic activity which required high levels of respiration and nutrient supply. It appeared that the encapsulation reduced the efficiency of respiration and nutrient supply affecting the conversion and viability of artificial seed produced. Therefore, the microshoots derived in an accelerating stage of growth were found to be the optimal for encapsulation.

3.3.2. Microshoot encapsulation

The use of sodium alginate for artificial seeds capsulation was found to be an efficient method producing good quality cauliflower artificial seeds in terms of size and solidity. However the use of sodium alginate was chosen due to its moderate thickness, fast gelatine, low toxicity to plant micropropagules and relatively cheap price (Saiprasad, 2001). Sodium alginate has been extensively used for artificial seed production in different species (Rai *et al*., 2009, Ara *et al*., 2000, Redenbaugh *et al*., 1987). However, since the rigidity of artificial seeds depends on the concentrations of both sodium alginate and $CaCl_2$,2H2O, it was necessary to

optimize the optimal concentration of these materials and to investigate their effects on the conversion rate and viability of artificial seeds.

The use of these materials (sodium alginate and $CaCl_2.2H_2O$) did not have any toxicity effects on the cauliflower microshoots whatever the concentrations used. However, an essential point for the technology of synthetic seeds is the evaluation of the effects of different concentrations of sodium alginate and calcium chloride on the size, texture and the form of capsules. The artificial seeds differed morphologically with respect to texture, shape and transparency with different combinations of sodium alginate and sodium chloride. The use of 2% (w/v) sodium alginate was found to be optimal producing 5-7 mm diameter cauliflower artificial seeds and allowing the easy emergence of shoot and root from the beads. In terms of $CaCl_2,2H_2O$, while the concentrations used did not affect the conversion rate of artificial seeds, the use of 15 g L^{-1} (100 mM) was found to be the optimal and more effective than using relatively lower concentrations. However, although the use of 2% of sodium alginate with a complexing solution containing 100 mM of $CaCl_2,2H_2O$ for 20-30 min mixing duration has been reported to be suitable for appropriate hardening of calcium alginate calcium working in different species (Ara *et al*., 1999, Ara *et al*., 2000, Saiprasad, 2001). Saiprasad (2001) mentioned that the use of 3 % sodium alginate upon complexation with 75 mM $CaCl_2.2H_2O$ for 30 min gives optimum bead hardness and rigidity for the production of viable synthetic seeds. Pattnaik *et al* (1995) reported that **t**he optimal gel complexation for the encapsulation of vegetative buds of mature mulberry trees was obtained using 4% sodium alginate with 75 mM $CaCl_2.2H_2O$. However, an important point to be mentioned here is that the optimal concentration of sodium alginate depends on its sterilising method

since there is a reduction in its gelling capacity when it is subsequently exposed to a high temperature during autoclaving (Naik and Chand, 2006, Larkin *et al*., 1998).

Since this study showed that sodium alginate concentrations had no significant effect on the artificial seed conversion rate, while a positive effect of using relatively high concentration of $CaCl_2$,$2H_2O$ was observed on the viability of artificial seeds, the use of 2 % sodium alginate with 100 mM of $CaCl_2$,$2H_2O$ was recommended for use in artificial seed production. In terms of calcium chloride concentration, the current results contradict reports that higher level of encapsulated somatic embryos conversion was obtained by increasing the concentration of sodium alginate and reducing the concentration and exposure time to calcium chloride (Cartes *et al*., 2009, Prewein and Wilhelm, 2003). It was reported that the high concentration or the long exposure of the embryos to the calcium chloride results in more absorption and penetration of $CaCl_2$ in the embryo, which can lead to growth inhibition (Redenbaugh *et al*., 1986). However, this inhibition role of calcium chloride was not observed in the current study and this could be attributed to the use of microshoots for artificial seed production instead of embryos. Cartes *et al* (2009) reported that the percentage of germination level reached by somatic embryos and zygotes of Rauli-Beech (Nothofagus alpine) was significantly different according to the concentration of sodium alginate (2%, 3% and 4%) and the period of exposure to the calcium chloride agent. Thus, the suitable gelling agent concentrations, seems to be dependent also on the plant species and on the part of plant used for the production of artificial seeds.

Microshoot encapsulation did not support root formation since the root development was only observed after the cultivation of artificial seeds in semi-solid media. However, since no rooted microshoots were used for artificial seed production and

since the root induction is considered as one of the key indicators of the successes of artificial seed production, the effect of gelling agent concentrations on microshoot rooting should be investigated further. Rady and Hanafy (2004) reported that the concentration of sodium alginate used for artificial seed production had an effect on rooting ability and while the use of 4 % of sodium alginate for *Gypsophila paniculata* artificial seed production induced high level of rooting (97 %), no root formation was observed when 2 % sodium alginate concentration was used. Cauliflower root inhibition caused by encaspsulation was also reported by Kieffer (1996) and he suggested that this phenomenon could be caused by respiration problems. Interestingly, however the insertion of air bubbles in the artificial seeds capsules, which was supposed to improve microshoots respiration, did not improve the rooting ability and it had no effect on the conversion rate and viability of artificial seeds.

Due to the lack of a nutrient tissue like the endosperm of the natural seed, 4.4 g L^{-1} MS, and 1 ml L^{-1} of PPM were used as an artificial endosperm and were found to be an efficient structure for maintaining the viability of artificial seeds and the majority of artificial seeds showed the ability for conversion after in semi-solid media. However, several studies have demonstrated the important role of artificial endosperm (nutrient, PGRs, carbon sources, and antimicrobial elements like antibiotics, fungicides) facilitating the survival and growth of micropropagules (Rai *et al*., 2009, Jain *et al*., 2005, Redenbaugh *et al*., 1987). On other hand, the addition of MS to the sodium alginate solution could have an effect on the root induction of microshoots. Rady and Hanafy (2004) reported that the level of root formation was higher when sodium alginate was dissolved in sterile distilled water than when it was used with MS salt solution. However, the effect of nutrient structure of cauliflower capsules on their rooting ability needs some more investigation. Interestingly, it was

demonstrated that there was no need for sucrose to be used with artificial seed matrixes and this could be a very important point to facilitate the use of artificial seeds under unsterilized conditions since sucrose could be one of the main contamination causes.

The use of PGRs (kinetin and auxins) incorporated with the matrix of artificial seed had a significant effect on its conversion rate. The addition of 1 mg L^{-1} of kinetin in the matrix of artificial seeds improved its conversion rate. Moreover, the presence of auxin PGR (IBA) had a great interaction with the kinetin used. However, Huda *et al* and Machii (2007, 1992) demonstrated that artificial seeds grew better when PGRs were added to their capsules. Soneji *et al* (2002) also mentioned that the germination of artificial seed seems to depend on the hormonal type and concentrations in the bead medium.

Although the nutrient and PGR combinations applied for cauliflower artificial seeds supported the growth of micropropagules, more studies are needed in order to improve microshoot rooting ability in this stage.

3.3.3. Artificial seed conversion (germination)

The use of S23 semi-solid media for artificial seed cultivation supported their conversion and growth. However, although the various auxin types and concentrations used with culture media did not have a significant effect on the conversion rate of artificial seeds, it had a great effect on their viability. This could be attributed to the significant effect of auxins on the micropropagules root establishment (Reed *et al*., 1998, Celenza *et al*., 1995, Kares *et al*., 1990). The growth of cauliflower artificial seed preferred the relatively high concentrations of IBA which considers a weak auxin compared with IAA and NAA. The use of IAA and NAA improved the viability of artificial seeds but led to a high level of callus

formation. The high level of callus formation observed could be caused by the high level of indigenous and exogenous auxin since the meristematic domes are considered to be the main resource for auxin creation in plant tissue. However, various plant types exert different effects on adventitious root initiation and elongation as a consequence of auxin treatment (Pandey *et al*., 2010).

A promising point for cauliflower artificial seeds is that their capacity to be sown in commercial substrates such as compost, perlite, vermiculite and sand. It was clear that the moistening solution structure used was the key factor for the success of these substances since the conversion of artificial seeds was observed to be quite low when the culture substances were irrigated with sterile distilled water. In agreement with current research, artificial seeds cultivated in sterilized soil moistened with distilled water weakened the response in encapsulated shoot tips of pomegranate (Naik and Chand, 2006) and , pineapple (Sonej *et al*., 2002). The use of maintenance S23 solution (MS + sucrose) had a positive effect on the conversion rate and viability of artificial seeds. Moreover, the use of PGR combination also had significant influence on the artificial seed conversion rate and viability. The use of high concentrations of strong PGRs (2 mg L^{-1} kinetin + 2 mg L^{-1} NAA) gave the optimal results in terms of artificial seed conversion rate and viability. The type of auxin used with the culture substances (2 mg L^{-1} NAA) was observed to be higher than those used with culture semi-solid media (2 mg L^{-1} IBA). This could be either due to presence of kinetin in the liquid media used for culture substrates moistening or because of physical structure of culture substances led to less transportation efficiency of PGR to the cauliflower artificial seeds. However, although the optimal conversion rate was obtained using perlite, the viability of artificial seeds was negatively affected by this substance and the growth of plantlets stopped at a certain

point. It was suggested that the cessation of growth could be caused by a lack of moistened liquid mixture supplied since the same volume of liquid mixture was used with the four culture substances and the signs of dehydration were observed with perlite in comparison with other substances. However, the use of higher amounts of maintenance liquid media did not improve the conversion and viability of artificial seeds cultivated in perlite.

The optimal artificial seed viability rate was obtained using compost as a culture substance supplemented with (2 mg L^{-1} kinetin +2 mg L^{-1} NAA). It seemed that the conversion rate and viability of artificial seed depends not only on the irrigation liquid mixture but also on the physical structure of the culture substances. However, it might be good idea to investigate the result of using a mixture of compost and perlile and its effects on the conversion rate and viability of artificial seeds since the optimal conversion rate and viability were obtained using perlite and compost respectively and considering the fact that 2 mg L^{-1} kinetin + 2 mg L^{-1} NAA PGR combination used with irrigating liquid media gave the optimal interaction with both of perlite and compost resulting in the optimal conversion rate and viability. However, several studies have investigated the possibilities of sowing artificial seeds in soil or commercial substances, reporting, for example, on the use vermiculite, sand and soil for the cultivation of mulberry artificial seeds by Machii and Yamanouchi (1993) and what was reported about the use of soil for as alfalfa artificial seed conversion substance by (Fujii *et al* (1989)).

Cauliflower artificial seed conversion rate and viability were affected by the use of PPM with irrigating liquid media. While the use of relatively high concentration of PPM was found to be the optimal in terms of the conversion rate, the low concentration of PPM used gave the best result in terms of the viability of artificial

seeds. Interestingly, considering the high interaction observed between PPM and the culture substances, the use of PPM at relatively low concentration with the use of sand gave the optimal results in terms of artificial seed conversion rate and viability. The positive effects of using PPM were reported by Lata *et al* (2009), who indicated that the addition of PPM had a positive result on overall seedling growth and a remarkable improvement in the regrowth and conversion rate of *Cannabis sativa* L artificial seeds.

However, since the optimization both of PGR combinations (types and concentrations) and PPM concentration had a positive effect on the artificial seed (conversion rate and viability), it might be worthwhile to investigate the effect of using combinations of PPM and PGRs on the artificial seed (conversion rate and viability) cultivated in various culture substrates.

3.3.4. Artificial seed preservation

Cauliflower artificial seed showed a high capacity for dehydration keeping its conversion rate and viability to be high even at low moisture content (4%). A high dehydration capacity of cauliflower artificial seeds opens new vistas for the improvement in the ability of using it for cauliflower artificial seed under *in-vivo* condition, short term storage (dehydrate-cold storage) and long term storage (dehydration-cryopreservation). However, although the application of dehydration-cryopreservation protocol for artificial seed preservation has been successfully investigated with several plant species such as grapevine (Wang *et al*., 2000), citrus (Gonzalez-Arnao *et al*., 2003) apple (Paul *et al*., 2000) and strawberry (Clavero-Ramirez *et al*., 2005), no capacity for dehydration- cryopreseravtion was observed for cauliflower artificial seeds at -196º (LN), -80º and -20º. Furthermore, the use of several cryoprotectant materials and the application of many cryopreservation

programmers did not improve the ability of cauliflower artificial seed cryopreservation. The lack of cauliflower cryopreservation ability could be attributed to the physiological structure of microshoots since it is young vegetative tissue containing very high level of water with high level of metabolism activity. However, there are some procedures which could be useful for improving the ability of cauliflower artificial seed cryopreservation viz:

1. The use of relatively higher freezing temperatures (-10º, -5 ºand -3ºC).
2. The use of a combination of both cryoprotectant materials incorporated with artificial seed matrix and artificial seed dehydration approaches in order to improve the capacity for artificial seed cryopreservation.
3. The use of a combination of encapsulation vitrification and dehydration techniques. In this system, microshoots are treated with cryoprotective materials followed by dehydration with vitrification solution and then subjected to freezing. Such protocols have been applied with different species such as strawberry (Larkin *et al*., 1998), potato (Hirai and Sakai, 1999) and cassava (Charoensub *et al*., 2004).

Cauliflower artificial seeds showed high capacity of cold storage. They kept their conversion capacity and viability over six months. This is considered to be an important point since it reduces the cost of maintaining cauliflower plant materials to the following season. A slow growth of cauliflower artificial seeds was observed under storage conditions suggestion that a lower temperature is needed to stop the germination of artificial seeds and that could prolong the storage period.

3.4. Conclusion

In this chapter, cauliflower artificial seed was produced successfully from encapsulated microshoots. Procedures were optimized and proper plantlets were obtained from cauliflower artificial seeds. This system has great advantages summarized as follow,

1. A cost effective delivery system for cauliflower microshoots minimizing the cost of plantlets produced by mean of this technique.
2. A simple methodology with high potential for mass production since thousands of microshoots can be produced (chapter 2) and encapsulated as artificial seeds.
3. A promising protocol for the direct use of cauliflower artificial seedlings *in vivo* since proper plantlets was obtained using commercial culture substances.
4. The high dehydration and cold storage capacity of cauliflower artificial seeds opened a new vista for improving the capability for the storage of elite genotype year round.

However, despite of the advantages of the protocol described, further experiments are required in the following points,

1. Further experiments are required in order to improve cauliflower microshoot root formation in the liquid culture media and in the artificial seed capsules. Early microshoots root formation could improve the efficiency of this protocol and the viability of plants produced.
2. More investigations are needed to improve the capacity of cauliflower artificial seed cultivation in commercial substrates and under non sterilized conditions.

This could be improved by the use of suitable types and concentrations antibiotics.

3. Further detailed research is needed for improvement in cauliflower artificial seed cryopreservation capacity.

Chapter 4: Cauliflower Artificial Cold Tolerance and The Up-regulation of The *CBF/DREB1* Gene

4.1. Introduction

Abiotic stress is one of the main causes of crop reduction globally. Abiotic stress causes loss in average yield production of more than 50 % in important crops (Hussain *et al*., 2011). Among the different abiotic stresses, cold is an essential factor that limits crop productivity worldwide. Low temperature affects the growth, development and distribution of agronomic species throughout the world (Pearce, 2001). The survival in plants to freezing temperatures depends on their capacity for cold acclimation (McKhann *et al*., 2008).

Low temperature affects a wide range of cellular components and plant metabolism. However, many studies have been conducted indicating that membrane structures are the main site of freezing injury in plant cells (Jan *et al*., 2009) and it has been demonstrated that severe dehydration linked with freezing causes the membrane damage. Freezing temperatures induce ice formation in intercellular spaces where the extracellular fluid has a lower osmotic potential (higher freezing point) than the intercellular fluid. The formation of ice in the extracellular spaces causes movement of water from inside the cell to the extracellular spaces because the water in its frozen state has a much lower water osmotic potential than the intercellular liquid at a given ice temperature (Guy, 1990b). The dehydration caused by freezing damages the cellular functions in multiple ways, including the structure of the plasma membrane and its function (Uemura *et al*., 1995) involving expansion- induced lysis, lamellar-to-hexagonal-II phase transition and fracture jump lesions (Jan *et al*., 2009).

It has been widely reported that the exposure of several types of plants to non-freezing low temperature for a period of time (7-14 days) increases their freezing tolerance and this process is known as cold acclimation (Thomashow, 1999).

Because of its importance to agriculture, great efforts have been made and thousands of experiments have been conducted to improve the understanding of this important phenomenon (Thomashow, 2001). Acclimation has an important effect on cold tolerance: it was demonstrated earlier that cold acclimated spinach and cabbage produced proteins which protect the thylakoid membrane against freeze injuries. Moreover, these type of proteins were not observed in non-acclimated plants (Hincha *et al*., 1989, Volger and Heber, 1975).

Multiple polygenic traits appear and various physiological and biochemical changes occur during the progress of acclimation. These changes often involve modifications in membrane lipid structure (Uemura and Steponkus, 1994, Lynch and Steponkus, 1987). Acclimation also causes an increase in the production of antioxidants, abscisic acid and the compatible osmolytes such as soluble sugars and proline (Uemura and Steponkus, 1994, Tao *et al*., 1998, Nomura *et al*., 1995, Murelli *et al*., 1995, Lynch and Steponkus, 1987, Koster and Lynch, 1992, Kishitani *et al*., 1994, Dörffling *et al*., 1997, Chen *et al*., 1993). However, the improvement of cold tolerance by acclimation involves broad reprogramming of gene expression and metabolism. Recent studies describing full genome transcripts and mutational and transgenic plant analysis have provided a great deal of information about the complex transcriptional system that functions under cold acclimation (Jan *et al*., 2009). Various regulatory pathways were found to be involved in the cold response when transcriptome profiling of around 8000 genes of *Arabidopsis* was carried out. It was observed that 300 genes were affected by cold, of which 218 showed an increase of transcripts expression and 88 showed a decrease of transcripts expression within 7 days of cold treatment (Fowler and Thomashow, 2002). The use of microarray systems has enabled researchers to demonstrate that a large number of genes are

induced under abiotic stresses (Seki *et al*., 2001, Kreps *et al*., 2002, Bray, 2004, Maruyama *et al*., 2004, Vogel *et al*., 2005, Jan *et al*., 2009). The genes affected by low temperature have been classified into two main groups (Seki *et al*., 2002): the first group involves the proteins that function as a response to cold stress such as, LEA proteins (Cushman and Bohnert, 2000); the second group contains proteins which have roles in the further regulation of gene expression in cold stress conditions. Some of these pathways are considered to have multiple functions since they have also been reported to be involved in stress responses to drought and high salinity (Seki *et al*., 2003).

It has been reported that there is a set of genes which are highly upregulated during the process of acclimation and these genes encode a specific family of proteins called cold-responsive (COR) proteins (Gilmour *et al*., 2004). Several types of cold-regulated (*COR*) genes have been recognized in both monocotyledonous and dicotyledonous plants (Sharma *et al*., 2005, Sun *et al*., 2009). It has been demonstrated that ABA-dependent and ABA-independent pathways are the main two pathways intermediating the induction of *COR* genes expression.

In the ABA-dependent pathway, the accumulation of endogenous ABA observed under the effect of cold could trigger the basic leucine zipper (b ZIP) transcription factor, and then induce ABA-dependent *COR* genes through ABA-regulated elements (Uno *et al*., 2000, Xiong *et al*., 2002). However, it was demonstrated that the cellular phytohormone abscisic acid (ABA) accumulates under the effects of environmental stresses such as, drought (Leung and Giraudat, 1998). The accumulation of ABA causes several physiological adaptations, for example, stomatal closure and growth inhibition. Moreover, ABA induced the expression of

several genes including *COR* genes (Kurkela and Franck, 1990, Lang and Palva, 1992, Mantyla *et al*., 1995).

In the ABA independent pathway, cold induces the expression of C-repeat binding factor (*CBF*) transcription factors. This family of genes has an essential role in activating downstream *COR* genes which in turn improves the freezing tolerance in plants (Sun *et al*., 2009). The *CBF* transcription factor has been identified and characterized in several plant species such as rape (*Brassica napus*), broccoli (*Brassica oleracea*), alfalfa (*Medicago sativa*), tomato (*Lycopersicon esculentum*), corn (*Zea mays*), rice (*Oryza sativa*), strawberry (*Fragaria ananassa*), soybeans (Glycine max) and barley (*Hordeum vulgare*) (Choi *et al*., 2002, Gao *et al*., 2002, Owens *et al*., 2002, Dubouzet *et al*., 2003, Vágújfalvi *et al*., 2003, Francia *et al*., 2004).

Both C-repeat binding factors (*CBF*s) and dehydration-responsive element binding factors (*DREBs*) are transcription factors which induce the expression of cold and dehydration stress regulated gene in plants (Gilmour *et al*., 1998, Liu *et al*., 1998, Shinwari *et al*., 1998). These transcription factors bind to specific regulatory sequences in the promoters of cold and dehydration responsive genes. These sequences are C-repeat (CRT: TGGCCCGAC) and dehydration-responsive elements (DRE: TACCGACAT). Both of these sequences contain the highly conserved cor 5-bp sequence of CCGAC, which has the capacity to regulate transcription under drought, temperature and salinity (Baker *et al*., 1994, Yamaguchi-Shinozaki and Shinozaki, 1994, Gao *et al*., 2007). Therefore, *CBF* induces the expression of *COR* genes (the genes which contain the *COR* sequence) and these genes play an essential role in the improvement of plant cold tolerance.

The accumulation of *CBF* transcription factors starts within just 15 min of exposing plants to low temperature, indicating that the cold signal transducers must be existent at warm temperature but in an inactive state. It was proposed therefore that there is a specific transcription factor which is present at warm temperature and acts as the promoter of *CBF*. This transcription factor was called ICE (inducer of *CBF* expression). ICE apparently binds to a cold regulatory element “ICE box” found in the promoter of *CBF*. After exposing the plants to low temperature, ICE is proposed to be activated and induces the expression of *CBF* (Gilmour *et al*., 1998, Teige *et al*., 2004). Constitutive expression of the *CBF* genes can increase the constitutive expression of the *CBF* regulon (CRT/DER-containing genes that are induced by CBF/DREB1 transcription factor) (Gilmour *et al*., 2004, Jaglo-Ottosen *et al*., 1998a, Liu *et al*., 1998). It was confirmed that products of *COR* gene increase cold and dehydration resistance in plants. COR15 protein has low molecular weight and it stays soluble upon boiling in aqueous solution. COR15 is rich in Ala and Gly (Glimour *et al*., 1996). Moreover, several *COR* genes have been characterized in plants such as, *COR6.6*, *COR15a*, *COR47* and *COR78* (Artus *et al*., 1996, Steponkus *et al*., 1998). A direct evidence of *COR15a* action mechanism was demonstrated by Artus *et al* and Steponkus *et al* (1996, 1998) . It was demonstrated that COR15a polypeptides decrease the propensity of cell membranes to form deleterious hexagonal **II** phase lipids upon freezing-induced dehydration (Steponkus *et al*., 1998). Moreover, CBF regulatory proteins were demonstrated to increase the level of proline and sugars under the effect of cold acclimation and therefore to contribute to the improvement of freezing tolerance (Thomashow, 2001, Gilmour *et al.*, 1998).

Cauliflower artificial seeds should show high cold tolerance in order to be a good alternative to the traditional seeds and, therefore, the main aim of the studies included in this chapter was to determine whether the cold acclimation process could be induced in microshoots and whether this system can be usefully used to study cold acclimation process control.

Objectives

1. To investigate the effect of cold acclimation on the cold tolerance of cauliflower artificial seeds (microshoots).
2. To optimise a PCR protocol to detect the expression of the *CBF/DREB1* gene in cauliflower.
3. To examine the effect of cold acclimation and drought on the induction of *CBF/DREB*1 gene expression in cauliflower at different developmental stages (microshoots and mature cauliflower plants).
4. To investigate the capacity of ABA (Abscsic Acid) applied with the culture media on the development, cold tolerance and *CBF/DREB1* gene expression induction in cauliflower artificial seeds (microshoots).
5. To identify the homology sequence of the *BoCBF/DREB1* gene within published databases.

4.2. General Material and Methods

4.2.1. Standard Operating Procedure for frost damage evaluation using relative electrical conductivity technique (REC %)($SOP_{EC\%}$)

4.2.1.1. $SOP_{EC\%}$ protocol used with cauliflower mature leaves ($SOP_{ECL\%}$)

The frost resistance was analysed using the electrical conductivity technique described by Fuller and Eed (2003). Four fully expanded upper cauliflower leaves were excised from treated plants. Fifteen leaf discs of 1 cm diameter each were cut

and placed in boiling tubes used for each acclimated or non-acclimated plants (Figure, 64). All the tubes were labelled in test tube racks and put in a Sanyo freezing chamber. A small piece of ice was added to each tube to ensure ice nucleation and the chamber was programmed to fall to various low temperatures (depending on the experiment) with a hold of two hours at each temperature. The samples were removed at the end of the 2 h hold at each temperature. All the samples were left at 4°C overnight to defrost. All the tubes were then transferred to the laboratory bench at ambient temperature and 20 ml distilled water was added to each tube using an automatic dispenser (Oxford, catalogue number 470A). The tubes were covered and left overnight at room temperature to allow leaching of electrolytes from cells damaged by the freezing. The post-freezing electrical conductivity (EC) of each solution was measured using a conductivity meter the following day. All the samples (tubes) were then autoclaved in order to rupture all the cells to get the total leachate measurement. After the samples were autoclaved, they were kept overnight and then the post-autoclaving EC was measured for all samples (tubes). To calculate the percentage of relative EC, the following formula was applied:

$$REC\% = \text{Post-freezing EC} / \text{Post-autoclaving EC} \times 100$$

Figure 64, general view of the set-up for measuring electrical conductivity (Bar = 10 cm).

4.2.1.2. $SOP_{EC\%}$ protocol with cauliflower microshoots ($SOP_{ECM\%}$)

The same procedure of $SOP_{ECL\%}$ was used with the only exception was that 10 g of treated microshoots (the treatment depends on the experiment) was placed in each boiling tube instead of the leaf discs.

4.2.2. Standard Operating Procedure for RNA extraction and purification (SOP_{RNAext})

The RNA extraction was carried out following the instructions provided by the RNA extraction kit manufacture (sigma cat, STRN50) and described as follows:

1. Plant tissue (leaves or microshoots) were ground to a fine powder in liquid nitrogen using a mortar and pestle.
2. After the liquid nitrogen had evaporated from the frozen tissue powder, 100 (90-110) mg from the leave tissue or 200 (190-210) mg for the microshoot tissue powders were weighed in 1.5 ml RNAse free microcentrifuge tube (Ambion). The samples were kept on ice until they were used in the next step.

3. Master mixture consisting of 10 µl of 2-mercaptoethanol and 1000 µl of lysis was prepared and mixed briefly to be used for each two sample preparations. 500 µl of the mixture was added to each RNA preparation sample in microcentrifuge tube and vortexed immediately and vigorously for at least 30 sec at the maximum speed (13.000 xg).
4. The samples were incubated in water bath for 3-5 min at 56 °C.
5. The samples were centrifuged at maximum speed (13000 xg) for 3 min in order to pellet cellular debris.
6. The obtained lysate supernatants were pipetted into a Filtration Columns (blue column) placed in a 2-ml free RNase collection tubes and centrifuged for 1 min to remove residual debris.
7. The blue columns were thrown away and 500 µl of binding solution was added to the clarified lysate of each sample. The mixture was mixed immediately by pipetting few times.
8. For each sample, 700 µl of the binding mixture was added to the binding column, (red column) placed in 2-ml collection tube and centrifuged for 1 min at the maximum speed. The flow through liquid was decanted and the column was returned to the collection tube.
9. Three steps of washing were applied:
    - 500 µl of the washing solution 1 was pipetted into each sample column followed by centrifuge at the maximum speed for 1 min. The flow through liquid was decanted and the column was returned to the collection tube.
    - 500 µl of the washing solution 2 was pipetted into the column followed by centrifuged at the maximum speed for 1 min. The flow through liquid was decanted and the column was returned to the collection tube.

- Another 500 µl of the washing solution 2 was pipetted into the column followed by centrifuge at the maximum speed for 1 min. The flow through liquid was decanted and the column was returned to the collection tube.

10. The column was centrifuged at the maximum speed for 1 min (dry centrifuge). The column tube was removed carefully and transfered to a new, clean 2-ml collection tube. 50 µl of elution solution was added directly onto the centre of the biding column. The cap was closed and left to set for 1 min. The tube containing the red column was centrifuged at the maximum speed for 1 min.
11. The obtained purified RNA was kept at -20 °C (short term) or -80 °C (long term) until it was used.

The purity and the concentration of RNA were determined by NanoDrop (NanoVue Pluce) analysis. The absorbance was measured using A_{260}/A_{280} ratio procedure (Warburg and Christian, 1942). However, nucleic acids have a higher absorbance at 260 nm than at 280 nm and therefore, the A_{260}/A_{280} ratio was expected to be ≥ 2 for the pure samples.

4.2.3. Standard Operating Procedure for *BoCBF/DREB1* regulatory gene identification ($SOP_{CBF/DREB1}$)

Two steps of PCR were applied for the synthesis and amplification of cDNA. In the first step cDNA was produced from mRNA by reverse transcription process.

4.2.3.1. mRNA reverse transcription

The RNA reverse transcription was carried out following the instructions provided by reverse transcription kit manufacture (sigma kit) and described as follows:

1. A specific volume from each RNA sample was used in order to obtain 1 µg of RNA (leave samples) or 2 µg of RNA (microshoot samples) which then were used for each reaction. The volume was calculated the following equation:

RNA volume =1000/RNA concentration.

The volume of RNA was completed to 8 µL using nuclease free water (sigma cat, w1754) in nuclease free 1.5 ml microcentrifuge tube.

2. A master mix consisting of 1 µl of randomnanomerase and 1 µl of dNTPs was prepared for each sample (the master mix was prepared for all samples together and 2 µL was used for each sample).
3. The samples were treated at 70 for 10 min followed by quick transfer to ice where they were left for 5 min. The samples were kept in ice for 5 min.
4. A master mix consisting of
    - 1 µl of reverse transcriptase
    - 2 µl of reverse transcription buffer
    - 7 µl nuclease free water

was prepared and added to each sample (the mixture was prepared for all samples together and 10 µl from the mix was used for each reaction)

5. Tubes containing the samples with the master mix were treated with PCR (Applied Biosystem, Veriti) thermal cycle as follows: 21°C for 10 min, 37°C for 50 min, 94 °C for 2 min and holding at 4°C. cDNA amplification was carried out using either ordinary PCR or quantitative PCR:

4.2.3.2. Standard Operating Procedure for cDNA amplification using ordinary PCR (SOP_{OPCR})

The first cDNA obtained at the end of reverse transcription process was amplified using Ordinary PCR protocol following the instructions provided by kit manufacture (sigma cat). Master mix consisting of (for each sample):

- 1 µl Red tag polymerase

- 2.5 µl Red tag polymerase buffer
- 0.5 µl forward primer
- 0.5 µl reverse primer
- 0.5 µl dNTPs
- 18 µl sterile free nucleus water (Sigma cat, w1754)

was prepared. The master mix was prepared for all samples together and 23 µl from the mixture was added to each 2 µl of each sample in free nuclease1.5 ml microcentrifuge tubs. Two gene specific (degenerate) forward and backward primers were used depending on the experiment applied and according to *B.oleraceae CBF/DREB1* gene: Primers (P_1) used were (Forward, 5-AAGAAGTTTCGTGAGACCCGTCAC-3, Reverse, 5-GGCAAAAGCATACCTTCCGCCAT-3) (eurofins mwg/operon. Germany) (Hadi *et al*., 2011) and primers (P2) (Forward 5-ACTTTCCTAACCGCCGAC, Reverse 5-TCTCAGCCTGAAAAGCCA-3) which was used with quantitative PCR experiments as well. The 25 µl reaction mixture was run in the PCR machine (Applied Biosystems, Veriti) under the following thermal cycle:

- Initial denaturation at 94°C for 2 min once.
- 40 cycles of (denaturation at 94 for 30 sec, annealing 57°C for 30 sec, extension at 72°C for 30 sec)
- Final extension at 72 °C for 5 min and then 4°C ∞.

The PCR products were analysed using 1.4 % high melting agarose (Fisher, EP1356-100)) gel melted in TAE (Tris-acetate + EDTA) and added with 0.005 % of syber safe. The PCR products were compared with a PCR 100bp low scale DNA

ladder (Fisher BioReagents, BP2581-200) consisting of 10 DNA fragments with sizes of 50, 100, 200, 300, 40, 500, 700, 1000, 1400, 1500, 2000 bp.

The same protocol was applied but using housekeeping (Actin1) gene primers (Forward, cccaaaggccaacagagagaag) (Reverse, caccagagtccagcacaatacc). Housekeeping gene bands were used in order to normalise the results of *CBF/DREB1* gene expression. The intensity proportions between *CBF/DREB*1 bands and *Actin*1 Bands were analysed using ImageJ software.

4.2.3.3. Standard Operating Procedure for cDNA amplification using quantitative PCR (SOP_{QPCR})

P2 primers were used with the quantitative PCR protocol after their suitability for CBF/DREB1 detection was confirmed using SOP_{QPCR}.

The quantitative PCR protocol used was according to manufacture instruction (sigma cat). In order to amplify the first stand cDNA obtained at the end of reverse transcription process. 2 µl of the stand cDNA was mixed with a master mix consisting of (for each sample):

- 12.5 µl Syber green master mix.
- 0.5 µl forward primer
- 0.5 µl reverse primer
- 0.25 µl reference dye
- 9.5 µl sterile free nucleus water (Sigma cat, w1754)

The 25.25 µl reaction mixture for each sample was run in the PCR machine under the following thermal cycle:

1. Holding stage at 94°C for 2 minutes.

2. Cycling stage which consists of 40 cycle of (94°C for 30 sec followed by 57°C for 30 sec followed by 72°C for 30 sec).
3. Melting stage which consists of 94°C for 15 sec followed by 57°C for 1 min followed by 94°C for 15 sec.

Actin1 Primers (Forward 5-CCCAAAGGCCAACAGAGAGAAG-3, Reverse 5-CACCAGAGTCCAGCACAATACC-3) (eurofins mwg/operon. Germany) were used to detected *Actine*1 as a housekeeping gene in order to normalize the gene expression obtained.

4.2.4. Standard operating procedure for protein extraction and purification SOP_{PEX}

Microshoots from -80°C freezer were thawed on ice and placed in a mortar. Extraction buffer consisting of (100 mM potassium phosphate KH2PO4 and pH was adjusted to 7.8 using KOH, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 10% glycerol)(Ni *et al*., 1996) was prepared. 2 mL of the extraction buffer was added to 1 g microshoot tissue/seed and ground.

1 mL of the liquid grindate was transferred to a microfuge tube (1.5 ml) and placed on ice. Samples were Centrifuged at 13000 xg for 15 minutes at 4 °C. The supernatant was transferred to a new microfuge tube (1.5 ml) and centrifuged for 10 minutes. The clear supernatant obtained was distributed in aliquots and stored at -80 °C until they were used in subsequent stages. The mortar and pestle were washed carefully between each two samples.

4.2.5. Standard Operating Procedure for protein estimation SOP_{PES}

The total protein content was evaluated by using BCA (bicinchoninic acid) assay kit (Pierce, product NO. 2161297A) (Thermo Scientific) following the manufacturer's microplate protocol. Working reagent was prepared by mixing BCA reagent A with B

at of 50:1 part respectively. 200 µL of the working reagent was added to 25 µl of each sample. Seed protein samples and microshoot protein samples were diluted with extraction buffer in a ratio of 1:50 and 1:20 (seed protein: extraction buffer) respectively. A standard curve was prepared using several dilutions of 2 mg mL^{-1} bovine serum albumin (BSA) stock as follow: 2000, 1500, 1000, 750, 500, 250, 125, and 25 µg mL^{-1} (Figure, 65). All samples and standards were used in triplicate in 96 well plates. The plates were incubated at 37°C Heraeus incubator for 30 min then plates were allowed to cool to room temperature. Sample protein concentrations were determined by calibration against the standard curve. The absorbance was measured at 562 nm using a plate reader (SpectraMax, Molecular Devices, Sunnyvale CA).

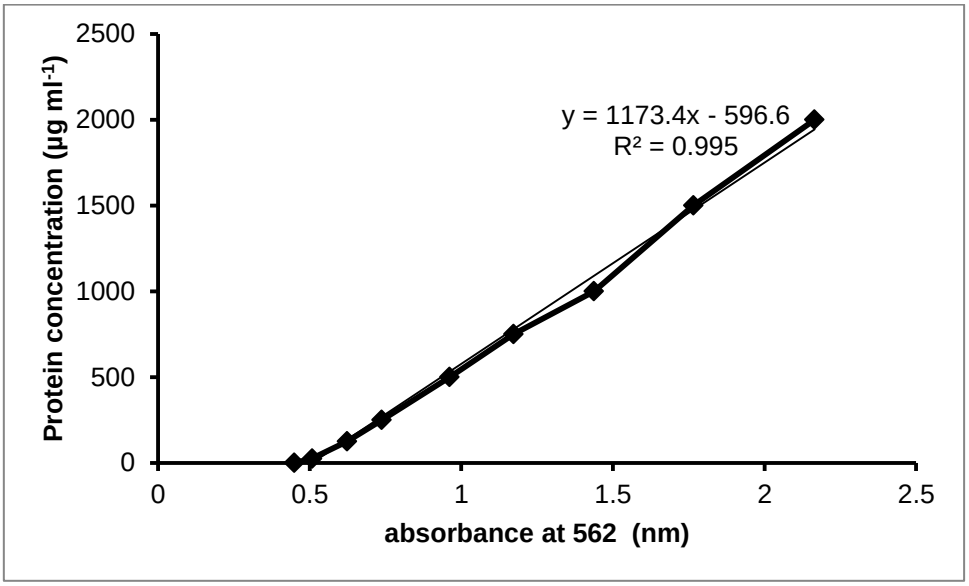


Figure 65. Standard curve of protein concentrations and absorbance at 562 nm

4.2.6. Standard Operating Procedure SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) SOP_{SDS}

SDS-PAGE analysis was carried out using a protocol modified from Laemmli (1970). Vertical 8.6 x 7.7 cm (WxL) polyacrylamide gels were prepared and run in a Bio-Rad

electrophoresis system. Two types of stock solutions were prepared. Solution B (15 mL of 2M Tris pH (8.8), 0.8 mL of 10 % w/v SDS and 4.2 mL H2O), Solution C (10 mL of 1M Tris pH (6.8), 0.8 mL of 10 % w/v SDS and 9.2 mL H2O). 5 X stock electrophoresis running buffer pH 8.3 was prepared (12 g Trizma base, 57.6 g Glycine and 4 g SDS were used to prepare 800 mL of the stock solution). Sample loading buffer (Tris HCl 0.5 M pH 6.8, SDS 10% w/v, bromophenol blue 0.1%, β-mercaptoethanol 0.5 ml/10 ml, Glycerol 20%) were also prepared. 12 % resolving gel was prepared by mixing 4 mL solution A (30% Acrylamide/Bis-acrylamide (Sigma cat # A3574)), 2.5 mL solution B, 3.5 mLH2O, 50 µL of 10% w/v APS (amonium persulfate) and 5 µL TEMED (tetramethylethylenediamine). 5% stacking gel was prepared by mixing 0.67mL solution A, 1.0 mL solution C, 2.3 mL H2O, 30 µL10% APS and 5 µL TEMED. APS (prepared fresh) and TEMED were added before pouring the gel. Resolving gel solutions were poured quickly into the gel casting system and space left about same size of combs for the stacking gel below the bottom of the comb. Any bubbles were removed by adding sterilised H_2O (Water for Molecular Biology, Sigma)to the top of the gel. Sterilised H_2O also prevented the gel from drying out. After 15 minutes, the gel was completely polymerized. The sterilised H_2O was washed away and the gel dried with blotting paper prior to pouring the stacking gel. The comb was added soon after pouring the stacking gel and allowed to polymerize completely for 30 min. Protein samples were diluted and adjusted to have the same concentration of 4 µg μL^{-1} of the total proteins. 50 µl of loading buffer was mixed with 50 µl of each sample. Therefore, the final concentration of protein in the loading buffer was 2 µg μL^{-1}. The samples were boiled at 95 °C (Techne, DRI-BLOCK) for 5 minutes then cooled and centrifuged at 13000 xg for 1 minute to remove any debris which could cause any blockage of gel pores and to remove also

air bubbles. 20 µl of the supernatant of each sample mixture was loaded into each well. With gel used for commassie staining, two types of standard protein markers, BlueEye Prestained Protein Marker (PS-104, Jena Bioscience) and HyperPAGE Prestained Protein Marker (Bioline, BIO-33065) were used depending on the experiment. Another two types of standard protein markers, Biotinylated Protein Ladder (Cell Signaling Technology, 7727) and Bio-Rad ladder (Precision Plus Protein Dula Xtra Standard 2-250 kDa) were used with western experiments. The gel was run at 200 volts for about 45 minutes. Staining solution was prepared by mixing 0.2% (w/v) commassie Brilliant Blue R-250, 30% (v/v) Methanol and 10% Acetic acid. Destaining solution was prepared by mixing 40% (v/v) methanol, 10 % acetic acid and 50 % dH_2O. Gels were stained in commassie blue solution overnight and they were then washed three times for 30 minutes at each for destaining. Photographs were made with a canon digital camera and stored for further analysis. A non-stained gel was used for protein transfer to polyvinylidene fluoride membrane (PVDF) for subsequent Western blotting.

4.2.7. Standard operating procedure for Western blotting SOP_{WB}

Protein samples resolved using SDS-PAGE were transferred to PVDF membrane and subjected to immunoblot analysis modified from Towbin *et al* (1979). The PVDF immobilon transfer membrane (pore size 0.2 µm) (Milipore, Cat.No ISE000010) was placed next to the gel and sandwiched between absorbent paper and sponge (-sponge/filter paper/gel/membrane/filter paper/sponge +). All were clamped firmly together after ensuring no bubbles have formed between the membrane and gel. The sandwich was submerged in transfer buffer. Transfer buffer was prepared by mixing 14.4 g L^{-1}, 3.03 g L^{-1} Trizma base, 200 mL methanol and 800 mL of dH2O. Tris and Glycine were dissolved first in dH2O and then methanol was added. The

transfer buffer was chilled overnight at 4°C. Protein transferred was carried out using Bio-Rad system. Electrical field was applied to be 100 volts for 27 minutes. Transfer time and voltage was optimised to get the optimal results since these conditions depend on the protein size. To prepare PBST solution; PBS tablets (Phosphate Buffered Saline Tablets, Fisher) were dissolved in dH2O (1 tablet in 100 ml water) and 0.05% Tween 20 was then added to the autoclaved PBS.

PVDF membrane containing the transferred protein was incubated in the blocking solution (5% Marvel semi skimmed milk (Iceland) dissolved in PBST solution) for one hour with gentle shaking at room temperature. Membranes were then washed two times each for 5 minutes with PBST and transferred to primary antibody solution. Hybridization for COR15a was achieved with primary antibodies obtained from the Prof. Michael Thomashow‟s Lab, (Michigan State University, USA). Membranes were incubated with COR15 primary antibodies (produced in rabbit). Antibodies were diluted (1:1000) in a total volume of 20 ml PBST and the membranes were incubated overnight at 4°C with gentle shaking. In the next day, the membranes were washed with PBST three times with a mild agitating, for 8 min each. Membranes were then incubated in goat anti rabbit IgG conjugated to horseradish peroxidase (Bio Rad, CaN: 172-1019) secondary antibody diluted 1:20,000 in PBST for 1 hour with mild shaking. The membranes were then washed in PBST three times at each for 8 minutes. Luminata crescendo western HRP substrate (Millipore, WBLUR0100) was used as a developing solution. 2.5 mL of this solution was added to the membrane and the membrane was incubated in dark for 5 minutes. Photos for the membranes were then taken using UVP gel documentation system. Each experiment was repeated 3-5 times.

In order to optimise the western blot protocol, 5 microshoots protein samples derived from acclimated and non-acclimated microhoots and from Mo treated and non-treated microshoots were transferred to PVDF membrane and the membrane was then inculpated in just secondary antibody (without incubation in the primary antibody) for 90 minutes at room temperature and they were then developed using the same procedures mentioned above. The aim of this step was to investigate if the secondary antibody reacts with the membrane proteins showing any sort of non-specific bands.

The same procedures were applied with all samples to detect Histone H3 protein (Agrisera, Art no: AS10 710). Histone Antibody was diluted (1:5000) in a total volume of 20 ml PBST. Histone protein bands were used to normalise the intensities of COR protein bands. Band intensities proportions between the COR bands and Histone bands were analysed using Image J software.

4.2.8. Standard Operating Procedure for statistical analysis (SOP_{SA})

Results are presented as means + standard error (S.E.). All data were subjected to analysis of variance (ANOVA) using Minitab soft-ware (version 15) and comparisons of means were made using the least significant difference test (LSD) at 5% level of probability.

4.3. Experiments

4.3.1. Experiment 1:

The effect of cold acclimation on the cold tolerance of cauliflower microshoots and up-regulation of *CBF/DREB1*

4.3.1.1. Aims and Objectives

The main aims of these experiments were to

1. Investigate the effect of cold acclimation on the cold tolerance of cauliflower microshoots.
2. Investigate the induction of *CBF/DREB*1 expression under the effect of low temperature (4 °C).
3. To investigate the effect of cold acclimation on the accumulation of COR15 protein in cauliflower microshoots.

4.3.1.2. Materials and Methods

Cauliflower microshoots (CV.Dionis) were obtained applying the Standard Operating Procedure for Culture System ($S.O.P._{CS}$) as described in chapter 2. The 26 day old cultures were divided into two parts:

The first part was transferred to the cold room at 4 °C supplied by cool white fluorescent tubes (spectral photon fluence 34 $\mu mol.m^{-2}.s^{-2}$) with a photo period of 16 hours for acclimation. Samples of microshoots, each consisting of 2 culture pots, were sampled at 0 (control), 1, 6, 12, 18, 24 hours after the transfer to the new temperature. The samples were kept at -80°C until the RNA was extracted using (SOP_{RNAext}) protocol. The cDNA of *CBF/DREB1* was produced and amplified using SOP_{OPCR} protocol. Three annealing temperatures (54, 57, 60 C°) of PCR primers were tested in order to determine the optimal temperature for obtaining optimal gene expression. Six samples, of which two were tested and found to have positive

*CBF/DREB*1 expression (P) and four found to have negative *CBF/DREB*1 expression (N), were used in this experiment. PCR was replicated three times. The rest of the cultures which were kept at 4°C for 15 days and were then used for protein extraction applying SOP_{PEX}. SDS PAGE and Western blotting experiments were carried out using SOP_{SDS} and SOP_{WB}.

The second group was used for the production of artificial seeds applying the Standard Operating Procedure for artificial seed production (SOP-ASP) described in chapter 3. Artificial seeds produced were divided into two groups. The first group was acclimated at 4°C for 15 days for acclimation and the second was used as a control (without low temperature treatments). Frost resistant analysis of the artificial seeds obtained using both acclimated and non-acclimated microshoots was carried out to test the effect of acclimation process. Artificial seeds were exposed to different temperatures as follows, 20, 0, -2, -4, -6, -8, and -10°C. The artificial seeds were placed in sterile petri dishes with a small piece of sterile ice to ensure ice nucleation. The petri dishes were placed in a Sanyo programmable chamber to various freezing temperatures with a hold of two hours at each temperature. Samples were removed at the end of the 2 h hold of each temperature. Samples were kept at 4°C overnight to thaw. Artificial seeds were then cultivated in maintenance semi-solid media S23. The conversion rates and the average weights of plantlets produced were assessed after 27 days of culture. 10 replicate culture pots, each cultivated with four artificial seeds were used with each treatment.

4.3.1.3. Results

The effect of acclimation on artificial seed cold tolerance

Acclimation improved the cold tolerance of artificial seeds. The conversion rate of non-acclimated artificial seeds significantly decreased at lower temperature

treatment than -4°C temperature treatment whilst the conversion rate of acclimated artificial seeds revealed 100% conversion rate down to -8°C. Significant differences appeared clearly at the temperature lower than -4°C (P<0.001) (Figure, 66).

Temperature treatments lower than -4°C significantly reduced the average weights of plantlets produced from both acclimated and non-acclimated artificial seeds (P<0.001) (Figure 67). In regards of the effect of acclimation on the plantlet average weights, it was observed that acclimation significantly decreased the average weights of plantlets derived from artificial seeds exposed to temperature higher than -4°C and significantly increased the average weights of plantlets derived from artificial seeds exposed to temperature lower than -4°C (P<0.001 (Figure 67).

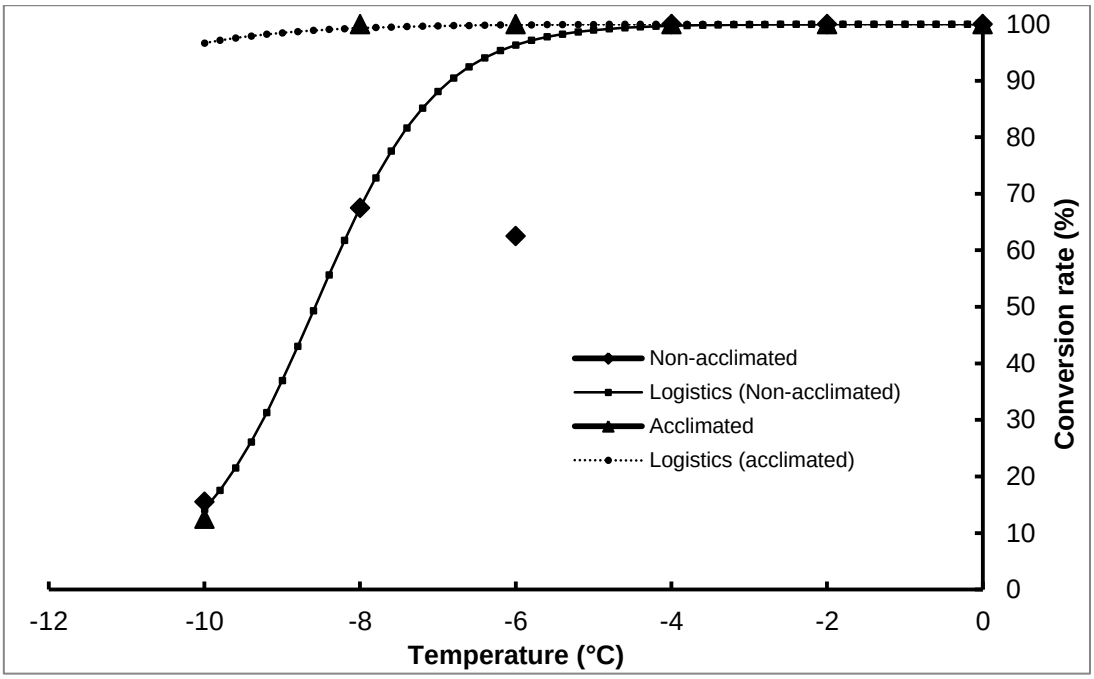


Figure 66. The effect of cold acclimation on artificial seeds cold tolerance assessed by their conversion rate at different low temperatures (LSD=10.11)

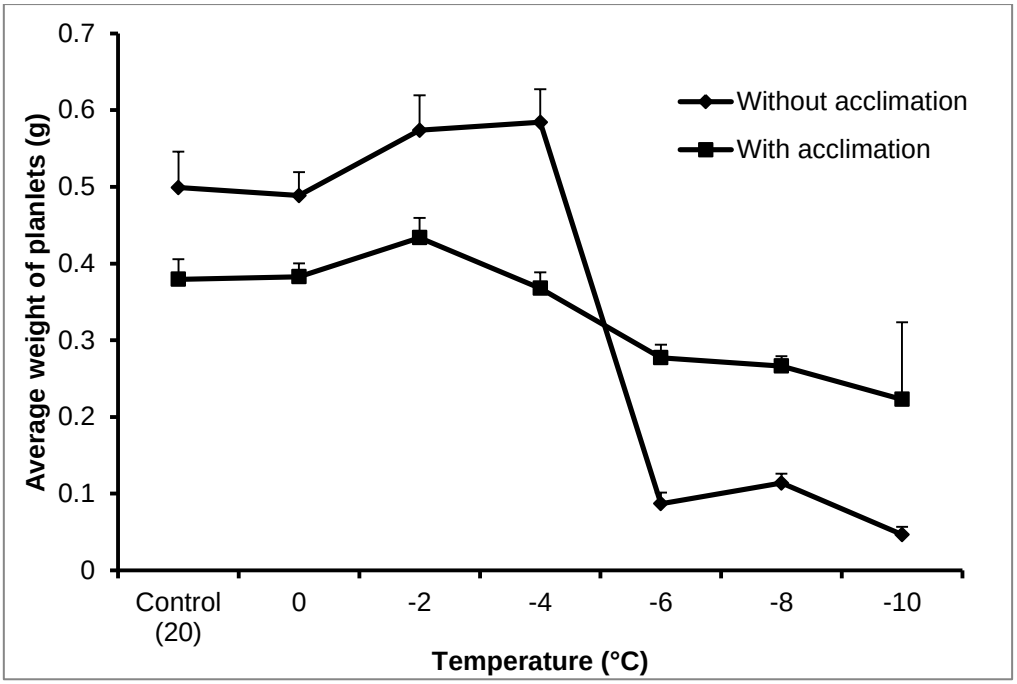
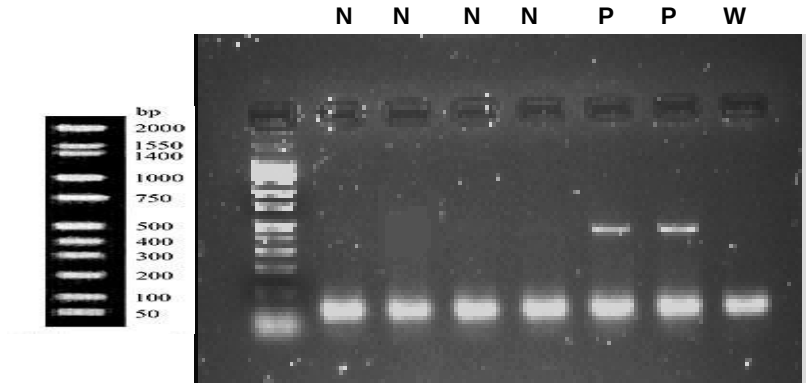


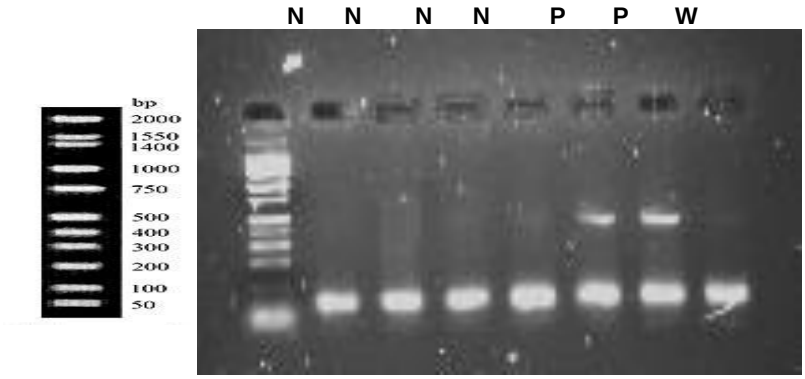
Figure 67. The effects of temperature treatments on the average weights of cauliflower plantlets produced from both acclimated and non-acclimated artificial seeds (LSD=0.0885)

1. The optimization of primer annealing temperature

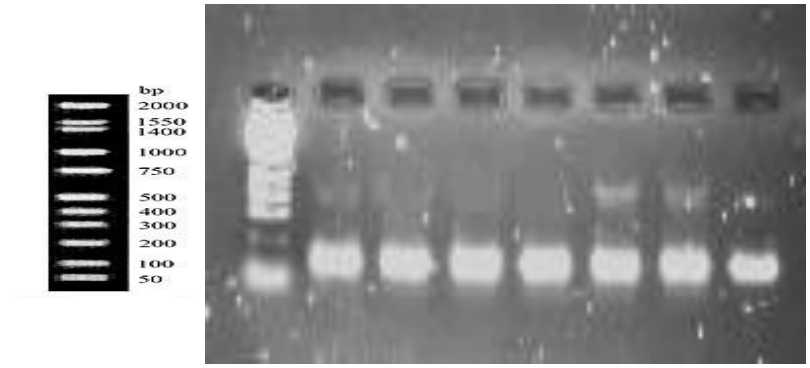
The PCR conditions were optimized empirically by adjusting annealing temperature. The optimal annealing temperature measured by means of expression band intensities was found to be 57ºC (P<0.001). However, although there were no significant differences between the use of 57 ºC and 54 ºC in terms of expression band intensities, the use of 57 ºC was recommended to be the optimal to avoid the production of any secondary PCR products (Figure, 68 and 69).



Annealing temperature: 54°C



Annealing temperature: 57°C



Annealing temperature: 60°C

Figure 68. The effect of annealing temperature on the expression of *CBF/DREB1* gene (N: sample tested to have negative *CBF/DREB1* expression and P: sample tested to have a positive *CBF/DREB1* expression)

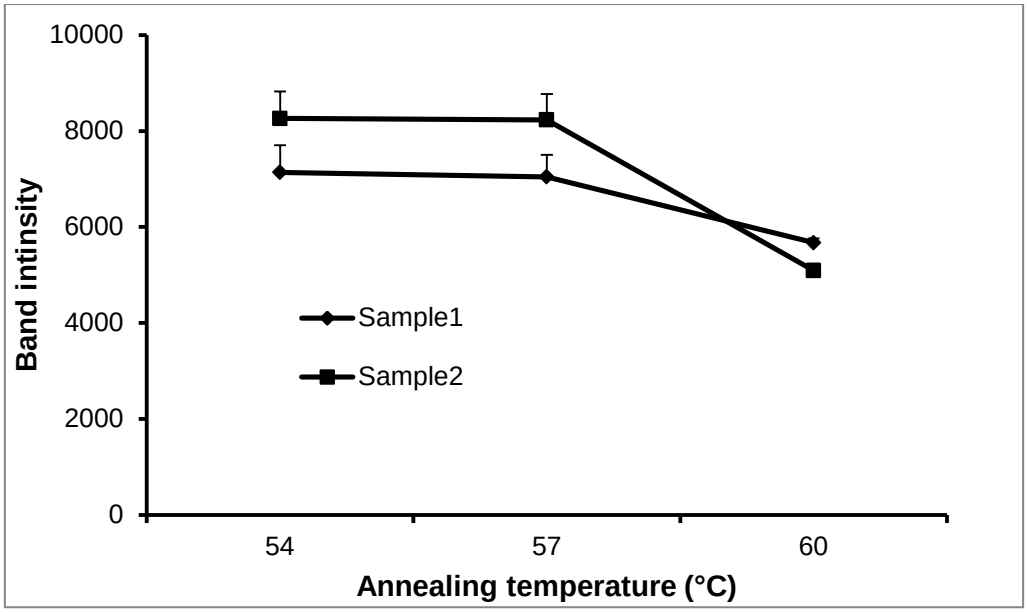


Figure 69. The effect of using three annealing temperature (54, 57, 60 ºC) on the expression of *CBF/DREB1* gene (band intensity proportions) in the samples which have positive gene expression (LSD=884.635).

2. The effect of cold acclimation on the induction of *CBF/DREB*1 expression in cauliflower microshoots

Low temperature treatments at 4°C induced the induction of *CBF/DREB1* after 1 hour of cold treatment. However, the maximum expression was observed after 1 hour of cold treatments at significant differences compared with the other treatments (*P*<0.001) (Figures 70, 71 and 72).

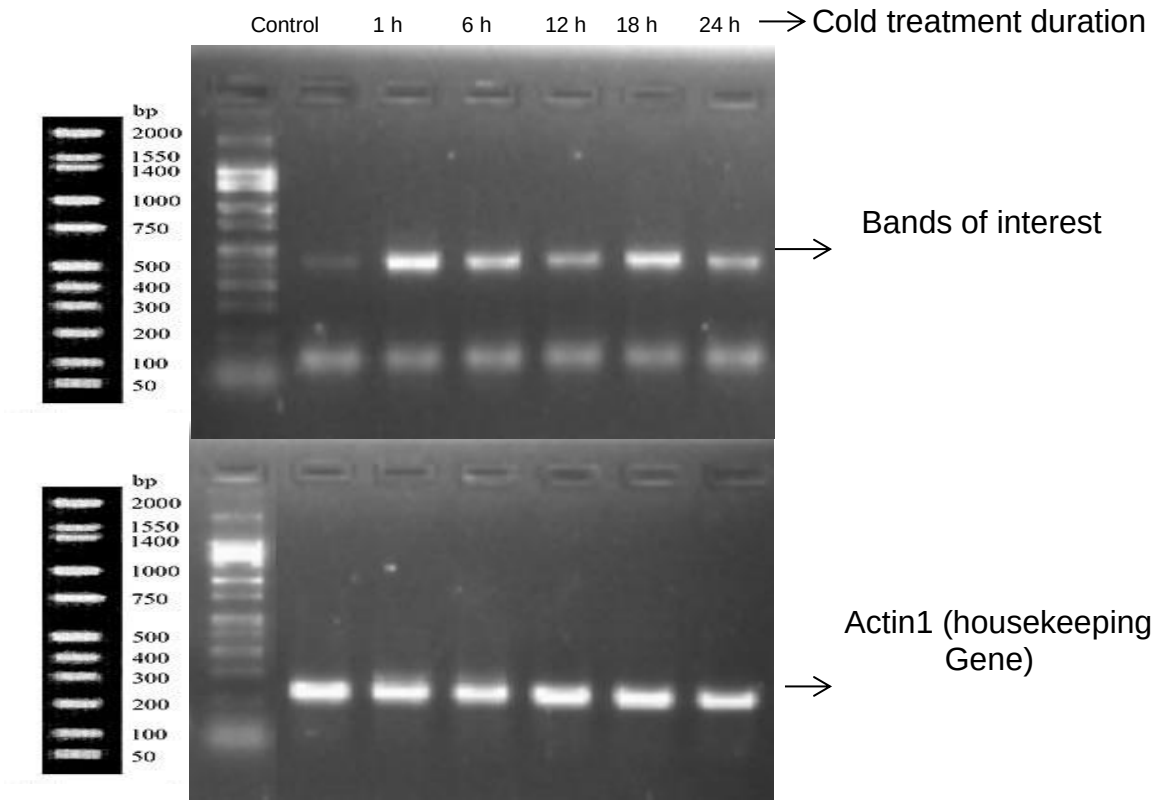


Figure 70. The effect of cold acclimation on the up-regulation of *CBF/DREB1* in cauliflower microshoots.

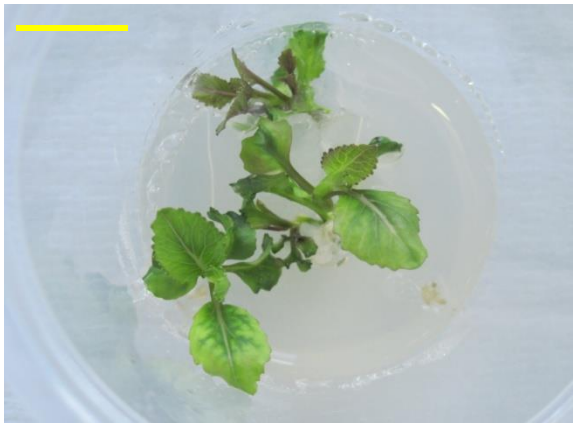


Figure 71. Plantlets converted from acclimated artificial seeds treated at -10°C (Bar = 1 cm).

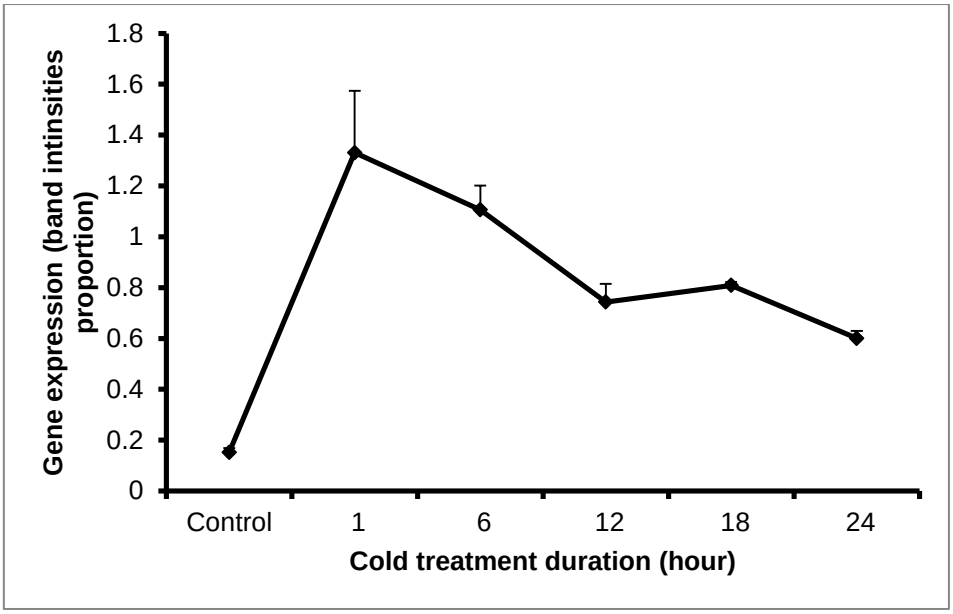


Figure 72. The effect of low temperature (4°C) at various exposure times on the induction of *CBF/DREB1* expression in cauliflower microshoots (LSD=0.285).

3. Detection of COR15 gene in acclimated and non-acclimated cauliflower microshoots

No protein (band) was detected (band) was detected when membrane was incubated with secondary antibody (without an incubation with primary (COR15) antibody).

The main single band detected using COR15 antibody was observed at about 23 kDa. This protein was observed in both acclimated and non-acclimated samples. However, no significant effect of acclimation was found to be on the amount (band intensity) of this protein (*P*= 0.376) (Figures, 73 and 74).

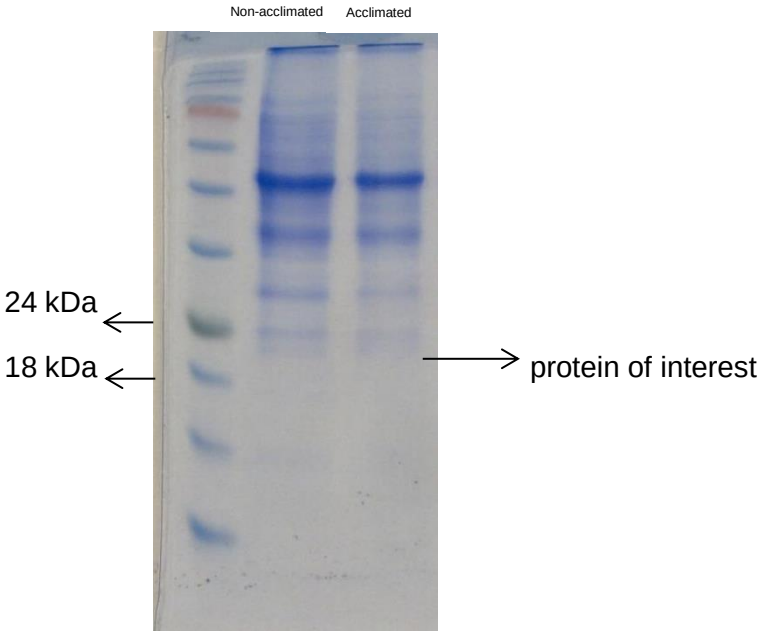
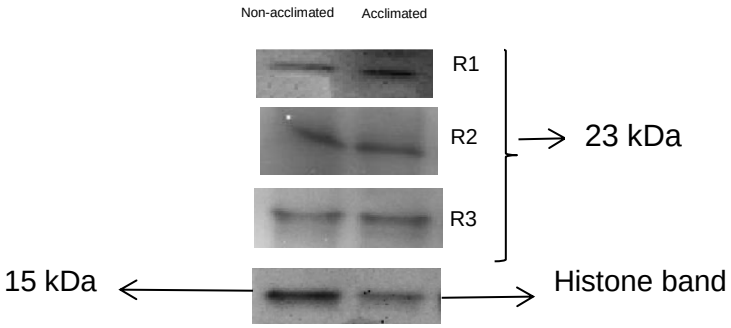


Figure 73. The SDS-PAGE analysis for protein samples derived from both acclimated and non-acclimated microshoots.



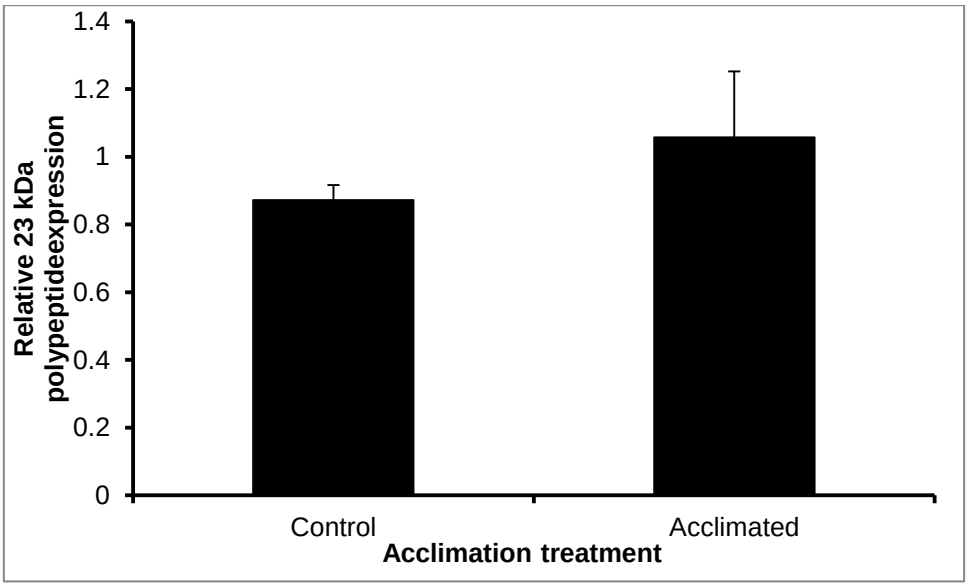


Figure 74. Western blot analysis for the detection of COR protein (23 kDa) under cold acclimation.

4.3.2. Experiment 2:

The effect of drought simulation on the artificial seeds (microshoots) cold tolerance and on the induction of *CBF/DREB*1 expression in the cauliflower microshoots

4.3.2.1. Aims and objectives

1. To investigate the effects of the simulation of drought conditions using mannitol in the microshoots culture media on the conversion rate and the development of cauliflower artificial seeds.
2. To assess the effect of mannitol treatments (drought simulation) on artificial seed cold tolerance.
3. To evaluate whether the simulation of drought obtained using a high osmotic potential mannitol solution has the capacity to induce the expression of *CBF/DREB*1 gene in cauliflower microshoots.

4.3.2.2. Material and Methods

The effect of mannitol on the development of cauliflower microshoots and artificial seed cold tolerance:

A standard curve for mannitol concentration and the osmotic potential of the culture liquid media was prepared using 0, 20, 50, 100, 200 g L^{-1} mannitol. The osmotic potentials of these solutions were measured using an osmometer (Osmomat R) (Figure, 75). Using the standard curve, culture media with several osmotic potentials were prepared by the addition of mannitol as follows, mannitol free culture media osmotic potential of -0.47 Osmol kg^{-1} (this media contained 3% of sucrose), -0.7 Osmol kg^{-1} (12.22 g L^{-1}), -1.15 Osmol kg^{-1} (48.98 g L^{-1}). -1.60 Osmol kg^{-1} (79.79 g L^{-1}), -2.05 Osmol kg^{-1} (113.55 g L^{-1}), -2.50 Osmol kg^{-1} (147.33 g L^{-1}), -2.95 (181.121 g L^{-1}) and -3.40 Osmol kg^{-1} (259.99 g L^{-1}).

The culture media prepared were used for the production of cauliflower microshoots (cv. Fremont) using the S.O.P_{CS}. The cultures were left in the shaker at room temperature (16 hours light provided by fluorescent lights, 80 µmol m^{-2} s^{-1}) for 28 days after which the number and average weight of microshoots were evaluated. Four culture pots (replicates) were used for each treatment.

Samples of 15 day-old microshoots derived from -0.47 (control), -2.05,-2.95 Osmol kg^{-1} treatments were used for the production of artificial seeds using SOP_{ASP}. The frost resistant analysis of the artificial seeds was carried out to test the effect of drought simulation on the artificial seed cold tolerance. Artificial seeds were tested at different sub-zero temperatures. The artificial seeds were placed in sterile petri dishes with small piece of a sterile ice to ensure ice nucleation and placed in a chamber Sanyo programmed to fall to temperature of 0, -2, -4, -6, -8, -10 and -12°C with a hold of two hours at each temperature. Samples were moved at the end of the 2 h hold of each temperature. Samples were kept at 4°C overnight to thaw. Artificial seeds were then cultivated in maintenance semi-solid media S23. Artificial seed conversion rate was evaluated after 20 days of culture. Five lines (replicates) of six artificial seeds in each were cultivated in small plastic containers (10, 10, 8 cm) containing 75 ml of maintenance semi-solid S23 media and were used with each treatment. Three lines were used per container. The lines were distributed randomly between the containers. Each line was considered as a replicate.

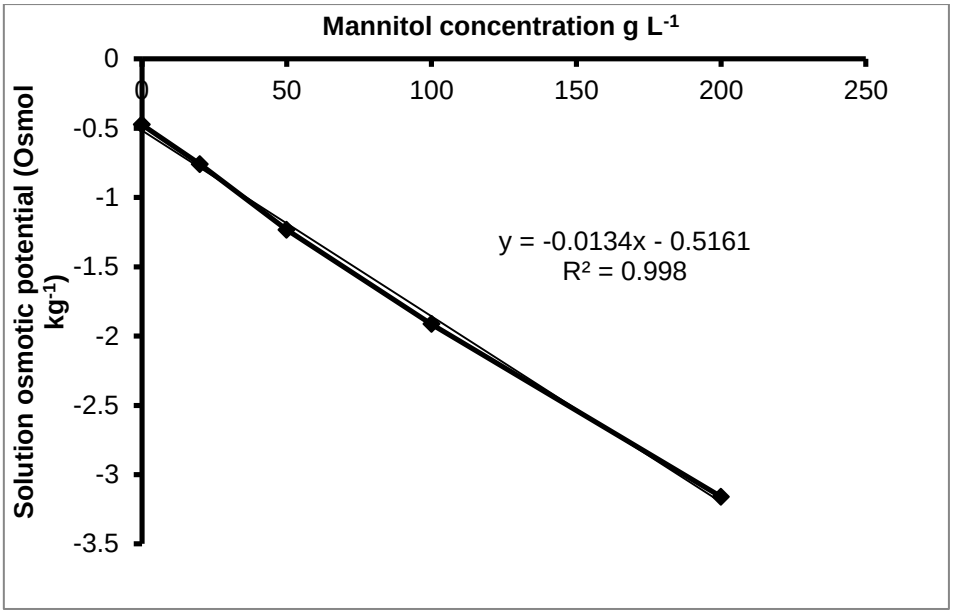


Figure 75. Standard curves for culture media osmotic potential obtained using different concentrations of mannitol.

The effect of mannitol on the induction of *CBF/DREB1* gene expression in cauliflower microshoots

Cauliflower microshoots (CV. Fremont) were produced applying S.O.P$_{CS}$ protocol and using the regular culture media. 25 day old microshoots produced were transferred to new culture medium containing several concentrations of mannitol -0.47 (control), -0.7, -1.15, -1.60, -2.05, -2.50, -2.95, -3.40 Osmol kg^{-1}. Samples of microshoots were derived from each mannitol concentration treatment after 0 (control), 1, 6, 12, 18, 24, 36 hours of the transfer to the new cultures. The samples were kept at -80°C until the RNA was extracted using SOP$_{RNAext}$ protocol. Synthesis and amplification of *CBF/DREB1* cDNA was carried out using the SOP$_{OPCR}$. Each PCR experiment was replicated three times.

4.3.2.3. Results

The effect of mannitol on the development of cauliflower microshoots

While various osmotic potential culture media had no significant effect on the number of growing microshoots (P=0.076), the effect on the average weights was highly significant (P<0.001). The higher the osmotic potential, the lower the average weight

of microshoots (Figures, 76 and 77). The use of relatively high concentration of mannitol (-2.95 Osmol kg^{-1}) negatively affected the growth of cauliflower microshoots and the colour of media changed when such a high concentration was used (Figure, 78).

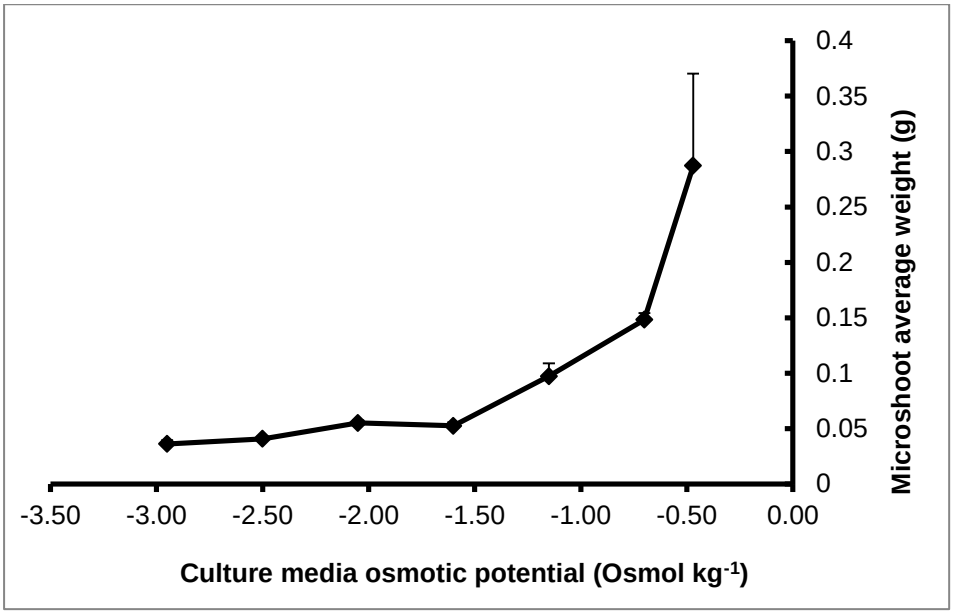


Figure 76. The effect of culture osmotic potential (mannitol concentration) on the average weight of cauliflower microshoots (LSD=0.016).

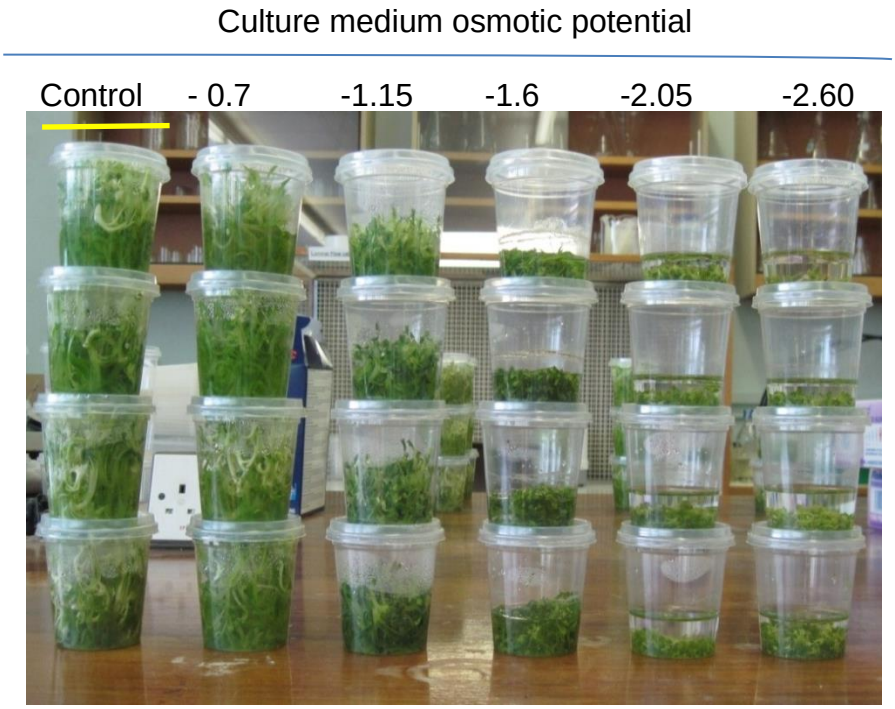


Figure 77. The negative effect of using high culture osmotic potential (mannitol concentration) on the development of cauliflower microshoots (Bar = 8cm).

Figure 78. The effect of using a high osmotic potential culture media (-2.95) on the development of cauliflower microoshots (Bar = 1.5 cm).

The effect of mannitol on artificial seed cold tolerance

Mannitol treatments had significantly positive effects on artificial seed cold tolerance when it was used at an osmotic potential of -2.05 Osmol kg^{-1} (147.33 g L^{-1}) (*P*<0001). While the artificial seeds produced using -2.05 Osmol kg^{-1} treated microshoots tolerated -10°C temperature, the conversion rate of the control artificial seeds decreased to less than 40% at this temperature. It was observed that the artificial seeds obtained from microshoots produced at -2.95 Osmol kg^{-1} were unable to be encapsulated as artificial seeds since the conversion rate for them was very low even without low temperature treatment (control) (Figure 79 and 80).

The effect of mannitol of the up-regulation of *CBF/DREB*1 gene in cauliflower microshoots

It was confirmed that none of the mannitol concentration used had the capacity to up-regulate *CBF/DREB1* gene whatever the exposure.

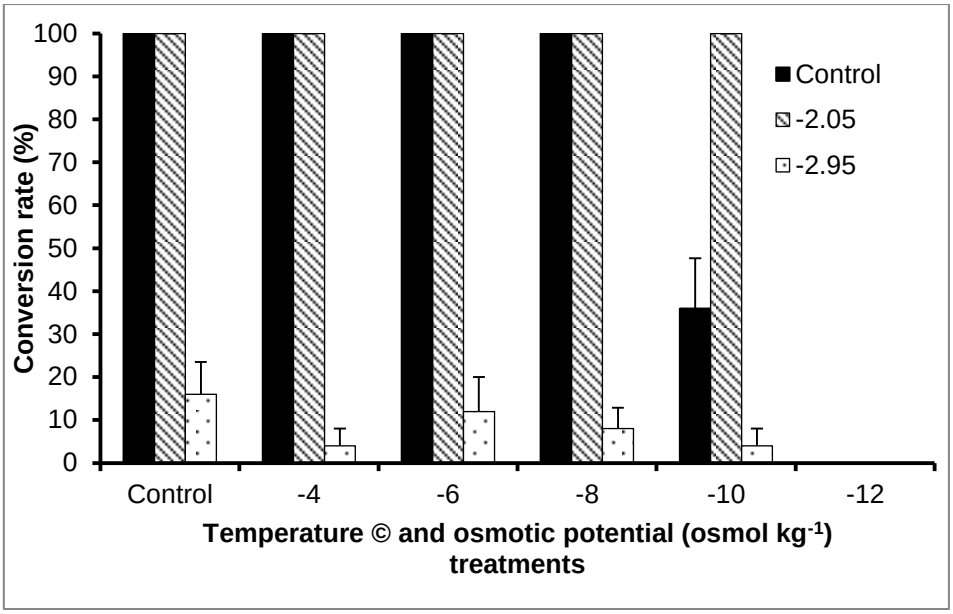


Figure 79. The effect of the culture osmotic potential (mannitol concentration) on the conversion rate of artificial seed treated with various low temperatures (LSD= 1.181).

Culture osmotic potential treatments → -2.95 Control -2.5

Figure 80. The effect of culture osmotic potential (mannitol concentration) on the conversion rate of artificial seeds treated with -10 °C (Bar= 1 cm).

4.3.3. Experiment 3:

The effect of cold acclimation on the cold tolerance and the induction of *CBF/DREB1* gene in mature cauliflower plants

4.3.3.1. Aims and Objectives

1. To determine the effect of acclimation on the cold tolerance in mature cauliflower plants.
2. To investigate the effect of low temperature on the induction of *CBF/DREB1* expression in mature cauliflower plants.

4.3.3.2. Material and Methods

The effect of low temperature treatment on the induction of *CBF/DREB1* gene expression

Eight cauliflower plants (cv. Aviso) were grown in pots placed in the greenhouse (Skarden Garden, Plymouth University) until they started forming curds. Four mature plants were transferred to Snijder cold cabinet at 4°C and 8 hours photoperiod while foure others were left in the greenhouse. Leaf samples, each consisting of 1 full leaf, were taken at 0 (control), 1, 6, 12, 18, 24 hours after transfer to the new temperature. The samples were kept at -80°C until the RNA was extracted using SOP-RNA$_{ext}$. Synthesis and amplification of *CBF/DREB1* cDNA was carried out using the SOP$_{OPCR}$. PCR was replicated three times.

The effect of cold acclimation on cauliflower mature plants cold tolerance

Two of the remaining mature plants were transferred from the greenhouse to the cold cabinet (the other two were kept as control) where they were kept for 15 days for acclimation and then the frost resistance was analysed using the electrical conductivity technique (SOP$_{ECL\%}$). Both acclimated and non-acclimated plants were tested at various sub-zeros temperatures as follows, control (20°C), -3, -6, -9, -12 °C.

Three tubes (replicates) were used with each temperature for both acclimated and non-acclimated plants (leaves).

4.3.3.3. Results

The effect of cold acclimation on cauliflower mature plant cold tolerance

It was observed that REC% increased frequently with the use of lower temperature and that was due to the increase in the cell damage. The REC% was significantly lower in the leave disk samples obtained from acclimated plants compared with that taken from non-acclimated when these samples were treated with various sub-zero temperature (*P*=0.016). Therefore, acclimation significantly improved the cold tolerance of cauliflower mature plants (Figure, 81).

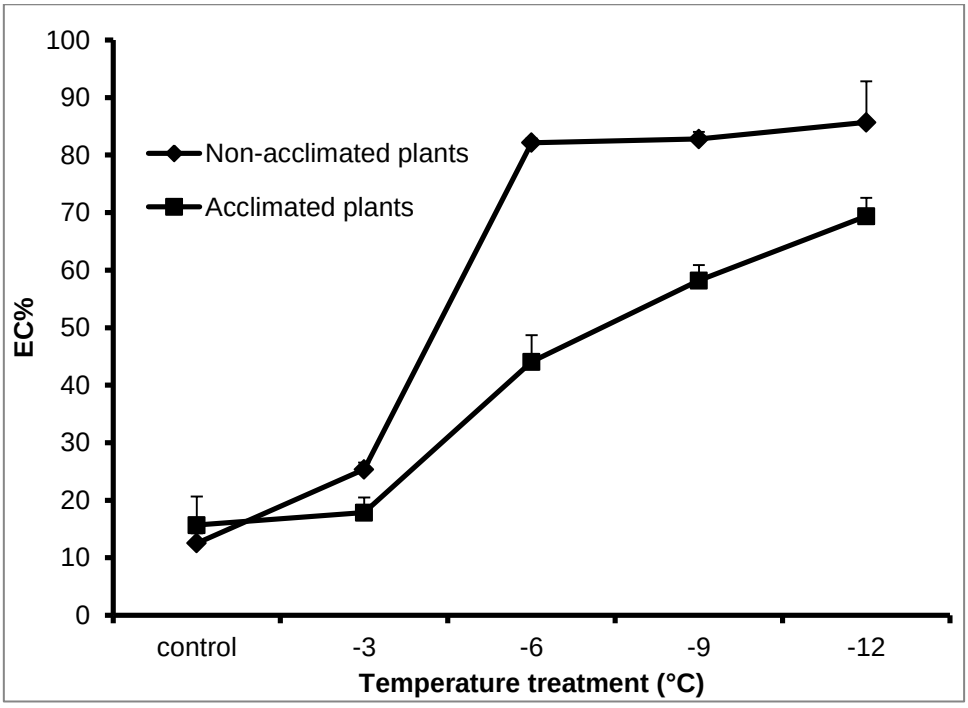
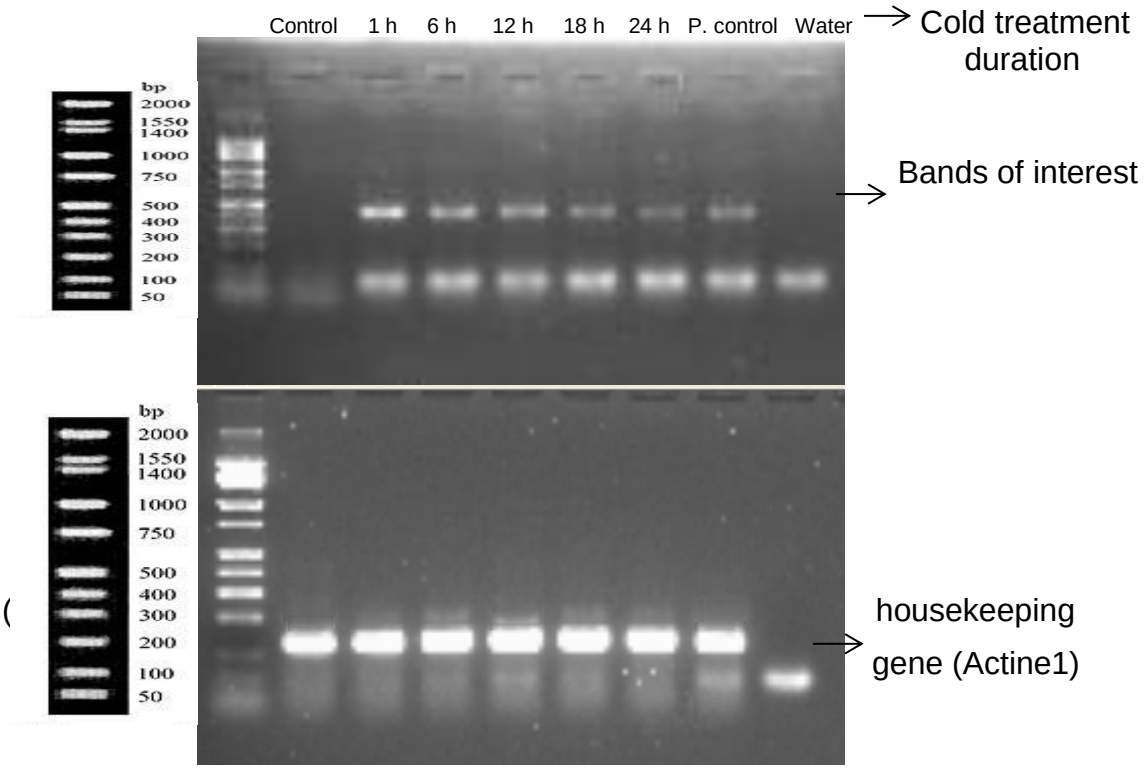


Figure 81. The effect of temperature treatment on the relative electrical conductivity (REC %) of both acclimated and non-acclimated leave disks derived from cauliflower mature plants (LSD=14.4).

The effect of cold acclimation on the induction of *CBF/DREB*1 gene expression in mature cauliflower plants

Cold acclimation had the capacity to induce the expression of *CBF/DREB1* gene after one hour of cold treatment. However the highest gene expression was observed after 12 h of cold treatment. (*P*=0.012)(Figure, 82).



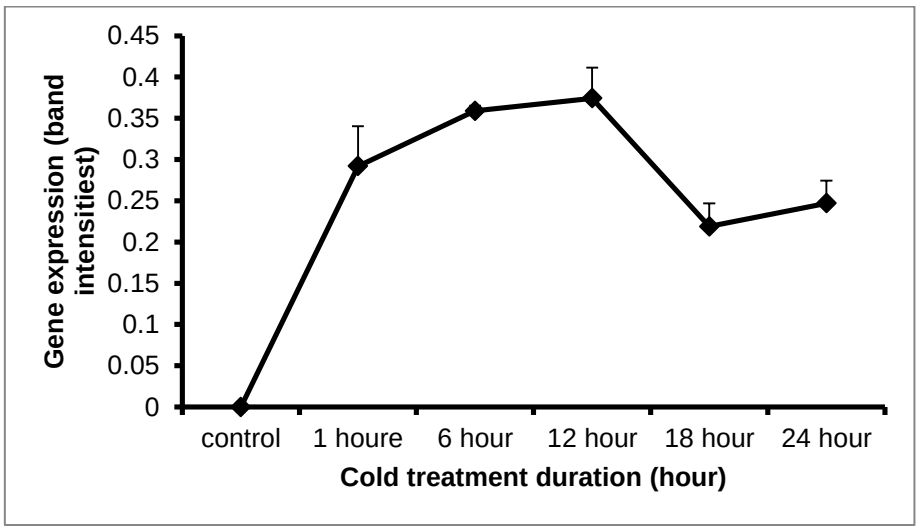


Figure 82. The effect of cold treatment at (4°C) treatments for different periods on the induction of CBF/DREB1 in mature cauliflower plants (LSD=0.098).

4.3.4. Experiment 4

The effect of drought on mature cauliflower plant cold tolerance and on the induction of *CBF/DREB1* gene expression in mature cauliflower plants

4.3.4.1. Aims and Objectives

1. To determine the effect of drought on the cold tolerance of mature cauliflower plants.
2. To investigate the capacity of drought to induce the expression of *CBF/DREB1* in mature cauliflower plants.
3. To determine the soil moisture level and cauliflower leaves stomatal conductance at which *CBF/BREB1* is up-regulated.

4.3.4.2. Materials and Methods

The effect of drought on the cold tolerance of cauliflower mature plants

Four cauliflower plants (cv. Aviso) were grown in pots in the green house (22 ± 2 °C) until they started forming curds. The plants were transferred to a Sanyo growth cabinet set at 23°Cand 16 hours light (177 µmol m^{-2} sec^{-1}). The plants were irrigated to field capacity. Two plants were then irrigated regularly every three days and the other two were left without irrigation for 10 days. The frost resistance of irrigated and non-irrigated plants was analysed using the electrical conductivity technique $SOP_{ECL\%}$. Both irrigated and non-irrigated were tested at different temperatures, control (20), -3, -6, -9, -12 °C. Three tubes (replicates) were used with each temperature for both irrigated and non-irrigated plants.

The effect of drought on the expression of *CBF/DREB1* in mature cauliflower plants

Four mature cauliflower plants (cv. Aviso) were cultivated in pots and they were transferred from the greenhouse to a Snijder growth cabinet set at 23°C and 8 hours

light when they started forming curds. All of these plants were irrigated to field capacity. One of the pots was placed on a balance (Toledo, model 4714) and weights were recorded of regular intervals. The weights and the soil voltage measured using a theta prob (Wavetek meterman, Delta T were recorded every two days. Three measurements of soil voltage were carried out each time. The field capacity soil (FC) was considered to be 100% moisture and the moisture content was determined each two days using the following equation:

Moisture content %= Soil weight at FC – recorded weight/ Soil weight at FC ×100

A standard curve of soil moisture content and the Theta probe reading was completed (Figure 83). Samples (each consisting of one full expanded leaf) from the other three plants were taken after 0, 1, 4, 8, 16, 24, 30 days of initial irrigation for RNA extraction. Theta probe readings were recorded when the samples were taken and using the standard curve, the Theta probe readings were used to calculate the moisture level at which the samples were obtained. At the same time stomatal conductance was measured using an automatic Porometer. Three measurements of soil voltage and stomatal conductance were carried out each time. However, the average was considered since the records were very similar at each time.The leaf samples were kept at -80°C until the RNA was extracted using SOP-RNA$_{ext}$. Synthesis and amplification of *CBF/DREB1* cDNA was carried out using the SOP$_{OPCR}$. Each PCR experiment was replicated 3 times.

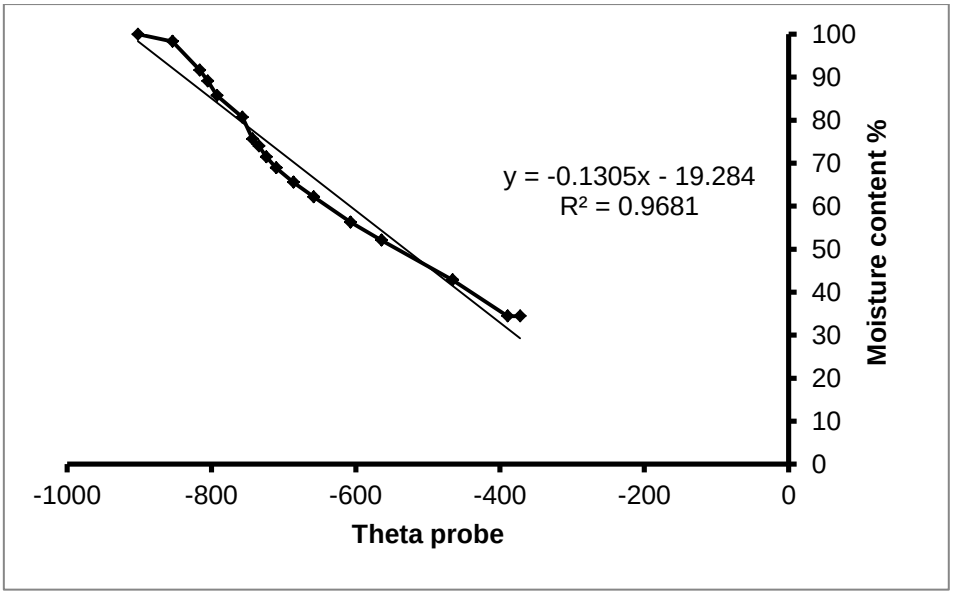


Figure 83. Standard curve of the relation between the Theta Probe and soil moisture content.

4.3.4.3. Results

Drought significantly reduced the REC% when the leaf disks were treated at -6°C (P<0.003) which seemed to be the critical temperature where the effect of drought on the REC% values (frost damage) was clear. The REC% was about 60 % from droughted plant at -6°C and it was about 90 % from irrigated plants (Figure 84). At temperature lower than this, a high increase in REC% occurred.

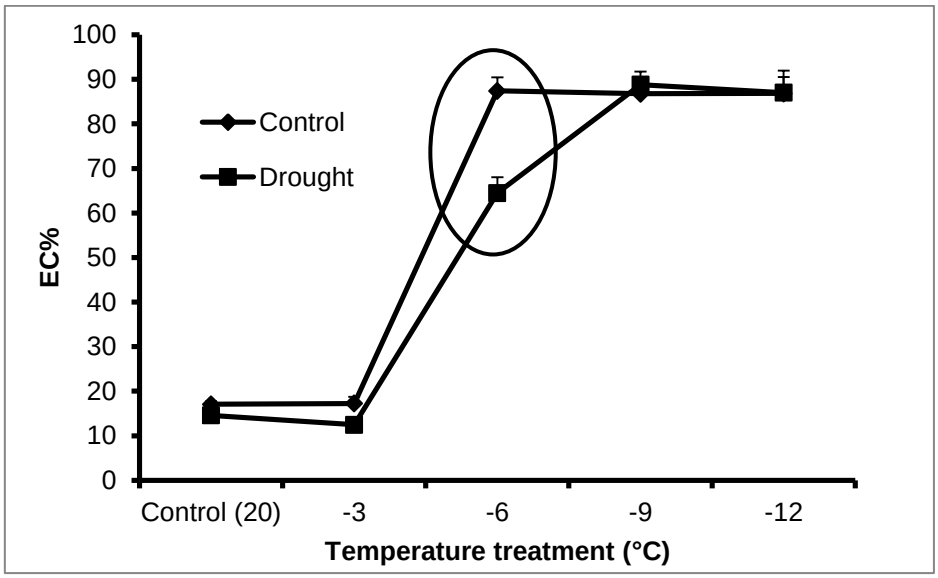


Figure 84. The effect of drought on the mature cauliflower frost damage under irrigation and drought (LSD=8.99 at -6°C).

Drought induced the expression of *CBF/DREB1* in cauliflower and it was observed that a reduction in moisture level to 70 % (4 days from the irrigation) was needed to induce the expression of *CBF/DREB1* (Figure 85).

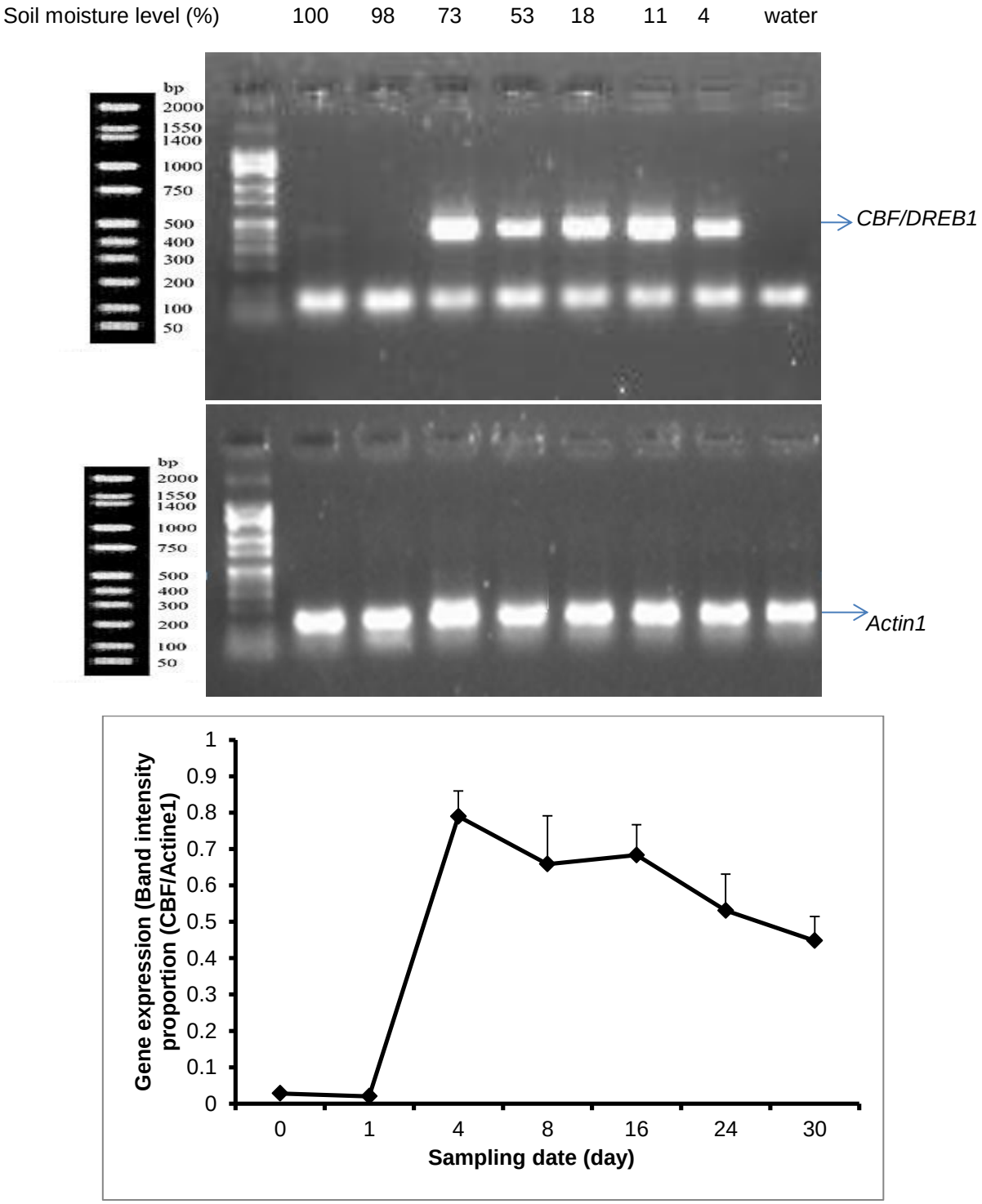


Figure 85. The effect of soil moisture level on the induction of *CBF/DREB1* expression in the cauliflower mature plants (LSD= 0.240).

It was confirmed that the lower the moisture level in the soil, the lower the stomatal conductance (Figure 86). The stomatal conductance, when the *CBF/DREB1* was up-regulated, was determined and found to be 0.889 mmol m^{-2} s^{-1} (Figure 86)

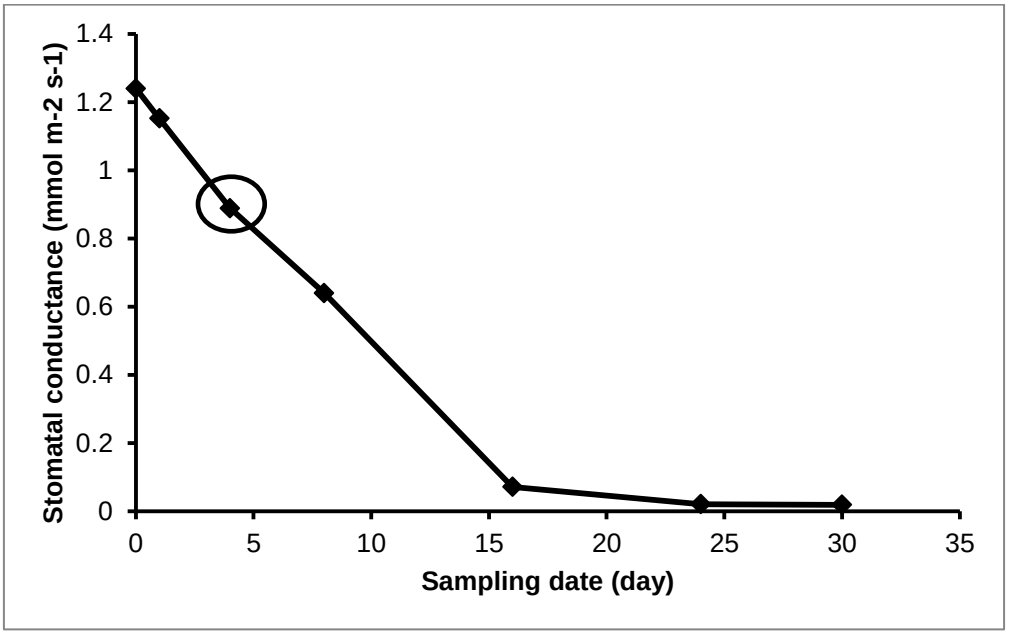


Figure 86. The effect of moisture level on the stomatal conductance of cauliflower full extended leaves.

4.3.5. Experiment 5

The effect of Abscisic acid (ABA) on the development of, cold tolerance and *CBF/DREB1* expression in cauliflower microshoots

4.3.5.1. Objectives

1. Investigate the effect of ABA on the number and average weight of cauliflower microshoots.
2. Assess the effect of ABA on the cold tolerance of acclimated and non-acclimated microshoots.
3. Evaluate the effect of ABA on the induction of *CBF/DREB1* gene expression.
4. Identify the cDNA sequence alignment of *BoCBF/DREB1* and compare it with those reported in other plant species.
5. Determine the sequence of *BoCBF/DREB1* amino acid alignments and compare these with those in other plant species.

4.3.5.2. Materials and methods

1. The effect of ABA on the development and cold tolerance of cauliflower microshoots

Cauliflower microshoots (cv. Cool) were produced using SOP_{CS} with five concentrations of ABA (sigma, A-7631) (0, 0.5, 1, 2, 4 mg L^{-1}) added to the culture medium. The number and average weights of microshoots produced were recorded after 21 days of culture. 8 culture pots were used for each treatment.

Another set of microshoots with same culture conditions were used to determine the effect of ABA on microshoots cold tolerance. This culture was divided into parts: in the first one, 21 day old microshoots were tested at, control (20°C), -4, -8 and -12 °C. The frost damage to microshoots was analysed using the $SOP_{ECM\%.}$ 3 tubs were use with each treatment at each temperature.

The second part of culture was transferred to a cold room at 4°C after 21 days of culture. They were left in at the new temperature for 15 days for acclimation. Microshoots cold tolerance (frost damage) was tested at control (20°C), -4, -8 and -12 °C. The frost damage to microshoots was analysed using the $SOP_{ECM\%.}$ 3 tubs were used with each treatment at each temperature.

2. The effect of ABA on the induction of *CBF/DREB1* gene expression

Cauliflower microshoots (cv. Cool) were produced using SOP_{CS} with the only exception that five concentration of ABA (0, 0.5, 1, 2, 4 mg L^{-1}) were used with the culture media. 21 day old microshoots were transferred to cold cabinet (Sanyo) set at 4°C for acclimation. Samples of microshoots were obtained after 0, 1, 6, 12, 24 hours of low temperature treatment. mRNAs were extracted using the (SOP_{RNAext}) protocol. The evaluation of *CBF/DREB*1 gene expression was carried out as follow:

1. SOP_{OPCR} protocol was used to detect the expression of *CBF/DREB1* gene using samples obtained from all ABA treatments exposed to low temperature 4°C for 6 hours.
2. SOP_{OPCR} protocol was used to investigate the expression of *CBF/DREB1* gene using samples obtained from the 4 mg L^{-1} ABA treatment exposed to 0, 1, 6, 12, 24 hours of low temperature (4°C).
3. Primers (P2) were with the SOP_{OPCR} protocol to investigate their suitability for *CBF/DREB*1 detection ordinary PCR. *CBF/DREB1* expression was investigated using samples obtained from 4 mg L^{-1} treatment and exposed to 0, 1, 6, 12, 24 hours of low temperature (4°C).

4. SOP_{QPCR} was used to detect *CBF/DREB1* expression using samples obtained from 4 mg L^{-1} treatment and exposed to different durations (0, 1, 6, 12, 24 hours) of low temperature (4°C).

Each PCR experiment was replicated three times.

3. cDNA sequencing

Microshoot samples that were investigated to have positive *CBF/DREB1* gene expression were used to yield cDNA of this gene using both types of primers P1 and P2. cDNA sequences of *CBF/DREB1* detected with both primers (P1 and P2) were purified using a cleaning kit protocol (Qiagen, Cat. no. 28004) described as follows:

- 500 µL of binding buffer was added and mixed with 100 µL of each PCR product samples obtained using primers P1 and P2.
- The mixtures were applied to high pure filter tubes and centrifuged at the maximum speed (13.000 xg) for 60 sec.
- The flow through was discarded and 500 µL of washing buffer was added to each of both filters. The filters were centrifuged at the maximum speed for 60 sec.
- The flow through was discarded and 200 µL of washing buffer was added to the filters. The filters were centrifuged at the maximum speed for 60 sec.
- The flow through was discarded and the filters were transferred to new collection tubes. 75 µL of molecular pure water was added to each filter centrifuged at the maximum speed for 60 sec to get the purified PCR product.

The purified DNA was subjected to sequencing by Eurofins MWG Operon (Germany). Multiple nucleotide sequence alignment and deduced amino acids

sequences of *BoCBF/DREB1* comparison between the sequences obtained using both primers (ordinary and quantitative PCR primers) and with other cold induced genes sequences were carried out using ClustalW 2. EMBL-EBI (Larkin *et al*., 2007) and BLAST (NCBI). Following this a phylogenic tree was constructed using sequence information.

4.3.5.3. Results

1. The effect of ABA on the number and viability of microshoots

While ABA had no significant effect on the number of developing microshoots (*P*=0.147), a highly significant effect on their average weights was observed. The higher the concentration of ABA used the lower their average weight (*P*<0.001) (Figure 87).

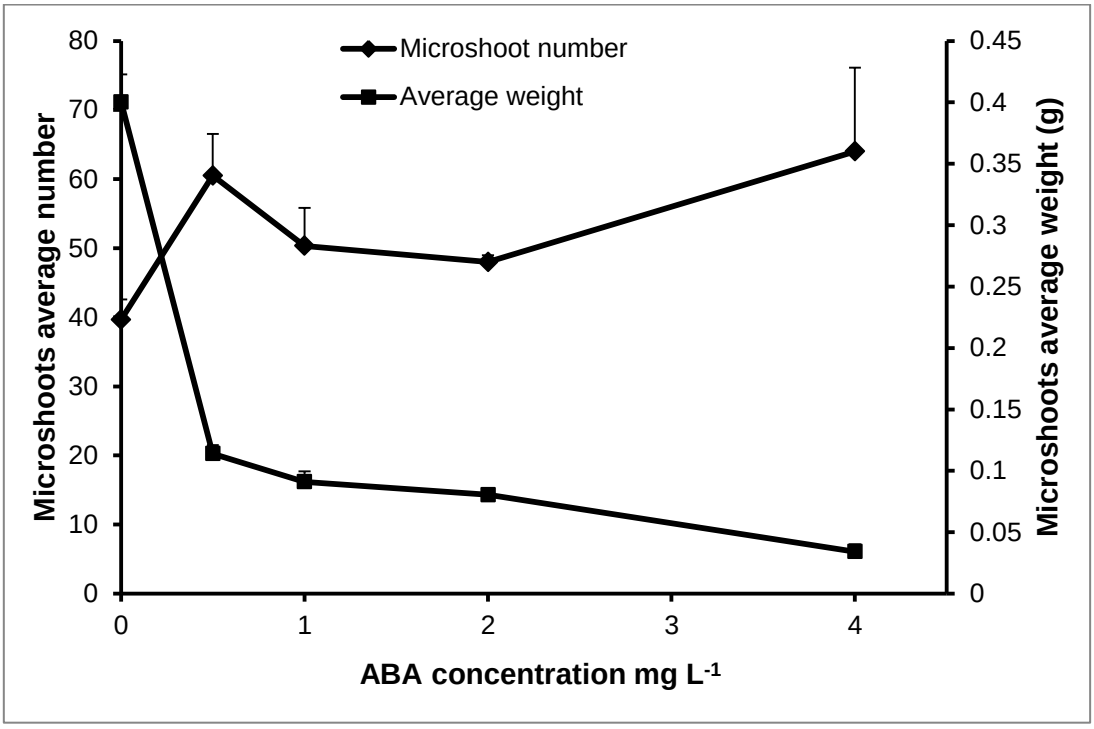


Figure 87. The effect of ABA on the development of microshoots (LSD=0.029 for the average weights of microshoots).

2. The effect of ABA on the microshoots cold tolerance

Low temperature treatment significantly increased the REC% (Figure 88) (P<0.001). While there was no significant effect of ABA on the REC% at control (20 ºC), -4 and -12 ºC (P=0.062, P=0.133 and P=0.870 respectively), A highly significant effect was observed at -8 ºC (P=0.003). The use of ABA at relatively low concentration (0.5 mg L^{-1}) significantly increased the REC% (frost damage). However, the higher the concentration of ABA the lower the increase in REC% compared with the control. Moreover, there was no significant increase in REC% between the control and 4 mg L^{-1} ABA treatments (Figure 89). -8 ºC seemed to be the sensitive temperature where the effect of ABA was clear. The use of lower than -8 ºC significantly increased the REC% in all ABA treatments.

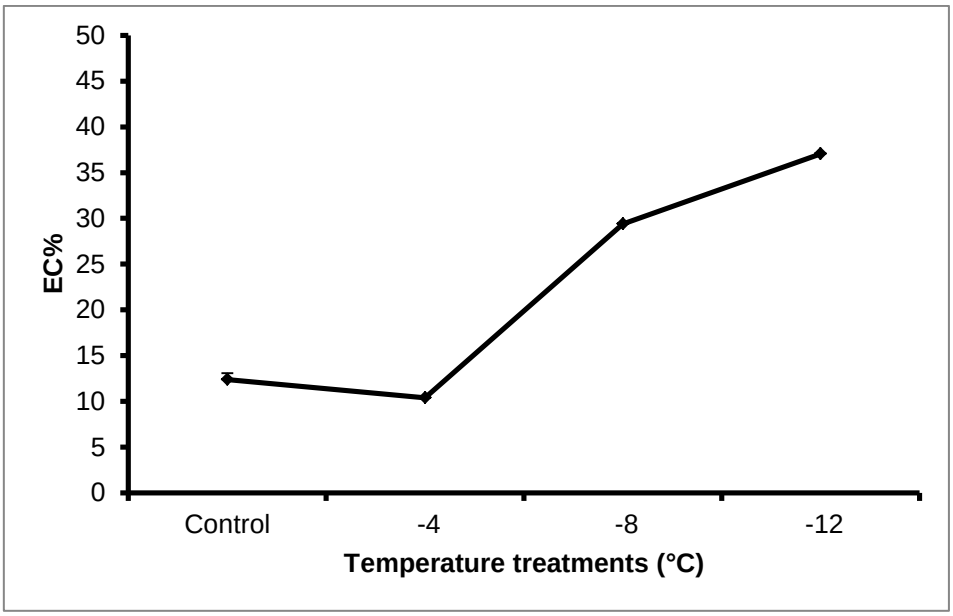


Figure 88. The effect of temperature treatments on the relative REC% (frost damage) in cauliflower microshoots (LSD=4.53).

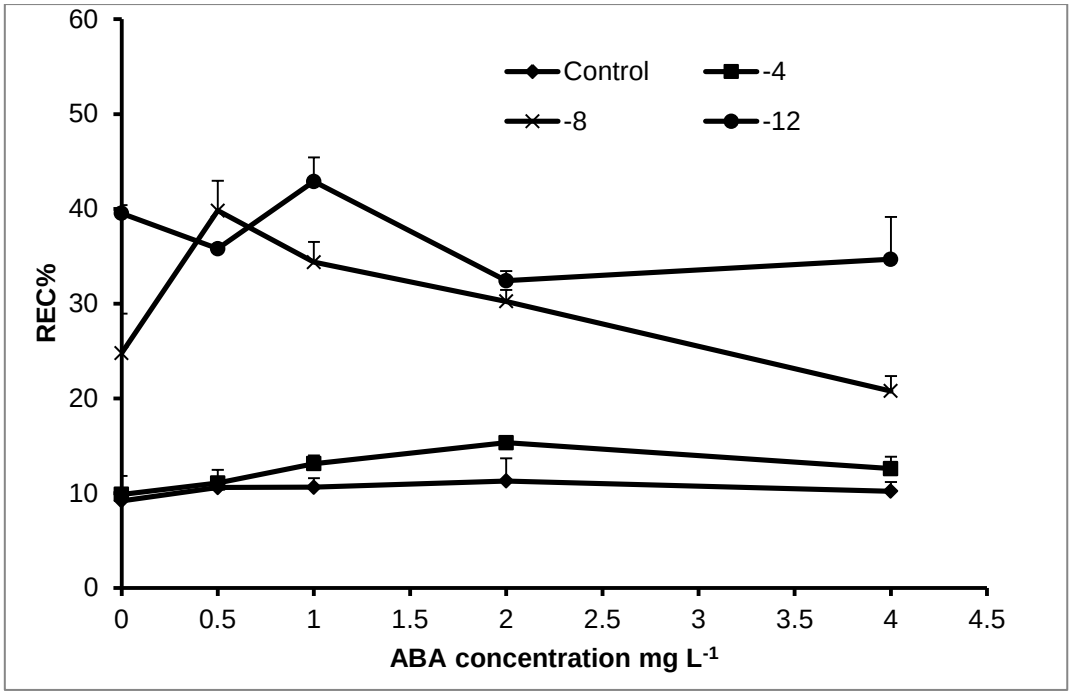


Figure 89. The effect of ABA on the REC % of microshoots treated with 20, -4, -8, and -12 ° C (LSD for REC% at -8 ° C= 6.7).

3. The effect of ABA on the cold tolerance of acclimated microshoots

There was no significant effect of ABA on the acclimated microshoots cold tolerance (REC%) when these microshoots were treated with 20 ºC (Control), -4 and -12 ºC (*P*= 0.214. *P*=0.062 and *P*=0.870 at 20, -4 and -12 treatments respectively). However, a highly significant effect of ABA on the acclimated microshoots frost damage was observed when microshoots were treated with -8 ºC. ABA significantly increased REC% (frost damage) at this temperature (*P*=0.003). Therefore, -8 ºC was confirmed to be the sensitive temperature where the effect of ABA was observed (Figure 90).

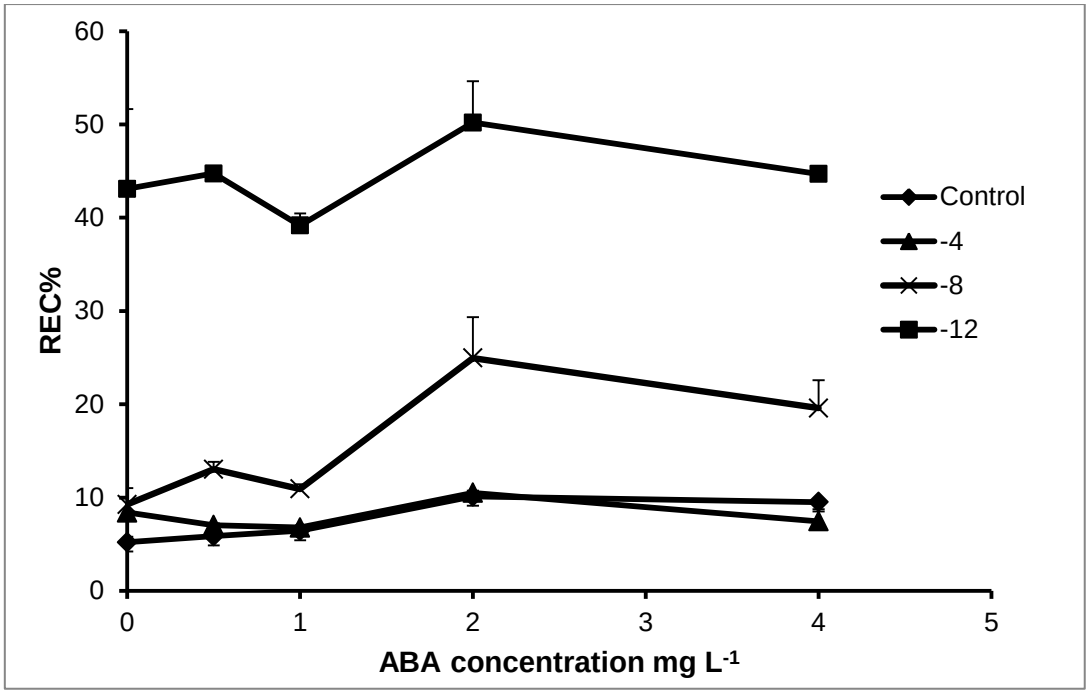


Figure 90. The effect of ABA on the REC % of microshoots treated with 20, -4, -8, and -12 ° C (LSD for the -8 treatment = 0.056).

4. The effect of ABA on the expression of *CBF/DREB1*

The concentration of RNA in the samples derived from microshoots treated with ABA was significantly higher than those in the samples derived from the ABA non-treated micoshoots (control) (P<0.001) (Figure 91) (the same fresh weight of microshoots was used for the RNA extraction).

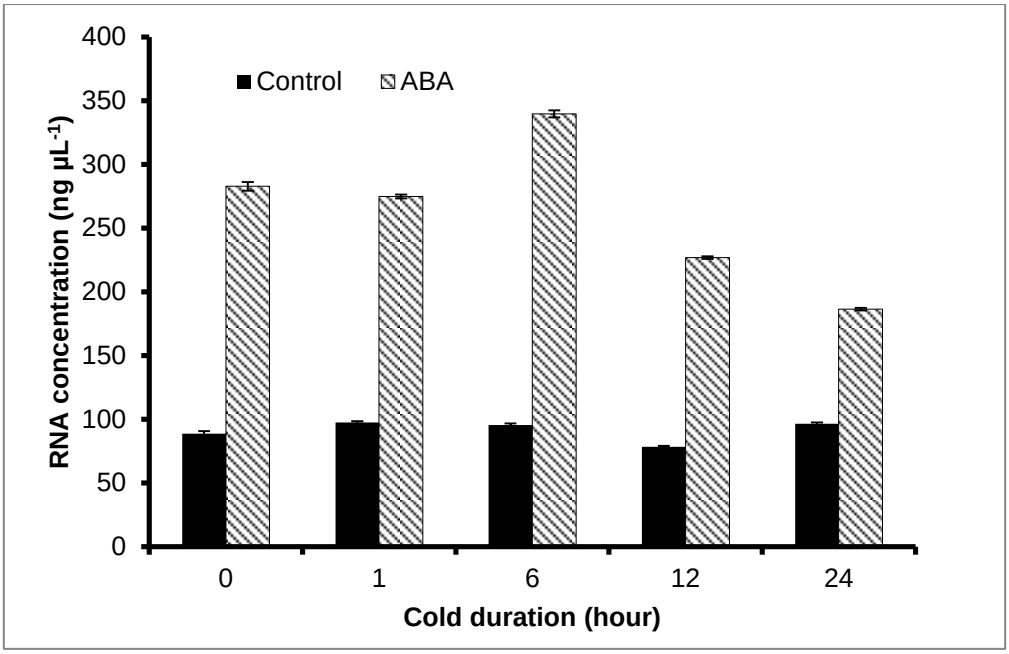
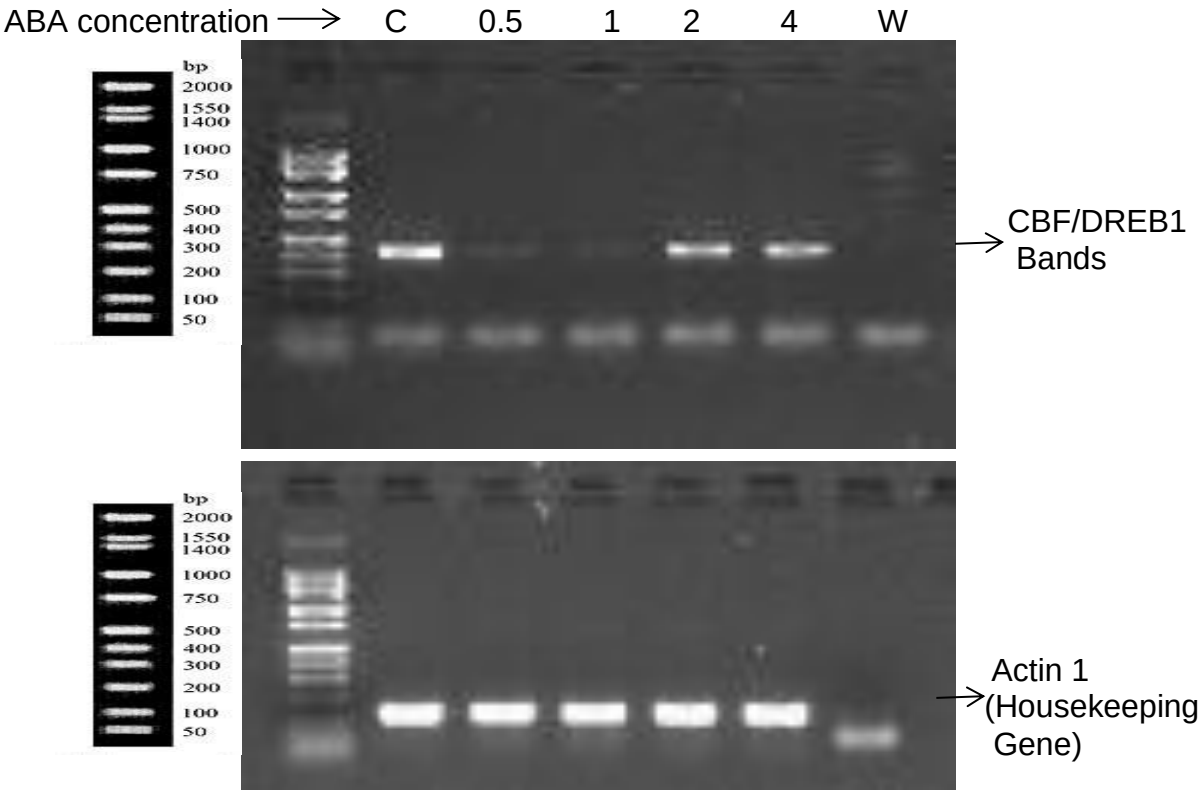


Figure 91. The effect of ABA on the concentration of mRNA derived from cauliflower microshoots treated with low temperature (4°C) for several durations (LSD=26.72).

Since the use of ABA appeared to reduce cold tolerance in both acclimated and non-acclimated microshoots, this experiment aimed to investigate the effect of this material on the expression of *CBF/DREB1*. The use of ABA with the liquid culture media significantly reduced the expression of *CBF/DREB*1 gene (P<0.001) in the cauliflower microshoots when they were treated at 4°C for 6 hours regardless of the concentration of ABA used. There was a relationship between the concentration of ABA used and *CBF/DREB1* expression; the higher the concentration of ABA was used, the higher the expression was observed. However, the expression of *CBF/DREB1* (Band intensity) was lower than the control even when it was used at relatively high concentration (4 mg L^{-1}) (Figure 92). It was not used at concentration higher than 4 mg L^{-1} due to the negative effect of such a concentration on the viability of microshoots (average weights).



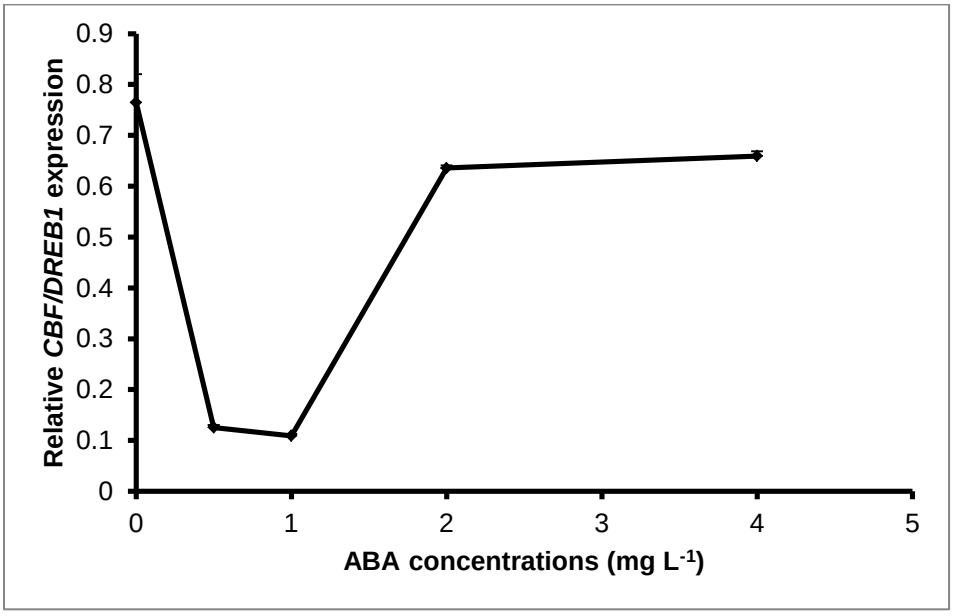


Figure 92. The effect ABA concentrations on the induction of CBF/DREB1 gene (CBF/Actin ratio) expression after 6 hours of low temperature treatment at 4°C (LSD=0.08).

The use of 4 mg L^{-1} of ABA in the cauliflower microshoots culture medium significantly reduced the expression of *CBF/DREB1* gene in the samples treated with low temperature at 4°C for 1, 6, 12, 24 hours (*P*=0.034) (Figure 93).

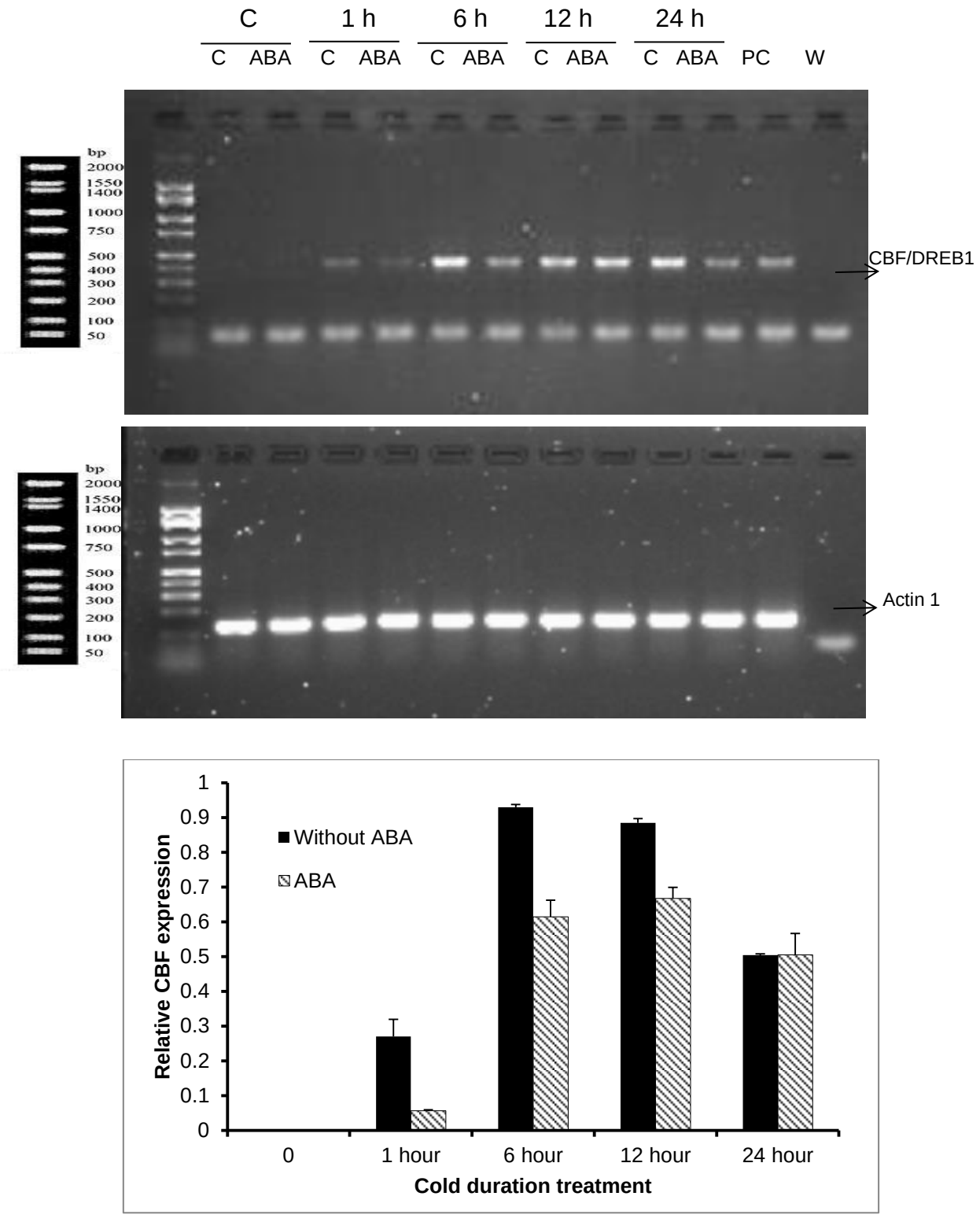
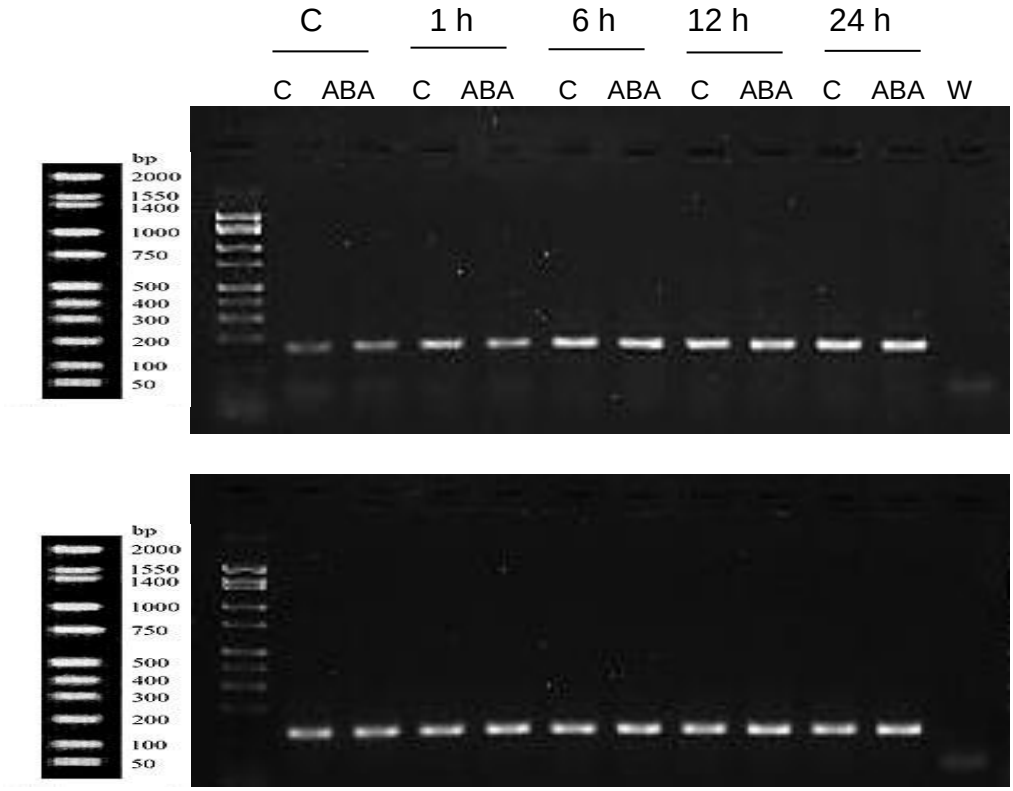


Figure 93. The effect of using 4 mg L^{-1} concentration of ABA on the expression of CBF/DREB1 in cauliflower microshoots exposed to several duration of low temperature at 4°C (LSD= 0.186).

5. The effect of ABA on the expression of *CBF/DREB1* using P2 primers

The results showed that there is no significant effect of ABA on the expression of *CBF/DREB*1 (*P*=0.863) when samples were treated with 4 mg L^{-1} ABA and subjected to 4°C for 0, 1, 6, 12, 24 hours (Figure 94). However, this contrast with the results obtained using (P1) primers. Therefore, it was necessary to determine the sequences of *CBF/BREB*1 genes detected using both to make sure that both of primers detect *CBF/DREB1*.



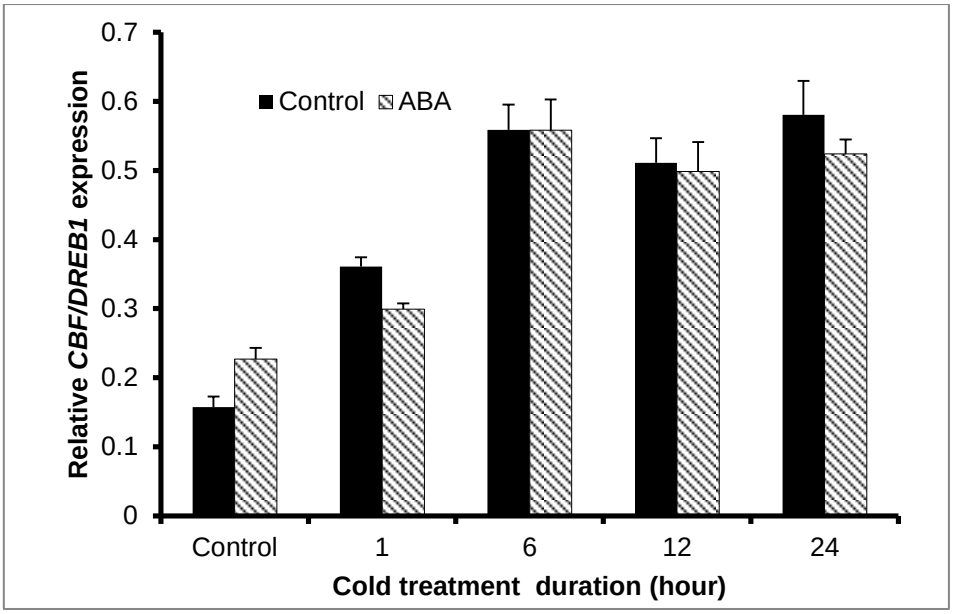


Figure 94. The effect of using 4 mg L^{-1} of ABA on the expression of *CBF/DREB1* (using P2 primers) in cauliflower microshoots treated to several duration of low temperature at 4°C compared to that in no-treated microshoots.

6. The effect ABA on the expression of *CBF/DREB*1 investigated using real time PCR

The investigation of the effect of using 4 mg L^{-1} of ABA on the expression of *CBF/DREB1* using microshoot samples exposed to low temperature at 4 °C for (0, 1 ,6, 12, 24 hours) and by applying quantitative PCR technique showed that the use of this material significantly reduced the expression of *CBF/DREB1* regardless the low temperature exposure time (P=0.006) (Figure 95).

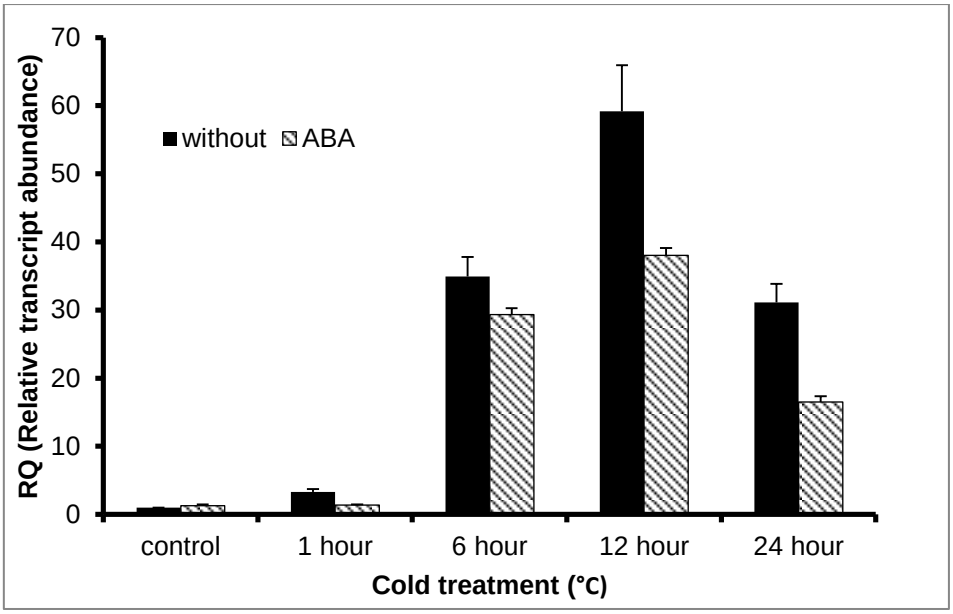


Figure 95. The effect of using 4 mg L^{-1} of ABA on the expression of *CBF/DREB1* using the quantitative (Real time) PCR in cauliflower microshoots exposed to several duration of low temperature at 4°C (LSD=9.87).

7. cDNA sequencing

The sequences of *CBF/DREB1* alignment obtained by using both P1 and P2 primers

1. The sequences of *CBF/DREB1* alignment obtained by using P1 primers

Forward

GAGGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCTCGGTACTTTCCTAACAGCCGAGATCGCAGCCCGT

GCTCACGACGTCGCCGCCATAGCCCTCCGCGGCAAATCAGCTTGTCTCAATTTTGCCGACTCCGCTTGGCGGCT

CCGTATCCCGGAGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGGC

TGAGATAAATAATACGACGGCGGATCATGGCATTGACGTGGAGGAGACGATCGTGGAGGCTATTTTCACGGA

GGAAAACAACGATGGTTTTTATATGGACGAGGAGGAGTCCATGTTCGGGATGCCGGCCTTGTTGGCTAGTAT

GGCGGAAGGTAGCTTTTGCC

Reverse

ATATGGACTCCTCCTCGTCCATATAAAAACCATCGTTGTTTTCCTCCGTGAAAATAGCCTCAACGATCGTCTCCT

CCACGTCAATGCCATGATCCGCCGTCGTATTATTTATCTCAGCCTGAAAAGCCACCGCGGCTTCAGCAGCCGCC

TTCTGAATCTCCTTGGGGCATGTTGTCTCCGGGATACGGAGCCGCCAAGCGGAGTCGGCAAAATTGAGACAA

GCTGATTTGCCGCGGAGGGCTATGGCGGCGACGTCGTGAGCACGGGCTGCGATCTCGGCTGTTAGGAAAGT

ACCGAGCCAAATCCTGGATTTCTTGTTTGGCTCCCTCACTTCACACACCCACTTACCTGAGTGTCTCAG

Figure 96. (*1CBF/DREB1*) sequence obtained using primers (P1) (BLAST (NCBI)) Fasta sequences :(F premix 52..433 of sequence) (R premix 25..385 of sequence).

2. The sequences of *CBF/DREB1* alignment obtained by using P2 primers

Forward

GTCGCCGCCATAGCCCTCCGTGGCAATCACGCTCTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCC

CGGAGACAACATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGGCTGAGAA

Reverse

CTGGATATCCTTGGGGCATGTTGTCTCCGGGATACGGAGCCGCCAAGCCGAGTCGGCGAAATTGAGGCAGGC

GGATTTGCCACGGAGGGCTATGGCGGCGACGTCGTGAGCACGAGCTGCGATCTCGGCGGTTAGGAAAGTC

Figure 97. (*2CBF/DREB1*) sequence obtained using primers (P2) (BLAST (NCBI)) Fasta sequences: (F premix 16...159 of sequence) (R premix 14...155 of sequence).

The nucleotide sequence of cDNA isolated from *Brassica oleracea* var. botrytis using both types of primers P1 and P2 were determined (Figures 96 and 97) and compared with each other. These sequences were also compared with *CBF/DREB1* DNA sequences reported for other plant species. The results showed significant resemblances with up to 93% sequence consensus between the sequences obtained using the primers P1 and the ones obtained using the primers P2.

When the sequences obtained using primers P1 were compared with *CBF/DREB*1 DNA sequences reported for other plant species, the results showed significant

similarities with some. The closest similarities between the DNA sequence was obtained using P1 primers:

1. *Brassica napus* CBF-like protein CBF16 (CBF16) mRNA, complete cds (98 % similarities)(GenBank: AF499033.1, 945 bp gene)(Gao *et al*., 2002)
2. *Brassica napus* DREB2-3 mRNA, complete cds (98 % similarities) (GenBank: AY444875.1, 648 bp)(Zhao *et al*., 2006).
3. *Nicotiana tabacum* DREB1 mRNA, complete cds (96% similarity) (GenBank: EU727155.1, 648 bp) (Liu and Feng, unpubished)
4. *Brassica napus* DREB2-1 mRNA, complete cds (96% similarities)(GenBank: AY437878.1, 648 bp) (Zhao *et al*., 2006)
5. Brassica napus DREB2-23 mRNA, complete cds (96% similarities) (GenBank:AY444876.1, 645 bp)(Zhao *et al*., 2006)
6. *Brassica juncea* DREB1B mRNA, complete cds (96% similarities) (GenBank:EU136731.1, 838 bp)(Cong *et al*., 2008)
7. *Raphanus sativus* CBF1 mRNA, complete cds (94% similarity) (GenBank: GQ866977.1, 923 bp) (Li and Gao, unpublished)
8. *Brassica rapa* subsp. pekinensis dehydration responsive element binding protein 2-19 gene, partial cds (94% similarity) (GenBank: EF219470.1, 646 bp)(Wang *et al*, unpublished)
9. *Brassica napus* CBF-like protein CBF7 (CBF7) mRNA, complete cds (94 % similarities) (GenBank:AF499032.1, 1061 bp) (Gao *et al*., 2002)
10. *Brassica napus* dehydration responsive element binding protein mRNA, complete cds (94% similarity) (GenBank: AF084185.1, 967 bp)(Zhou, unpublished)

When the sequences obtained using primers P2 compared with *CBF/DREB*1 sequence reported for other species, the results showed significant similarities found to be 98% sequence consensus in several plant species described as follow:

1. *Brassica napus* DREB2-23 mRNA, complete cds (GenBank:AY444876.1, 645 bp)(Zhao *et al*., 2006)
2. *Raphanus sativus* CBF1 mRNA, complete cds (GenBank: GQ866977.1) (Li and Gao, unpublished)
3. *Brassica napus* CBF mRNA, partial cds (GenBank: HM235815.1, 437 bp)(Zhao *et al*, 2010)
4. *Brassica rapa* subsp. pekinensis DREB-like protein 2 (DREB2) mRNA, complete cds. (GenBank: GQ122331.1) (Wang *et al*, Unpublished)
5. *Brassica rapa* subsp. pekinensis DREB-like protein 1 (DREB1) mRNA, complete cds (GenBank: EU924266.1, 645 bp) (Wang *et al*, Unpublished)
6. *Brassica rapa* subsp. chinensis DREB2 mRNA, partial cds (GenBank: EF495249.1, 429 bp)(Han *et al*, 2008)
7. *Chorispora bungeana* isolate A6 CRT/DRE-binding factor CBF-like protein mRNA, partial cds (GenBank: AY994128.1) (Yang,T *et al*, published)
8. *Chorispora bungeana* isolate A4 CRT/DRE-binding factor CBF-like protein mRNA, partial cds (GenBank: AY994126.1) (Yang,T *et al*, published)
9. *Chorispora bungeana* isolate A3 CRT/DRE-binding factor CBF-like protein mRNA, partial cds (GenBank: AY994125.1) (Yang,T *et al*, published)
10. *Brassica napus* DREB2-2 mRNA, complete cds (GenBank: AY444874.1, 645 bp) (Zhao *et al*., 2006)

It was observed from the results of sequencing obtained using both primers P1 and P2 that the nucleotide sequences were similar to different *CBF/DREB*1

genes in the Brassicase. However, Brassica napus DREB2-23 mRNA, (GenBank:AY444876.1, 645 bp) (Zhao *et al*., 2006) was found to have the best similarity with both sequences obtained using P1 and P2 primers (Figures 98 and 99)

```
AF499033.1        ------------------------------------------------------------
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        ------------------------------------------------------------
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        ------------------------------------------------------------
AF084185.1        ------------------------------------------------------------
EF219470.1        ------------------------------------------------------------
AF499032.1        AAATTATACAAAAAGAGTTCGAGCTCTTGATTACTTAATTAAAAACAAATTTTACTTCTC 60

AF499033.1        ------------------------------------------------------------
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        ------------------------------------------------------------
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        ------------------------------------------------------------
AF084185.1        ------------------------------------------------------------
EF219470.1        ------------------------------------------------------------
AF499032.1        TTTTTTTTATGGTCTTTTGTCATGATCTGAAATACGGTCTTTGTTGATAAATCATATATG 120

AF499033.1        ---------AATTCGGCACGAGCTGAGAGATAAATTAAACATTTATCAAACCAACGAAAC 51
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        ---------AATTCGGCACGAGCTGAGAGATAAATTAAACATTTATCAAACCAACGAAAC 51
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        ------------------------------------------------------------
AF084185.1        --------------------GGCACGAGGATAAATTAAACATTTATCAAACCAACGAAAC 40
EF219470.1        ------------------------------------------------------------
AF499032.1        CCGATTTTGATTTTTAATCCACCTGAGAGATAAATTAAACATTTATCAAACCAACGAAAC 180

AF499033.1        ATAGATCTTTGTACTTACTATACTTCACCTTATCCAGTTTTATTTTTTTATTTATAAAGA 111
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        ATAGATCTTTGTACTTACTATACTTCACCTTATCCAGTTTTATTTTTTTATTTATAAAGA 111
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        ------------------------------------------------------------
AF084185.1        ATAGATCTTTGTAGTTACTTATCCAGTT---------TATTTTTTAAAAAATTATAAAGA 91
EF219470.1        ------------------------------------------------------------
AF499032.1        ATAGATCTTTGTAGTTACTTATCCAGTT---------TATTTTTTAAAAAATTATAAAGA 231

AF499033.1        GTTTTCAACAATGACCTCATTTTCTACCTTTTCTGAACTGTTGGGCTCCGAGCATGAGTC 171
```

```
AY444875.1          ----------ATGACCTCATTTTCTACCTTCTCTGAACTGTTGGGCTCCGGGCATGAGTC 50
1BoCBF/DREB1        ------------------------------------------------------------
2BOCBF/DREB1        ------------------------------------------------------------
AY444876.1          ----------ATGACCTCATTTTCTGCCTTTTCTGAAATGATGGGCTCCGAGAACGAGTC 50
GQ866977.1          GTTTTCAACAATGACCTCATTTTCTACCTTTTCTGAACTGTTGGGCTCCGAGCATGAGTC 171
EU727155.1          ----------ATGACCTCATTTTCTGCCTTTTCTGAACTGTTGGGCTCCGAGCATGAGTC 50
AY437878.1          ----------ATGACCTCATTTTCTGCCTTTTCTGAACTGTTGGGCTCCGAGCATGAGTC 50
EU136731.1          ------AACAATGACCTCATTTTCTACCTTTTCTGAAATGTTGGGCTCCGAGTATGAGTC 54
AF084185.1          GATTTCAACAATGACCTCATTTTCTACCTTTTCTGAAATGTTGGGCTCCGAGTATGAGTC 151
EF219470.1          -----ATGCGATGACCTCATTTTCTGCCTTCTCTGAAATGTTGGGCTCCGAGTACGAGTC 55
AF499032.1          GATTTCAACAATGACCTCATTTTCTACCTTTTCTGAAATGTTGGGCTCCGAGTATGAGTC 291

AF499033.1          TCCGGTTACATTAGGCGAAGAGTATTGTCCGAAGCTGGCCGCAAGCTGTCCGAAGAAACC 231
AY444875.1          CCCGGTTACGTTAGGCGAAGAGTATTGTCCGAAGCTGGCCGCAAGCTGTCCGAAGAAACC 110
1BoCBF/DREB1        ------------------------------------------------------------
2BOCBF/DREB1        ------------------------------------------------------------
AY444876.1          TCC---TGCATTAAGCGGGGAGTATTGTCCGACGCTGGCCGCGAGCTGTCCGAAGAAACC 107
GQ866977.1          TCCGGTTACATTAGGCGAAGAGTATTGTCCGAAGCTGGCCGCAAGCTGTCCGAAGAAACC 231
EU727155.1          TCCGGTTACATTAGGCGAAGAGTATTGTCCGAAGCTGGCCGCAGGCTGTCCGAAGAAACC 110
AY437878.1          TCCGGTTACATTAGGCGAAGAGTATTGTCCGAAGCTGGCCGCAAGCTGTCCGAAGAAACC 110
EU136731.1          TCCGGTTACATTAGGCGGTGAGTATTGTCCGACGCTGGCCGCGAGCTGTCCGAAGAAACC 114
AF084185.1          TCCGGTTACGTTAGGCGGAGAGTATTGTCCGAAGCTGGCCGCGAGCTGTCCGAAGAAACC 211
EF219470.1          TCC---TACATTAAGCGGGGAGTATTGTCCGACGCTGGCCGCGAGCTGTCCGAAGAAACC 112
AF499032.1          TCCGGTTACGTTAGGCGGAGAGTATTGTCCGAAGCTGGCCGCGAGCTGTCCGAAGAAACC 351

AF499033.1          AGCCGGCCGGAAGAAGTTTCGAGAGACGCGTCACCCAGTTTACAGAGGAGTTCGTCTGAG 291
AY444875.1          AGCCGGTCGGAAGAAGTTTCGAGAGACGCGTCACCCAGTTTACAGAGGAGTTCGTCTGAG 170
1BoCBF/DREB1        ------------------------------------------------------------
2BOCBF/DREB1        ------------------------------------------------------------
AY444876.1          TGCGGGTCGGAAGAAGTTTCGGGAGACGCGTCACCCAATTTACAGAGGAGTTCGTCAGAG 167
GQ866977.1          AGCCGGTCGGAAGAAGTTTCGGGAGACGCGTCACCCAATTTACAGAGGAGTTCGTCTGAG 291
EU727155.1          AGCCGGTCGGAAGAAGTTTCGAGAGACGCGTCACCCAGTTTACAGAGGAGTTCGTCTGAG 170
AY437878.1          AGCCGGTCGGAAGAAGTTTCGAGAGACGCGTCACCCAGTTTACAGAGGAGTTCGTCTGAG 170
EU136731.1          TGCGGGTCGGAAGAAGTTCCGGGAGACGCGTCACCCAATTTACAGAGGAGTTCGTCTGAG 174
AF084185.1          AGCCGGTCGTAAGAAGTTTCGGGAGACGCGTCACCCAGTTTATAGAGGAGTTCGTCTGAG 271
EF219470.1          TGCGGGTCGGAAGAAGTTTCGGGAGACGCGTCACCCAATTTACAGAGGAGTACGTCTGAG 172
AF499032.1          AGCCGGTCGTAAGAAGTTTCGGGAGACGCGTCACCCAGTTTATAGAGGAKTTCGTCTGAG 411

AF499033.1          AAACTCAGGTAAGTGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 351
AY444875.1          AAACTCAGGTAAGTGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 230
1BoCBF/DREB1        ----------------------GAGGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 38
2BOCBF/DREB1        ------------------------------------------------------------
AY444876.1          ACACTCAGGTAAGTGGGTGTGCGAGGTGAGAGAGCCAAACAAGAAATCCAGGATTTGGCT 227
GQ866977.1          AAACTCAGGCAAGTGGGTGTGTGAGGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 351
EU727155.1          AAACTCAGGTAAGTGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 230
AY437878.1          AAACTCAGGTAAGTGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 230
EU136731.1          AAACTCAGGTAAGTGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCT 234
AF084185.1          AAACTCAGGTAAATGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCCAGGATTTGGCT 331
EF219470.1          AAACTCAGGTAAGTGGGTGTGTGAGGTGAGGGAGCCAAACAAAAAGTCTAGGATTTGGCT 232
AF499032.1          AAACTCAGGTAAATGGGTGTGTGAAGTGAGGGAGCCAAACAAGAAATCCAGGATTTGGCT 471

AF499033.1          CGGTACTTTCCTAACAGCCGAGATCGCAGCCCGTGCTCACGACGTCGCCGCCATAGCCCT 411
AY444875.1          CGGTACTCTCCTAACAGCCGAGATCGCAGCCCGTGCTCACGACGTCGCCGCCATAGCCCT 290
1BoCBF/DREB1        CGGTACTTTCCTAACAGCCGAGATCGCAGCCCGTGCTCACGACGTCGCCGCCATAGCCCT 98
2BOCBF/DREB1        ------------------------------------------GTCGCCGCCATAGCCCT 17
AY444876.1          CGGTACTTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCT 287
GQ866977.1          CGGCACTTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCT 411
EU727155.1          CGGTACTTTCCTAACAGCCGAGATCGCAGCCCGTGCTCACGACGTCGCCGCCATAGCCCT 290
AY437878.1          CGGTACTTTCCTAACAGCCGAGATCGCAGCCCGTGCTCACGACGTCGCCGCCATAGCCCT 290
EU136731.1          CGGTACTTTCCTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCT 294
AF084185.1          CGGTACTTTCTTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCT 391
EF219470.1          CGGTACTTTCTTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCT 292
AF499032.1          CGGTACTTTCTTAACCGCCGAGATCGCAGCTCGTGCTCACGACGTCGCCGCCATAGCCCT 531
                                                    ****************
```

```
AF499033.1        CCGCGGCAAATCAGC-TTGTCTCAATTTTGCCGACTCCGCTTGGCGGCTCCGTATCCCGG 470
AY444875.1        CCGCGGCAAAACAGC-TTGTCTCAATTTTGCCGACTCCGCTTGGCGGCTCCGTATCCCGG 349
1BoCBF/DREB1      CCGCGGCAAATCAGC-TTGTCTCAATTTTGCCGACTCCGCTTGGCGGCTCCGTATCCCGG 157
2BOCBF/DREB1      CCGTGGCAATCACGCTCTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGG 77
AY444876.1        CCGTGGCAAATCCGC-CTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGG 346
GQ866977.1        CCGTGGCAAATCCGC-CTGCCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGG 470
EU727155.1        CCGCGGCAAATCAGC-TTGTCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGG 349
AY437878.1        CCGCGGCAAATCAGC-TTGTCTCAATTTCGCCGACTCGGCTTGGCGGCTCCGTATCCCGG 349
EU136731.1        CCGCGGCAAATCAGC-TTGTCTCAATTTTGCTGACTCGGCTTGGCGGCTCCGTATCCCGG 353
AF084185.1        CCGCGGCAAATCAGC-TTGTCTCAATTTTGCTGACTCGGCTTGGCGGCTCCGTATCCCGG 450
EF219470.1        CCGCGGCAAATCAGC-TTGTCTCAATTTTGCTGACTCGGCTTGGCGGCTCCGTATCCCGG 351
AF499032.1        CCGCGGCAAATCAGC-TTGTCTCAATTTTGCTGACTCGGCTTGGCGGCTCCGTATCCCGG 590
                  *** *****    **  ** ******** ** ***** ********************

AF499033.1        AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTAAGG 530
AY444875.1        AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTAAGG 409
1BoCBF/DREB1      AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGG 217
2BOCBF/DREB1      AGACAACATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGG 137
AY444876.1        AGACAACATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGG 406
GQ866977.1        AGACAACATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGG 530
EU727155.1        AGACAACATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGG 409
AY437878.1        AGACAACATGCCCCAAGGATATCCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGG 409
EU136731.1        AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCCTTGGCTTTTCAGG 413
AF084185.1        AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCCTTGGCTTTTCAGG 510
EF219470.1        AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCCTTGGCTTTTCAGG 411
AF499032.1        AGACAACATGCCCCAAGGAGATTCAGAAGGCGGCTGCTGAAGCCGCCTTGGCTTTTCAGG 650
                  ****************** ** ************************  ******** ***

AF499033.1        CTGAGATAAATAATACGACGGCGGATCATGGCATTGACGTGGAGGAGACGATCGTTGAGG 590
AY444875.1        CTGAGATAAATAATACGACGGCGGATCATGGCATTGACGTGGAGGAGACGATCGTTGAGG 469
1BoCBF/DREB1      CTGAGATAAATAATACGACGGCGGATCATGGCATTGACGTGGAGGAGACGATCGTGGAGG 277
2BOCBF/DREB1      CTGAGAA---------------------------------------------------- 144
AY444876.1        CTGAGATAAATGATACGACGGCGGATCATGGCATTGACGTGGAGGAGACGATCGTTGAGG 466
GQ866977.1        CTGAGATAAATGATACGACGACGGATCATGGCTTGGACGTGGAGGAGACGATCGTGGAGG 590
EU727155.1        CTGAGATAAATGATACGACGACGGATCATGACTTCGACGTGGAGGAGACGATCGTGGAGG 469
AY437878.1        CTGAGATAAATGATACGACGACGGATCATGGCCTGGACGTGGAGGAGACGATCGTGGAGG 469
EU136731.1        CTGAGATAAATAATACGACGACGGATCATGGCCTGGACATGGAGGAGACGATCGTGGAGG 473
AF084185.1        CTGAGATAAATAATACGACGACGGATCATGGCCTGGACATGGAGGAGACGATCGTGGAGG 570
EF219470.1        CTGAGATAAATAATACGACGACGGATCATGGCCTGGACATGGAGGAGACGATCGTGGAGG 471
AF499032.1        CTGAGATAAATAATACGACGACGGATCATGGCCTGGACATGGAGGAGACGATCGTGGAGG 710
                  ******

AF499033.1        CTATTTTCACGGAGGAAAACAACGATGGTTTTTATATGGACGAGGAGGAGTCCATGTTCG 650
AY444875.1        CTATTTTCACGGAGGAAAACAACGATGGTTTTTATATGGACGAGGAGGAGTCCATGTTCG 529
1BoCBF/DREB1      CTATTTTCACGGAGGAAAACAACGATGGTTTTTATATGGACGAGGAGGAGTCCATGTTCG 337
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        CTATTTTCACGGAGGAAAACAACGATGGTTTTTATATGGACGAGGAGGAGTCCATGTTCG 526
GQ866977.1        CTATTTTTACGGAGGAAAACAGCGATGGGTTTTATATGGACGAGGAGGAGTCCATGTTCG 650
EU727155.1        CTATTTTTACGGAGGAAAACAACGATGGGTTTTATATGGACGAGGAGGAGTCCATGTTCG 529
AY437878.1        CTATTTTTACGGAGGAAAACAACGATGGGTTTTATATGGACGAGGAGGAGTCCATGTTCG 529
EU136731.1        CTATTTTCACGGAGGAAAACAACGATGTGTTTTATATGGACGAGGAG---TCCATGTTAG 530
AF084185.1        CTATTTTCACGGAGGAAAACAACGATGTGTTTTATATGGACGAGGAG---TCCATGTTAG 627
EF219470.1        CTATTTTCACGGAGGAAAACAACGATGTGTTTTATATGGACGAGGAG---TCCATGTTAG 528
AF499032.1        CTATTTTCACGGAGGAAAACAACGATGTGTTTTATATGGACGAGGAG---TCCATGTTAG 767


AF499033.1        GGATGCCGGCCTTGTTGGCTAGTATKGCTGAAGGAATGCTTTTGCCGCCTCCGTCCGTAC 710
AY444875.1        GGATGCCGGCCTTGTTGGCTAGTATGGCTGAAGGAATGCTTTTGCCGCCTCCGTCCGTAC 589
1BoCBF/DREB1      GGATGCCGGCCTTGTTGGCTAGTATGGCGGAAGGTA-GCTTTTGCC-------------- 382
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        GGATGCCGGCCTTGTTGGCTAGTATGGCTGAAGGAATGCTTTTGCCGCCTCCGTCCGTAC 586
GQ866977.1        GGATGCCGACCTTGTTGGCTAGTATGGCTGAAGGAATGCTTTTGCCGCCTCCGTCCGTAC 710
EU727155.1        GGATGCCGTCCTTGGTGGCTAGCATGGCGGAAGGTATGCTTTTGCCGCCACCGTCGGTAC 589
AY437878.1        GGATGCCGTCCTTGGTGGCTAGCATGGCGGAAGGGATGCTTTTGCCGCCACCGTCGGTAC 589
EU136731.1        AGATGCCGGCCTTGTTGGCTAGTATGGCGGAAGGAATGCTTTTGCCGCCGCCGTCCGTAC 590
AF084185.1        AGATGCCGGCCTTGTTGGCTAGTATGGCGGAAGGAATGCTTTTGCCGCCGCCGTCCGTAC 687
EF219470.1        AGATGCCGGCCTTGTTGGCTAGTATGGCGGAAGGAATGCTTTTGCCGCCGCCGTCCGTAC 588
```

```
AF499032.1        AGATGCCGGCCTTGTTGGCTAGTATGGCGGAAGGAATGCTTTTGCCGCCGCCGTCCGTAC 827


AF499033.1        AATTCGGACATACCTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAGT 770
AY444875.1        AATTCGGACATACCTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAA- 648
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        AATTCGGACATACCTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAG- 645
GQ866977.1        AATTCGGACATACCTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAGT 770
EU727155.1        GATTCGAACATAACTATGACTTTGACGGAGATGCCGACGCGTCCCTTTGGAGTTATTAA- 648
AY437878.1        GATTCGAACATAACTATGACTTTGACGGAGATGCCGACGTGTCCCTTTGGAGTTATTAA- 648
EU136731.1        ATTTCGGACATAACTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAGT 650
AF084185.1        ATTTCGGACATAACTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAGT 747
EF219470.1        ATTTCGGACATAACTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTT-- 646
AF499032.1        ATTTCGGACATAACTATGACTTTGACGGAGATGCTGACGTGTCCCTTTGGAGTTATTAGT 887


AF499033.1        ACAAAGATTTTTTTATTTCCATTTTTGGTATAATACTTCTTTTTGATTTTCGGATTCTACC 830
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        ACAAAGATTTTTTTATTTCCATTTTTGGTATAATACTTCTTTTTGATTTTCGGATTCTACC 830
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        GCAAAG-TTTTTTTTTCAATTTTTTCGTATAATACATCTTTTGGATTTTCGGATTCTGCC 709
AF084185.1        GCAAAG-GTTTTTTTTCAATTTTTTCGTATAATACTTCTTTTGGATTTTCGGATTCTGCC 806
EF219470.1        ------------------------------------------------------------
AF499032.1        GCAAA--TTTTTTTTTCAATTTTTTCGTATAATAT-TCTTTTGGATTTTCGGATTCTGCC 944


AF499033.1        TTTTTATGGGTATCATTTTTTTTTTTAGGTAACGTGGAAGCTGAGTGTAAATGTTTGAACA 890
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        TTTTTATGGGTATCATTTTTTTTTTTAGGTAACGTGGAAGCTGAGTGTAAATGTTTGAACA 890
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        TTTTTATGGGAATCTTTTTTTTTTTTTGGTAATGTGGAAGCTGAGTGTGAATGTTTAAACA 769
AF084185.1        TTTTTATGGGAATCTTTTTTTTTTTT-GGTAATGTGGAAGCTGAGTGTGAATGTTTAAACA 865
EF219470.1        ------------------------------------------------------------
AF499032.1        TTTTTATGGGAATCTTTTTTTTTTTT-GGTAATGTGGAAGCTGAGTGTGAATGTTTAAACA 1003


AF499033.1        ATTGTGTTATAAAATGCTAGTATTTTTGTGTGCAAAAAAAAAAAAAAAAAAAAAA----- 945
AY444875.1        ------------------------------------------------------------
1BoCBF/DREB1      ------------------------------------------------------------
2BOCBF/DREB1      ------------------------------------------------------------
AY444876.1        ------------------------------------------------------------
GQ866977.1        ATTGTGTTATAAAATGCTAGTATTTTTGTGTGC---------------------------- 923
EU727155.1        ------------------------------------------------------------
AY437878.1        ------------------------------------------------------------
EU136731.1        ATTGTGTTATCAAATGCTAGTATTTTTTTGTGCAGCAAAAT-----------------AA 812
AF084185.1        ATTGTGTTATCAAATGCTAGTATTTTTTTGTGCAGCATAATCATCTTATTGGCTCTCCAA 925
EF219470.1        ------------------------------------------------------------
AF499032.1        ATTGTGTTATCAAATGCTAGTATTTTTTTGTGCAGCCTCGTG--------------CCGA 1049


AF499033.1        ------------------------------------------
AY444875.1        ------------------------------------------
1BoCBF/DREB1      ------------------------------------------
2BOCBF/DREB1      ------------------------------------------
AY444876.1        ------------------------------------------
GQ866977.1        ------------------------------------------
EU727155.1        ------------------------------------------
AY437878.1        ------------------------------------------
EU136731.1        AAAAAAAAAAAAAAAAAAAAAAAAAA---------------- 838
```

```
AF084185.1        AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 967
EF219470.1        ----------------------------------------
AF499032.1        ATCCTGCAGCCC------------------------------ 1061
```

Figure 98. Nucleotide sequences (cDNA) alignment.

Alignments were made using ClustalW2 EMBL-EBI (Larkin *et al*., 2007). Consensus symbols denoted as: "*" means that the nucleotides in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, "." means that semi-conserved substitutions are observed. The AP2 region is indicated by overline.1 BoCBF/DREB1 and 2 BoCBF/DREB1 sequence isolated from Brassica oleracea v. botrytis using both primers P1 and P2, Brassica napus DREB2-23 mRNA (GenBank:mRNA AY444876.1, 645 bp), Raphanus sativus CBF1 mRNA (GenBank:GQ866977.1), Brassica napus CBF mRNA (GenBank: HM235815.1, 437 bp), Brassica rapa subsp. pekinensis DREB-like protein 2 (DREB2) mRNA (GenBank: GQ122331.1), Brassica rapa subsp. pekinensis DREB-like protein 1 (DREB1) mRNA (GenBank: EU924266.1, 645 bp), Brassica rapa subsp. chinensis DREB2 mRNA (GenBank: EF495249.1, 429 bp), Chorispora bungeana isolate A6 CRT/DRE-binding factor CBF-like protein mRNA (GenBank: AY994128.1), Chorispora bungeana isolate A4 CRT/DRE-binding factor CBF-like protein mRNA (GenBank: AY994126.1), Chorispora bungeana isolate A3 CRT/DRE-binding factor CBF-like protein mRNA (GenBank: AY994125.1), Brassica napus DREB2-2 mRNA (GenBank: AY444874.1, 645 bp).

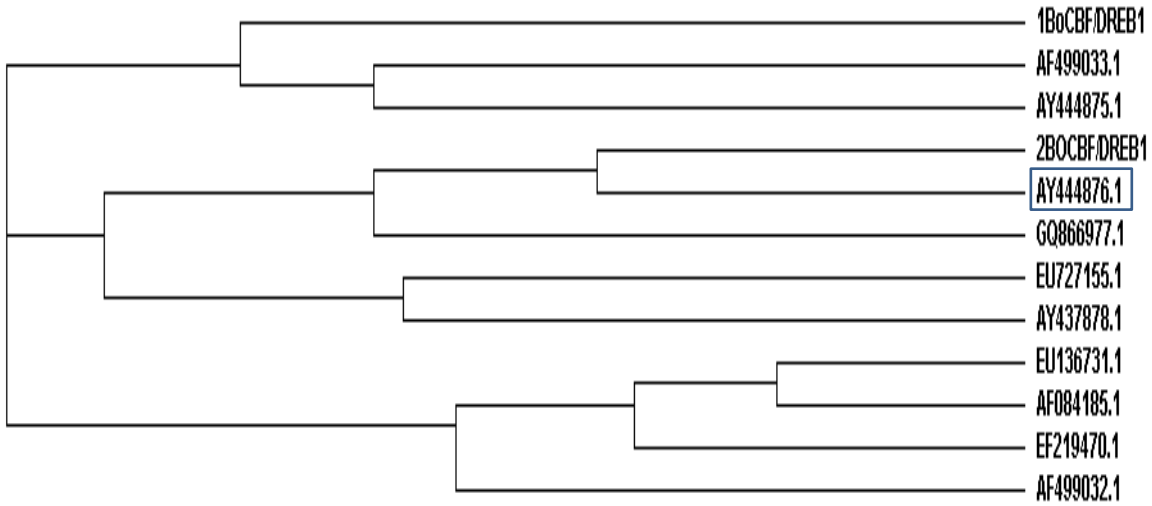


Figure 99. Phylogenic relation of the BoCBF/DREB1 cDNA.

The phylogram is based on the alignment of DNA sequence of *Brassica oleracea v*. botrytis (1*BoCBF/DREB*1and 2*BoCBF/DREB1*) and the following sequences from the members of Brassicacea and other families: Brassica napus DREB2-23 (mRNA AY444876.1, 645 bp), Raphanus sativus CBF1 mRNA (GenBank:GQ866977.1), Brassica napus CBF mRNA (GenBank: HM235815.1, 437 bp), Brassica rapa subsp. pekinensis DREB-like protein 2 (DREB2) mRNA (GenBank: GQ122331.1), Brassica rapa subsp. pekinensis DREB-like protein 1 (DREB1) mRNA (GenBank: EU924266.1, 645 bp), Brassica rapa subsp. chinensis DREB2 mRNA (GenBank:

EF495249.1, 429 bp), Chorispora bungeana isolate A6 CRT/DRE-binding factor CBF-like protein mRNA (GenBank: AY994128.1), Chorispora bungeana isolate A4 CRT/DRE-binding factor CBF-like protein mRNA (GenBank: AY994126.1), Chorispora bungeana isolate A3 CRT/DRE-binding factor CBF-like protein mRNA (GenBank: AY994125.1), Brassica napus DREB2-2 mRNA (GenBank: AY444874.1, 645 bp). The values show tree graph distances. The tree was constructed with ClustalW2 EMBL-EBI (Larkin *et al*., 2007).

Amino Acid sequence

The cDNA sequence obtained using the primers (P1) was translated to amino acid sequence (Figures 100 and 101). The amino acid sequence was blasted using NCBI software and compared with the amino acid sequence in other plants. The results indicated a high similarity with different plant species. The most similar sequences are described as follow:

1. DREB2-23 [*Brassica napus]* (98 % similarity) (GenBank: AAR20499.1, 214 aa) (Zhao et al., 2006)
2. CBF-like protein CBF16 [*Brassica napus*] (98 % similarity) (GenBank: AAM18960.1, 215 aa)(Gao *et al*., 2002)
3. CBF-like protein CBF5 [*Brassica napus*] (96 % similarity) (GenBank: AAM18958.1, 214 aa) (Gao *et al*., 2002)
4. CBF [*Brassica napus*] (95 % similarity) (GenBank: ADN28047.1, 146 aa) (Zhao and Song, unpublished)
5. DREB2-3 [*Brassica napus*] (98 % similarity) (GenBank: AAR20498.1, 215 aa) (Zhao *et al*., 2006)
6. DREB2-1 [*Brassica napus*] (95 % similarity) (GenBank: AAR11858.1, 215 aa)(Zhao *et al*., 2006)
7. DREB2-2 [*Brassica napus*] (95 % similarity) (GenBank: AAR20497.1, 214 aa) (Zhao *et al*., 2006)
8. DREB-like protein 1 [*Brassica rapa* subsp. *pekinensis*] (95 % similarity) (GenBank: ACL12046.1, 214 aa) (Wang *et al*, unpublished)

9. CBF1 [*Raphanus sativus*] (95 % similarity) (GenBank: ACX48435.1, 215 aa) (Li and Gao, unpublished)
10. CBF-like protein [*Brassica rapa* subsp. *pekinensis*] (94 % similarity) (GenBank: AAY43345.1, 214 aa) (Zhang *et al*., 2006b)
11. DREB1 [*Nicotiana tabacum*] (94 % similarity) (GenBank: ACE73693.1, 215 aa) (Liu and Feng, unpublished)

```
AAR20497.1      MTSFSAFSEMLGSEYESP-TLSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 59
ACL12046.1      MTSFSTFSEMLGSEYESP-TLSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 59
AAY43345.1      MTSVSAFSETLGSEYESP-TLSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 59
ADN28047.1      -------------------------------------------ETSHPIYRGVRLRNSG 16
ACX48435.1      MTSFSTFSELLGSEHESPVTLGEEYCPKLAASCPKKPAGRKKFRETRHPIYRGVRLRNSG 60
AAR11858.1      MTSFSAFSELLGSEHESPVTLGEEYCPKLAASCPKKPAGRKKFRETRHPVYRGVRLRNSG 60
ACE73693.1      MTSFSAFSELLGSEHESPVTLGEEYCPKLAAGCPKKPAGRKKFRETRHPVYRGVRLRNSG 60
AAR20499.1      MTSFSAFSEMMGSENESP-ALSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRQRHSG 59
AAM18958.1      MTSFSAFSEMMGSENESP-ALSGEYCPTLAASCPKKPAGRKKFRETRHPIYRGVRQRHSG 59
AAM18960.1      MTSFSTFSELLGSEHESPVTLGEEYCPKLAASCPKKPAGRKKFRETRHPVYRGVRLRNSG 60
AAR20498.1      MTSFSTFSELLGSGHESPVTLGEEYCPKLAASCPKKPAGRKKFRETRHPVYRGVRLRNSG 60
BoCBF/DREB1     ------------------------------------------------------------

AAR20497.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
ACL12046.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
AAY43345.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
ADN28047.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 76
ACX48435.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
AAR11858.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
ACE73693.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
AAR20499.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
AAM18958.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 119
AAM18960.1      KWVCEVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 120
AAR20498.1      KWVCEVREPNKKSRIWLGTLLTAEIAARAHDVAAIALRGKTACLNFADSAWRLRIPETTC 120
BoCBF/DREB1     ----EVREPNKKSRIWLGTFLTAEIAARAHDVAAIALRGKSACLNFADSAWRLRIPETTC 56
                    ***************:********************:******************

AAR20497.1      PKDIQKAAAEAAVAFQAEINDTTKDHGLDVEETIVEAIFTEENSDGFYMDEEESMFGMPT 179
ACL12046.1      PKDIQKAAAEAAVAFQAEINDTTKDHGLDVEETIVEAIFTEENSDGFYMDEEESMFGMPT 179
AAY43345.1      PKDIQKAAAEAAVAFQAEINDTTKDHGLDVEETIVEAIFTEEDSDGFYMDEEESMFGMPT 179
ADN28047.1      PKDIQKAAAEAAVAFQAEINDTTTDHGLDVEETIVEAIFTEENSDGFYMDEEESMFGMPT 136
ACX48435.1      PKDIQKAAAEAAVAFQAEINDTTTDHGLDVEETIVEAIFTEENSDGFYMDEEESMFGMPT 180
AAR11858.1      PKDIQKAAAEAAVAFQAEINDTTTDHGLDVEETIVEAIFTEENNDGFYMDEEESMFGMPS 180
ACE73693.1      PKDIQKAAAEAAVAFQAEINDTTTDHDFDVEETIVEAIFTEENNDGFYMDEEESMFGMPS 180
AAR20499.1      PKDIQKAAAEAAVAFQAEINDTTADHGIDVEETIVEAIFTEENNDGFYMDEEESMFGOSMOL KG-
1 179
AAM18958.1      PKDIQKAAAEAAVAFQAEINDTTTDHGLDVEETIVEAIFTEENNDGFYMDEEESMFGMPS 179
AAM18960.1      PKEIQKAAAEAAVAFKAEINNTTADHGIDVEETIVEAIFTEENNDGFYMDEEESMFGOSMOL KG-
1 180
AAR20498.1      PKEIQKAAAEAAVAFKAEINNTTADHGIDVEETIVEAIFTEENNDGFYMDEEESMFGOSMOL KG-
1 180
BoCBF/DREB1     PKEIQKAAAEAAVAFQAEINNTTADHGIDVEETIVEAIFTEENNDGFYMDEEESMFGOSMOL KG-
1 116
                **:************:****:** **.:**************:.***************:

AAR20497.1      LLASMAEGMLLPPPSVQFEYNYDFDGDTDVSLWSY 214
ACL12046.1      LLASMAEGMLLPPPSVQFEYNYDFDGDTDVSLWSY 214
AAY43345.1      LLASMAEGMLLPPPSVQFEYNYDFDGDTDISLWSY 214
ADN28047.1      LLASMAEGIL------------------------ 146
ACX48435.1      LLASMAEGMLLPPPSVQFGHTYDFDGDADVSLWSY 215
AAR11858.1      LVASMAEGMLLPPPSVRFEHNYDFDGDADVSLWSY 215
```

```
ACE73693.1        LVASMAEGMLLPPPSVRFEHNYDFDGDADASLWSY 215
AAR20499.1        LLASMAEGMLLPPPSVQFGHTYDFDGDADVSLWSY 214
AAM18958.1        LLASMAEGMLLPPPSVRFEHXYDFDGDAXVSLWSY 214
AAM18960.1        LLASXAEGMLLPPPSVQFGHTYDFDGDADVSLWSY 215
AAR20498.1        LLASMAEGMLLPPPSVQFGHTYDFDGDADVSLWSY 215
BoCBF/DREB1       LLASMAEGSFC---------------------- 127
                  *:** *** :
```

Figure 100. Multiple alignment of the BoCBF/DREB1 deduced amino acids sequence with members of Brassicacea family and other plant families.

Brassica oleracea v. botrytis BoCBF/DREB1,. DREB2-23 [Brassica napus], CBF-like protein CBF16 [Brassica napus], CBF-like protein CBF5 [Brassica napus], CBF [Brassica napus], DREB2-3 [Brassica napus], DREB2-1 [Brassica napus], DREB2-2 [Brassica napus], DREB-like protein 1 [Brassica rapa subsp. pekinensis], CBF1 [Raphanus sativus], DREB1 [Nicotiana tabacum], CBF-like protein [Brassica rapa subsp. pekinensis], Different symbols such as + indicate the CBF signature motif DSAWR,"*" indicate the identical nucleotides in all sequences. ":" indicate conserved substitutions, "." indicate semi-conserved substitutions and CRT/DRE binding AP2 domain is indicated by oveline.

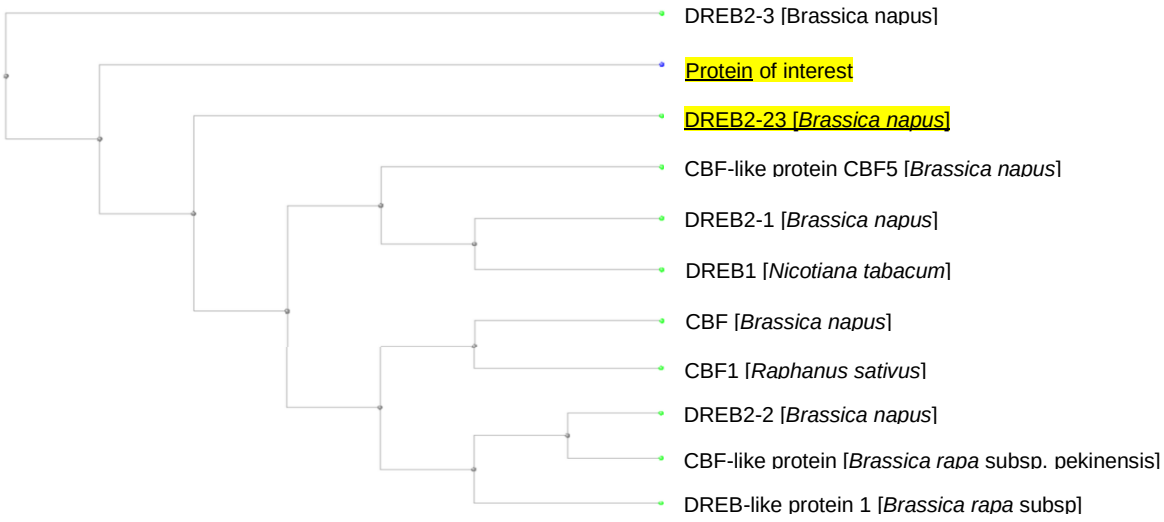


Figure 101. Phylogenic relation of the BoCBF/DREB1 proteins.

The phylogram is based on the alignment of amino acids sequence of *Brassica oleracea* v. botrytis BoCBF/DREB1 and the following proteins from the members of Brassicacea and other families. DREB2-23 [Brassica napus], CBF-like protein CBF16 [Brassica napus], CBF-like protein CBF5 [Brassica napus], CBF [Brassica napus], DREB2-3 [Brassica napus], DREB2-1 [Brassica napus], DREB2-2 [Brassica napus], DREB-like protein 1 [Brassica rapa subsp. pekinensis], CBF1 [Raphanus sativus], DREB1 [Nicotiana tabacum], CBF-like protein [Brassica rapa subsp. pekinensis]. The values show tree graph distances. The tree was constructed with ClustalW2 EMBL-EBI (Larkin *et al*., 2007).

The effect of ABA treatment on accumulation of COR protein in cauliflower microshoots

The only band that was detected using COR15 protein antibody was at 23 kDa size.

It was observed that the amount of 23 polypeptide was significantly affected by the

ABA treatments (*P*= 0.009). It was significantly increased with the increase of ABA concentration in the liquid media until it was stabilized at concentrations higher than 1 mg L^{-1} (Figures 102 and 103).

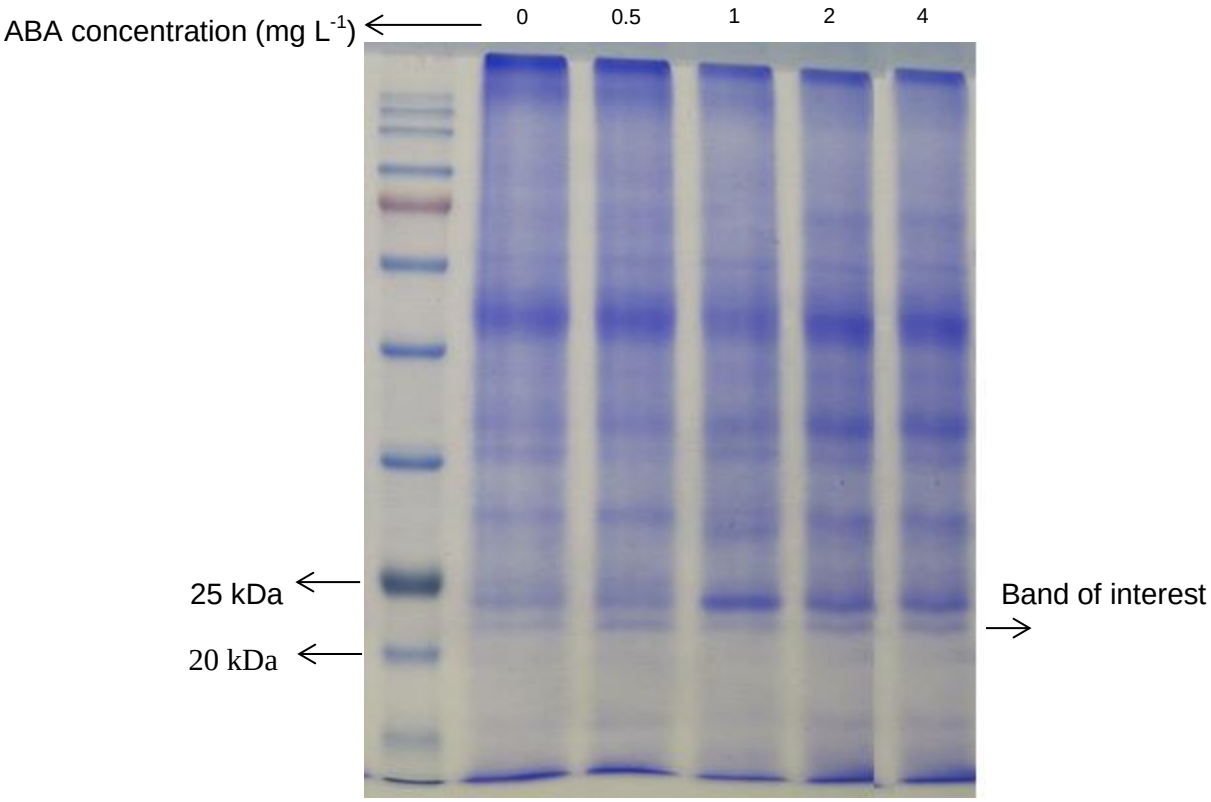
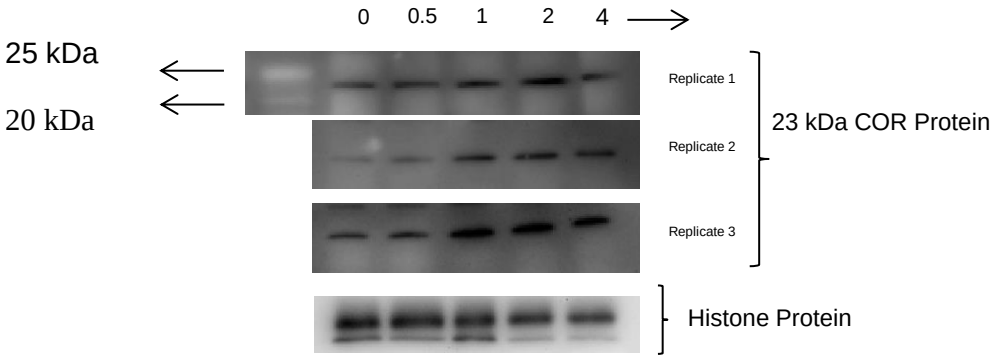


Figure 102. The SDS-PAGE analysis for protein samples derived from both ABA treated and non-treated samples.



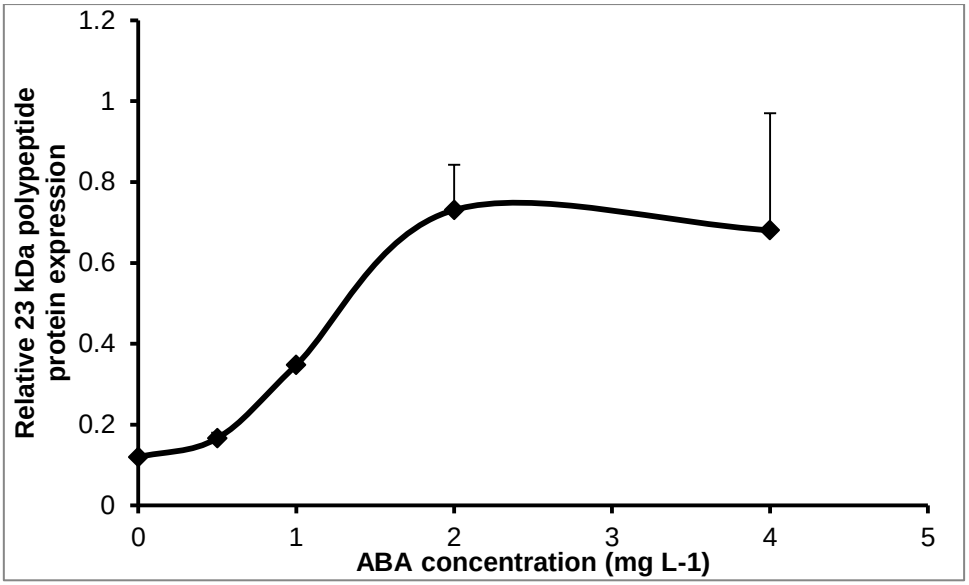


Figure 103. Western blot analysis for the detection of COR protein (23 kDa) from ABA treated and non-treated microshoot samples (LSD=0.29).

4.4. Discussion

Cold acclimation which is defined as the exposure of plant to low, non-freezing temperature, has been reported to increase the cold tolerance in several plant species (Jan *et al*., 2009, Thomashow, 2001, Gilmour *et al*., 2000, Thomashow, 1999, Shinozaki and Yamaguchi-Shinozaki, 1996). In terms of *Brassica olearaceae* var *botrytis*, the experiment conducted demonstrated the capacity of cauliflower microshoots to be cold acclimated and that the acclimation increased the cauliflower artificial seeds cold tolerance. Cold acclimation causes several biochemical and physiological changes in plant tissues including an increase in the sugars, proline and soluble protein concentrations. It also embraces some modifications in the lipid composition of the cell membranes. These changes culminate in the effect of cold acclimation significantly improve the cold tolerance of plants (McKhann *et al*., 2008, Thomashow, 1999). At the molecular level, cold acclimation requires recognition of low temperature by cells through a signalling process and as a consequence large modifications of gene expression take place in order to eventually enable the plants to survive the low temperature (Lee *et al*., 2005, Seki *et al*., 2002). However, several

studies have demonstrated that the *CBF/DREB1* (CRT/DER binding factor) genes are the central pathway participating in the cold acclimation (Sung *et al*., 2003, Shinozaki *et al*., 2003, Xiong *et al*., 2002, Stitt and Hurry, 2002, Smallwood and Bowles, 2002, Francia *et al*., 2004, Vágújfalvi *et al*., 2003, Dubouzet *et al*., 2003, Owens *et al*., 2002, Gao *et al*., 2002, Choi *et al*., 2002). The current results showed that the *CBF/DREB1* gene was upregulated under the effect of low temperature and that the peak of gene expression was observed one hour after transfer to the new environmental condition. These results confirm the important role of "CBF regulon" in the improvement of cold tolerance in cauliflower microshoots. However, as *CBFs/DREBs* are upstream transcription factors which bind to promoter cis element CRT/DER and activate the expression of cold responsive genes (*COR*), the effect of acclimation on the accumulation of COR 15 protein was investigated. Although several approaches of western blotting experiment (several concentrations of the COR15 antibody, protein sample concentrations, incubation time, transfer times…etc) have been investigated, COR15 bands were not detected with any of the samples. Our results contrast with what was reported by Hadi *et al* (2011) who reported that COR15 protein accumulates under the effect of acclimation in mature cauliflower plants. However, this could be due to the effect of development stage and culture conditions as microshoots derived from the liquid media culture system were used here instead of mature plants. However, CBF regulatory proteins were demonstrated not just to up-regulate the expression of COR proteins but also to increase the level of proline and sugars under the effect of cold acclimation and therefore to contribute to the improvement of freezing tolerance (Thomashow, 2001, Gilmour *et al*., 1998) and this could explain the positive role of *CBF/DREB1* in improving the microshoots cold tolerance without leading to the accumulation of COR15 protein.

The main band detected using COR15 antibody appeared at 23 kDa in both acclimated and non-acclimated microshoots. The detection of a 23 kDa polypeptide using COR15 antibody suggests that there is a homology between the 23 kDa protein peptide sequence and the peptide sequence of COR15 protein. The amount of this protein (band intensity) was not significantly affected by acclimation and this was in agreement with Rao *et al* (1993) who mentioned that a 23 kDa polypeptide in rice was not responsive to cold treatment. However, our findings contrast with what was reported by Meza-Basso *et al* (1986) who reported that the production of 22-23 kDa protein in *Brassica napus* was inhibited by cold stress. Fabijanski *et al* (1987) and Atkinson *et al* (1989) indicated that the 23 kDa polypeptide increases under the effect of heat shock in *Brassica oleracea* L. (Broccoli) and maize respectively. Ben-Hayyim *et al* (1993) and Uma *et al* (1995) demonstrated that the 23 kDa polypeptide is responsible for salt tolerance in *Citrus* and *Eleusine coracana* (Finger Millet) respectively. In contrast, It was indicated that 23 kDa polypeptide is not responsive to NaCl in rice (Rao *et al*., 1993). Indeed it seems that the response and role of 23 kDa polypeptide depends on plant species, plant developmental stages and the type of abiotic stress applied.

The current experiments demonstrated that the treatment of cauliflower artificial seeds (acclimated and non-acclimated) with low non-lethal temperature improved the growth of plantlets produced in the subsequent stages. It was proposed that the cold shock increased the level of cold shock proteins (CSPs) which had a positive effect on the growth rate of cauliflower artificial seeds. It was confirmed that bacterial CSPs enhanced stress adaptation in multiple plant species by demonstrating improved stress tolerance in maize and rice. The expression of *CspA* and *CspB* in transgenic rice improved the growth of plants under the effect of a number of abiotic

factors like cold, heat, and water deficit. The expression of bacterial *CSPs* increased cold tolerance in transgenic Arabidopsis (Castiglioni *et al*., 2008, Nakaminami *et al*., 2006, Karlson and Imai, 2003). However the effect of cold shock on the development of cauliflower artificial seeds needs further investigation.

Dehydration is one of the main mechanisms which imposes stress on plants at low temperature. When the temperature of certain plant species drops below freezing point, ice formation begins in the extracellular spaces of the plant tissue. As a consequence, the water moves from inside the cell to the extracellular spaces since the chemical potential of ice is less than that in liquid water. Therefore, the freezing injury could be caused by the effect of plant cell dehydration (Thomashow, 2001). Thus, it is obvious that the freezing and drought tolerance could include the action of shared genes. Many studies have reported that the induction of *CBF* expression had positive impact not just on the cold tolerance but also on the drought and salinity tolerance (Kasuga *et al*., 1999, Liu *et al*., 1998). In view of this finding, the capacity of increasing the osmotic potential (drought simulation) on both cold tolerance and up-regulation of *CBF/DREB1* gene was investigated using different concentrations of mannitol. The mannitol had negative effects on the growth rate of microshoots and that was mainly through the increase of culture osmotic potential. It was observed that the use of mannitol in the culture media (the increase of culture osmotic potential) improved the cold tolerance of the cauliflower artificial seeds when it was used to obtain -2 Osmol kg^{-1} culture media osmotic potential. The simulation of drought did not have the capacity to induce the expression of *CBF/DREB1* regardless of the concentration of mannitol used. However, it has been reported that a multifaceted network of genes is involved in cold tolerance and that the CBF regulon only cannot clarify all differences in phenotype cold tolerance (McKhann *et*

al., 2008). The cold stress cause changes in expression of hundreds of genes resulting in the increase of hundreds of metabolites some of which are known to have an important effect in the improvement of plant cold tolerance (Jan *et al*., 2009).

The failure of mannitol (drought simulation) to induce the expression of *CBF/DREB1* gene in cauliflower microshoots raised an important question that was the failure of *CBF/DREB1* gene up-regulation under the effect of drought due to the developmental stage of cauliflower (microshoots) or is it related to the plant species, drought being unable to induce the expression of *CBF/DREB1* gene in cauliflower regardless of the developmental stage? It was therefore necessary to investigate the effect of cold acclimation and drought on the up-regulation of *CBF/DREB1* gene in mature cauliflower plants in order to investigate the effect of cauliflower developmental stage on the expression of this gene.

The technique of EC (electrical conductivity) was used to analyse the frost resistance in acclimated and non-acclimated mature cauliflower plants since the cellular membrane systems are the main place of freeze-induced injury caused by severe cellular dehydration which occurs upon ice formation in the extracellular spaces (Hadi *et al*., 2011, Thomashow, 2001, Fuller *et al*., 2006). The injury of cell membranes is the principle on which the electrical conductivity test is based. It is supposed that individual cells become progressively leakier under the increase of frost stress, therefore the electrical conductivity is used to measure the collective average of cell damage caused by freezing. The electrolyte leakage evaluation contains the measurement of electrical conductivity of pure water in which detached samples have been located after a freezing thaw cycle (Linden, 2002). The use of EC% using leaf discs derived from mature cauliflower leaves was found to be an effective methodology for evaluating frost damage in cauliflower mature plants. The

positive effect of acclimation on the frost tolerance of cauliflower was confirmed. The effect of low temperature was demonstrated to induce the expression of *CBF/DREB1* gene which resulted in the increase of the cauliflower cold tolerance.

The effect of drought on the cold tolerance of cauliflower was investigated using EC% technique. It was confirmed that drought had a significantly positive influence on cauliflower cold tolerance. Moreover, the current results showed that the drought had the capacity to induce the expression of *CBF/DREB1* gene in mature cauliflower plants. The moisture level and the stomatal conductance points, in which the *CBF/DREB1* was up-regulated, were determined. However, leaf stomatal conductance has been considered a good selection criterion for drought resistance (Ashraf and Oleary, 1996). It was reported that the fast stomatal response could be a drought resistance mechanism to save soil water for later use and to maintain a high leaf water potential (Jones, 1974). What was found to be interesting in the current study was that a small reduction on the soil moisture (about 70% of the field capacity) had the capacity to up-regulate the *CBF/DREB1* gene in mature cauliflower plants. Furthermore, the stomatal conductance was relatively high at the point of which *CBF/DREB1* was up-regulated. The relatively high level of stomatal conductance allows a high level of gas exchange and as a consequence a high level of photosynthesis was maintained resulting in the proper growth of the plants (Mediavilla and Escudero, 2004). The up-regulation of *CBF/DREB1,* as mentioned, led to increase in sugars, proline and many other solutes in plant tissue resulting in high potential osmotic required to keep the stomata open and maintain gas exchange and growth under relatively drier condition (Pérez-Pérez *et al*., 2009).

The current study showed that cold acclimation had the capacity to induce the expression of *CBF/BREB1* gene in both microshoots and mature plants, and the

capability of drought to up-regulate this gene in mature plant but not in cauliflower microshoots. It seems that the cauliflower developmental stage and the culture environment have an effect of *CBF/DREB1* up-regulation. This result agreed with Beck et a (2004) who reported that the plant injury caused by low temperature depends on the plant developmental stage. Prasil *et al* (2004) indicated that cold tolerance of wheat depends on the growth stage. They demonstrated that the cold tolerance of wheat decreases significantly after vernalisation and that is due to the failure in the up-regulation of *CBF* genes after this stage of growth.

The use of liquid culture media in the described cauliflower micropropagation system facilitates the application of various chemical materials with this media in order to improve the characteristics of microshoots produced and consequently the quality of cauliflower artificial seeds. It was supposed that ABA could be an important material for improving the microshoots abiotic stress (cold). ABA is an essential controller of the plant growth and development and it is an important regulator for stress resistance (Kempa *et al*., 2008, Fait *et al*., 2008). Hirayama and Shinozaki (2007) proposed that ABA accumulates in response to various abiotic stresses, for example: cold, drought and high salinity. However, the use of ABA with the cauliflower culture media significantly reduced the viability of microshoots assessed by the mean of their average weights. This is in agreement with Tuteja (2007) who reported that the use of ABA mimics the effect of abiotic stress in plants which in turn slow the growth and development of plants.

The current study showed a significantly higher concentration of mRNA extracted from the same weights of microshoots treated with ABA compared with that extracted from non-treated microshoots. This could be attributed to higher number of microshoots (higher number of cells) used per the ABA treated microshoot weight

unite than that used with the control since ABA reduced growth speed of microshoots. It could also be attributed to critical role of ABA on plant growth and development (Tuteja, 2007) as ABA works as an endogenous messenger in the plant's water status regulation and the adaptive process of plants to abiotic stress such as, cold, drought and salinity, is controlled by ABA (Swamy and Smith, 1999). ABA is an essential element involved in intermediating different environmental stresses and plants react to different conditions with overlapping but separate physiological mechanisms. Therefore, it is logical to suppose that an ABA-dependent signalling pathway acts in concert with ABA-independent stress specific pathways to express suitable metabolic response (Kempa *et al*., 2008). The use of ABA with cauliflower culture media was found to have negative effects on the microshoots cold tolerance. The higher the concentration of ABA, the lower the EC% (less frost damage). However, the frost damage was higher in the ABA treated microshoots compared with non-treated microshoots, even when ABA was used at relatively high concentration. This finding raises the important question of the effect of using this phytohormone on the induction of *CBF/DREB*1 gene expression, since this family of transcriptional factors plays a key role in the cold acclimation and on the improvement of plant cold tolerance (Hadi *et al*., 2011, Jan *et al*., 2009, McKhann *et al*., 2008, Thomashow, 2001, Thomashow, 1999). The use of ABA with culture media reduced the expression *CBF/DREB1*. Although the increase of ABA concentration increased the expression of *CBF/DREB1*, the expression was found to be the highest in the control treatment. In contrast with the current results, *Knight et al* (2004) demonstrated that the application of ABA increases the level of *CBF1-3* transcript. Narusaka *et al* (2003) indicated that ABA application does not activate DER (CRT) cis element. However, the mechanism by which the abiotic stresses

induces the expression of ABA biosynthesis genes is not well understood (Tuteja, 2007). Indeed, it seems that the up-regulation of these genes is controlled by the age and the condition of plants. Furthermore, these conditions might affect flux through different branches of the signalling network (Knight *et al*., 2004). In terms of the microshoots culture system, it seems that the use of liquid media has a significant effect on the capacity of the culture to respond to the use of ABA. Knight (2004) reported that water relations during the growing period may be effective element that influence the response of plant to ABA application and the capacity of this element to up-regulate CBF genes. They also mentioned that whether or not the plants have been cultured on soil or tissue culture might critically affect their sensitivity to ABA.

In terms of the effect of ABA on the accumulation of COR15 proteins, no bands were detected at 15 kDa. However, the increase of 23 kDa polypeptide caused by ABA treatment and observed in this study agreed with Pla *et al* (1989) who reported that 23-25 kDa protein is a class of protein that are involved in generalized plant ABA responses in maize. Rao *et al* (1993) has also indicated that 23 kDa polypeptide is an ABA responsive. Although ABA treatments increased the accumulation of 23 kDa polypeptide, they decreased cold tolerance of microshoots. This suggests that 23 kDa is not involved with cold tolerance of cauliflower microshoots.

The cDNA sequences of *CBF/DREB1* were done using two types of primers with samples isolated from form control micoshoot samples treated with low temperature at 4°C for 12 hours. On comparison using bioformatics tools, the parts of *CBF/DREB1* gene sequences gained using both primers P1 and P2 were similar to each other with homology level assessed to be up to 93 %. The comparison of the isolated partial sequences isolated from cauliflower (*Brassica olearceae* var botrytis) with the *CBF/DREB1* sequences in other brassica species such as, *Brassica napus*

*CBF*16, *Brassica napus DREB*2-3, *Brassica napus* DREB2-1, *Brassica napus* CBF mRNA, *Brassica rapa* subsp.pekinensis(*DREB*2) and *Brassica rapa* subsp. pekinensis(*DREB*1), showed high similarity (more than 90%). However, the most similar *CBF/DREB1* gene alignment sequence in Brassica species to that in cauliflower *CBF/DREB1* fragments obtained using both primers P1 and P2 was found to be *Brassica napus DREB*2-23 mRNA (GenBank:AY444876.1, 645 bp)(Zhao *et al*., 2006). The sequence of this gene showed up to 96% similarity with the partial sequence of cauliflower *CBF/DREB*1 obtained using P1 primers (the primers used with ordinary PCR) and similarity up to 98 % with the partial sequence of cauliflower *CBF/DREB1* obtain using P2 primers. The similarity between the *BoCBF/DREB1* partial sequence and the *CBF/DREB1* sequences in other brassica species confirms that this gene is in the genome of *Brassica oleraceae*. However, further investigation is required to identify the remaining *BoCBF/DREB1* sequence.

Deduced amino acid sequence of the *BoCBF/DREB1* partial sequence in comparison with other Brassica species showed 90% homology and showed an identical conserved AP2 domain. The AP2 domain may play a crucial role in recognition of DNA binding sequence in the promoter of cold responsive genes (Sakuma *et al*., 2002, Liu *et al*., 1998). Among the six member *Brassica* species in the triangle of U (U, 1935), the sequence from *B. oleracea* showed high resemblance with the species *B. napus*, *B. juncea*, and *B. rapa*. For the remaining two species, *B. nigra* and *B. carinata*, no CBF genes have been reported in the literature. However, this homology was found to be more than 90% when compared with plants other than *Brassicas* such as *Nicotiana tabacum*. Therefore, this sequence is not highly conserved in *Brassicas* in contrast with what was reported by Hadi *et al* (2011). The high level of similarity between cDNA sequences obtained using both primers P1

and P2 and the sequence of *Brassica napus DREB*2-23 mRNA and the high level of homology between the amino acid sequence of *Brassica oleracea* with that in *Brassica napus* suggests that the closest structure of *CBF/DREB1* in *Brassica oleracea* is *Brassica napus DREB*2-23 mRNA.

Since the two types of primers P1 and P2 were confirmed to detect the same gene (*CBF/DREB1*), the difference in the expression observed using the ordinary PCR protocol could be caused by the different product size of cDNA fragments detected by these primers. The cDNA product size detected using the primers P1 is bigger than that detected using the primers P2. Therefore, the reaction components were utilized faster using the P1 primers than when the P2 primers where used since 40 thermal PCR thermal cycles were used with both primers. Thus, the difference in the *CBF/DREB1* expression was clearer using P1 than that when P2 primers were used with the ordinary PCR.

4.5. Conclusion

The studies reported in this chapter indicated that cauliflower could be cold acclimated regardless their developmental stage (microshoots and mature plants) and that cold acclimation increased the physiological cold tolerance of mature plants and microshoots of cauliflower. It was also confirmed the capacity of low temperature to up-regulate *CBF/DREB1* in both microshoots and mature plants. The up-regulation of *CBF/DREB1* transcription factor did not lead to up-regulation of *COR* genes and the COR15 protein was not detected in acclimated cauliflower microshoots. Acclimation also did not have a significant effect on the accumulation of the 23 kDa polypeptide suggesting that there is no an important role to this polypeptide in plant cold tolerance. It was confirmed that Brassica napus DREB2-23

mRNA (GenBank:AY444876.1, 645 bp) nucleotide sequence was the most similar sequence to the cDNA isolated from *Brassica oleracea* var. *botrytis* using two sets of primers P1 and P2.

While drought improved the cold tolerance in cauliflower microshoots and mature plants and it had the capacity to up-regulate *CBF/DREB1* in mature plants, this was not evident in microshoots. However, the level of moisture and the stomatal conductivity at which *CBF/DREB1* was up-regulated in mature cauliflower plants, were determined. It was demonstrated that a small reduction in the soil moisture (70%) had the capacity to up-regulate *CBF/DREB1* in cauliflower mature plants. This is considered to be an important finding that could have significant practical applications in the field of agriculture.

ABA had a negative effect on cauliflower microshoots cold tolerance. Moreover, ABA significantly decreased the expression of *CBF/DREB1* under the effect of low temperature. However, ABA significantly increased the amount of a 23 kDa polypeptide in cauliflower microshoots and this confirms that this polypeptide has no role in cauliflower cold tolerance.

Chapter 5: The Effect of Molybdenum on the Cauliflower Artificial Seed Production System and the Molecular Mechanism of Cold Tolerance

5.1. Introduction

Molybdenum is an essential element for healthy growth of higher plant and plays an important role in many plant physiological and biomedical processes (Sun *et al*., 2009, Mendel and Hansch, 2002). Molybdate is the dominate form of molybdenum available to plants. Although molybdenum participate in various redox reactions, it is required at very low levels (Kaiser *et al*., 2005) and the required amount of molybdenum is considered to be the among the lowest essential micronutrients for plant growth (Zimmer and Mendel, 1999). Molybdenum is an important element for more than 40 enzymes four of which have been found in plants (Mendel and Hansch, 2002) and include nitrate reductase (NR) which is involved in nitrogen fixation and assimilation, xanthine dehydrogenase/oxidase (XDH) which plays an important role in purine catabolism, aldehyde oxidase (AO) that plays an essential part in the synthesis of indole-3 acetic acid (IAA) and abscisic acid (ABA), and sulphite oxidase (SO) which has a role in sulphur metabolism (Kaiser *et al*., 2005). XDH and AO contain a monoxo-Mo co-factor which needs Moco insertion and then consequent sulfuration of the Mo centre to activate the Moco protein complex. SO and NR have a dioxo-Mo co-factor which motivates the protein when it is inserted into the protein complex (Mendel and Hansch, 2002).

AO is a key enzyme for catalysing the final step of ABA production. ABA is a molecular made from carotenoid and the production of this compound involvs cleavage of 9-cis-epoxycarotenoid to produce xanthoxin. The xanthoxin is then catalysed by 9-cis-epoxycarotenoid dioxygenase (NCED) in plastids (Huang *et al*., 2009). Xanthoxin is further changed to produce abscisic aldehyde in the cytosol via alcohol dehydrogenase (González-Guzmán *et al*., 2002) and AO then catalyses abscisic aldehyde to synthesis ABA (Cutler and Krochko, 1999). Several studies

have reported that the absence of AO cause deficiency in *in-planta* ABA (Sagi *et al*., 2002, Bittner *et al*., 2001).

It has previously been proposed that molybdenum can be involved in amelioration of frost damage (Li *et al*., 2001, Du *et al*., 1994) and freezing tolerance in wheat (Sun *et al*., 2006a). Furthermore, since *COR* genes have an essential role on plant cold tolerance, several studies have instigated the effect of molybdenum on *COR* gene expression (Sun *et al*., 2009). ABA-dependent and ABA-independent pathways both mediate *COR* gene expression. Molybdenum plays an important role in the synthesis of ABA which in turn activates basic leucine zipper (bZIP) transcription factors and bZIP activates ABA-dependent *COR* genes through ABA responsive elements (ABREs) (Xiong *et al*., 2002, Uno *et al*., 2000). In terms of the ABA-independent pathway, cold activates the expression *CBF* transcription factors which in turn up-regulate *COR* genes resulting in an increase of plant cold tolerance (Thomashow, 1999). Sun *et al* (2009) and Al-Issawi *et al* (2013) reported that molybdenum treatment improved the expression of *CBF* that was regulated by low temperature treatment.

This study aimed to investigate the effect of molybdenum on the cold tolerance of cauliflower artificial seeds (microshoots) and the molecular mechanism of this effect.

Objectives

- To determine the effect of molybdenum (Mo) used with microshoot liquid culture media on the growth and development of cauliflower microshoots (number and average weight).
- To investigate the effect of molybdenum (Mo) used with artificial seed matrix and artificial seed conversion medium on the growth and development of cauliflower artificial seeds.

- To investigate the effect of Mo on cauliflower artificial seed cold tolerance.
- To evaluate the effect of Mo on the cold tolerance of microshoots in combination with low temperature.
- To investigate the effect of Mo on the induction of *CBF/DREB*1 expression.
- To investigate the effect of Mo on the accumulation of COR15 protein in acclimated cauliflower microshoots.

5.2. Materials and Methods

5.2.1. The effect of Mo on the development of microshoots

Cauliflower microshoots (cv. Fremont) were produced using SOP_{CS} (standard operating procedure for culture system) the only exception being that four concentrations of Mo (0, 15, 30, 45 and 60 ppm) were used with the culture media. The molybdenum was derived from ammonium molybdate compound (VWR, Prod 271874B). Therefore, the amounts used from ammonium molybdate were (0, 27, 57, 81 and 108 ppm) since the Mo forms 54% from this compound. The effect of Mo on the number and viability (assessed as the average of microshoot fresh weight) was evaluated after 28 days of culture. Five culture pots (replicates) were used with each Mo treatment.

5.2.2. The effect of Mo used with artificial seed capsules and artificial seed conversion media

Cauliflower microshoots (cv. Diwan) were produced using SOP_{CS}. 15 day old microshoots were encapsulated in sodium alginate capsules supplemented with 0, 15 and 30 ppm of Mo using SOP_{ASP} (standard operating procedure for artificial seed production). Artificial seeds were cultivated in culture pots each containing 30 mL of maintenance S23 media. Cultures were incubated in a Snijder growth cabinet at 22 °C and light intensity (177 µmol m^{-2} sec^{-1}). The effect of Mo on both artificial seed

conversion rate and viability, assessed as plantlet average weight, was evaluated after 29 days of culture. 15 culture pots with 5 artificial seeds were used with each treatment.

Another set of cultures with the same culture conditions were produced. 15 day old micoshoots were encapsulated in free Mo sodium alginate capsules using SOP_{ASP}. Artificial seeds were cultivated in culture pots which each contained 30 mL of S23 media supplemented with 0, 15 and 30 ppm of molybdenum. The effect of Mo on both artificial seed conversion rate and viability was assessed after 29 days of culture. 15 culture pots with 5 artificial seeds were used with each treatment.

5.2.3. The effect of Mo on the cold tolerance of cauliflower artificial seeds

Cauliflower microshoots (cv. Fremont) were produced using SOP_{CS} with the exception that two concentrations of Mo (0 and 15 ppm) were used with the culture media. These concentrations were used because higher concentrations had negative effects on the growth and development of microshoots. Samples of 15 days old microshoots were encapsulated in sodium alginate matrix using the SOP_{ASP} protocol in order to produce artificial seeds.

Frost resistant analysis of the artificial seeds was carried out to test the effect of molybdenum (Mo). The artificial seeds were placed in sterile petri dishes with a small piece of a sterile ice to ensure ice nucleation and placed in a Sanyo chamber programmed to 0, -2, -4, -6, -8, -10 and -12°C with a hold of two hours at each temperature. Samples were taken at the end of each 2 h hold. Samples were kept at 4°C overnight to thaw. Artificial seeds were then cultivated in maintenance semi-solid media S23 in a growth cabinet at 23°C and 16 hours photoperiod provided by fluorescent lighting (177 µmol m^{-2} sec^{-1}). The conversion rate and the average

weights of plantlets were evaluated after 28 days of culture. Five replicate culture pots, each containing 30 mL of maintenance S23 semi-solid media and cultured with 5 artificial seeds, were used with each treatment.

5.2.4. The effect of Mo on the induction of *CBF/DREB1* expression and on the cold tolerance of acclimated cauliflower microshoots

Cauliflower microshoots (cv. Fremont) were produced using SOP_{CS} with two concentrations of Mo (0 and 15 ppm) and cultured in the culture media. After 25 days growth at 20 °C samples for mRNA extraction were taken and then the shaker containing the microshoot cultures was transferred from room temperature to the cold room at 4°C to induce acclimation and after 12 and 18 hours after transfer to the cold room mRNA samples were taken again in order to investigate the induction of *CBF/DREB1* expression. The samples were kept at -80°C until the RNA was extracted. The evaluation of *CBF/DREB1* gene expression was done using $SOP_{CBF/DREB1}$ protocols in two steps:

1. In the first part SOP_{QPCR} was applied using the primers (P2) (Forward 5-ACTTTCCTAACCGCCGAC, Reverse 5-TCTCAGCCTGAAAAGCCA-3).
2. In the second part SOP_{QPCR} for quantitative PCR was applied using the primers (P2) in order to confirm the results of part 1.

Actin1 expression (constitutively expressed gene) was assessed as a housekeeping gene and *CBF/DREB1* gene expression was normalised against Actin1 expression in both ordinary and quantitative PCR. Each PCR treatment was replicated three times.

The microshoot cultures were left at 4°C for a total of 15 days for full acclimation. Samples from the molybdenum treatments were then taken and used for protein extraction using SOP_{PEX}. Protein estimation was carried out using SOP_{PES}. Sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was carried out applying SOP_{SDS}. SOP_{WB} was applied for COR15 protein detection. Each experiment was replicated 3-5 times.

The cold tolerance of acclimated microshoots was tested by measuring electrical conductivity of samples after exposure to 20 (control), -2, -4, -6, -8, -10 and -12 °C applying $SOP_{ECM\%}$. 3 replicates (tubes of which contained 10 g microshoots) were used for each Mo treatment at each temperature.

5.3. Results

5.3.1. The effect of Mo used with microshoots culture media on the development of microshoots

When culture media were autoclaved, it was noticed that the media which contained molybdenum had an increasing darker colour the higher the concentration of Mo used (Figure 104). In terms of the effect of Mo on the number and viability of microshoots, concentrations higher than 30 ppm had negative effects on the explants and no microshoots were observed to grow when these concentrations were applied. When 30 ppm of Mo was used with the culture media, a highly significant decrease in the number of growing microshoots was observed (P<0.001). In terms of the effect of Mo on microshoots average weight, although the use of 30 ppm Mo significantly increased the average weight (P = 0.021), the number was relatively very low (Figure 105).

Colour differences between microshoots derived from different Mo treatments were observed and the higher the Mo concentration, the darker the colour of microshoots (Figure 106).

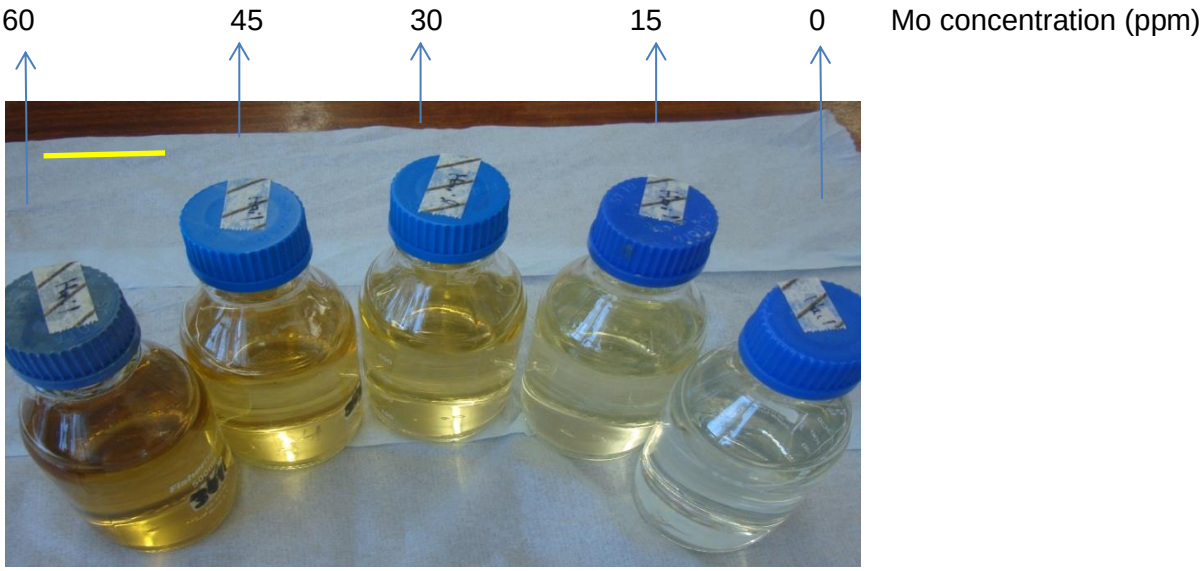


Figure 104. The change of media colour with the change of molybdenum concentration in the culture media (Bar = 5 cm).

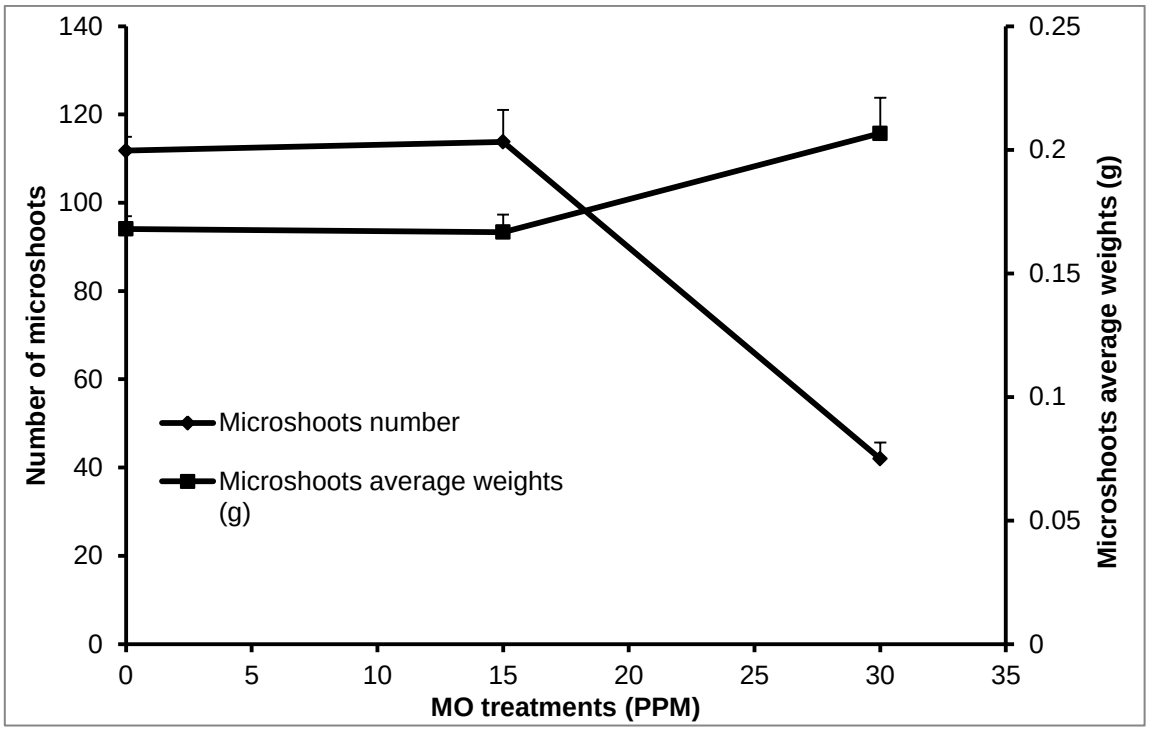


Figure 105. The effect of using Mo used with the culture media on the number and average weight of microshoots produced (LSD=15.53 for the number of microshoots, LSD=0.029 for the microshoots average weights).

Figure 106. The effect of Mo concentration used with the culture liquid media on the colour of microshoots produced (A and B were derived from 30ppm of MO treatment, C microshoots were derived from Mo free culture media) (Bar = 0.5 cm for photo A, bar = 2 cm for photo B and bar= 1 cm for photo C).

5.3.2. The effect of Mo applied to artificial seed matrix

The use of Mo with artificial seed matrices significantly reduced the conversion rate of artificial seeds (P = 0.015). However, there was no significant effect of Mo on the artificial seed viability (P = 0.505) (Figure 107)

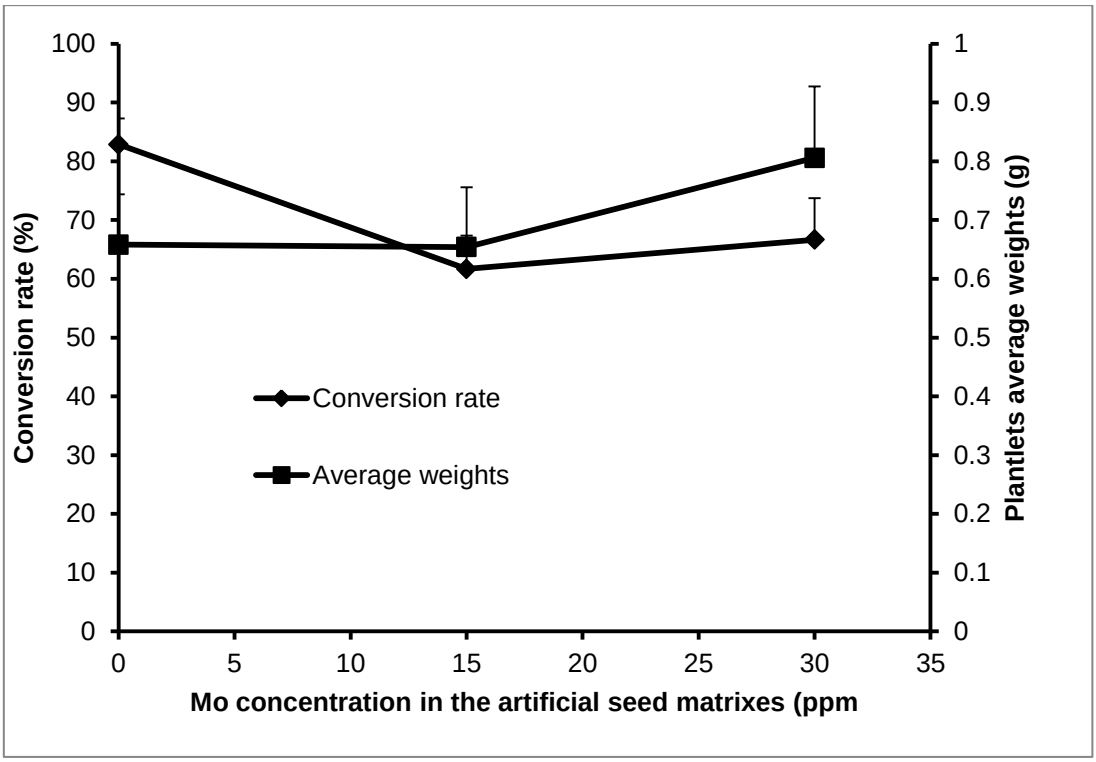


Figure 107. The effect of Mo used with artificial seed matrix on their conversion rate and viability (LSD=13.26 for artificial seed conversion rate).

5.3.3. The effect of molybdenum applied with artificial seed conversion media

While there was no significant effect of Mo used with the artificial seed conversion media (P = 0.237), Mo significantly reduced the viability of cauliflower artificial seed (P < 0.001) (Figures 108 and 109).

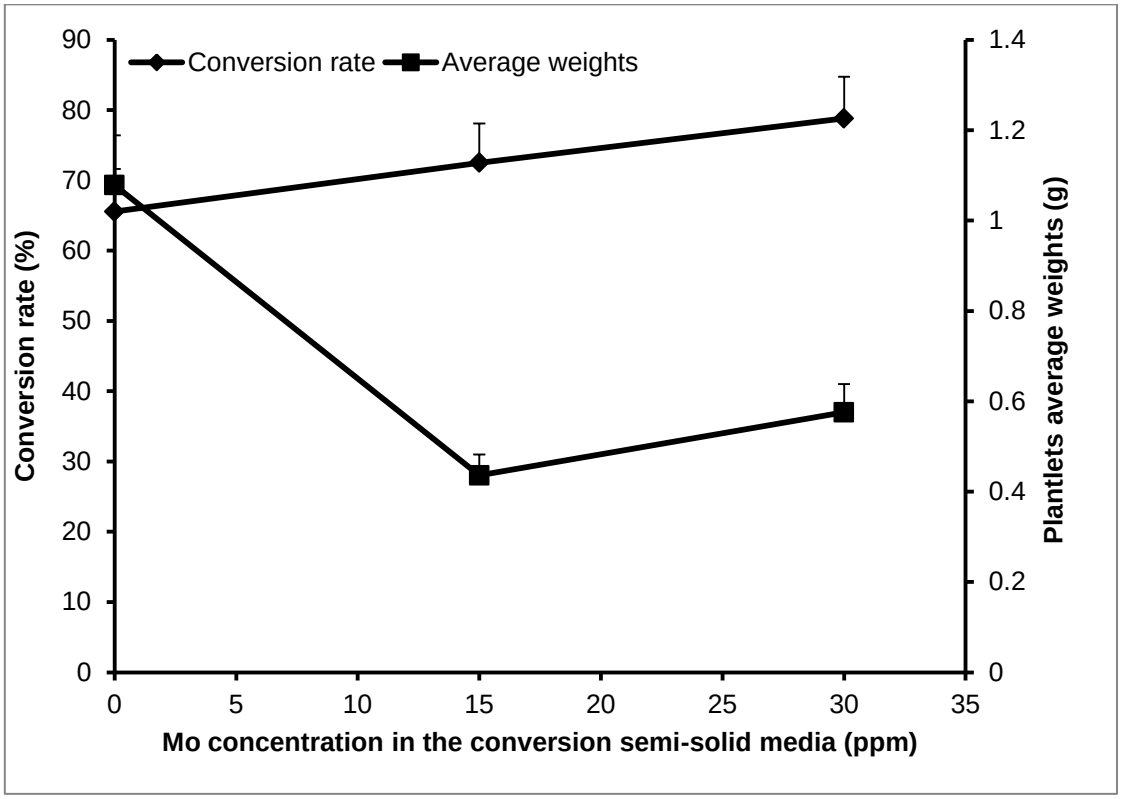


Figure 108. The effect of Mo used with artificial seed conversion media on their conversion rate and viability (LSD= 0.20 for artificial seed viability).

Mo concentrations 0 (ppm) 15 (ppm) 30 (ppm)

Figure 109. The effect of Mo used with artificial seed conversion media on the growth of plantlets (Bar = 2 cm).

5.3.4. The effect of Molybdenum on the cold tolerance of artificial seeds of cauliflower

Molybdenum had significantly positive effect on artificial seeds cold tolerance ($P < 0.001$) at both -10 and -12 °C treatments. While the conversion rates decreased significantly at temperature lower than -8°C in the artificial seeds derived from non Mo treated microshoots, the conversion rate of the artificial seeds produced from microshoots treated with Mo tolerated low temperature down to -12 °C (Figure 110).

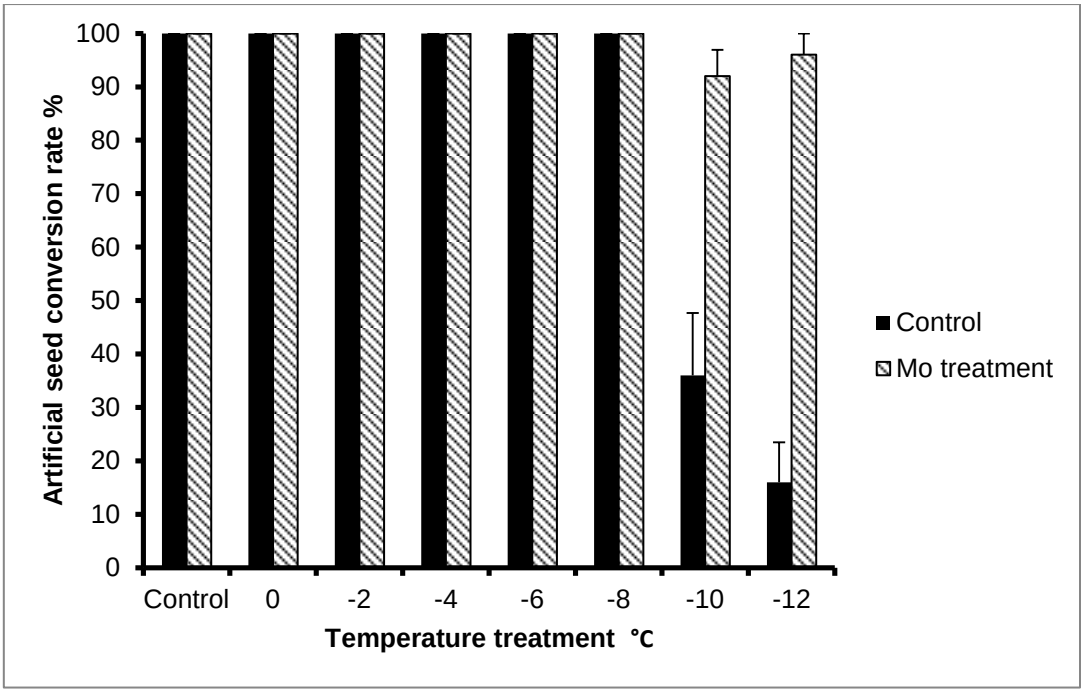


Figure 110. The effect of using 15 PPM of Mo with microshoots culture media on the artificial seeds cold tolerance (LSD= 25.78).

5.3.5. The effect of low but non damaging temperature on the viability of artificial seeds derived from both MO treated and non-treated microshoots

The acclimation (4°C) treatment had a positive effect on the viability of artificial seeds assessed as plantlets produced average weights. However, while Mo-non-treated artificial seeds exposed to -6°C showed the highest viability (plantlets average weight) (P = 0.018), Mo-treated artificial seed exposed to -2°C gave the highest viability (P < 0.001) (Figure 111).

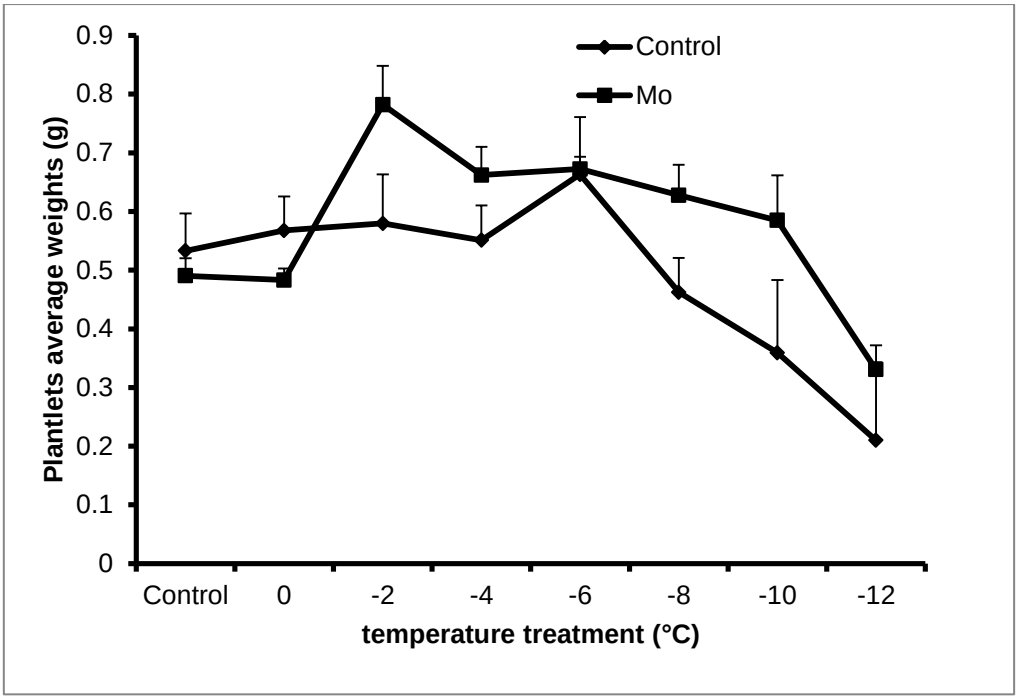


Figure 111. The effect of temperature treatments (°C) on the average weight of plantlets derived from the conversion of encapsulated microshoots produced in free MO liquid media (LSD= 0.22) and liquid media supplemented with 15 ppm Mo (LSD= 0.15).

5.3.6. The effect of molybdenum treatment on the cold tolerance of acclimated cauliflower microshoots

It was found that the use of 15 ppm of Mo significantly improved the cold tolerance of acclimated microshoots (P = 0.022) when they were frost tested at -10°C (Figure 112) with the EC% being significantly lower in the Mo treated microshoots compared to the controls. However, -10°C was a critical temperature since this was where a significant difference between Mo and non-Mo treated artificial seeds was observed (Figure 49).

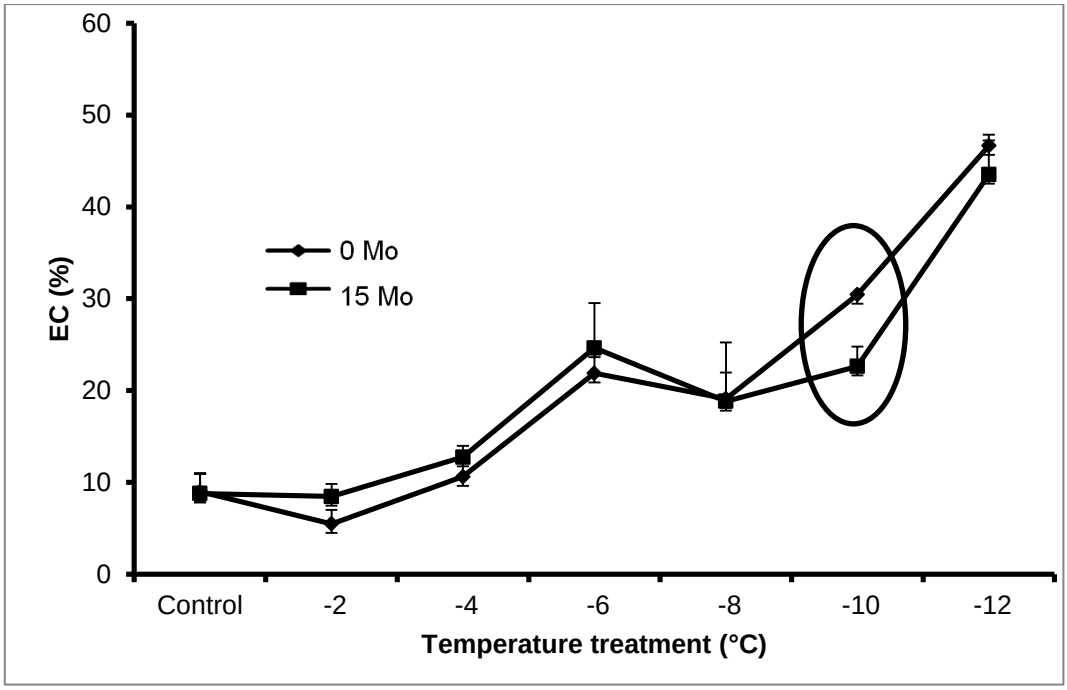


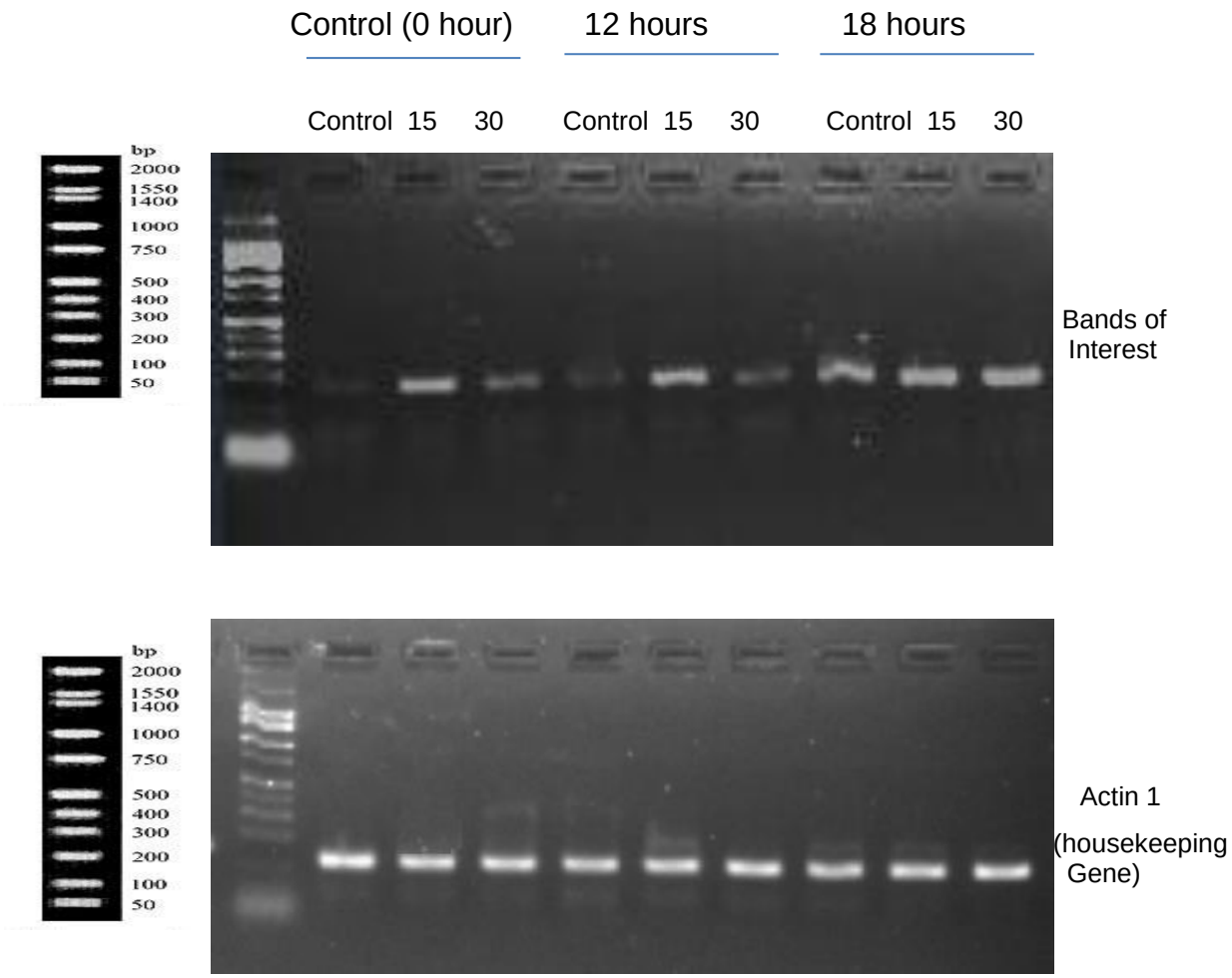
Figure 112. The effect of Mo treatment on the cold tolerance assessed as EC% of acclimated cauliflower microshoots (LSD = 4.30 at -10°C).

5.3.7. The effect of molybdenum treatment on the induction of *CBF/DREB1* gene expression

It was observed using both ordinary and quantitative PCR that the use of molybdenum significantly increased *CBF/DREB1* gene expression when applied in conjunction with acclimating temperature ($P < 0.001$). Also, it was shown that Mo had the capacity to up-regulate *CBF/DREB1* without cold acclimating treatment (Figure 113 and 114). The use of a relatively high concentration of Mo (30 ppm) decreased the expression of *CBF/DREB1* and 15 ppm Mo was found to be the optimal concentration in terms of its effect on the induction of *CBF/DREB*1 gene expression among the concentrations investigated (Figures 113 and 114).

While no significant differences were observed in terms of the effect of the duration of acclimation (4°C) treatment on the expression of *CBF/DREB1* gene ($P = 0.335$) using ordinary PCR (Figure 113), a significant effect of the acclimation duration ($P =$

0.03) (Figure 114) was found using quantitative PCR. It was demonstrated that the level of expression increased with time.



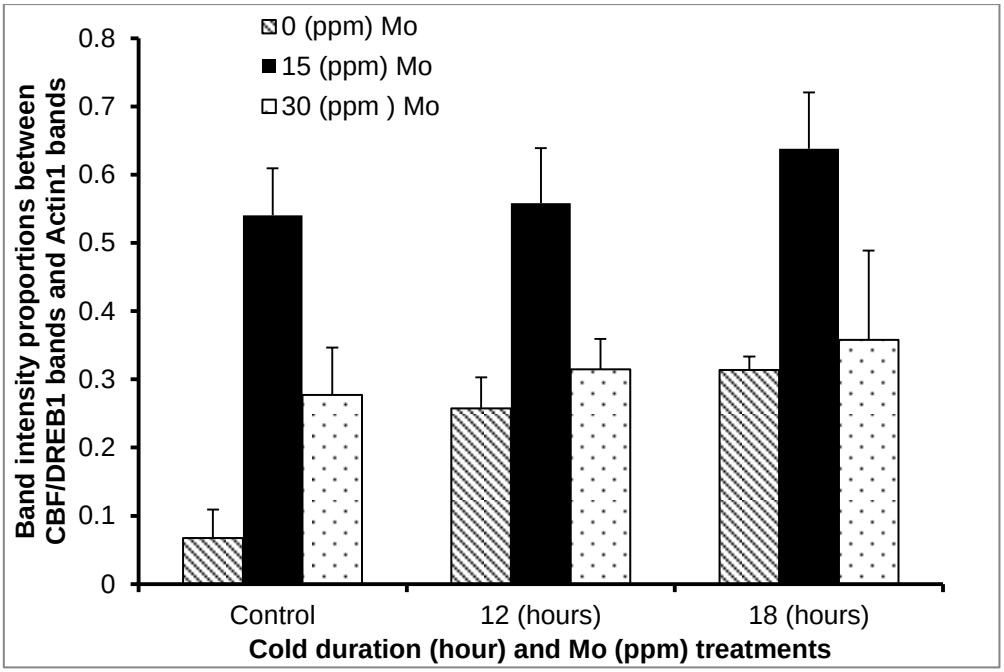


Figure 113. The effect of different concentrations of Mo and acclimation treatment on the expression of *CBF/DREB1* gene observed using ordinary PCR (LSD = 0.175 for Mo treatment).

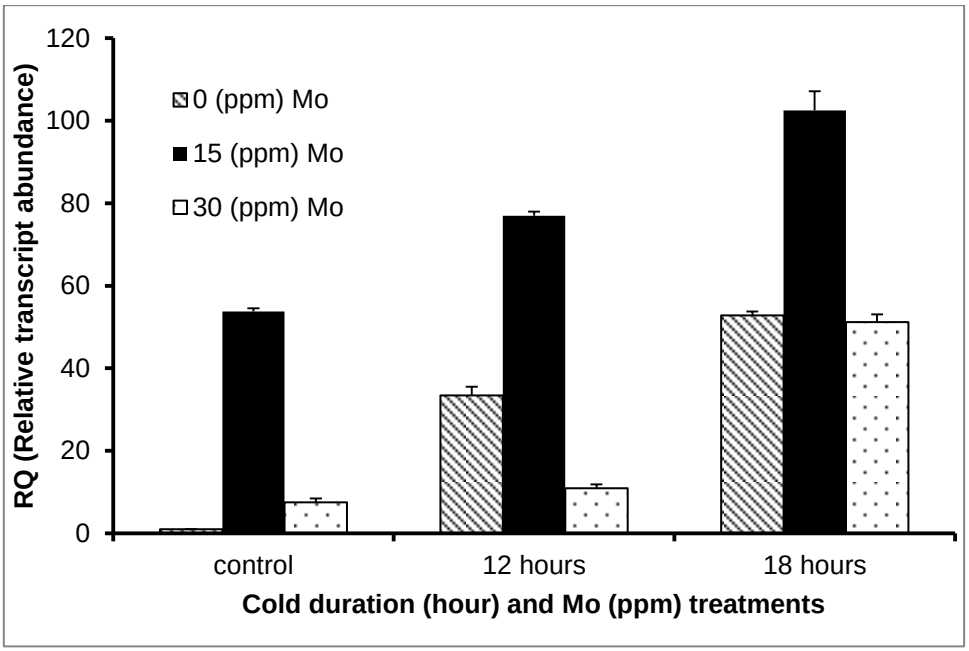


Figure 114. The effect of different concentrations of Mo (PPM) and acclimation treatmens on the expression of CBF/DREB1 observed using quantitative PCR (LSD = 30.31 for Mo treatment, LSD = 36.98 for the temperature effect).

5.3.8. Detection of COR15 protein in molybdenum treated cauliflower microshoots

One band was detected using the COR15 antibody at 23 kDa. However, the amount of this polypeptide (Band intensity) was significantly affected by Mo treatment (P = 0.035). The highest amount of this 23 kDa protein was observed when 15 ppm Mo was used with the microshoot culture media (Figures 115 and 116). The use of higher concentrations than 15 ppm however had a negative effect on the amount of the 23 kDa polypeptide (Figures 115 and 116).

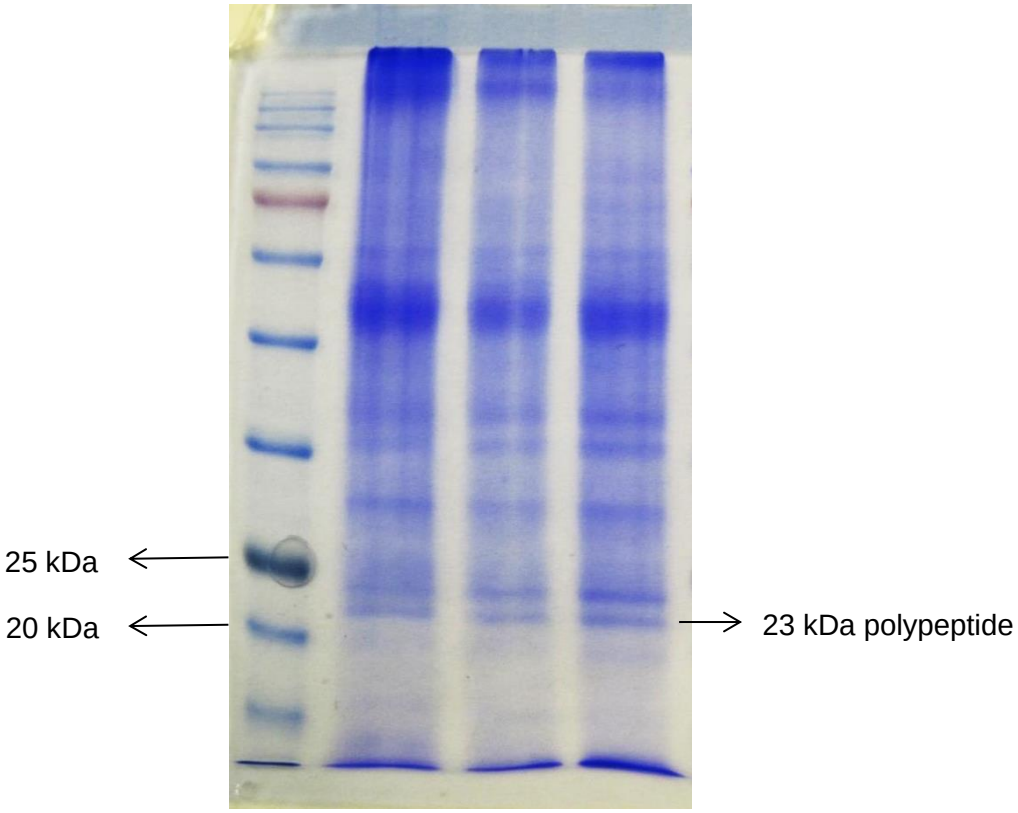
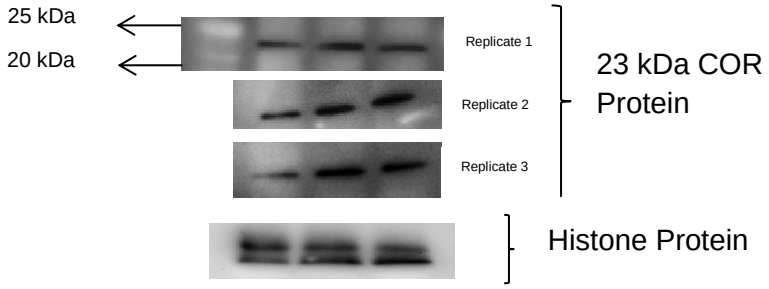


Figure 115, the SDS-PAGE analysis for protein samples derived from both Mo treated and non-treated samples.



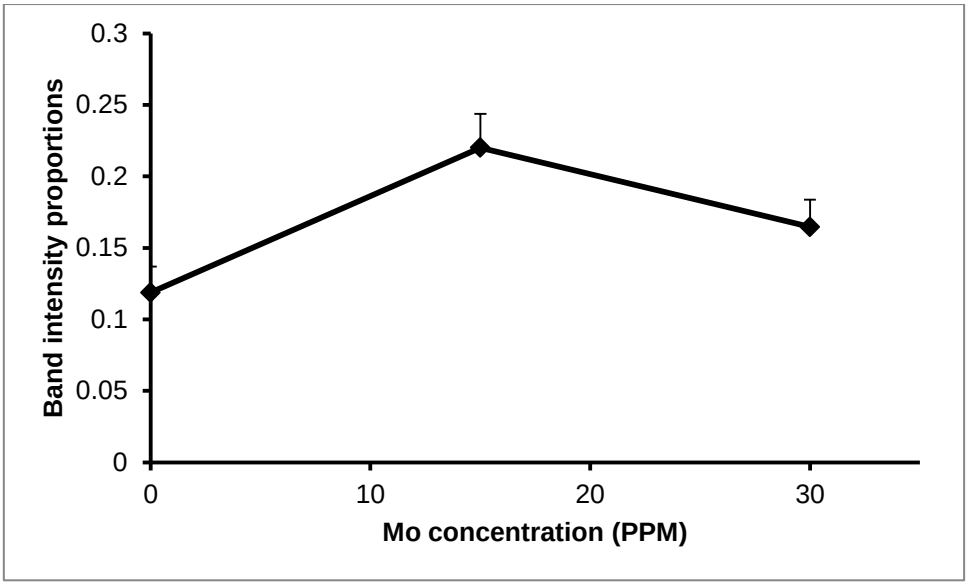


Figure 116. Western blot analysis for the detection of COR15 protein (23 kDa) from Mo treated and non-treated microshoot samples (LSD = 0.054).

5.4. Discussion

It was shown in 4.3.5.chapter 4 (experiment 5) that ABA had no capacity to improve the cold tolerance of cauliflower artificial seeds (microshoots) nor up-regulate *CBF/DREB1*, it was decided to investigate the effect of Molybdenum (Mo) for use with culture media to improve cauliflower microshoots cold tolerance. Mo is a rare element available from the soil and is essential for the growth of plants (Kaiser *et al*., 2005). A concentration higher than 30 ppm Mo was found to have a negative effect on the cauliflower microshoots since no growth was observed using these concentrations. Moreover, the use of 30 ppm of Mo significantly reduced the viability of microshoots. However, Mo, like other rare elements, is required at low concentration and the high concentrations of many of these rare elements have a negative effect on the growth of plants.

It was observed that Mo changed the colour of media and the higher the concentration of Mo, the darker the colour of the media. It appeared that Mo increased the level of sugar (sucrose) caramelization and this could indicate an important role of Mo in enzyme activities. Sun *et al* (2009) reported that more than 40 Mo-enzymes catalysing various redox reactions have been found in all organisms. Interestingly molybdenum also increased the darkness of cauliflower microshoots and this suggests that molybdenum has an important role on the biosynthesis of chlorophyll or the synthesis of anthocyanins. In agreement with the current results, it has been reported that the net photosynthetic rate (Pn) reduced and chlorophyll biosynthesis was inhibited in Mo-deficient winter wheat under the influence of cold stress (Sun *et al*., 2006a). Chlorophyll biosynthesis can be described as follows, glutamate (Glu) $\longrightarrow$ aminolaevulinic acid (ALA) $\longrightarrow$ porphobilinogen (PBG) uroporphyrinogen$\longrightarrow$ 111 (Uro I11) $\longrightarrow$ protoporphyrin IX (Proto IX) $\longrightarrow$ Mg-

protoporphyrin JX (Mg-Proto IX) → protochlorophyll (Pchl) → chlorophyll a (Chl a) chlorophyll b (Chl b)(Porra, 1997). Yu *et al* (2006) demonstrated that Mo deficiency blocked the conversion of ALA to Uro111 causing a decrease in chlorophyll biosynthesis.

It was observed that the use of Mo with artificial seed matrices significantly decreased artificial seed conversion rate and the use of this compound with artificial seed conversion media significantly reduced the viability of artificial seeds even when it was used at relatively low concentrations (15 ppm). However, the use of Mo at 15 ppm concentration with liquid culture media had no negative effect on the development of cauliflower microshoots and therefore the effect of Mo on microshoot cold tolerance was investigated. It seems that the optimal stage for Mo introduction in cauliflower artificial seed production system is the microshoot liquid culture media. The differences observed in terms of the effect of Mo applied at various steps of cauliflower artificial seed production could be attributed to the physical structure of the media used in these stages (semi-solid with the conversion media and solid structure artificial seed capsules). However, more investigations are required to find out the causes of the negative influences of Mo on cauliflower artificial seeds conversion rate and viability when it was used with artificial seed capsules and conversion media respectively.

The application of Mo with the liquid culture media improved the cold tolerance of cauliflower artificial seeds (microshoots) without acclimation at 4°C. Furthermore, the use of Mo enhanced the cold tolerance of acclimated cauliflower microshoots. Other reports have indicated that molybdenum could have a role in the amelioration of frost damage (Li *et al*., 2001, Du *et al.*, 1994) and freezing (Al-Issawi *et al*., 2013, Sun *et al*., 2006a) and several hypothesises have been examined to investigate the Mo

effect mechanism on plant cold tolerance. Sun *et al* (2006a) mentioned that Mo could increase the anti-oxidative defence in plants by increasing the activity of the anti-oxidative enzymes such as SOD, CAT and POX resulting in an increase in plant cold tolerance. That is argued because cold stress normally induces oxidation stresses, producing Reactive Oxygen Species (ROS) such as superoxide radicals ($.O_2$), hydroxyl radicals (.OH) and hydrogen peroxidase (H_2O_2). ROS makes complexes with DNA, lipids and proteins causing cellular damage (Sattler *et al*., 2000). At the molecular level, several studies have investigated the mechanism by which Mo improves plant cold tolerance. For example it has been reported that Mo induces the expression of *COR* genes due to ABA dependent pathway since aldehyde oxidase (AO), which is an Mo-enzyme, catalyses the final steps in the conversion of indole-3-acetaldehyde to indole-3-abscisic acid and the oxidation of abscisic aldehyde to abscisic acid (Kaiser *et al*., 2005, Zdunek-Zastocka, 2008, Barabas *et al*., 2000, Akaba *et al*., 1999). This could then lead to the accumulation of endogenous abscisic acid which activates the basic leucine zipper (bZIP) transcription factors which in turn induce the expression of ABA-dependent *COR* genes through ABA receptive elements (Xiong *et al*., 2002, Uno *et al*., 2000). However, it appears that this is not the case for cauliflower microshoots because, as was mentioned in 4.3.5.chapter 4 (experiment 5), ABA did not have the capacity to improve cold tolerance in this system. This could be due to either a failure in the induction of ABA-dependent *COR* gene signalling system or possibly that the exogenous ABA application in the experiments in 4.3.5.chapter 4 (experiment 5) did not have an endogenous ABA effect. This was justification for testing the effect of Mo on the ABA-independent *COR* gene induction pathway through the the up-regulation of *CBF/DREB1* in cauliflower microshoots. The results confirmed that the use of Mo

at 15 ppm improved the expression of *CBF/DREB1* under the effect of low temperature treatments. In agreement with this finding, Sun *et al* (2009) and Al-Issawi *et al* (2013) indicated that the expression of *CBF/DREB1* transcription factor genes was significantly higher in Mo-fertilized winter wheat than in non-Mo-fertilized. It has been also demonstrated that Mo application increased the transcripts level of C*bf*14 (Thomashow, 2010, Jaglo-Ottosen *et al*., 1998b, Stockinger *et al*., 1997). However, the current study is the first which confirms the capacity of Mo to induce the expression of the *CBF/DREB1* gene under warm temperatures (20 ^{o}C) and the cold tolerance in cauliflower microshoots without cold acclimation. This result could have great practical importance in the field. However, the role played by Mo in cold tolerance needs further investigations using cauliflower mature plants under field conditions.

In terms of Mo effects on the accumulation of COR15 proteins in cauliflower microshoots, no bands were detected at the normal COR15 size (15 kDa) but consistent bands were recorded at 23 kDa. This suggests that *CBFs* could play a role as a transcription factor for other types of genes similar to COR15 and picked up by the COR15 antibody and that these genes play an important role in plant cold tolerance. The polypeptide band observed at 23 kDa must have a high homology with part of the sequence of COR15 protein because it was detected using the COR15 antibody.

The use of Mo significantly increased the amount of 23 kDa polypeptides when it was used at 15 ppm concentrations whilst higher concentrations than 15 ppm significantly decreased the amount of the 23 kDa polypeptide. Several studies have confirmed that a 23 kDa polypeptide is increased under the effect of the heat shock in *Brassica oleracea* var *gemmiferea* L. (Broccoli) (Fabijanski *et al*., 1987) and in

maize (Atkinson *et al*., 1989). Also, Ben-Hayyim *et al* (1993) and Uma *et al* (1995) demonstrated that a 23 kDa polypeptide is responsible for salt tolerance in *Citrus* and Finger Millet (*Eleusine coracana*) . Given these findings, it is conceivable that Mo could have an important role in other plant stress systems including heat shock and salt tolerance but this point needs more investigation.

5.5. Conclusion

It was demonstrated that the microshoot liquid culture media was the optimal stage for molybdenum mediated induction in the cauliflower artificial seed system. The use of molybdenum in the artificial matrixes or in the conversion media had negative effects on artificial seed conversion media and viability respectively. Mo significantly improved cauliflower microshoot cold tolerance and the expression of *CBF/DREB1* in acclimated cauliflower microshoots. Importantly, this study is the first to report the positive effect of Mo on the improvement of cauliflower cold tolerance without acclimation and to demonstrate the capacity of Mo to up-regulate *CBF/DREB1* without any cold treatment. The results reported in this chapter could have an important application in the improvement of cauliflower artificial seed abiotic stress tolerance which in turn will raise the effectiveness of this technique.

Chapter 6: An Analysis of the Development of Cauliflower Seed as A Model to Improve Artificial Seed

6.1. Introduction

Seed development involves a series of changes from ovule fertilization to seed maturation that is genetically controlled. This development comprises a series of morphological, physiological and biochemical changes occurring from ovule fertilization to the time when seeds become physiologically independent of the parent plant (Delouche, 1971). Fellows *et al* (1979) found that the physiological maturity of orthodox-seeds generally occurs when the moisture content declines to about 50-60% and that at this stage the seeds exhibit the highest level of viability, vigour and dry weight. Seed maturity is normally accompanied by noticeable changes in seed and fruit colouration (Nkang, 2002).

Seed development can be divided into four different stages: embryo patterning, embryo growth, seed filling and seed desiccation (Fei *et al*., 2007). After the completion of embryo growth, major increases include accumulation of seed storage products such as protein, oil and carbohydrate. The seed filling stage is followed by the desiccation stage during which seeds acquire drought tolerance (Bewley and Black, 1994). Coelho and Benedito (2008) divided the development of seeds into three main stages according to the dry components accumulation: the first stage is characterised by a relatively slow mass accumulation during embryogenesis. The second stage is the maturation stage, characterized by a continuous and high increase in dry matter. This stage ends by reaching the maximum dry matter content at physiological maturity. Seed dehydration is the third phase of the seed development. This stage is characterised by biological mechanisms leading to the embryos desiccation resistance.

Hormones are crucial to the seed development process since they play a fundamental role from the start of embryo creation, tissue expansion, reserve accumulation and its utilization during the germination. High levels of plant growth regulators, such as auxins, cytokinins, abscisic acid and gibberellins have been observed with their levels varying at different developmental stages of the seeds. Cytokinins are observed at the beginning of seed development, controlling the intense cellular division process. Auxins are present from the initial stages of seed development and are responsible for seed filling compound assimilation from the mother-plant. Gibbrellins are associated with cell expansion. ABA weakens early germination in the pod (viviparity) and is related to LEA (late embryogenesis abundant) protein production and desiccation tolerance in seeds (Ali-Rachedi *et al*., 2004, Koornneef *et al*., 2002).

LEA proteins were first described in wheat and cotton (Goyal *et al*., 2005). LEA proteins are synthesised in abundance during seed development and can comprise up to 4 % of cellular proteins (Roberts *et al*., 1993). LEA proteins have been linked to the embryo capability for withstanding dehydration, although the mechanism of action is still not clear (Coelho and Benedito, 2008). One of the mechanisms proposed with regards to LEA functions was that these proteins might act as protectors of macro-molecular and/or cellular structures during water deficit since they preferentially network with the available water molecules and deliver a hydration shell to protect the “integrity” and function of these macro-molecules (Olvera-Carrillo *et al*., 2010, Hoekstra *et al*., 2001, Garay-Arroyo *et al*., 2000). LEA proteins also, together with oligosaccharides and perhaps small heat shock proteins, (sHSPs), participate in the formation of the “glassy state” of the seed and its stabilization (Kalemba and Pukacka, 2008). LEA proteins increase hydrogen bonding and

thereby the average strength of the amorphous matrix and the glass transition temperature (Wolkers *et al*., 2001).

LEA protein genes have been described in different plant species and six different groups of these proteins have been characterised according to their expression patterns and gene sequence. The main classes are group 1, group 2 and group 3 (Wise, 2003, Bray, 1993). Group 1 consists of LEA proteins, of which Em protein is the form sequence. They are found only in plants and are unstructured protein in solution. This group of proteins has a preserved 20-residue amino acid motif, most often in one copy (Goyal *et al*., 2005). Group 2 LEA proteins, which are known as dehydrins, are mainly found in plants (Close *et al*., 1989). This group of proteins is classified in three sequence motifs described as K-domains (lysine-rich), the Y-domain (DEYGNP) and the S-segment (poly-serine stutter) (Kalemba and Pukacka, 2008). However, the K segment, which contains the consensus amino acids sequence EKKGIMDKIKELPG, is the only segment present in all types of dehydrins (Close, 2006a). Based on the existence and combinations of segments, dehydrins are categorized into (the) Y_nSK_2, K_n, K_nS, SK_n and Y_2K_n classes (Kalemba and Pukacka, 2008). Although dehydrins show some α-helical content, they are considered to be unstructured proteins (Lisse *et al*., 1996, Ceccardi *et al*., 1994). Group 3 LEA proteins are defined by a repeated 11-mer amino acid motif. The consensus sequence of this motif has been widely described as ΦΦE/QXΦKE/QKΦXE/D/Q (where Φ characterizes a hydrophobic residue). This group of proteins has been identified in the homologues of organisms other than plants (Dure, 2001).

It is has been widely proposed that both freezing stress and drought cause desiccation of the plant cell protoplasm (Steponkus *et al.*, 1980). Freezing stress

causes ice formation in the intercellular spaces and, since ice has a lower osmotic potential than water, water moves from inside the cells to the ice in the intercellular space resulting in desiccation of the cell protoplasm (Thomashow, 1999). Several studies suggest that plants have similar approaches to desiccation resistance regardless of whether the desiccation is caused by drought or freezing stress. Several genes have been found to respond to both drought and freezing stresses (Shinozaki and Yamaguchi-Shinozaki, 2000). It seems to be that there is a cross adaptation where the exposure to one stress can improve the tolerance to other stresses (Parmentier-Line *et al*., 2002). It has been demonstrated that drought can predispose plants to cold tolerance (Anisko and Lindstrom, 1996) and vice-versa (Levitt, 1960). It has also been demonstrated that groups of dehydrin proteins are produced by conditions which have a dehydrative constituent such as drought, salinity, cold and ABA (Close, 2006a).

The studies reported in this chapter aimed to investigate the developmental stages of cauliflower seeds in terms of the accumulation of reserved compounds (lipids, carbohydrates, minerals and proteins) and in terms of the changes in the amount of dehydrin protein which occurs. Furthermore it was an aim to improve artificial seeds by mimicing traditional cauliflower seeds and investigating the effect of certain treatments on the accumulation of dehydrin proteins in cauliflower microshoots. This study also aimed to find out whether dehydrin proteins play any part in the cold tolerance of cauliflower artificial seeds (microshoots).

Objectives

The main objectives of the studies reported in this chapter were

1. To determine the developmental stages of cauliflower seeds in terms of the changes that occur in moisture, lipids, carbohydrates, proteins and ash level from the start of seed formation to the end of seed maturation.
2. To confirm the accumulation of dehydrin proteins during the development of artificial seeds and during the reduction of seed moisture content.
3. To investigate the effect of mannitol used with cauliflower microshoot culture media on the accumulation of dehydrin proteins in microshoots.
4. To assess the effect of cold acclimation on the accumulation of dehydrin proteins in cauliflower microshoots.
5. To investigate the effect of ABA used with culture media on the accumulation of dehydrin proteins in acclimated microshoots.
6. To evaluate the effect of molybdenum used with culture media on the accumulation of dehydrin proteins in acclimated microshoots.
7. To investigate the possible role of dehydrin proteins in the cold tolerance of cauliflower microshoots.

6.2. Material and methods

6.1.1. Plant materials

Twenty cauliflower plants (vc Medallion) were obtained from a field in Cornwall, courtesy of Simmonds & Sons Ltd, and replanted in pots in Scarden Garden greenhouse at the University of Plymouth. The plants were grown according to good commercial practice. 5 other plants of the variety Fleet were cultured between the Medallion plants to ensure sufficient open pollination between the two varieties in view of the self-incompatibility cauliflower and the necessity for external pollinators. When the plants started flowering, they were covered with fleece bags for 8 days to

ensure that there was a significant amount of flowers available for fertilization. The bags were then removed for a week to allow open pollination between cauliflower plants. The plants were then re-covered to stop further pollination (Figure 117). Giving a limited period for open pollination helped to increase the level of synchronization in cauliflower seed maturation. A month later, the first sample of cauliflower seeds was collected. Subsequently, seed samples were collected five times at 15 day intervals (Figure 118). It was difficult to collect any more seeds after that since they had become very dry and the seeds started falling from the plants. Samples were placed in nylon bags and transferred quickly to a fridge at 4 °C and the subsequent analyses were done on the day of the sample collection or on the following day.

Figure 117. The growth stages of cauliflower plants used for seed production (Bar = 30cm for the photos A,C and D. Bar = 5cm for photo B)

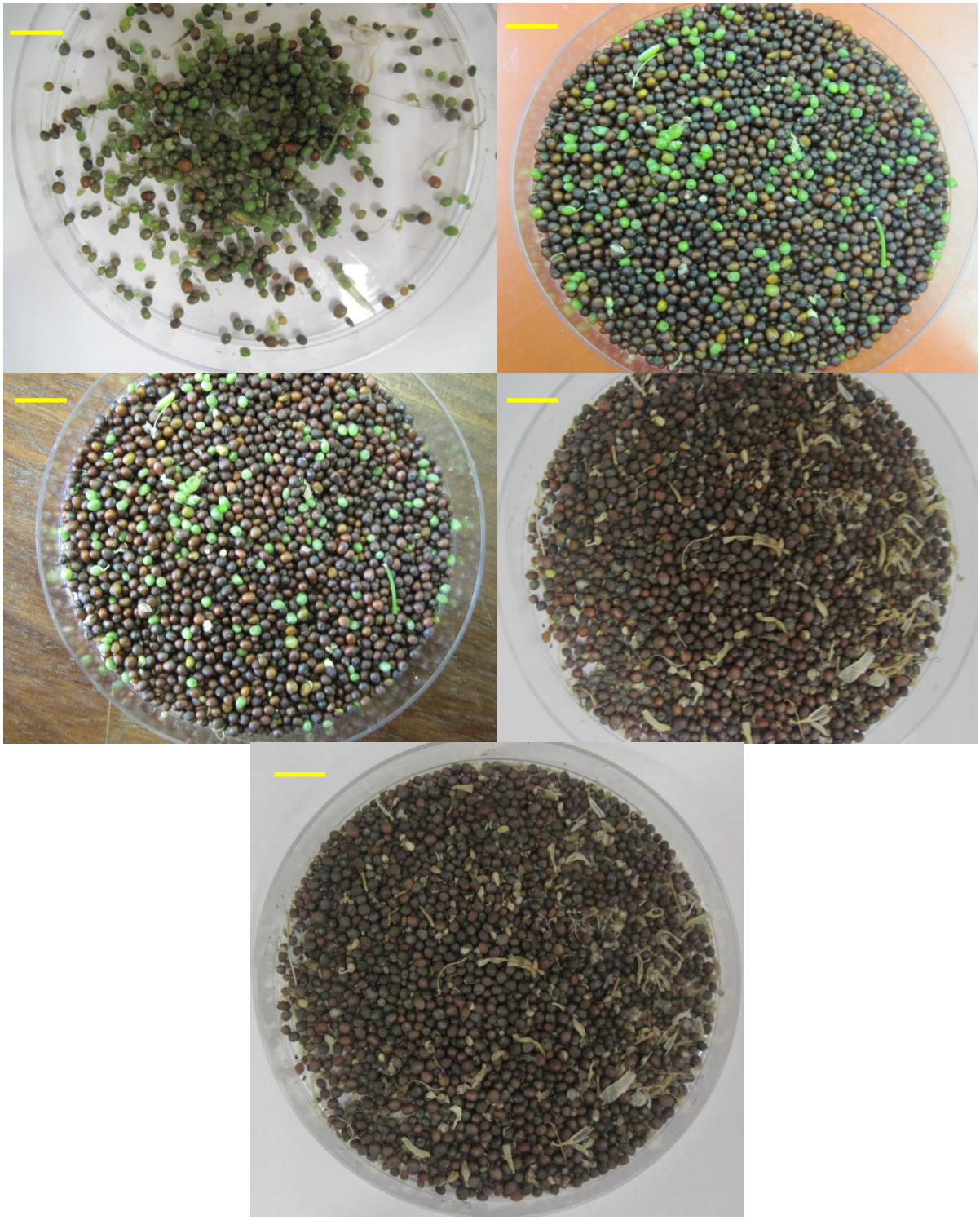


Figure 118. Cauliflower seed samples at different developmental stages (Bar = 1 cm).

6.1.2. Determination of moisture content

Three replicates, each consisting of 5 g seeds, were used from each seed sample. Seeds were dried to a constant weight in an oven at 130 °C. The moisture level was calculated using the following equation:

$$\%\ \text{Moisture} = \frac{\text{W1} - \text{W2}}{\text{W1}} * 100$$

W_1: Seed fresh weight.

W_2: Seed dry weight.

6.1.3. Determination of lipid content (rapid soxhlet extraction)

The free lipid content consists essentially of neutral fats (triglycerides) and free fatty acids. These can be determined by extracting the dried and ground material with a suitable solvent in a continuous extraction apparatus.

Well ground cauliflower seeds were weighed into cellulose thimbles and solvent was added. The solvent was heated to boiling point. The vapours were condensed and hot liquid solvent washed through the sample in the thimble in a continuous process. The non-volatile solutes were extracted into the solvent (Appendix 1). After a period of time the solvent was recovered and the extracted lipids was quantified by weighing using the following equation:

$$\%\ \text{FAT} = \frac{\text{final weight of beaker} - \text{ initial weight of beaker}}{\text{Initial weight of sample}} * 100$$

Three replicates, each consisting of 0.6 g of ground seeds, were used with each seed sample.

6.1.4. Determination of nitrogen content (Kjeldahl method)

Detailed materials and methods are attached in (Appendix 2). Three replicates (of which consisted) each consisting of 0.12 g of ground seeds were used with each sample.

6.1.5. Determination of ash content

Samples were dried at 550 °C for 8 hours (Appendix 3). The ash level was calculated using the following equation:

$$\% \text{ Ash } = \frac{\text{weight of residue(g)}}{\text{Sample weight (g)}} * 100$$

6.1.6. Determination of carbohydrate level

The carbohydrate content in the dried cauliflower seeds was determined by subtraction:

% Carbohydrate = 100 - (%moisture + % Ash + %Protein + %Lipids).

All the seed components analysis was carried out in the nutrition laboratory at Plymouth University.

6.1.7. The accumulation of dehydrin proteins during the development of cauliflower seeds (standard operating procedure for western blotting SOP_{WBD})

SOP_{PEX} was used for seed protein extraction, SOP_{PES} was used for protein estimation and SOP_{SDS} was used for sodium dodecyl sulphate polyacrylamide gel electrophoresis. A modified protocol from SOP_{WB} was applied. The modifications are described as follows: the electrical field applied was 100 volts for 35 minutes. The PVDF immobilon transfer membrane (pore size 0.4 µm) (Milipore, Cat.No ISE000010) was placed next to the gel and sandwiched between absorbent paper and sponge (-sponge/filter paper/gel/membrane/filter paper/sponge +). Hybridization for the antibody raised against the K-segment of dehydrins was achieved with primary

antibodies obtained from the Dr Michael Wisniewski's Lab, (USDA-ARS, Appalachian Fruit Research Station, USA). Membranes were incubated with dehydrin primary antibodies (produced in rabbit). Antibodies were diluted (1:1000) in a total volume of 20 ml PBST and the membranes were incubated overnight at 4°C with gentle agitation.

To ensure the specificity of the dehydrin antibodies, antibodies were blocked by peptide-salt containing the dehydrin K-segment consensus peptide TGEKKGIMDKIKEKLPGQH provided kindly by Prof. Timothy J.Close (Riverside University, California, USA). 0.5 g of the peptide-salt was disolved in PBS. Equal volume of the peptide salt (5 mg mL^{-1}) and dehydrin antibody serum (1:1000) were mixed and left for 30 min. Antigen-blocked serum was then used with a membrane containing seed sample proteins. The same protocol steps were then followed.

The same procedures were applied with all samples to detect histone H3 protein (Agrisera, Art no: AS10 710) as a house keeping protein. Histone Antibody was diluted (1:5000) in a total volume of 20 ml PBST. Histone protein bands were used to normalise the intensities of dehydrin protein bands. Band intensity proportions between the dehydrin bands and Histone bands were analysed using Image J software.

6.1.8. The effect of acclimation on the accumulation of dehydrin proteins in cauliflower microshoots

Cauliflower microshoots (cv. Dionis) were obtained applying SOP_{cs} described in chapter 2. The 26 day old cultures were transferred to the cold room at 4 °C for acclimation. Cauliflower cultures were left in the cold room for 15 days for acclimation and the accumulation of dehydrin protein was investigated using SOP_{WBD}. This experiment was replicated three times.

6.1.9. The effect of mannitol used with the culture media on the accumulation of dehydrin proteins in cauliflower microshoots

Cauliflower microshoots (cv. Fremont) were produced using the SOP_{CS}. Culture media were prepared to have several osmotic potentials by the addition of mannitol as follows,: mannitol free culture media osmotic potential of -0.47 Osmol kg^{-1} (this media contained 3% of sucrose), -0.7 Osmol kg^{-1}, -1.15 Osmol kg^{-1}, -1.60 Osmol kg^{-1}, -2.05 Osmol kg^{-1}, -2.50 Osmol kg^{-1} and -2.95 Osmol kg^{-1}. 28 day old microshoots were used to investigate the accumulation of dehydrin proteins using SOP_{WBD}. The experiment was replicated three times.

6.1.10. The effects of ABA on the accumulation of dehydrin proteins in acclimated cauliflower microshoots

Cauliflower microshoots (cv. Cool) were produced using SOP_{CS} with five concentrations of ABA (sigma, A-7631) (0, 0.5, 1, 2, 4 mg L^{-1}) added to the culture medium. 21 day cauliflower microshoots cultures were transferred to the cold room at 4 °C for 15 days for acclimation. Dehydrin protein accumulation in the cauliflower microshoots was assessed using SOP_{WBD}. The experiment was replicated three times.

6.1.11. The effect of molybdenum on the accumulation of dehydrin proteins in acclimated cauliflower microshoots

Cauliflower microshoots (cv. Cool) were produced using SOP_{CS} with the sole difference that three concentrations of Mo (0, 15 and 30 ppm) were used with the culture media. The shaker containing the 25 day old microshoots was transferred from room temperature to the cold room at 4°C for 15 days for acclimation. The accumulation of dehydrin proteins in the cauliflower microshoots was assessed using SOP_{WBD}. The experiment was replicated three times.

6.3. Results

6.3.1. The development of cauliflower seed structure (moisture, lipids, carbohydrate and ash)

Seed moisture level was quite high at the beginning of seed formation (76 %) and it started decreasing gradually with the development of the seeds. At the end of seed maturation the moisture level was about 8%. At that point the seed pods had become very dry and were starting to fall from the plants. The level of ash increased from about 1.2 % to about 5 % and the level of protein increased to 28.7% after 75 days of pollination (20% moisture level) (physiological maturation) followed by a decrease in the level of total protein to about 23% (Figure 119).

The level of lipids increased from about 4.5 % at the beginning of seed development to reach about 24 % at the end of seed development. The level of carbohydrate was about 9 % at the beginning of seed development and reached about 39% at the end of seed maturation (Figure 120).

.

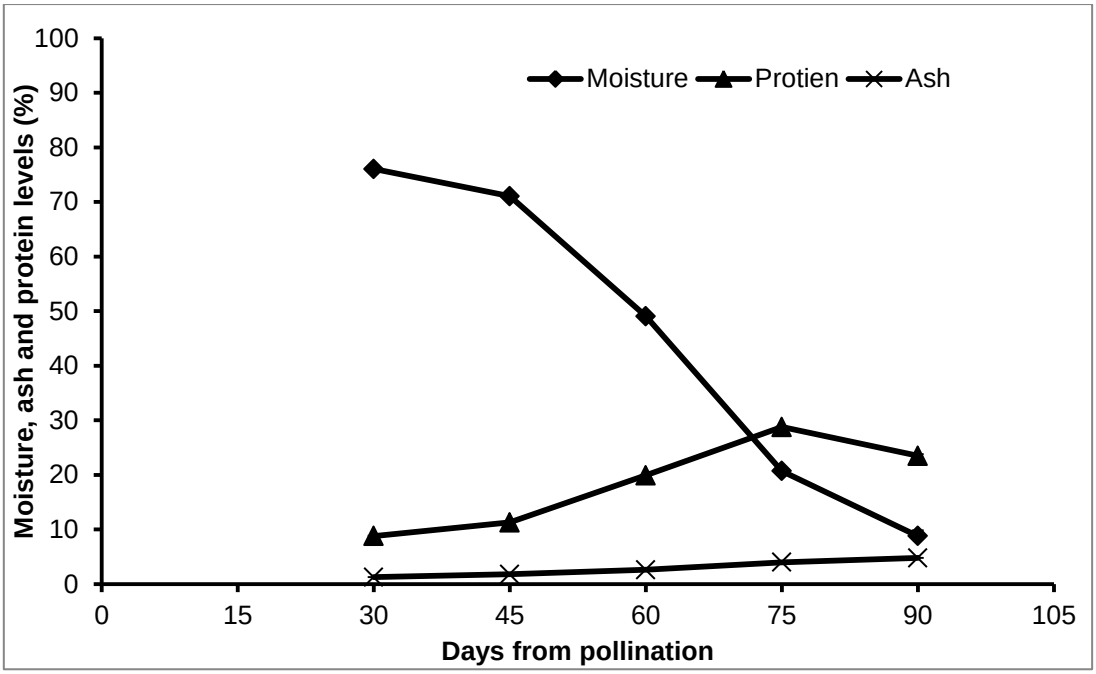


Figure 119. Moisture, protein and ash level changes during the development of cauliflower seeds.

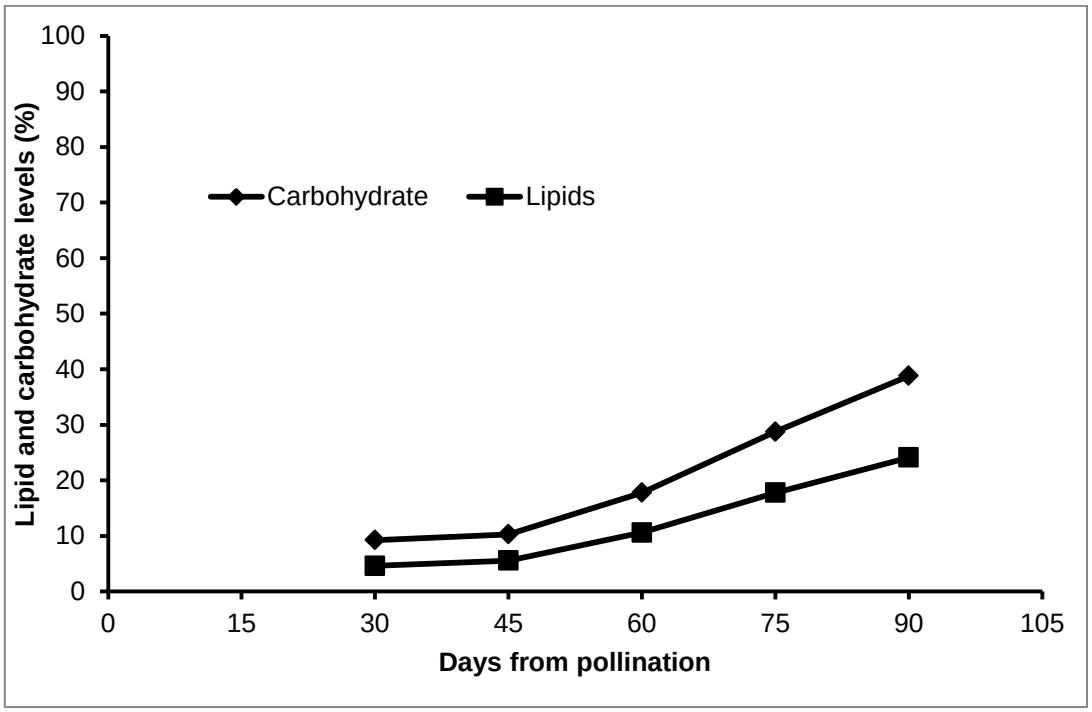


Figure 120. Carbohydrate and lipid level changes during the development of cauliflower seeds.

6.3.2. The accumulation of dehydrin proteins during the development of cauliflower seeds

No dehydrin bands were detected when blocked dehydrin antibody was used as a primary antibody (Figure 122) illustrating the specificity of the antibody. Proteins at several molecular weights were detected by the k-segment dehydrin antibody at 12, 17, 26, 50 and 78 kDa. The results demonstrated that dehydrin proteins at 12, 17 and 26 molecular weights displayed a very highly significant increase during the development of cauliflower seeds (P<0.001 for protein at 12 and 17 kDa size classes and P=0.002 for protein at size class 26 kDa). The amount of these proteins was stabilised at a specific stage of seed development when the moisture level was assessed to be about 50% (60 days from pollination) (Figures 121, 123 and 124). The amounts of high molecular weight dehydrin proteins at sizes 45 and 78 kDa were significantly decreased during the development of cauliflower seeds (P<0.001 for protein at size class 78 kDa and P=0.015 for protein at size class 48 kDa). However, the relative amounts of these proteins are very low compared to the proteins observed at the small molecular weight sizes (12, 17 and 26 kDa) (Figures 121, 123 and 124).

Dehydrin protein at size class 78 kDa was observed in the western blotting experiment but not in the SDS PAGE analysis and this could have been due to the low amount of this protein in the seed sample and because western blotting is considered as being a more sensitive approach for detecting a specific type of protein.

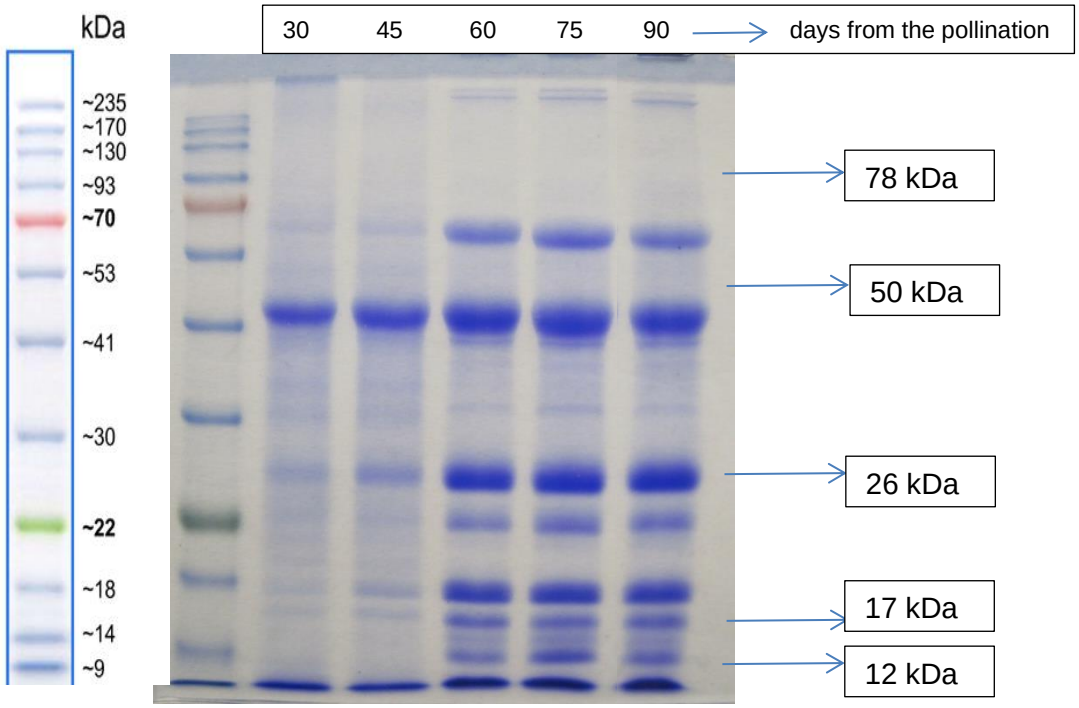


Figure 121. The SDS-PAGE analysis for protein samples derived from cauliflower seeds at different developmental stages.

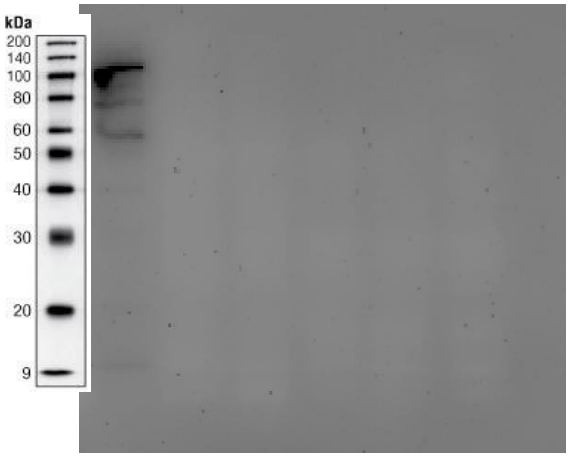


Figure 122. Western blot analysis for the detection of dehydrin proteins from cauliflower artificial seeds at different developmental stages using a blocked dehydrin k-segment antibody.

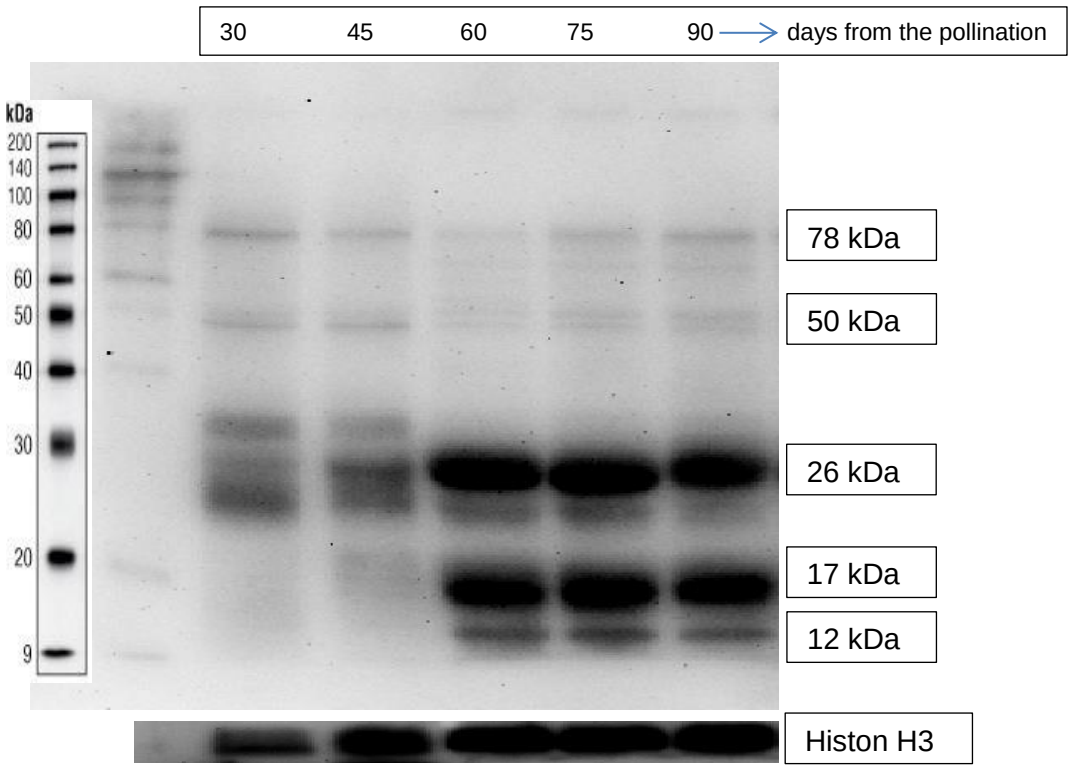


Figure 123. Western blot analysis for the detection of dehydrin proteins from cauliflower artificial seeds at different developmental stages using a k-segment dehydrin antibody.

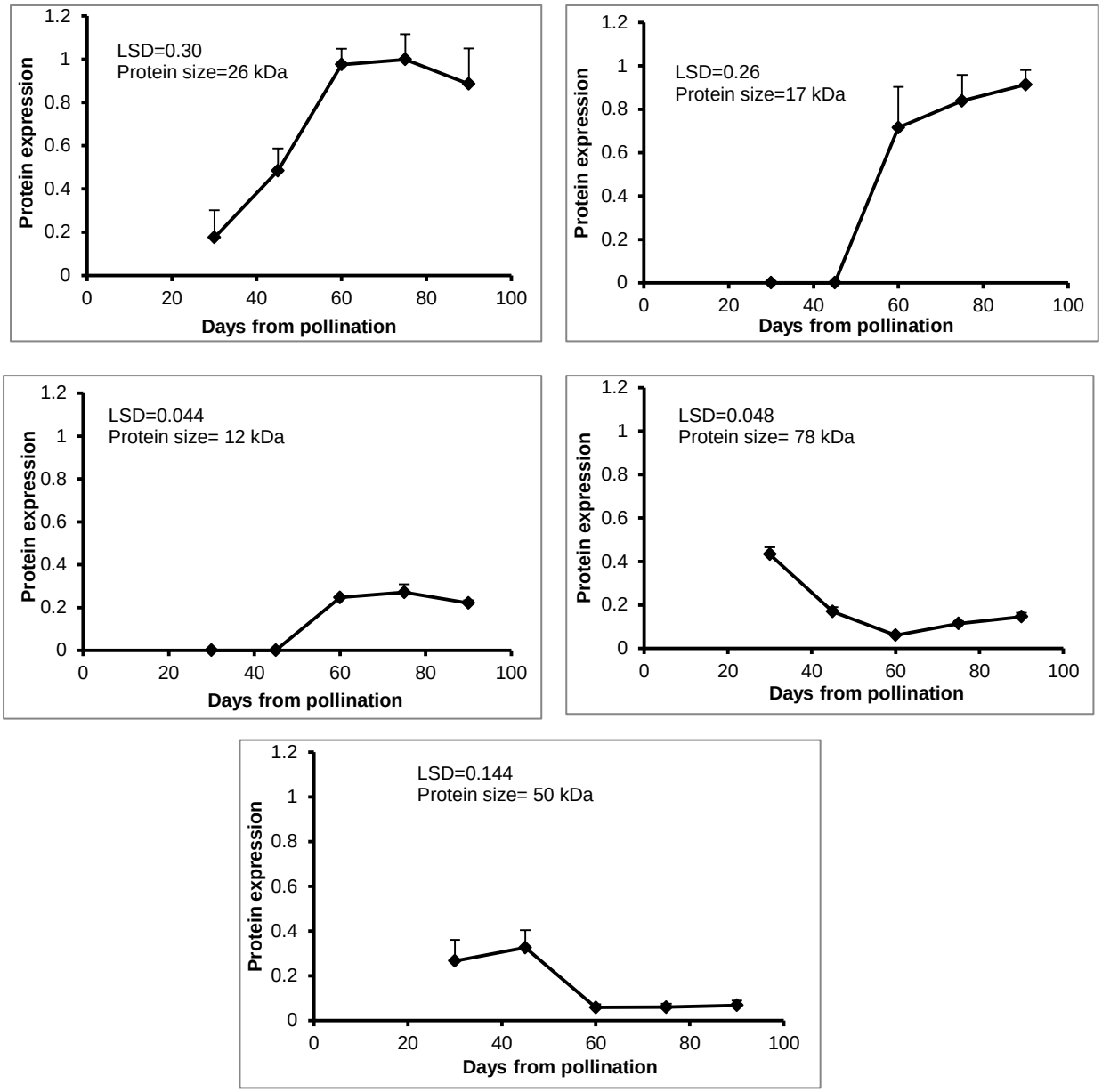


Figure 124. The accumulation of dehydrin proteins during the development of cauliflower seeds.

6.3.3. The effect of mannitol on the accumulation of dehydrin proteins in cauliflower microshoots

The use of mannitol with the cauliflower microshoots liquid culture media significantly increased the amount of dehydrin protein at 35 kDa size class (P=0.001). However, the amount of this protein stabilized using the liquid culture media with osmotic pressure lower than -0.7 Osmol kg^{-1}. In terms of the effect of mannitol on the accumulation of dehydrin protein at 78 and 170 kDa size classes, it was observed that mannitol significantly increased the accumulation of this protein, reaching a peak at -2.05 Osmol kg^{-1} liquid culture media osmotic pressure (P<0.001) followed by a significant decrease at lower culture osmotic pressures. However, the microshoots produced from culture media that have an osmotic pressure lower than -2.05 Osmol kg^{-1} were not capable of being encapsulated as artificial seeds (conversion rate = 0 at this osmotic pressure i.e. lower than -2.05) (Figures 125, 126 and 127) which suggests that these proteins are an important in regard to the viability of cauliflower microshoots.

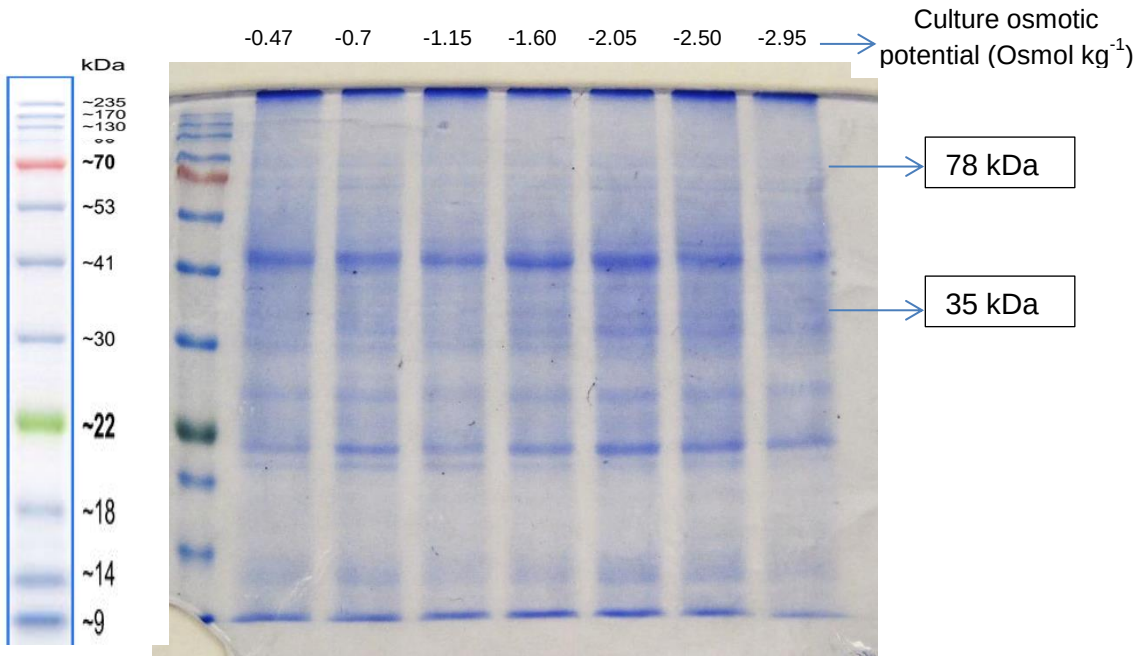


Figure 125. SDS-PAGE analysis for protein samples derived mannitol treated microshoots.

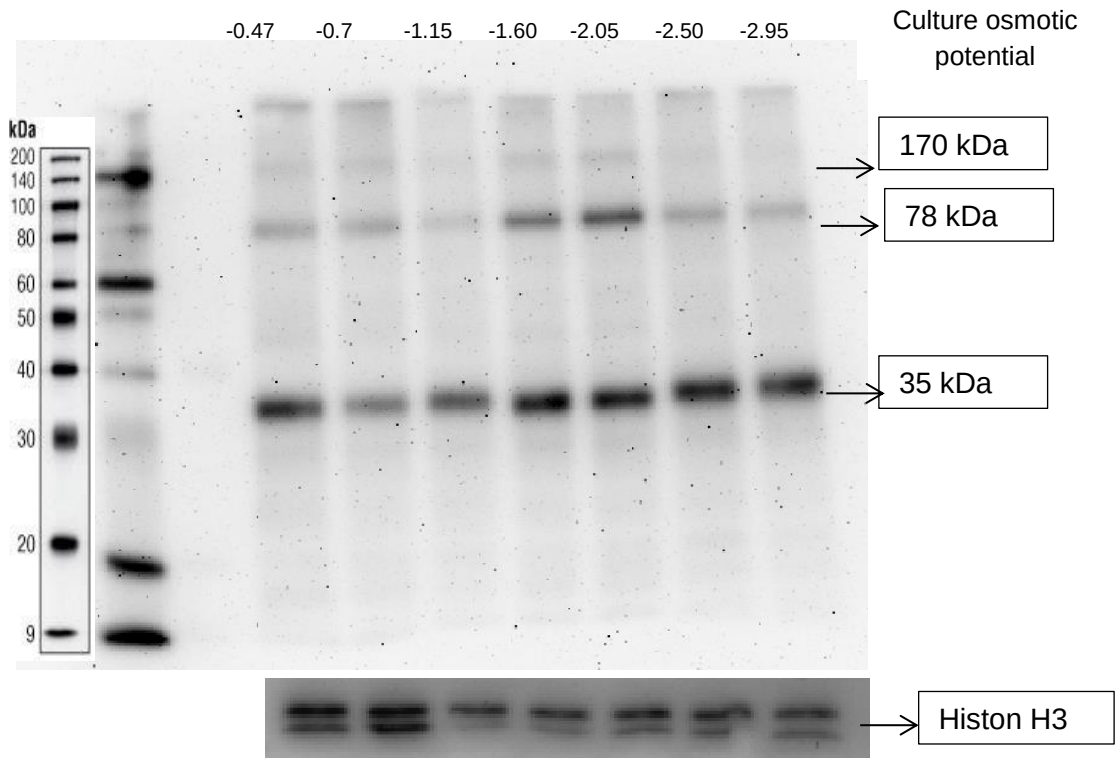


Figure 126, Western blot analysis for the detection of dehydrin proteins from cauliflower mannitol treated microshoots using a k-segment dehydrin antibody.

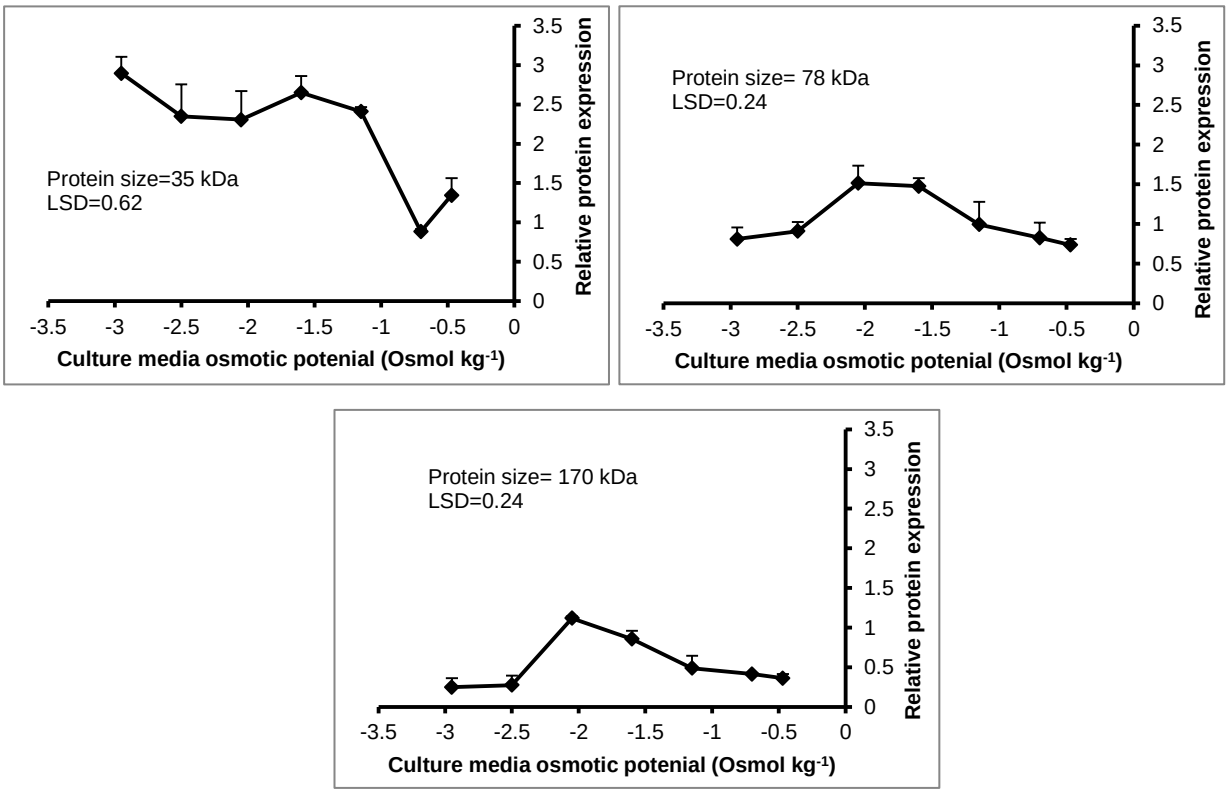


Figure 127, The accumulation of dehydrin proteins in mannitol treated microshoots.

6.3.4. The effect of cold acclimation on the accumulation of dehydrin protein in cauliflower microshoots

Acclimation was observed to have no significant effect on the amount of dehydrin proteins at 79, 22, 35 and 37 kDa size classes (*P*=0.110 for protein at size class 79 kDa, *P*= 0.265 for protein at size class 22, *P*=0.219 for the protein at size class 35 kDa and *P*=0.125 for protein at size class at 37 kDa). Moreover, acclimation significantly decreased the amount of dehydrin protein at 28 kDa size class (*P*=0.004) (Figures 128, 129 and 130).

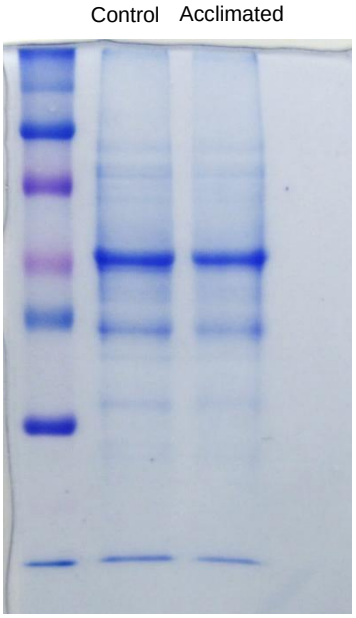


Figure 128. The SDS-PAGE analysis for protein samples derived from acclimated and non-acclimated microshoots.

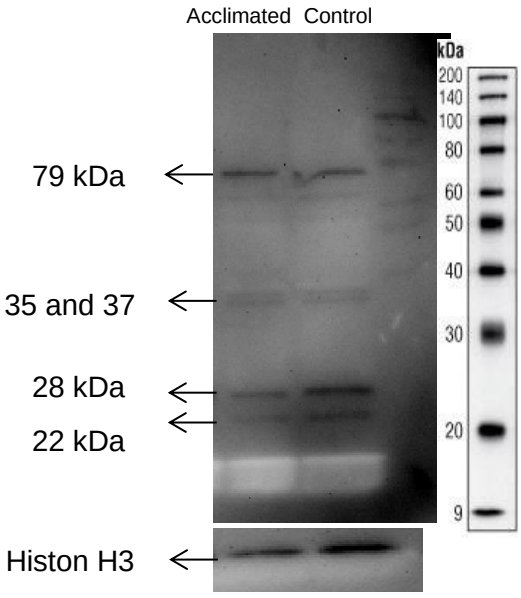


Figure 129. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated and non-acclimated microshoots using a k-segment dehydrin antibody.

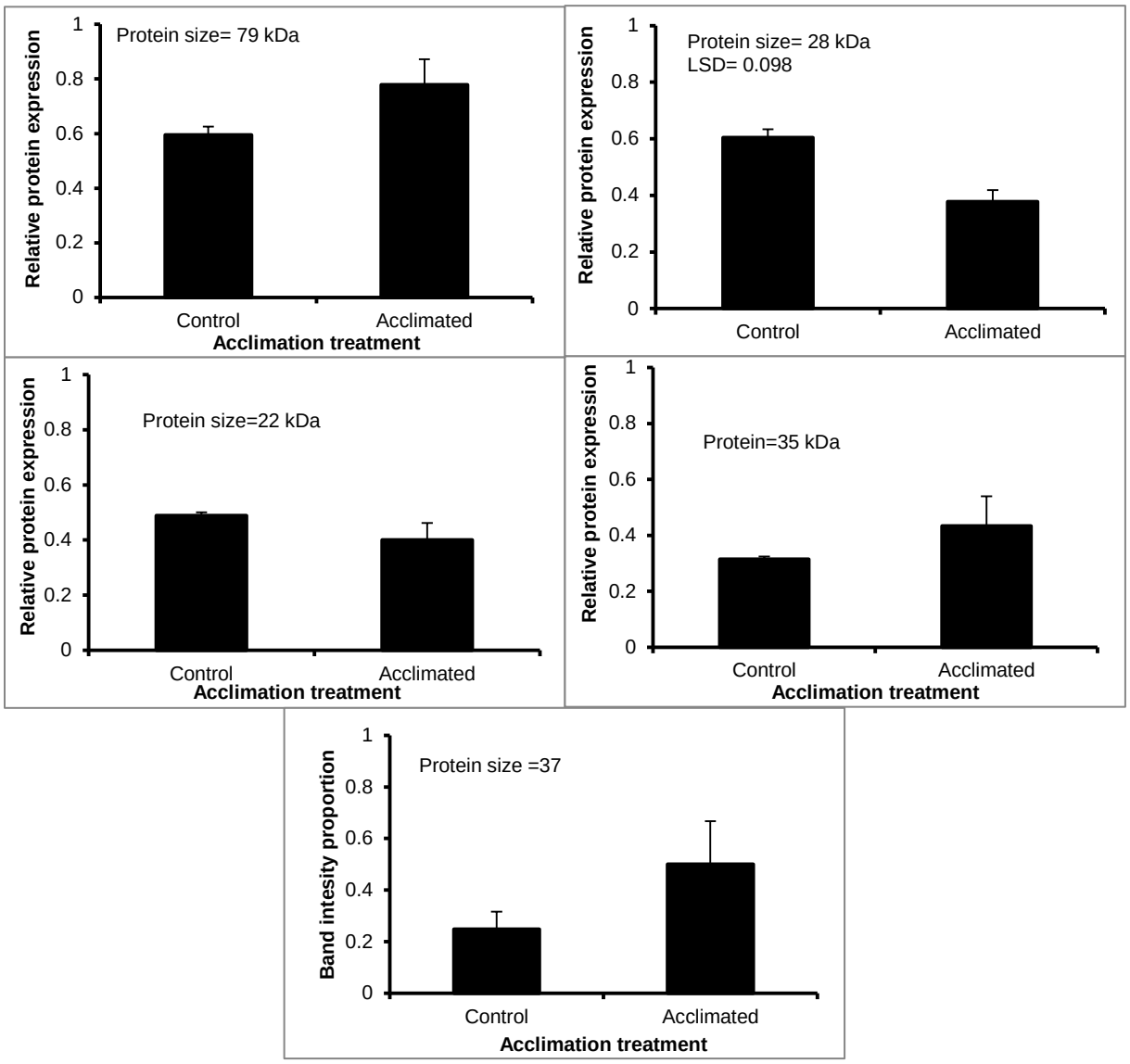


Figure 130. The effect of cold acclimation on the accumulation of dehydrin proteins in cauliflower microshoots.

6.3.5. The effect of ABA on the accumulation of dehydrin protein in acclimated cauliflower microshoots

The use of ABA significantly increased the accumulation of dehydrin proteins at 78, 53, 50 and 35 kDa size classes (P=0.008, P=0.001, P=0.010 and P<0.001 at 35, 78, 50 and 53 kDa protein size classes respectively) (Figures 131, 132 and 133). However, while the amount of dehydrin protein at size 35 kDa stabilized after a specific concentration of ABA assessed to be 1 mg L^{-1}, the amount of dehydrin proteins at 78, 50 and 35 kDa significantly decreased using concentrations of ABA higher than 2 mg L^{-1} with proteins at size classes 53 and 78 kDa and with concentration higher than 1 mg L^{-1} with protein at size class 50 kDa (Figures 131, 132 and 133). Moreover, the use of 2 mg L^{-1} increased the level of dehydrin protein at 62 kDa which was not observed clearly using the other ABA treatments. Therefore, the use of 2 mg L^{-1} of ABA was recommended to stimulate the maximum accumulation of dehydrin proteins in the cauliflower microshoots.

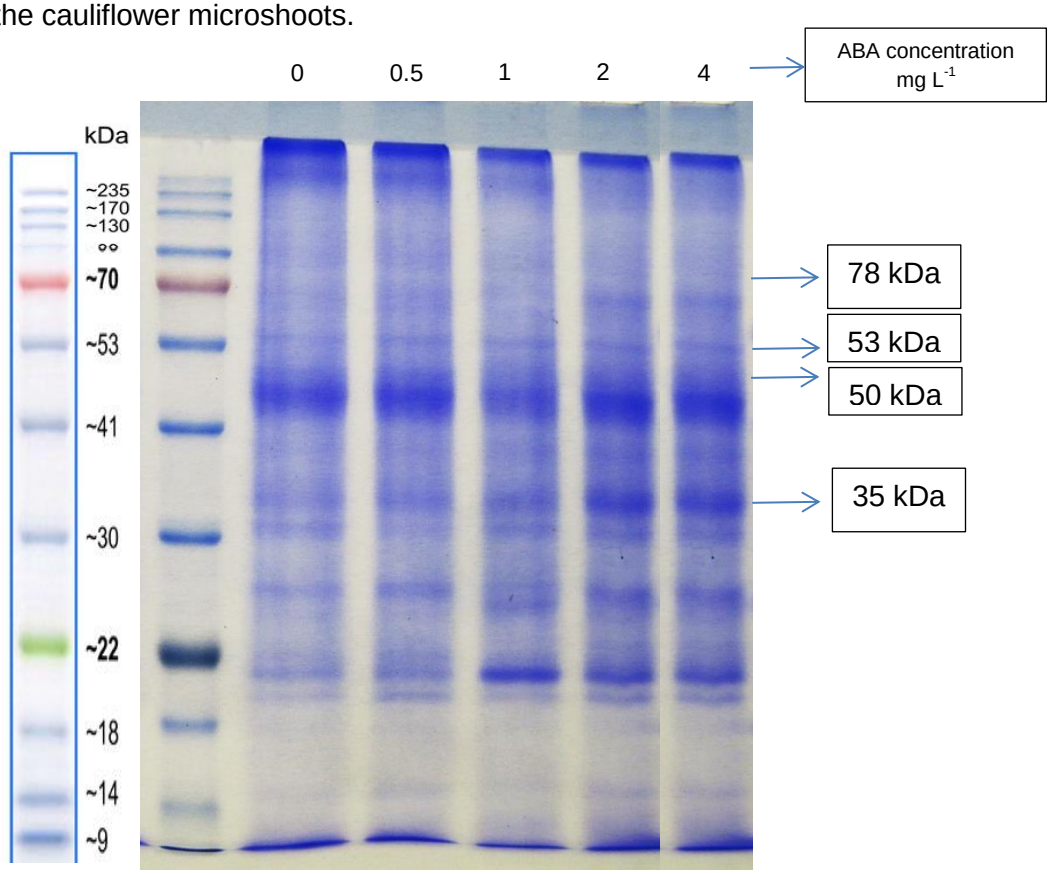


Figure 131. SDS-PAGE analysis for protein samples derived acclimated cauliflower microshoots treated with ABA.

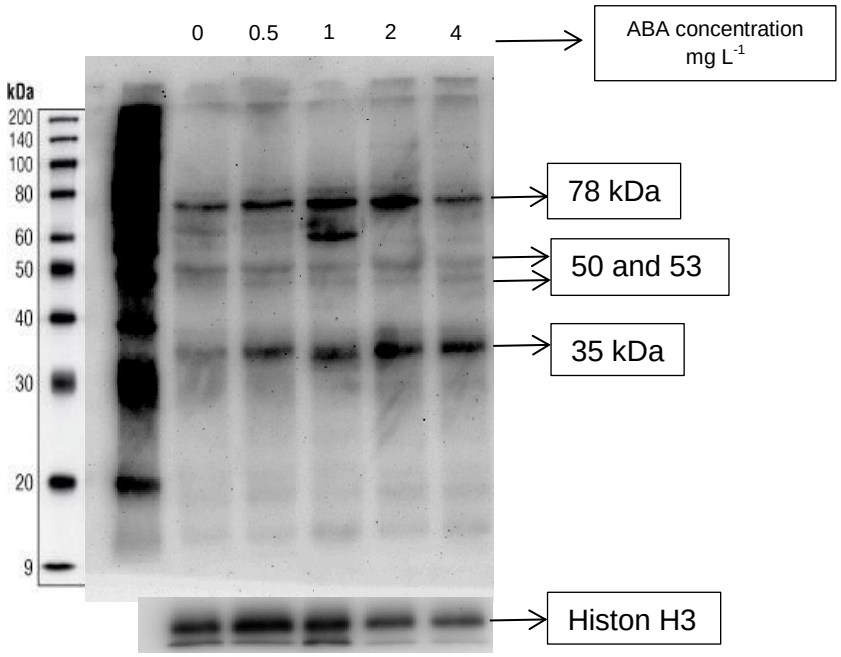


Figure 132. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated microshoots treated with ABA using a k-segment dehydrin antibody.

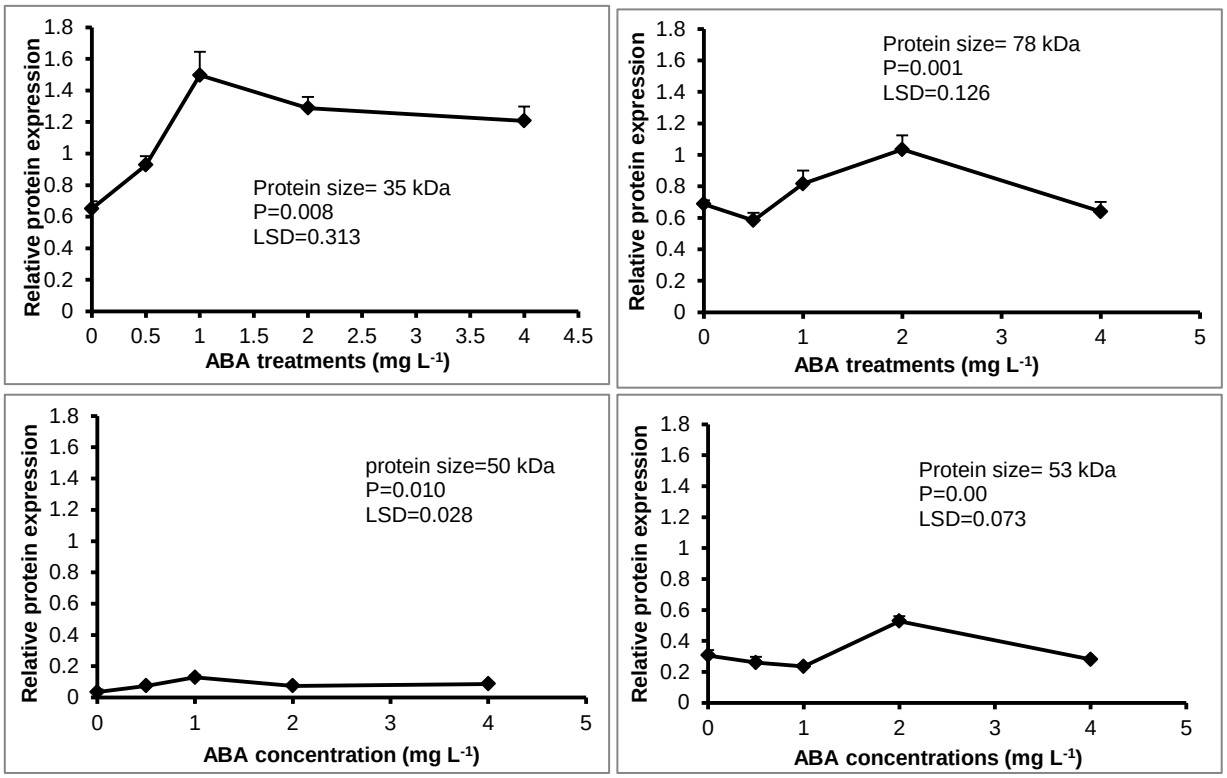


Figure 133. the effect of ABA used with cauliflower microshoots liquid culture media on the accumulation of dehydrin proteins in the acclimated cauliflower microshoots.

6.3.6. The effect of molybdenum on the accumulation of dehydrin proteins in acclimated cauliflower microshoots

The use of molybdenum in acclimated cauliflower culture media significantly decreased the amount of dehydrin proteins at 78, 35 and 50 kDa (P<0.001 for protein at size class 78 kDa, P=0.001 for protein 35 kDa and P=0.040 for protein at size class 50 kDa). However, the effect of Mo on the amount of dehydrin protein at size class 53 kDa was found to be not significant (P=0.087) (Figures 134, 136 and 137).

No dehydrin protein band was detected using a blocked dehydrin antibody. Therefore it can be concluded that all the bands detected by the k-segment antibody belong to the dehydrin protein family (Figure 135).

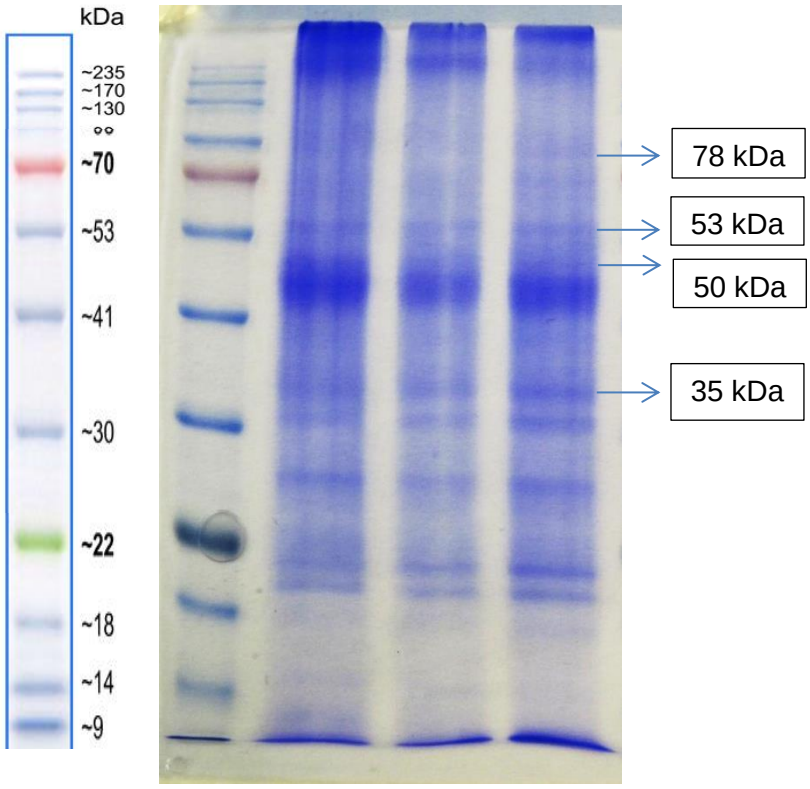


Figure 134. SDS-PAGE analysis for protein samples derived acclimated cauliflower microshoots treated with Mo.

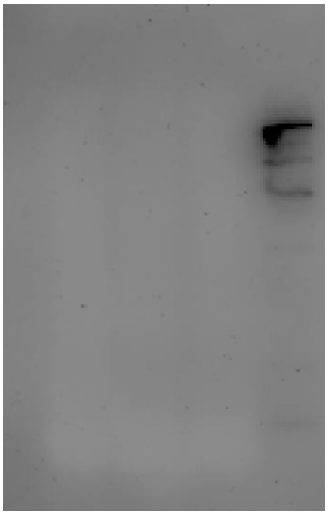


Figure 135. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated microshoots treated with Mo using blocked k-segment dehydrin antibody.

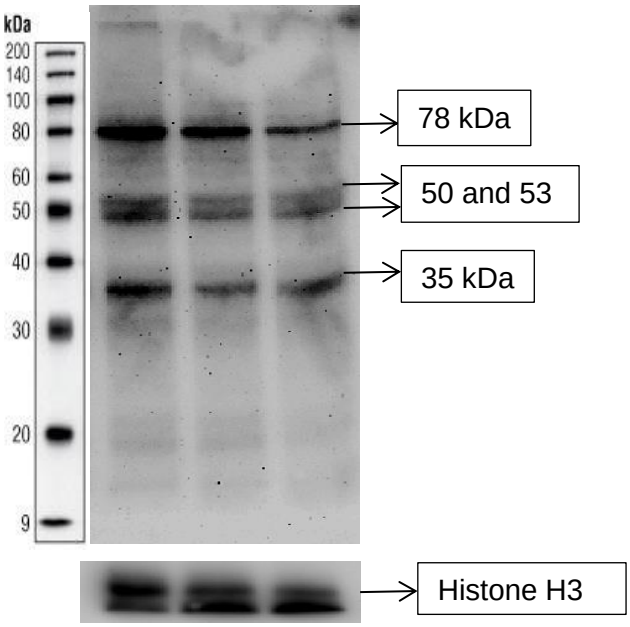


Figure 136. Western blot analysis for the detection of dehydrin proteins from cauliflower acclimated microshoots treated with Mo using a k-segment dehydrin antibody.

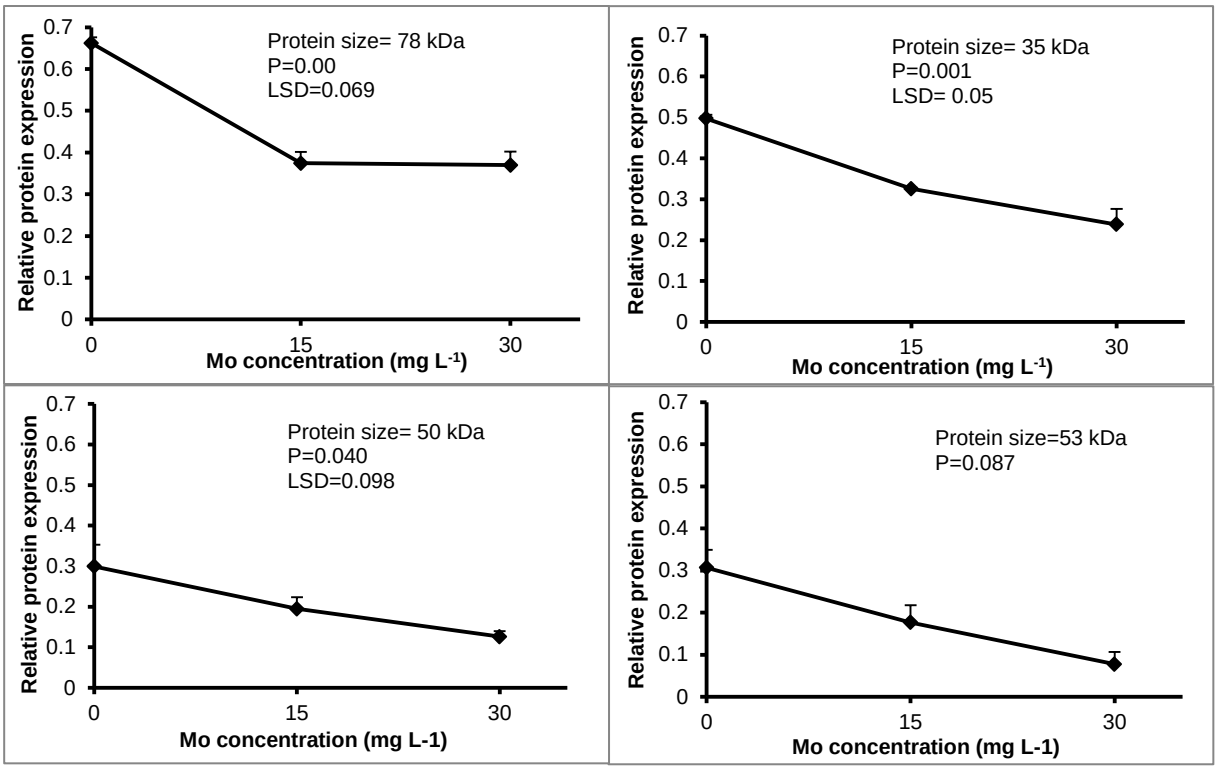


Figure 137. The effect of Mo used with cauliflower microshoots liquid culture media on the accumulation of dehydrin proteins in the acclimated cauliflower microshoots.

6.4. Discussion

Seed moisture content at the early stages of seed development was high (about 80%) and decreased during maturation to a final level of 8%. The high level of moisture is very necessary, especially at the early stages of seed development since water is the vehicle for transferring nutrients from the parent plant to the developing embryo and cotyledons.

The process of seed development comprises consecutive stages that may be considered as preparation for successful future germination. Seed development is characterized by synthesis and accumulation of food reserves and the development of the embryo. One of the main metabolic activities that occurs during the development of seeds is the conversion of sucrose via glycolysis to oil and lipids (Wei *et al*., 2008, Baud *et al.*, 2008). However, the lipid synthesis during the development of seeds depends on the supply of several precursors such as, sucrose

acetate (Vigeolas and Geigenberger, 2004), glucose 6-phosphate, pyruvate, phosphoenol pyruvate and malate (Rawsthorne, 2002). The developing embryo accumulates lipids in several forms such as, triacylglycerols (TAG), fatty acids and ester of glycerol when the early morphogenesis stage is completed. The fatty acid biosynthesis pathway during the development of seeds includes various subcellular compartments and is well understood (Baud *et al*., 2002). Lipids are stored in cytosolic oil bodies that form almost 60 % of the cell volume in the cotyledons of mature embryos of *Arabidopsis thaliana* which is a crucifer like cauliflower (Mansfield and Briarty, 1992). The accumulation of lipids is necessary to supply the required energy for seedling growth after germination (Siloto *et al*., 2006). During germination the oil is degraded, providing energy and carbon to seedlings in their early growth stages through the consecutive operation of β-oxidation, the glyoxylate cycle, partial tricarboxylic acid (TCA) cycle and gluconeogenesis (Chia *et al*., 2005). However, although several studies have reported that the level of lipids could decrease during the seed maturation of oil seeds such as *Crambe abyssinica, Nicotiana tabacum*, Arabidopsis and *B. napus* (Tomlinson *et al*., 2004, Baud *et al*., 2002, Norton and Harris, 1975, Gurr *et al*., 1972), this does not seem to be the case with cauliflower seeds since no decrease in the lipid level was observed during seed maturation. Moreover, the lipids accumulated to reach about 24 % of the seed component when the seed moisture was assessed to be around 8% which accords with the findings of Gurusamy (1998).

Soluble carbohydrates can act as cryprotectants, antioxidants and osmotic regulators in salt and drought stressed plants (Nkang, 2002). Sucrose has a fundamental function in the development of seeds as a main form of transportation of energy and carbon for most plant species and as a regulator of gene expression (da

Silva Bonome *et al*., 2011). Seeds are non-photosynthetic structures and are therefore very dependent on sucrose importation. However, as the first step for the production of storage products is the hydrolysis of sucrose, the cleavage of sucrose to hexose is essential for plants (Koch, 2004). There are two enzyme pathways for the sucrose hydrolysis in plants and these pathways are catalysed by sucrose synthase (SuSy) and invertases. It has been suggested that SuSy and invertases play an essential role for the transitions of seed development phases (Weschke *et al*., 2003, Baud *et al*., 2002). The acid invertase activity and the hexose/sucrose ratio were observed to be high in the cell wall in the early stages of the seed development of many cereals such as rice (Hirose *et al*., 2002) and barley (Weschke *et al*., 2000). However, while the invertase activity increases following the transition of the embryonic expansion stage to the storage stage, the SuSy activity increases in the storage stage and this induces the synthesis of the cell wall, starch and other reserve components (Winter and Huber, 2000). Several researchers have shown that the gene expression for the invertases happen predominantly in the first stages of seed development while the expression of SuSy genes occurs in the intermediate and final stages of the development. These changes could control the transition from the division and expansion stage to the storage of reserve compounds (Borisjuk *et al*., 1998).This could explain the relatively sharp increase in the carbohydrates level in the final developmental stages of cauliflower seeds and this is in agreement with Nkang (2002) who reported that carbohydrates such as starch and sucrose increase through the development of *E. caffra* seeds with a sharp increase during the final stages of the development.

Seed proteins are mainly produced in the cotyledons throughout the mid to late development stages of seed development. Amino acids are transferred from the

phloem into the maternal seed tissue from where they are transferred to the seed and consequently taken up by the embryo (Golombek *et al*., 2001). The level of cauliflower seed total proteins increased to reach the maximum (assessed to be about 28%) at seed moisture level around 20 %. After that the level of protein decreased to about 23 % at moisture level around 8 %. However, the small reduction in the level of protein in the late stages of cauliflower seed developments is in agreement with that reported by Gurusamy and Thiagarajan (1998) who found that the small decrease in the cauliflower seed protein content observed at the late stages of seed development could be caused by its utilization in growth.

Although several studies have reported the important role of carbohydrate in seed desiccation tolerance, recent studies have demonstrated that carbohydrates alone are insufficient to protect against desiccation (Bettey *et al*., 1998). It has been suggested that a set of heat-stable, late-embryogenesis-abundant (LEA) proteins have an essential role in the seed desiccation capacity (Blackman *et al*., 1991, Dure *et al*., 1989).

A very highly significant increase in the amount of relatively low molecular weight dehydrin proteins (12, 26 and 6 kDa) was observed here during the development of cauliflower seeds, especially when the moisture level dropped below 50 %. However, although a slightly significant decrease in the amount of dehydrin proteins at relatively high molecular weights was found, this was considered to be unimportant since these proteins were in much lower abundance than the low molecular weight dehydrin proteins. The current results accord with the findings of Kalemba and Pukacka (2008) who reported that dehydrins are produced during the development of seeds as an element of the embryogenesis program and that they accumulate in response to seed desiccation. Dehydrins were observed in different plant species,

such as several recalcitrant seeds of *Castanospermum australe* L., *Clausena lansium* (Lour.) and the orthodox woody plant seeds of *Acer platanoides* L. (Finch-Savage and Blake, 1994). Dehydrins are thought to bind and stabilise cell lipids and membrane structure under abiotic stresses (Rorat, 2006). Farrant *et al* (2004) and Close (2006a) demonstrated that acquisition of desiccation tolerance of orthodox seeds is linked with accumulation of late embryogenesis abundant (LEA) proteins. Goyal *et al* (2005) indicated that LEA proteins could play an essential part in protecting the cellular or molecular structure from the damaging effect of water loss. It was also demonstrated that dehydrin proteins protect cells under drought stress by protecting the structure of proteins and preserving their water binding (Garnczarska *et al*., 2008, Farrant *et al*., 2004). The amino acid composition of dehydrin proteins with a high content of charged and polar residues could support their specific protective roles under environments of cell dehydration (Rorat, 2006, Allagulova *et al*., 2003).

Considering the important role of dehydrin proteins in seed abiotic stress tolerance, several experiments were conducted here aimed at increasing the amount of these proteins in cauliflower microshoots which in turn could improve cauliflower artificial seed abiotic stress tolerance. Moreover, the cauliflower micropropagation system applied in the current studies facilitates the investigation of molecular response of specific stressor or protective elements such as mannitol, Mo and ABA, applied with the liquid culture media taking advantage of the huge number of microshoots that can be produced from the culture system. However, culture systems have been widely used for molecular and physiological studies since they have potential advantages over whole plants (Tal, 1983) including the homogeneity of the plant culture population, the capacity of easily repeating the experiments under controlled

conditions and the possibility of isolating the culture response from the whole plant response (Parmentier-Line *et al*., 2002).

The effect of various materials on the accumulations of dehydrin proteins in cauliflower microshoots was investigated. However, the protein bands detected were observed to be at different molecular sizes depending on the cauliflower variety used and this could be due to the various genetic backgrounds of these varities. In agreement with the hypothesis put forward here, different studies have reported dehydrin proteins at different size classes, for example, while Battaglia *et al* (2008) reported that most of the described *Brassica* dehydrins range from19 to 22 kDa with the only exception of 31 kDa ERD10 dehydrin of *Brassica* napus, Rurek (2010) was able to detect dehydrin proteins at several other size classes such as 40, 50, 55 and 70 kDa.

The effect of drought stimulation using a high osmotic pressure solution of mannitol with culture liquid media was determined. 78 and 170 kDa proteins seem to play an essential role in relation to the viability of cauliflower microshoots since the artificial seeds using microshoots derived from lower than -2 Osmol kg^{-1} osmotic pressure showed no germination capacity. However, dehydrin proteins have been reported to increase in response to dehydration stress (Cellier *et al*., 1998, Han and Kermode, 1996, Welin *et al*., 1994) and osmotic agents such as mannitol, which reduce the osmotic potential of the culture media and are widely used to induce the osmotic potential of the cultured cell in woody and herbaceous plants (Leustek and Kirby, 1990, Fallon and Phillips, 1989, Leustek and Kirby, 1988, Bhaskaran *et al*., 1985).

Several studies have reported the increase of dehydrin proteins resulting from low temperature treatments and acclimation (Welin *et al*., 1994). Muthalif and Rowland

(1994) and Panta *et al* (2001) reported the accumulation of dehydrin proteins in whole blueberry plants under the effect of low but non-freezing temperature while Parmentier-Line *et al* (2002) reported the accumulation of these proteins in cold treated blueberry cell cultures. However, although several studies have reported the accumulation of dehydrin proteins during the acclimation of *Arabidopsis, Fragaria, Brassica, Miscanthus* and woody plant species of genus *Prunus, Cydonia* (BANIULIS *et al*., 2012, Rugienius *et al*., 2009, Lukoševičiūtė *et al*., 2009, Nishizawa *et al*., 2008, Patton *et al*., 2007, Wisniewski *et al*., 2006, Welling *et al*., 2004), the amount of these in cauliflower microshoots was not significantly affected by cold acclimation. Moreover, the acclimation significantly decreased the amount of 28 kDa dehydrin protein. This could be due to the culture conditions as a liquid media and reproductive tissue have been used for cauliflower microshoot production and these conditions may have affected the cauliflower microshoots' response to cold acclimation. Kovalchuk *et al* (2009) reported that the composition of culture media plays an essential role in the acclimation of plant microshoots and cold tolerance under *in vitro* conditions.

As cold acclimation was reported here to have no role in the accumulation of dehydrin proteins in cauliflower miroshoots and as the process of acclimation was reported to improve the cold tolerance of cauliflower microshoots, an important question was raised about the role of dehydrin proteins in cauliflower microshoots cold tolerance. Therefore, the effect of some materials, which were reported to have negative (ABA) or positive (Mo) effects on acclimated cauliflower microshoots cold tolerance (ABA), on the accumulation of dehydrin proteins in cauliflower microshoots was investigated. The use of ABA at 1 and 2 mg L^{-1} significantly increased the level of dehydrin proteins at various dehydrin size classes. However, Zhang *et al* (2006a)

indicated that ABA is one of the most central stress signal transduction pathways of plant responses to abiotic stress. In agreement with the current results, several studies have demonstrated the positive responses of several dehydrin proteins to exogenous ABA application or diverse stresses that involved ABA as regulator (Close, 2006b, Nylander *et al*., 2001). Schroeder *et al* (1987) and Kao *et al* (1996) have also demonstrated that the accumulation of dehydrin proteins could be induced by the application of exogenous ABA in cultured embryos. Moreover, working with ABA deficient mutants in *A. thaliana* (LeonKloosterziel *et al*., 1996, Paiva and Kriz, 1994, Nelson *et al*., 1994) and maize (Mori and Muto, 1997, Pla *et al*., 1993), the significant effect of endogenous ABA on the induction of LEA protein gene expression was demonstrated. Although exogenous ABA significantly increased the accumulation dehydrin proteins, it did not improve their cold tolerance (as it was reported in experiment 5 of chapter 4) indicating that there is no significant role of dehydrin proteins in the cold tolerance of cauliflower microshoots. However, more research is still required to determine the effect of dehydrin proteins on the cold tolerance of cauliflower.

The application of molybdenum to the cauliflower culture media decreased the accumulation of dehydrin proteins at different size classes. This indicates again that dehydrin proteins have no significant effect on the cold tolerance of cauliflower microshoots since molybdenum was confirmed earlier in this thesis to significantly improve the cold tolerance of the microshoots. However, although molybdenum has an essential role on the synthesis of ABA (Huang *et al*., 2009) and ABA was confirmed to have a positive effects on the accumulation of dehydrine in cauliflower microshoots, molybdenum has a negative effect on the accumulation of these proteins. It should be mentioned that molybdenum has an essential role in several

enzymes in plants such as nitrate reductase, aldehyde oxidase, xanthine dehydrogenase and sulphite oxidase (Mendel and Hansch, 2002). However, to the best of our knowledge, this is the first study to determine the effect of molybdenum on the accumulation of dehydrin proteins in plants and further investigations are required to determine the mechanism which molybdenum decreases the accumulation of dehydrins.

6.5. Conclusion

The chemical changes that occur during the development of cauliflower seeds were determined and the accumulation of dehydrin proteins during the development of the seeds was confirmed. Several chemical materials were applied to the cauliflower microshoots culture media with the aim of increasing the level of dehydrin proteins in order to simulate the structure of cauliflower traditional seeds in artifical seeds. The addition of these chemical materials also enabled an investigation into the possible contribution made by dehydrins to microshoot cold tolerance. While mannitol and ABA increased the accumulation of dehydrins in the cauliflower microshoots when they were used at suitable concentrations, cold acclimation had no significant effect on the accumulation of these proteins. However, molybdenum significantly decreased the level of dehyrin proteins. The current results suggest that dehydrin proteins make no significant contribution to cauliflower microshoots cold tolerance since cold acclimation and molybdenum improved the cold tolerance of microshoots while having no or negative effects on the accumulation of the microshoots dehydrin proteins. Moreover, while ABA decreased the cold tolerance of cauliflower microshoots, it significantly increased the accumulation of dehydrins.

Chapter 7: General Discussion

Cauliflower micropropagation

Cauliflower is an open pollinated species and there are technical challenges to producing in-bred lines with reliable self-incompatability or male sterility necessary for producing F1 hybrids, particularly amongst the winter-heading maturity subgroup. In view of this characteristic, the micropropagation techniques have been suggested as a possible alternative for cauliflower propagation or at least for facilitating its breeding programmes. As a consequence, the most innovative procedure facilitating the production of cauliflower propagules from fractionated and graded curd was designed by Kieffer *et al* (2001). The protocol takes advantage of the huge number of meristems (millions) available in each cauliflower curd, most of which have the capacity to develop and grow into cauliflower microshoots and plantlets in later stages. However this protocol required further optimization and was too labour intensive to be cost effective. Therefore, a revised protocol for cauliflower propagule production was optimized in the research undertaken for this thesis using both micropropagation and artificial seed production techniques. The cauliflower microshoot production system was optimised by means of:

1. Optimization of cauliflower explant production in order to obtain the highest amount and quality of cauliflower explants (by blending and sieving steps).
2. Studying the developmental stages of cauliflower microshoots in order to improve the understanding of physiological aspects of their development.
3. Optimization of the explant culture conditions through improving the culture media composition including the carbohydrate type, concentration and source, and plant growth regulators.

It was important to optimise the blending step in the described protocol since meristematic tissues are quite sensitive and could be damaged by long blending durations. This is counterbalanced bythe use of short blending durations which could reduce the efficiency of the protocol in terms of the amount of the explants produced.

According to several studies conducted with various plant species (Gawel and Robacker, 1990, Hammerschlag, 1982; Jones and Petolino, 1988), the use of liquid media for cauliflower microshoot production has great advantages in reducing the unit production cost of tissue culture and facilitating the automation of the production system. Several experiments have been conducted during the current research in order to maximise the benefits gained from using liquid media whilst avoiding negative symptoms, such as hyperhydricity, that could affect the efficiency of using this protocol. These experiments were as follows:

1. Several agitation methods were tried to induce the growth of cauliflower microshoots and the use of an orbital shaker was found to be the superior method, in agreement with what was reported with Kieffer *et al* (2001).
2. Plant growth regulators (PGRs) were confirmed to be an essential requirement for the induction of cauliflower microshoots. Various types and concentrations of cytokinins and auxins were also used with culture media to get the highest number of best quality microshoots. PGRs were found to be a vital requirement for cauliflower micropropagation, as for plant micropropagation in most plant species (Vandemoortele *et al*., 2001, Feito *et al*., 1994, Vankova *et al*., 1991, Kataeva *et al*., 1991). No microshoot root formation was observed in the cauliflower liquid culture media, in contrast to what was reported by Kieffer *et al*.(1995) counterclaiming the capacity of NAA to induce the root formation of cauliflower microshoots in the liquid media.

This point still needs more investigation and more experiments are still required to improve the root formation of cauliflower microshoots during culture at the liquid media stage.

3. Carbohydrates (sugars) are essential as a source of carbon to provide the energy for biosynthesis in *in-vitro* cultures (Amiri and Kazemitabar, 2011) and they also have a stabilizing osmotic effect. In the current research, testing various types (sucrose, glucose, fructose and maltose) and concentrations of sugars, a significant finding was the superior effect of sucrose derived from sugar cane in improving the efficiency of the protocol. The number of microshoots produced using 4.5 g L^{-1} of sucrose derived from sugar cane was more than double that produced using sucrose derived from sugar beet (used in previous protocols). This finding could reduce the cost of the culture units by as much as 50 % of the initial cost. These results support those reported by Sul and Korban (1998) and Kodym and Zapata-Arias (2001) on the significant effect of using sugar cane juice with both *Pinus sylvestris* and Grand Naine bananas respectively. The current study is the first to report this effect of sugar cane sucrose for cauliflower micropropagation. However, further analysis is still required to to determine the constituent that has the reported positive effect of sugar cane.

The revised micropropagation protocol involves several mechanical processes such as blending and sieving, and a large number of explants are cultivated in each culture pot. Therefore, there was a high risk of contamination during the protocol, even when strict aseptic conditions were applied. Contamination by bacteria, fungi and yeasts is a constant challenge that frequently threatens plant tissue cultures during the culture phase (Leifert and Cassells, 2001). Thus, several experiments

have been conducted during this research using Plant Preservative Mixture (PPM) with the aim of reducing the risk of contamination without affecting the efficiency of the cauliflower micropropagation protocol. PPM was chosen as a heat stable material which targets specific multiple enzyme sites of several microorganism genera (Lunghusen, 1998b). However, as is to be expected with any anti-microbial compound, there were phyto-toxic effects to plant growth during *in-vitro* culture of cauliflower microshoots when this material was used in relatively high concentration. Although PPM has been used for controlling the contamination in the culture systems of different plant species such as sweet orange (*Citrus sinensis* L.) (Niedz, 1998) and european beech (*Fagus sylvatica* L.) (Kraj and Dolnicki, 2003), it was used at different concentrations with each species. Therefore, the optimal concentration of PPM for controlling the contamination without reducing the efficiency of the designed protocol was determined empirically in this research.

Cauliflower artificial seed production

It was reported that labour cost accounts for about 60 % of the overall cost of cauliflower micropropagation and that about 29 % of the total is attributable to greenhouse costs (Kieffer *et al*., 2001). Therefore, it is suggested that the use of artificial seed techniques could reduce the unit production costs of cauliflower propagules. Artificial seeds are more durable for handling, transporting and storage. Artificial seeds are also a useful technique as clonal propagation systems in terms of direct delivery to the field, low cost and fast reproduction of plants (Saiprasad, 2001). The current research sought to optimise a full protocol for cauliflower artificial seed production, starting with the production of cauliflower microshoots suitable for encapsulation and followed by the optimization of encapsulation procedures and the

structure/constitution of their artificial endosperm. Artificial seed conversion in both semi-solid media and commercial substrates were also optimised. Several experiments were also conducted in order to investigate and to improve the storage ability of these artificial seeds.

This research confirmed the suitability of cauliflower microshoots for encapsulation as artificial seeds and adds cauliflower to the long list of plant species that are suitable for artificial seeds which includes fruits, cereals, medicinal plants, vegetables, ornamentals, forest trees and orchids (Bapat and Rao, 1988, Mathur *et al*., 1989, Corrie and Tandon, 1993, Sarkar and Naik, 1998, Mandal *et al*., 2000, Nyende *et al*., 2003, Chand and Singh, 2004, Naik and Chand, 2006, Micheli *et al*., 2007, Rai and Jaiswa, 2008, Singh *et al*., 2009, Sundararaj *et al*., 2010). The developmental stages of cauliflower microshoots were studied and it was confirmed that the acceleration stage of microshoots was the optimal stage suitable for encapsulation in sodium alginate matrices. One significant finding was that the PGRs suitable for use with culture media to produce microshoots suitable for encapsulation were different from the PGR combination used to obtain the highest number with the highest average weight as microshoots. This research therefore highlights the importance of optimising all stages of the designed protocol in the early stages and whether it is used for micropropagation only or for artificial seeds at a later stage.

Cauliflower seed artificial endosperm (nutrients, PGRs, carbon source etc) was optimised in view of the important role of this component of artificial seeds in facilitating the survival in storage and the growth of micropropagules during conversion (Rai *et al*., 2009, Jain *et al*., 2005). A promising feature of cauliflower artificial seeds was that there was no need for sugar to be present in their matrices. This could open new perspective for future use of these seeds under non-sterilie

condition, since sugar is favourable to bacteria and other contaminants and its omission from the matrix raises the potential success of conversion in the field. This also reduces the cost of encapsulation, thus increasing the efficiency of the protocol. The PGR combinations used with the artificial seed matrices were optimised to gain the optimal conversion rate and viability. However, several studies have highlighted the important role played by of PGRs in the growth and development of propagules in such matrixes (Micheli *et al*., 2007, Soneji *et al*., 2002).

Once the experiments aimed at optimising the production of cauliflower artificial seeds had been carried out, more research was conducted with the aim of optimising the environment most suitable for their conversion. This was carried out in two stages a) artificial seed conversion in semi-solid media b) artificial seed conversion in commercial substrates such as compost, perlite, vermiculite and sand. Very high conversion rates and viability were obtained using S23 semi-solid medium supplemented with a relatively high concentration of ABA. In view of these results and considering that to be a promising alternative for their use in agriculture, cauliflower artificial seeds capacity to be sown into commercial substrates such as compost, perlite, vermiculite or sand needs to be established. It was confirmed that the moistening solution is a key factor affecting the conversion and growth of artificial seeds in various *in-vivo* substrates. This finding supports that reported by (Naik and Chand, 2006). It should be mentioned that PGRs had a significant effect on the conversion rate and viability of cauliflower artificial seeds and that the use of artificial seed in commercial substrates requires higher concentrations of PGR combinations. The type of culture substrate had a significant impact on the conversion and subsequent development of propagules from artificial seeds. It is believed that the current results will have very important effects in increasing the economic

importance of cauliflower artificial seed production techniques and will open new vistas for the commercial use of the protocol described, since it reduces labour, media, acclimation, and greenhouse costs. However, all the experiments were conducted under sterilized conditions since contamination was considered to constitute a high risk to artificial seeds. Therefore, PPM was suggested for use with the moistening solution as this material has been proven to have an important effect in controlling contamination in the cauliflower microshoots culture system. Significant effects of PPM and significant interaction between PPM and culture substrates on both conversion rate and viability of artificial seeds were observed. This could be attributed to the effect of substrate physical structure or of cation exchange capacity of the substrates. PPM seems to have a role not only in the control of contamination in the culture substrate but also on the development and growth of artificial seeds and Rihan *et al* (2011) indicated that the optimal substrate for cauliflower artificial seeds growth was compost when supplemented with S23 with PGRs used as the irrigation solution; while the optimal substrate was found to be sand when PPM was used with the irrigation solution. However, many studies have investigated the possibilities of sowing artificial seeds in soil or commercial substrates, for example those of Machii and Yamanouchi (1993) and Fujii *et al* (1989). The positive effects of using PPM were also reported by Lata *et al* (2009).

Artificial seed storage capacity was also investigated in this research using two approaches. The first involved using liquid nitrogen -196 ^{o}C, and then deep freezers at either -80 or -20 ^{o}C for long term storage since this technique is considered an important approach to plant germaplasm storage. However, cauliflower artificial seeds showed low tolerance of this sort of low temperature. The second approach was cold storage at 4 ^{o}C. Cauliflower artificial seeds showed high suitability for cold

storage and they kept their conversion rate potential and viability for about 6 months. This is an important factor because it is much easier to store artificial seeds than to store cultured plants in S23 media, in terms of both cooled space and culture cost.

Molecular analysis of cauliflower abiotic stress tolerance

The reported protocol in this research enables the production of thousands of cauliflower micropropagules/artificial seeds from one cauliflower curd at a low cost per culture unit. Over 100 cauliflower microshoots can be produced for each culture pot containing a relatively small amount of liquid culture media (30 mL). This facilitates the investigation of molecular responses to specific stressor or protective elements such as mannitol, Mo and ABA, applied with the liquid culture media. Molecular studies included in the current research aimed to achieve two main goals, 1) to improve the abiotic stress tolerance of cauliflower artificial seeds; 2) To make use of the designed cauliflower micropropagation protocol for enhancing the understanding of abiotic stress tolerance levels in plants and the effect of specific treatments and materials on this tolerance. Three aspects of abiotic stresses and protective materials effect mechanisms were investigated in this research: a) The effect on the development of plant material (cauliflower microshoots or artificial seeds); b) The effect on abiotic stress tolerance; c) Confirmation of the results at the molecular level (gene and/or protein expression).

The cold acclimation process has been demonstrated to have a positive impact on plant cold tolerance and up-regulation of *CBF/DREB1* genes in several plant species. The existence of this pathway and its role in increasing the cold tolerance of cauliflower microshoots was also confirmed in *in-vitro* grown cauliflower. However, it

is difficult to control the environmental conditions in the field and there is also difficulty in providing low but not freezing temperatures (acclimation condition) before the freezing effects take place on the plant. Also, in the view of the reported cross talk between different abiotic stresses including the role of drought in increasing plant cold tolerance and in the up-regulation of *CBF* genes (Kasuga *et al*., 1999, Liu *et al*., 1998), it was suggested that the simulation of drought conditions in the liquid culture media using mannitol could increase the cold tolerance of microshoots and up-regulate *CBF/DREB1* gene. However, although the high osmotic potential increased the cold tolerance of cauliflower microshoots, it did not induce the expression of *CBF/DREB1* transcription factors. This suggests that drought could up-regulate other sets of genes that play a part in microshoot cold tolerance. Moreover, cold stress affects the expression of several genes, and not just *CBF,* resulting in the increase of hundreds of metabolites, some of which are known to have an important effect in the improvement of plant cold tolerance. It was also reported that the *CBF* regulon alone cannot clarify all differences in phenotypic cold tolerance (Jan *et al*., 2009, McKhann *et al*., 2008). In view of this finding, another experiment was conducted with mature *in-vivo* grown cauliflower plants, with the aim of discovering the effect of drought on the cold tolerance and up-regulation of *CBF*/*DREB1* in cauliflower.

Both cold and drought increased the cold tolerance and induced the expression of *CBF/DREB1* in cauliflower microshoots. However, the capacity of drought to induce the regulation of *CBF/DREB1* in mature plants and not in the microshoots could be due to the developmental stage, media structure (liquid media) or culture conditions. This point requires further investigations. In agreement with our results, the effect of developmental stages on the expression of *CBFs* in other plant species has been

reported (Prasil *et al*., 2004). The induction of *CBF/DREB1* expression was demonstrated in mature cauliflower plants through a small reduction of the soil moisture content and it is believed that this could have an important practical application in the field because such a reduction in the moisture content will not have a significant negative effect on the crop yields.

In view of the fact that mannitol (high osmotic potential) did not up-regulate the expression of *CBF/DREB1*, an alternative inducing material was investigated. ABA as an essential controller of plant growth and development and it is an important regulator for stress resistance was therefore investigated (Kempa *et al*., 2008, Fait *et al*., 2008). Unexpectedly, ABA decreased cold tolerance and the expression of *CBF/DREB1* in both acclimated and non-acclimated microshoots. However, the effect of this material on the expression of *CBF/DREB1* is controversial and needs more investigation. Shinozaki and Yamaguchi-Shinozaki (2000) reported that although ABA levels increase transiently under the effect of low temperature, ABA does not continue to accumulate in response to cold. This observation has suggested that ABA does not play a role in low temperature signalling. The CBF signalling pathway leading to CRT/DRE activation has been described as an ABA-independent route leading to *COR* gene expression (Liu *et al*., 1998). However, Knight *et al* (2004) reported that this pathway could also be up-regulated by ABA. In contrast to Knight *et al* (2004) and in agreement with our results, Medina *et al* (1999) and Liu *et al* (1998) reported that ABA has no significant effect upon *DREB1A* (*CBF3*) and *DREB2A.* These differences in the effect of ABA on the expression of *CBFs* could be attributed to the culture conditions as Knight *et al* (2004) reported that water relations during the growing period could have a very important effect on the

response to ABA, and whether or not the plants have been gown on soil or in tissue culture may significantly affect their responsiveness to ABA.

When mannitol and ABA were found to have either no effect or a negative effect on the expression of *CBF/DREB1* respectively, molybdenum was suggested for use with cauliflower culture media as it has been reported by Al-Issawi *et al* (2013) to have a positive effect on the cold tolerance and the up-regulation of *CBF* genes. A very significant finding was reported in this research concerning the positive effect of molybdenum on the cold tolerance of both acclimated and non-acclimated cauliflower microshoots and on the expression of *CBF/DREB1*. Moreover, to the best of our knowledge, this study is the first to report the capacity of this element to up-regulate *CBF* without acclimation. It is believed that this finding could have a very positive impact in the field of agriculture. However, the effect of molybdenum on the cold tolerance of cauliflower still needs to be confirmed under field conditions. The mechanism by which molybdenum up-regulates the *CBF* gene also needs further investigation. Moreover, although molybdenum increased the cold tolerance and the expression of *CBF* when it was used at a suitable concentration, it did not, unlike mannitol and ABA, have a negative effect on the growth and development of cauliflower microshoots. This increases the significance that the application of molybdenum could have.

Several experiments have been conducted throughout this research aiming to detect COR15 protein, as this protein is used as a representative member of the CBF regulon. However, this protein was difficult to detect in cauliflower microshoots, which suggests that *CBF* genes could have a role in the up-regulation of other sets of genes apart from *COR* genes and these genes could play an essential role in the cold tolerance of cauliflower. The question remains: how does expression of the CBF

regulon bring about the cold tolerance effects on plants if it is not through the normal regulon? To answer this question, several studies have attempted to identify the genes that comprise the CBF regulon and to recognize genes that are up-regulated in response to *CBF* overexpression (Vogel *et al*., 2005, Maruyama *et al*., 2004, Fowler and Thomashow, 2002, Seki *et al*., 2001) cited from (Matthew, 2005). These studies have proved that 109 genes have been assigned to the CBF regulon. These genes were demonstrated to comprise a varied range of functional classes and they were categorized into four groups, depending on the nature of the proteins that they encode. Unknown function proteins are the biggest group, forming up to 50% of the proteins. The other three groups are cryoprotective proteins which include COR proteins, regulatory proteins and biosynthetic proteins (Matthew, 2005).

A 23 kDa polypeptide was been detected using the COR15 antibody indicating that there was homology between the sequence of COR15 protein and this unknown 23 kDa polypeptide. However, the current study suggests that this polypeptide plays no significant role in cold tolerance since there was no significant relation between cauliflower microshoots cold tolerance and the accumulation of this polypeptide. Moreover, no relationship was observed between the accumulation of this polypeptide and the expression of *CBF/DREB1,* although this polypeptide was affected by various treatments used throughout the current research. The results here agree with those of Rao *et al* (1993) who reported that 23 kDa polypeptide in rice was not responsive to cold treatment.

Normal cauliflower seeds show high level of abiotic stress tolerance, including drought and this may provide some clues to manipulating artificial seed stress tolerance. Several studies have indicated that the accumulation of dehydrin proteins during the development of these seeds is one of the main reasons behind their

tolerance capacity (Garnczarska *et al*., 2008, Rorat, 2006, Farrant *et al*., 2004, Allagulova *et al*., 2003). Therefore, several experiments were conducted throughout this research in order to determine the chemical changes that occur during the development of cauliflower seed, focusing on the changes in the accumulation of dehydrin proteins. Dehydrin accumulation was successfully monitored in the development of true seeds and following this several experiments were conducted with cauliflower microshoots aiming to:

1. Increase the accumulation of dehydrins in cauliflower microshoots.
2. Investigate the possible role of dehydrins on the cold tolerance of cauliflower microshoots using the refined cauliflower micropropagation system. This was because the refined system has several advantages over whole plants such as the homogeneity of the plant culture population, the capacity for easily repeating the experiments under controlled conditions and the possibility of isolating the culture response from the whole plant response (Parmentier-Line *et al*., 2002) and could offer an important test facility to investigate the physiology of cold hardiness in this species.

The treatments which were used to induce the cold tolerance and up-regulate *CBF/DREB1* gene were also applied to investigate the increase in the accumulation of dehydrin proteins in microshoots relying on the hypothesis that there is a cross talk between different abiotic stress tolerance mechanisms in plants. The results showed that whereas some treatments such as mannitol and ABA succeeded in increasing the accumulation of dehydrins when it was used at sensible concentrations, others either did not have a significant effect (cold acclimation treatment) or significantly decreased the accumulation of these proteins (molybdenum treatment). This research suggests that there is no

significant role of dehydrins in the cold tolerance of cauliflower. This clearlycontradicts several reported studies in the literature (Rugienius *et al*., 2009, Lukoševičiūtė *et al*., 2009, Nishizawa *et al.,* 2008, Wisniewski *et al*., 2006, Parmentier-Line *et al.,* 2002, Panta *et al*., 2001). However, it should be mentioned that this contradiction could be due to the fact that the mechanism of dehydrin accumulation in cauliflower microshoots is affected by several factors, such as the structure of culture media (liquid), plant species, and developmental stage.

Future work

1. More experiments could be conducted to improve cauliflower microshoot root formation in liquid culture medium and during the encapsulation stage. This research could follow various routes, for example by investigating the effect of new PGRs or by changing the culture media with kinetin free media after the induction of cauliflower microshoot growth i.e. phased PGR treatments.
2. More research is still needed to optimise the use of cauliflower artificial seeds in commercial substrates and under non-sterilie conditions. This could be through the investigation of the effects of using combinations of different commercial substrates and investigating different antibiotics with the moistening solutions.
3. The research also provides a base for further investigations to explore the molecular mechanisms and gene expression in cauliflower associated with increased frost resistance under cold acclimation. The micro-array technique is recommended for the exploration of the full genome expression during cold acclimation.

4. Full *CBF/DREB1* gene sequences in cauliflower need to be determined and this gene could be isolated and transferred to other plants by transgenosis to investigate increases in their cold tolerance capacity.
5. This study confirmed the role of *CBF* in the improvement of cauliflower microshoot cold tolerance although *CBF* did not lead to the production of COR proteins (as represented by COR15). Therefore, the role of the *CBF* transcription factor in the up-regulation of other genes, apart from *COR* genes, and the role of these gene in the plant abiotic stress tolerance are subjects for future research.
6. The role of molybdenum in the cold tolerance of cauliflower microshoots and the up-regulation of *CBF/DREB*1 gene were confirmed in this research. The mechanism by which molybdenum improved the cold tolerance and induced the expression of *CBF/DREB1* could also be a subject of future work. This could be done through the investigation of different enzymatic activities/influences in which molybdenum is implicated in order to discover which pathway is involved in the up-regulation of *CBF/DREB1*.
7. It was demonstrated in this research that some materials have the capacity to increase the accumulation of dehydrin proteins in cauliflower microshoots. However, more research could be conducted to find out the mechanism by which these materials have their effects. This could be done through the investigation of the effects of these compounds on the up-regulation of specific dehydrin genes in cauliflower microshoots.
8. It was demonstrated that developmental stages have a significant impact on the induction of *CBF/DREB1* in cauliflower. Therefore, it might be useful to investigate whether this is the case with other crops.

9. It is important also to investigate the effects of various treatments used throughout this research to induce abiotic stress tolerance in cauliflower microshoots on mature cauliflower plants under field conditions. Therefore, the effect of the culture environment could be determined and positive responses could be obtained with mature plants.

Appendixes

Appendix 1: Determination of ash content in cauliflower seeds

Equipment

5 ml medium form porcelain crucibles- 1 per sample

Heat proof porcelain tray

Small spatula

Drying oven set at 120°C

Desiccator with drying agent in base (silica gel)

4 decimal place balance

Muffle furnace set at 550°C

Method

1. A clean porcelain crucible was placed in drying oven and heated to 100°C for 1 hour and it was then allowed to cool in a desiccator.
2. 500 mg +/- 100 mg well mixed dry sample was accurately weighed (4 decimal places) into a pre-weighed crucible.
3. Crucible was placed on to porcelain tray.
4. Muffle furnace was ignited at 550°C for 8 hrs until light grey ash was resulted.
5. Samples were cooled in a desiccator and weighed soon after reaching room temperature.

Calculation

$$\% \text{ ash} = \frac{(\text{weight of crucible + residue}) - \text{weight of crucible (g)}}{\text{Sample weight (g)}} \times 100\%$$

Appendix 2: Determination of lipid content in cauliflower seeds using rapid Soxhelt extraction

Principle

The free lipid content of seeds consists essentially of neutral fats (triglycerides) and free fatty acids. These can be determined by extracting the dried and ground material with a suitable solvent in a continuous extraction apparatus.

The Soxhlet method

Well ground seed samples were weighed into cellulose thimbles and solvent were added. The solvent was heated to boiling point, the vapours are condensed and hot liquid solvent was washed through the sample in the thimble in a continuous process. The non-volatile solutes were extracted into the solvent. After a period of time the solvent was recovered and the extracted lipids was quantified by weighing.

A. Sample preparation

The preparation of samples prior to extraction can be a critical factor in the accuracy of the Soxhlet extraction procedure.

- To ensure the Soxtherm runs correctly, samples were dried prior to extraction.
- Preparation was done in the way that allows for maximum contact between the sample and the solvent.
- Seed samples were ground to allow penetration of the solvent.

B. Apparatus

1. 6 place Soxtherm Extraction Unit x 2.
2. Multistate Unit to control process.

3. Extraction Beakers x 1 (Rack for beakers).
4. Tongs (Wire Thimble Supports).
5. Extraction Thimbles (Anti bump granules).
6. Balance- 3 decimal places (Spatula, weighing boats).
7. Cotton/Glass Wool (Drying oven 103°C).
8. 40-60 Petroleum Ether or other solvent with automatic dispenser.

For accurate analysis it was avoided to handle the glassware with bare hands. It was handled either using tongs or protective gloves.

C. Extraction

1. A pinch of granules was placed in each clean, numbered dry beaker.
2. The beaker was weighted on a 3 decimal place balance and the weight was recorded.
3. 2-3 g of prepared sample was accurately weighted into extraction thimble and the top was plugged with cotton wool.
4. The thimble was placed into a wire support and insert into beaker.
5. 140 ml of solvent was added to each beaker using dispenser in a fume cupboard.
6. The beakers were placed on the Soxtherm unit and run the correct program.

D. Calculation

The increase in beaker weight was compared to the original sample weight to determine the extractable material.

$$\% \text{ FAT} = \frac{\text{final weight of beaker- initial weight of beaker}}{\text{Initial weight of sample}} \times 100$$

Appendix 3: Determination of nitrogen and protein content by Kjeldahl methods

Principle

The Kjeldahl method is a means of determining the nitrogen content of organic and inorganic substances. The Kjeldahl method may be divided into 3 stages.

1. Digestion stage

a) Apparatus

- Insert rack
- 40 x 100ml Kjeldahl boiling tubes
- 4 decimal place Balance
- Weighing boats
- Spatula

b) Methods

1. Extraction hood was turned on.
2. Scrubber Unit – The lid of the 15% Sodium Hydroxide solution was unscrewed carefully and a few drops of the Universal Indicator were added. The lid was Re-tightened. The Scrubber Unit was turned on and it was ensured that the Sodium Hydroxide solution remains pale blue and there was active turbulence.
3. Kjeldatherm digestion block was turned on.
4. Heating temperature was set to 105°C and it was allowed the block to preheat while preparing samples.
5. The composition of the batch of samples for digestion was as follows:
    - For Control purposes: 2 tubes with reagents only were used. These were the blanks used to correct the results for any influence the reagents might have on the procedure.

- To determine the efficiency of the total procedure in terms of total Nitrogen recovery: 2 samples Acetanilide standard (theoretical Nitrogen content=10.36%) were used. Sample size was 100-150 mg. The calculated efficiency was used to correct the final results.
- 2 samples were processed of a standard reference material (casein) with a declared stated, validated nitrogen and protein content in the run (100-150 mg).
- 11/12 Samples were in triplicate-Weighed (100-150 mg) of seed sample into weighing boat. Weights were recorded in milligrams.

6. 1 catalyst tablet was added to each tube.
7. 10 ml low nitrogen, 98% Sulphuric ($H_2SO_{4)}$ was added to each tube (automatic dispenser was used in fume cupboard with protective clothing, eye protection and nitrile gloves).
8. When all samples were ready, they were placed on Kjeldatherm Digestion Block.
9. Samples were left to heat at 105°C for 15mins.
10. Temperature was raised to 225°C for 60 mins.
11. Temperature was then raised to 380°C for 45.
12. Samples were left to cool.

Samples were then ready, after cooling, for Vapodest Distillation and titration.

2. Vapodest distillation stage

3. Calculation

1. To determine Nitrogen

The following information was used:

- Sample weight in g or mg (about 100 mg per seed sample).

- Volume in mls of titrant (0.1 M H_2SO_4).
- Acid normality (N=0.2).
- Average volume (mls=0.14898) of 0.1M H_2SO_4 used to titrate the Blank samples.
- MW of nitrogen (1400.67) (sample weight was in mg).
- Conversion factor for protein (6.25).

Formula:

% Nitrogen =

$$\frac{(\text{mls sample Titrant - mls Blank Titrant}) \times \text{Acid Normality} \times \text{MW Nitrogen}}{\text{Sample wt}}$$

2. To Determine Protein Content

%Protein = %Nitrogen x Conversion Factor

3. To determine Efficiency of Nitrogen recovery from samples

The theoretical nitrogen content of acetanilide is 10.36%

$$\% \text{ efficiency of procedure} = \frac{100}{10.36} \times \text{acetanilide sample result}$$

4. To Correct Nitrogen results of sample to 100% efficiency

$$\text{Corrected Value} = \frac{\% \text{ Nitrogen}}{\% \text{ efficiency}} \times 100$$

References

Ahmad, T. Abbasi, N. A. Hafiz, I. A. & Ali, A. 2007. Comparison of sucrose and sorbitol as main carbon energy sources in micropropagation of peach rootstock GF-677. *Pakistan Journal of Botany,* 39**,** 1269-1275.

Akaba, S. Seo, M. Dohmae, N. Takio, K. Sekimoto, H. Kamiya, Y. Furuya, N. Romano, T. & Koshiba, T. 1999. Production of Homo- and Hetero-Dimeric Isozymes from Two Aldehyde Oxidase Genes of Arabidopsis thaliana. *Journal of Biochemistry,* 126**,** 395-401.

Akhter Zobayed, S. M. Armstrong, J. & Armstrong, W. 1999. Cauliflower shoot-culture: effects of different types of ventilation on growth and physiology. *Plant science,* 141**,** 209-217.

Al-Issawi, M. Rihan, H. Z. Woldie, W. A. Burchett, S. & Fuller, M. P. 2013. Exogenous application of molybdenum affects the expression of CBF14 and the development of frost tolerance in wheat. *Plant Physiology and Biochemistry,* 63**,** 77-81.

Ali-Rachedi, S. Bouinot, D. Wagner, M. H. Bonnet, M. Sotta, B. Grappin, P. & Jullien, M. 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta,* 219**,** 479-488.

Allagulova, C. R. Gimalov, F. Shakirova, F. & Vakhitov, V. 2003. The plant dehydrins: structure and putative functions. *Biochemistry (Moscow),* 68**,** 945-951.

Amiri, S. & Kazemitabar, S. K. 2011. Enhancement of callus induction and regeneration efficiency from embryo cultures of Datura stramonium by adjusting carbon sources and concentrations. *African Journal of Biotechnology,* 10**,** 10101-10107.

Amutha, S. Ganapathi, A. & Muruganantham, M. 2003. *In vitro* organogenesis and plant formation in *Vigna radiata* (L.) Wilczek. *Plant Cell, Tissue and Organ Culture,* 72**,** 203-207.

Anderson, C. & Carstens, J. B. 1977. Tissue culture propagation of broccoli, Brassica oleracea (italica group), for use in F1 hybrid seed production. *Journal of the American Society of Horticultural Science,* 102**,** 69-73.

Anisko, T. & Lindstrom, O. M. 1996. Seasonal Changes in Cold Hardiness of Rhododendron L.Catawbiense Boursault'Grown under Continuous and Periodic Water Stress. *Journal of the American Society for Horticultural Science,* 121**,** 301-306.

Ara, H. Jaiswal, U. & Jaiswal, V., S. 2000. Synthetic seed: Prospects and limitation. *Current science,* 78**,** 1438-1444.

Ara, H. Jaiswal, U. & Jaiswal, V. S. 1999. Germination and plantlet regeneration from encapsulated somatic embryos of mango (Mangifera indica L.). *Plant Cell Reports,* 19**,** 166-170.

Arora, N. Yadav, N. R. Yadav, R. C. Chowdhury, J. B. & 1997. Role of IAA and BAP on plant regeneration in cultured cotyledons of cauliflower. *Cruciferae Newsl,* 19**,** 41-42

Artus, N. N. Uemura, M. Steponkus, P. L. Gilmour, S. J. Lin, C. T. & Thomashow, M. F. 1996. Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. *Proceedings of the National Academy of Sciences of the United States of America,* 93**,** 13404-13409.

Asghar, R. Fenton, R. Demason, D. A. & Close, T. 1994. Nuclear and cytoplasmic localization of maize embryo and aleurone dehydrin. *Protoplasma,* 177**,** 87-94.

Ashraf, M. & Oleary, J. W. 1996. Effect of drought stress on growth, water relations, and gas exchange of two lines of sunflower differing in degree of salt tolerance. *International Journal of Plant Sciences,* 157**,** 729-732.

Atkinson, B. G. Liu, L. Goping, I. S. & Walden, D. B. 1989. Expression of the genes encoding hsp73, hsp18, and ubiquitin in radicles of heat-shocked maize seedlings. *Genome,* 31**,** 698-704.

Attree, S. M. Pomeroy, M. K. & Fowke, L. C. 1994. Production of vigorous, dessication tolerant white spruce (Picea-glauca (moench) voss) synthetic seeds in a bioreactor. *Plant Cell Reports,* 13**,** 601-606.

Baker, S. S. Wilhelm, K. S. & Thomashow, M. F. 1994. The 5′ -region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Molecular Biology,* 24**,** 701-713.

Baniulis, D. Stepulaitiene, I. Lukoseviciute, V. Blazyte, A. Stanys, V. Rugienius, R. & Sasnauskas, A. 2012. Accumulation of dehydrin-like proteins in pear (*Pyrus communis* L.) microshoots during cold acclimation.

Bapat, V. A. Mhatre, M. & Rao, P. S. 1987. Propagation of *Morus indica* L. (Mulberry) by encapsulated shoot buds. *Plant Cell Reports,* 6**,** 393-395.

Bapat, V. A. & Rao, P. S. 1988. Sandalwood plantlets from synthetic seeds. *Plant Cell Rep,* 7**,** 434–436.

Bapat, V. A. & Rao, P. S. 1990. Invivo growth of encapsulated axillary buds of mulberry (*Morus-indical L*.). *Plant Cell Tissue and Organ Culture,* 20**,** 69-70.

Barabas, N. K. Omarov, R. T. Erdei, L. & Lips, S. H. 2000. Distribution of the Mo-enzymes aldehyde oxidase, xanthine dehydrogenase and nitrate reductase in maize (Zea mays L.) nodal roots as affected by nitrogen and salinity. *Plant Science,* 155**,** 49-58.

Barrett, C. & Cassells, A. C. 1994. An evaluation of antibiotics for the elimination of Xanthomonas campestris cv. Pelargonii (Brown) from *Pelargonium domesticum* cv. `Grand Slam. *Plant Cell Tiss. Organ Cult,* 36**,** 169-175.

Bartels, D. Singh, M. & Salamini, F. 1988. Onset of desiccation tolerance during development of the barley embryo. *Planta,* 175**,** 485-492.

Bartels, D. & Sunkar, R. 2005. Drought and salt tolerance in plants. *Critical Reviews in Plant Sciences,* 24**,** 23-58.

Baskaran, P. & Jayabalan, N. 2005. Role of basal media, carbon sources and growth regulators in micropropagation of *Eclipta alba* – a valuable medicinal herb. *Kmitl. Sci. J,* 5**,** 469-482.

Battaglia, M. Olvera-Carrillo, Y. Garciarrubio, A. Campos, F. & Covarrubias, A. A. 2008. The enigmatic LEA proteins and other hydrophilins. *Plant Physiology,* 148**,** 6-24.

Baud, S. Boutin, J. P. Miquel, M. Lepiniec, L. & Rochat, C. 2002. An integrated overview of seed development in *Arabidopsis thaliana* ecotype WS. *Plant Physiology and Biochemistry,* 40**,** 151-160.

Baud, S. Dubreucq, B. Miquel, M. Rochat, C. & Lepiniec, L. 2008. Storage reserve accumulation in Arabidopsis: metabolic and developmental control of seed filling. *The Arabidopsis Book/American Society of Plant Biologists,* 6.

Beck, E. H. Heim, R. & Hansen, J. 2004. Plant resistance to cold stress: Mechanisms and environmental signals triggering frost hardening and dehardening. *Journal of Biosciences,* 29**,** 449-459.

Ben-Hayyim, G. Faltin, Z. Gepstein, S. Camoin, L. Donny Strosberg, A. & Eshdat, Y. 1993. Isolation and characterization of salt-associated protein in Citrus. *Plant science,* 88**,** 129-140.

Bernier, G. 1988. The control of floral evocation and morphogenesis. *Annual Review of Plant Physiology and Plant Molecular Biology,* 39**,** 175-219.

Bettey, M. Sinniah, U. M. a. R. Finch-Savage, W. & Ellis, R. 1998. Irrigation and seed quality development in rapid-cycling Brassica: Accumulation of stress proteins. *Annals of Botany,* 82**,** 657-663.

Bewley, J. D. & Black, M. 1994. *Seeds: physiology of development and germination*, Springer.

Bhalla 1998. Comparison of shoot regeneration potential from seedling explants of Astralian cauliflowers (*Brassica oleracea* var botrytis). *Australia journal of agricultural research,* 49**,** 1261.

Bhalla, P. & De Weerd, N. 1999. *In vitro* propagation of cauliflower, *Brassica oleracea* var. botrytis for hybrid seed production. *Plant Cell, Tissue and Organ Culture,* 56**,** 89-95.

Bhaskaran, S. Smith, R. H. & Newton, R. J. 1985. Physiological changes in cultured sorghum cells in response to induced water stress I. Free proline. *Plant physiology,* 79**,** 266-269.

Bies-Etheve, N. Gaubier-Comella, P. Debures, A. Lasserre, E. Jobet, E. Raynal, M. Cooke, R. & Delseny, M. 2008. Inventory, evolution and expression profiling diversity of the LEA (late embryogenesis abundant) protein gene family in Arabidopsis thaliana. *Plant molecular biology,* 67**,** 107-124.

Bittner, F. Oreb, M. & Mendel, R. R. 2001. ABA3 is a molybdenum cofactor sulfurase required for activation of aldehyde oxidase and xanthine dehydrogenase in *Arabidopsis thaliana*. *Journal of Biological Chemistry,* 276**,** 40381-40384.

Blackman, S. A. Wettlaufer, S. H. Obendorf, R. L. & Leopold, A. C. 1991. Maturation proteins associated with desiccation tolerance in soybean. *Plant physiology,* 96**,** 868-874.

Blakely, L. M. Blakely, R. M. Colowit, P. M. & Elliott, D. S. 1988. Experimental Studies on Lateral Root Formation in Radish Seedling Roots: II. Analysis of the Dose-Response to Exogenous Auxin. *Plant Physiol.,* 87**,** 414-419.

Boerjan, W. Cervera, M. T. Delarue, M. Beeckman, T. Dewitte, W. Bellini, C. Caboche, M. Onckelen, H. V. Montagu, M. V. & Inze, D. 1995. superroot, a Recessive Mutation in Arabidopsis, Confers Auxin Overproduction. *Plant Cell,* 7**,** 1405-1419.

Booij, R. & Struik, P. 1990. Effects of temperature on leaf and curd initiation in relation to juvenility of cauliflower. *Scientia Horticulturae,* 44**,** 201-214.

Borisjuk, L. Walenta, S. Weber, H. Mueller-Klieser, W. & Wobus, U. 1998. High-resolution histographical mapping of glucose concentrations in developing cotyledons ofVicia fabain relation to mitotic activity and storage processes: glucose as a possible developmental trigger. *The Plant Journal,* 15**,** 583-591.

Bornman, C. H. & Vogelmann, T. C. 1984. Effect of rigidity of gel medium on benzyladenine-induced adventitious bud formation and vitrification *in vitro* in Picea abies. *Physiologia Plantarum,* 61**,** 505-512.

Bottcher, I. Zoglauer, K. & H. Goring 1988. Induction and reversion of vitrification of plants cultured *in vitro*. *Physiol Plant.,* 72**,** 560-564.

Böttcher, K. Z., K. And Göring, H 1988. Induction and reversion of vitrification of plants cultured *in vitro*. *Physiol Plant,* 72**,** 560-564.

Böttgor, M. 1974. Apical dominance in roots of *Pisum sativum* L. *Planta (Ber1),* 121 253–261.

Bravo, L. A. Gallardo, J. Navarrete, A. Olave, N. Martínez, J. Alberdi, M. Close, T. J. & Corcuera, L. J. 2003. Cryoprotective activity of a cold‐induced dehydrin purified from barley. *Physiologia Plantarum,* 118**,** 262-269.

Bray, E. A. 1993. Molecular responses to water deficit. *Plant physiology,* 103**,** 1035.

Bray, E. A. 2004. Genes commonly regulated by water-deficit stress in *Arabidopsis thaliana*. *Journal of Experimental Botany,* 55**,** 2331-2341.

Bubu, O. & Fuller, M. 2005. Production, storability, dehydration and regeneration of micro-shoots of cauliflower encapsulated in soduim alginate beads. *.2005.botanyconference.* Salon A - Gov Ballroom/Hilton.

Buiattii, E. T. Baroncelli, S. Bennici, A. Pagliai, M. & Tesi, R. 1974. Genetics of growth and differentiation *in vitro* of *Brassica oleracea* var Botrytis. II. An *in vitro* analysis of a diallel cross. *Z. Pflanzenzuchtung,* 72**,** 269–274.

Burn, J. E. Bagnall, D. J. Metzger, J. D. Dennis, E. S. & Peacock, W. J. 1993. DNA methylation, vernalization, and the initiation of flowering. *Proceedings of the National Academy of Sciences of the United States of America,* 90**,** 287-291.

Campbell, S. A. & Close, T. J. 1997. Dehydrins: genes, proteins, and associations with phenotypic traits. *New Phytologist,* 137**,** 61-74.

Cao 2003. Transgene expression in broccoli (*Brassica oleracea* var Italica) clones propagated *in vitro* via leaf explants. *Plant Cell Reports,* 21**,** 789.

Capuano, G. Piccioni, E. & Standardi, A. 1998. Effect of different treatments on the conversion of M.26 apple rootstock synthetic seeds obtained from encapsulated apical and axillary micropropagated buds. *Journal of Horticultural Science & Biotechnology,* 73**,** 299-305.

Cartes, P. Castellanos, H. Rios, D. Saez, K. Spierccolli, S. & Sanchez, M. 2009. Encapsulated somatic embryos and zygotic embryos for obtaining artificial seeds of rauli-beech (*Nothofagus alpina* (Poepp.&Endl.) oerst.). *Chilean Journal of Agricultural Research,* 69**,** 112-118.

Castiglioni, P. Warner, D. Bensen, R. J. Anstrom, D. C. Harrison, J. Stoecker, M. Abad, M. Kumar, G. Salvador, S. D'ordine, R. Navarro, S. Back, S. Fernandes, M. Targolli, J. Dasgupta, S. Bonin, C. Luethy, M. H. & Heard, J. E. 2008. Bacterial RNA chaperones confer abiotic stress tolerance in plants and improved grain yield in maize under water-limited conditions. *Plant Physiology,* 147**,** 446-455.

Ceccardi, T. L. Meyer, N. C. & Close, T. J. 1994. Purification of a maize dehydrin. *Protein Expression and Purification,* 5**,** 266-269.

Celenza, J. L. Grisafi, P. L. & Fink, G. R. 1995. A pathway for lateral root formation in *Arabidopsis thaliana*. *Genes & Development,* 9**,** 2131-2142.

Cellier, F. Conéjéro, G. Breitler, J. C. & Casse, F. 1998. Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of

sunflower accumulation of dehydrin transcripts correlates with tolerance. *Plant Physiology,* 116**,** 319-328.

Chamandoosti, F. 2010. The relationship between plant growth regulators for organogenesis and phenolic compound in cotton (*Gossypium hirsutum* L.). *Asian J. Dev. Biol,* 2**,** 16-22.

Chand, S. & Singh, A. K. 2004. Plant regeneration from encapsulated nodal segments of *Dalbergia sissoo* Roxb., a timber-yielding leguminous tree species. *Journal of Plant Physiology,* 161**,** 237-243.

Chapman, J., S & Diehl, M., A 1995. Methylchloroisothiazolone-induced growth inhibition and lethality in *Escherichia coli*. *Appl Bacteriol,* 78**,** 134-141.

Charoensub, R. Hirai, D. & Sakai, A. 2004. Cryopreservation of *in vitro*-grown shoot tips of cassava by encapsulation–vitrification method. *Cryo-Lett* 25**,** 51–58.

Chen, C. C. 1978. Effects of sucrose concentration on plant production in anther culture of rice. *Crop Science,* 18**,** 905-906.

Chen, H. H. Brenner, M. L. & Li, P. H. 1993. Involvement of abscisic acid in potato cold acclimation. *Plant Physiology,* 71**,** 362–365.

Chen, H. H. Li, P. H. & Brenner, M. L. 1983. Involvement of abscisic acid in potato cold acclimation. *Plant physiology,* 71**,** 362-365.

Chia, T. Y. P. Pike, M. J. & Rawsthorne, S. 2005. Storage oil breakdown during embryo development of *Brassica napus* (L.). *Journal of experimental botany,* 56**,** 1285-1296.

Chinnusamy, V. Ohta, M. Kanrar, S. Lee, B. Hong, X. Agarwal, M. & Zhu, J. K. 2003. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. *Genes & development,* 17**,** 1043-1054.

Choi, D. W. Rodriguez, E. M. & Close, T. J. 2002. Barley Cbf3 gene identification, expression pattern, and map location. *Plant Physiology,* 129**,** 1781-1787.

Christey, M. C. & Earle, E. D. 1988. Use of peduncle explants for high efficiency regeneration of Brassica oleracea L. - potential for transformation. *Cruciferae Newsletter,* 13**,** 94-95.

Christey, M. C. & Earle, E. D. 1991. Regeneration of *Brassica-oleracea* from peduncle explants. *Hortscience,* 26**,** 1069-1072.

Clavero-Ramirez, I. Galvez-Farfan, J. Lopez-Aranda, J. M. Gonzalez-Benito, M. E. & 2005. Apex Cryopreservation of several strawberry genotypes by two encapsulation–dehydration methods. *Cryo-Lett,* 26**,** 17–24.

Close, T. J. 2006a. Dehydrins: a commonalty in the response of plants to dehydration and low temperature. *Physiologia Plantarum,* 100**,** 291-296.

Close, T. J. 2006b. Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. *Physiologia Plantarum,* 97**,** 795-803.

Close, T. J. Kortt, A. A. & Chandler, P. M. 1989. A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant molecular biology,* 13**,** 95-108.

Coelho, C. M. M. & Benedito, V. A. 2008. Seed development and reserve compound accumulation in common bean (Phaseolus vulgaris L.). *Seed Sci. Biotechnol,* 2**,** 42-52.

Compton, M. & Koch, J. 2001. Influence of plant preservative mixture (PPM)^{TM} on adventitious organogenesis in melon, petunia, and tobacco. *In Vitro Cellular and Developmental Biology - Plant,* 37**,** 259-261.

Cong, L. Chai, T.-Y. & Zhang, Y.-X. 2008. Characterization of the novel gene BjDREB1B encoding a DRE-binding transcription factor from Brassica juncea L. *Biochemical and Biophysical Research Communications,* 371**,** 702-706.

Cooke, R. C. 1979. Homogenization as an aid in tissue culture propagation of *Platycerium* and *Davallia*. *HortScience,,* 14**,** 21-22.

Corrie, S. & Tandon, P. 1993. Propagation of Cymbidium giganteum wall through high frequency conversion of encapsulated protocorms under *in vivo* and *in vitro* conditions. *Indian J Exp Biol,* 31**,** 61–64.

Crisp, P. & Gray, A. R. 1979. Successful selectionfor curd quality in cauliflower, using tissue-culture. *Horticultural Research,* 19**,** 49-53.

Crisp, P. & Walkey, D. G. A. 1974. The use of aseptic meristem culture in cauliflower breeding. *Euphytica,* 23**,** 305-313.

Cushman, J. C. & Bohnert, H. J. 2000. Genomic approaches to plant stress tolerance. *Current Opinion in Plant Biology,* 3**,** 117-124.

Cutler, A. J. & Krochko, J. E. 1999. Formation and breakdown of ABA. *Trends in Plant Science,* 4**,** 472-478.

Da Silva Bonome, L. T. Moreira, S. a. F. De Oliveira, L. E. M. & De Jesus Sotero, A. 2011. Metabolism of carbohydrates during the development of seeds of the brazilian rubber tree [*Hevea brasiliensis* (Willd. Ex Adr. de Juss) Muell.-Arg.]. *Acta Physiologiae Plantarum,* 33**,** 211-219.

Dalal, M. Tayal, D. Chinnusamy, V. & Bansal, K. C. 2009. Abiotic stress and ABA-inducible Group 4 LEA from *Brassica napus* plays a key role in salt and drought tolerance. *Journal of Biotechnology,* 139**,** 137-145.

Danso, K. E. & Ford-Lloyd, B. V. 2003. Encapsulation of nodal cuttings and shoot tips for storage and exchange of cassava germplasm. *Plant Cell Reports,* 21**,** 718-725.

Dash, P. K. Sharma, R. P. & Kumar, P. A. 1995. Shoot regeneration in the genotypes of cauliflower. *Cruciferae Newsletter,* 17**,** 26-27.

Datta, K. B. Kanjilal, B. & De Sarker, D. 1999. Artificial seed technology: Development of a protocol in *Geodorum densiflorum* (Lam) Schltr. - An endangered orchid. *Current Science,* 76**,** 1142-1145.

Daud, M. Taha, M. Z. & Hasbullah, A. Z. 2008. Artificial seed production from encapsulated micro shoots of *Sainpaulia ionantha* Wendl. (African Violet). *Journal of Applied Science,* 8**,** 4662-4667.

David, C. & Margara, J. 1979. *In vitro* development of cauliflower meristems *Brassica-oleracea* L.Var botrytis *Comptes Rendus Hebdomadaires Des Seances De L Academie Des Sciences Serie D,* 288**,** 1529-1535.

De Block, M. Debrouwer, D. & Tenning, P. 1989. Transformation of Brassica napus and Brassica oleracea using *Agrobacterium tumefaciens* and the expression of the bar and neo Genes in the transgenic plants. *Plant Physiology,* 91**,** 694-701.

Deane, C. 1994. *Whole plant and tissue culture studies on frost tolerance in cauliflower.* PhD, The National University of Ireland.

Debergh, P. Aitken-Christie, J. Cohen, D. Grout, B. Arnold, S. Zimmerman, R. & Ziv, M. 1992. Reconsideration of the term 'vitrification' as used in micropropagation. *Plant Cell, Tissue and Organ Culture,* 30**,** 135-140.

Debergh, P. C. 1988. Micropropagation of woody species-state of the art on *in vitro* aspects. *Acta Hort. (ISHS),* 227**,** 287-295.

Delouche, J. 1971. Seed maturation. *HANDBOOK of seed technology. Mississipi: Mississipi State University***,** 17-21.

Delpierre, N. & Boccongibod, J. 1992. An extensive hairy root production in protoplast-derived calli of calliflower (*Brassica-oleracea* var botrytis). *Plant Cell Reports,* 11**,** 351-354.

Denesik, T. J. 2007. *Quantitative expression analysis of four low-temperature-tolerance-associated genes during cold acclimation in wheat (Triticum aestivum L.).* Master of Science, University of Saskatchewan.

Deng, S. Y. Heap, I. M. & Klein, T. A. 1991. *In vitro* vegetative propagation of chinese cabbage. *Plant Cell, Tissue and Organ Culture,* 26**,** 135-139.

Deng, W. Luo, K. Li, Z. & Yang, Y. 2009. A novel method for induction of plant regeneration via somatic embryogenesis. *Plant Science,* 177**,** 43-48.

Digonzelli, P. Díaz Y, L. & Carrizo De Bellone, S. 2005. Use of PPM (Plant Preservative Mixture) to control bacterial contaminants in the multiplication *in vitro* of sugarcane. *Rev. Fac. Agron. (LUZ),* 22**,** 20-29.

Doherty, C. J. Van Buskirk, H. A. Myers, S. J. & Thomashow, M. F. 2009. Roles for Arabidopsis CAMTA transcription factors in cold-regulated gene expression and freezing tolerance. *The Plant Cell Online,* 21**,** 972-984.

Dörffling, K. Dörffling, H. Lesselich, G. Luck, E. Zimmermann, C. Melz, G. & Jürgens, H. U. 1997. Heritable improvement of frost tolerance in winter wheat by *in vitro*-selection of hydroxyproline-resistant proline overproducing mutants. *Euphytica,* 93**,** 1-10.

Du, Y. Q. Wang, Y. H. Wei, W. X. & Wang, Z. R. 1994. Effect of Molybdenum Fertilizer Application on the Nitrogen and Molybdenum Nutritions of Wheat. *Journal of Huazhong Agriculture.,* 13(4)**,** 384-389.

Dubouzet, J. G. Sakuma, Y. Ito, Y. Kasuga, M. Dubouzet, E. G. Miura, S. Seki, M. Shinozaki, K. & Yamaguchi-Shinozaki, K. 2003. OsDREB genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. *The Plant Journal,* 33**,** 751-763.

Dure, I. 2001. Occurrence of a repeating 11-mer amino acid sequence motif in diverse organisms. *Protein and Peptide Letters,* 8**,** 115-122.

Dure, L. 1993. Structural motifs in LEA proteins. *In:* CLOSE, T. J. (ed.) *Plant responses to cellular dehydration during environmental stress.* Rockville: American Society of Plant Physiologists.

Dure, L. Crouch, M. Harada, J. Ho, T. H. D. Mundy, J. Quatrano, R. Thomas, T. & Sung, Z. 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Molecular Biology,* 12**,** 475-486.

Eckardt, N. A. 2009. CAMTA proteins: a direct link between calcium signals and cold acclimation? *The Plant Cell Online,* 21**,** 697-697.

Eriksen, E. N. 1974. Root Formation in Pea Cuttings III. *Physiologia Plantarum,* 30**,** 163-167.

Fabijanski, S. Altosaar, I. & Arnison, P. G. 1987. Heat Shock Response of Brassica oleracea L. (Broccoli). *Journal of Plant Physiology,* 128**,** 29-38.

Fabre, J. & Dereuddre, J. 1990. Encapsulation dehydration-a new approach to cryopreservation of Solanum shoot-tips. *Cryo-Letters,* 11**,** 413-426.

Faisal, M. Ahmad, N. & Anis, M. 2006. *In vitro* plant regeneration from alginate-encapsulated microcuttings of *Rauvolfia tetraphylla* L. *American-Eurasian J,* 1.

Faisal, M. & Anis, M. 2007. Regeneration of plants from alginate-encapsulated shoots of *Tylophora indica* (Burm. f.) Merrill, an endangered medicinal. *plant, J Hortic Sci Biotechnol,* 82**,** 351–354.

Fait, A. Fromm, H. Walter, D. Galili, G. & Fernie, A. R. 2008. Highway or byway: the metabolic role of the GABA shunt in plants. *Trends in Plant Science,* 13**,** 14-19.

Fallon, K. & Phillips, R. 1989. Responses to water stress in adapted and unadapted carrot cell suspension cultures. *Journal of Experimental Botany,* 40**,** 681-687.

Fao. 2011. *http://faostat.fao.org/site/339/default.aspx* [Online]. Available: http://faostat.fao.org/site/339/default.aspx [Accessed 10, 1, 2010 2009].

Farrant, J. M. Bailly, C. Leymarie, J. Hamman, B. Côme, D. & Corbineau, F. 2004. Wheat seedlings as a model to understand desiccation tolerance and sensitivity. *Physiologia Plantarum,* 120**,** 563-574.

Fei, H. Tsang, E. & Cutler, A. J. 2007. Gene expression during seed maturation in *Brassica napus* in relation to the induction of secondary dormancy. *Genomics,* 89**,** 419-428.

Feito, I. Rodríguez, A. Centeno, M. Sánchez-Tamés, R. & Fernández, B. 1994. Effect of the physical nature of the culture medium on the metabolism of benzyladenine and endogenous cytokinins in *Actinida deliciosa* tissues cultured *in vitro*. *Physiologia Plantarum,* 91**,** 449-453.

Fellows, R. Pfeiffer, T. Egli, D. Tekrony, D. & Balles, J. 1979. Physiological maturity in soybean. *Agronomy Journal,* 71**,** 771-775.

Finch-Savage, W. E. & Blake, P. S. 1994. Indeterminate development in desiccation-sensitive seeds of Quercus robur L. *Seed Science Research,* 4**,** 127-133.

Finkelstein, R. R. Gampala, S. S. L. & Rock, C. D. 2002. Abscisic acid signaling in seeds and seedlings. *The Plant Cell Online,* 14**,** S15-S45.

Fowler, S. & Thomashow, M. F. 2002. Arabidopsis transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell,* 14**,** 1675-1690.

Francia, E. Rizza, F. Cattivelli, L. Stanca, A. M. Galiba, G. Tóth, B. Hayes, P. M. Skinner, J. S. & Pecchioni, N. 2004. Two loci on chromosome 5H determine low-temperature tolerance in a 'Nure' (winter) × 'Tremois' (spring) barley map. *TAG Theoretical and Applied Genetics,* 108**,** 670-680.

Fransz, P. F. Leunissen, E. H. M. & Colijn-Hooymans, C. M. 1993. 2,4-Dichlorophenoxyacetic acid affects mode and frequency of regeneration from hypocotyl protoplasts of *Brassica oleracea*. *Protoplasma,* 176**,** 125-132.

Fujii, J. Slade, D. & Redenbaugh, K. 1989. Maturation and greenhouse planting of alfalfa artificial seeds. *In Vitro Cellular & Molecular Biology - Plant,* 25**,** 1179-1182.

Fuller, M., P. & Eed, M. 2003. The development of multiple stress-resistance cauliflower using mutagenesis in conjunction with a microshoot tissue culture technique. *Acta Horticulturae,* 618**,** 71-76.

Fuller, M. P. Metwali, E. M. R. Eed, M. H. & Jellings, A. J. 2006. Evaluation of abiotic stress resistance in mutated populations of cauliflower (*Brassica oleracea* var. Botrytis). *Plant Cell Tissue and Organ Culture,* 86**,** 239-248.

Fuller, M. P. Turton, S. & Grout, B. W. W. 1990. Anther culture of winter-heading cauliflower. *In:* JANICK, J. & ZIMMERMAN, R. H. (eds.) *Proceedings of the First Ishs Symposium on in Vitro Culture and Horticultural Breeding.*

Galau, G. A. Hughes, D. W. & Dure, L. 1986. Abscisic acid induction of cloned cotton late embryogenesis-abundant (Lea) mRNAs. *Plant molecular biology,* 7**,** 155-170.

Ganapathi, T. R. Suprasanna, P. Bapat, V. A. & Rao, P. S. 1992. Propagation of banana through encapsulated shoot tips. *Plant Cell Reports,* 11**,** 571-575.

Gao, J. P. Chao, D. Y. & Lin, H. X. 2007. Understanding abiotic stress tolerance mechanisms: recent studies on stress response in rice. *Journal of Integrative Plant Biology,* 49**,** 742-750.

Gao, M.-J. Allard, G. Byass, L. Flanagan, A. M. & Singh, J. 2002. Regulation and characterization of four CBF transcription factors from *Brassica napus*. *Plant Molecular Biology,* 49**,** 459-471.

Garay-Arroyo, A. Colmenero-Flores, J. M. Garciarrubio, A. & Covarrubias, A. A. 2000. Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *Journal of Biological Chemistry,* 275**,** 5668-5674.

Garnczarska, M. Zalewski, T. & Wojtyla, Ł. 2008. A comparative study of water distribution and dehydrin protein localization in maturing pea seeds. *Journal of plant physiology,* 165**,** 1940-1946.

Gaspar, T. & Bajaj, Y. P. S. 1991. Vitrification in micropropagation. *Biotechnology in Agriculture and Forestry,* 17**,** 117-126.

Gaspar, T. Kevers, C. Bisbis, B. Franck, T. & Crevecoeur, M. 2000. Loss of plant organogenic totipotency in the course of neoplastic progression. *In Vitro Cell. Dev. Biol. Plant,* 36.

Gaspar, T. Kevers, C. Franck, T. Bisbis, B. & Billard, J. P. 1995. Paradoxical results in the analysis of hyperhydric tissues considered as being under stress: Questions for a debate. *Bulg. J. Plant Physiol,* 21**,** 80-97.

Gawel, N. J. & Robacker, C. D. 1990. Somatic embryogensis in 2 Gossypium-hirsutum genotypes in semisolid versus lequid proliferation media. *Plant Cell Tissue and Organ Culture,* 23**,** 201-204.

Ghosh, B. & Sen, S. 1994. Plant-regeneration from alginate encapsulated somatic embryos of *Asparagus-cooperi-baker. Plant Cell Reports,* 13**,** 381-385.

Gibson, S. I. 2001. Plant sugar-response pathways. Part of a complex regulatory web. (vol 124, pg 1532, 2000). *Plant Physiology,* 125**,** 2203-2203.

Gilbert, J. E. Shohet, S. & Caligari, P. D. S. 1991. The use of antibiotics to eliminate latent bacterial-contamination in potatotissue-culture. *Annals of Applied Biology,* 119**,** 113-120.

Gilmour, S. J. Fowler, S. G. & Thomashow, M. F. 2004. Arabidopsis Transcriptional Activators CBF1, CBF2, and CBF3 have Matching Functional Activities. *Plant Molecular Biology,* 54**,** 767-781.

Gilmour, S. J. Sebolt, A. M. Salazar, M. P. Everard, J. D. & Thomashow, M. F. 2000. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. *Plant Physiology,* 124**,** 1854-1865.

Gilmour, S. J. Zarka, D. G. Stockinger, E. J. Salazar, M. P. Houghton, J. M. & Thomashow, M. F. 1998. Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. *Plant Journal,* 16**,** 433-442.

Glendening, T. M. & Sjolund, R. 1988. *In vitro* propagation of kohlrabi from leaf explants. *HortScience,* 23**,** 772.

Glimour, S. Lin, C. & Thomashow, M. F. 1996. Purification and properties of *Arabidopsis thaliana* COR (cold-regulated) gene polypeptides COR15am and COR6. 6 expressed in *Escherichia coli*. *Plant physiology,* 111**,** 293-299.

Goday, A. Jensen, A. B. Culiáñez-Macià, F. A. Albà, M. M. Figueras, M. Serratosa, J. Torrent, M. & Pagès, M. 1994. The maize abscisic acid-responsive protein

Rab17 is located in the nucleus and interacts with nuclear localization signals. *The Plant Cell Online,* 6**,** 351-360.

Golombek, S. Rolletschek, H. Wobus, U. & Weber, H. 2001. Control of storage protein accumulation during legume seed development. *Journal of plant physiology,* 158**,** 457-464.

Gonzalez-Arnao, M. T. Juarez, J. Ortega, C. Navarro, L. & Duran-Vila, N. 2003. Cryopreservation of ovules and somatic embryos of citrus using the encapsulation–dehydration technique. *Cryo-Lett,* 24**,** 85–94.

González-Guzmán, M. Apostolova, N. Bellés, J. M. Barrero, J. M. Piqueras, P. Ponce, M. R. Micol, J. L. Serrano, R. & Rodríguez, P. L. 2002. The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *The Plant Cell Online,* 14**,** 1833-1846.

Goodwin, P. B. & Morris, S. C. 1979. Application of phytohormones to pea roots after removal of the apex: effect on lateral root production. *Aust J Plant Physiol,* 6**,** 195–200.

Goyal, K. Walton, L. J. & Tunnacliffe, A. 2005. LEA proteins prevent protein aggregation due to water stress. *Biochemical Journal,* 388**,** 151.

Gresshof.Pm & Doy, C. H. 1972. Development and differentiation of haploid *Lycopersicon-esculentum* (tomato). *Planta,* 107**,** 161-&.

Grout, B. W. W. & Crisp, P. 1977. Practical aspects of the propagation of cauliflower by meristem culture. *Acta Horticulturae,* 78**,** 289-296.

Gupta, P. K. & Durzan, D. J. 1987. Somatic embryos from protoplasts of *Loblolly-pine proembryona*l cells. *Bio-Technology,* 5**,** 710-712.

Gupta, S. K. Singh, P. K. Sawant, S. V. Chaturvedi, R. & Tuli, R. 2000. Effect of light intensity on *in vitro* multiple shoot induction and regeneration of cotton (Gossypium hirsutum L. cv Khandawa-2). *Indian J. Exp. Biol,* 38**,** 399-401.

Guri, A., Z & Patel, K. N. 1998. Compositions and methods to prevent microbial contamination of plant tissue culture media. *United States Patent,* 5**,** 398-402.

Gurr, M. I. Blades, J. & Appleby, R. S. 1972. Studies on seed-oil triglycerides-composition of *Crambe-abyssinica* triglycerides during seed maturation. *European Journal of Biochemistry,* 29**,** 362.

Gurusamy, C. & Thiagarajan, C. 1998. The pattern of seed development and maturation in clauliflower (*Brassica oleracea* L. var. botrytis). *Phyton,* 38**,** 259-268.

Guy, C. Haskell, D. & Li, Q. B. 1998. Association of proteins with the stress 70 molecular chaperones at low temperature: evidence for the existence of cold labile proteins in spinach. *Cryobiology,* 36**,** 301-314.

Guy, C. L. 1990a. Cold Accelimation and Freezing Stress Tolerance: Role of Protein Metabolism. *Annual Review of Plant Biology,* 41**,** 187-223.

Guy, C. L. 1990b. Cold Accelimation and Freezing Stress Tolerance: Role of Protein Metabolism. *Annual Review of Plant Physiology and Plant Molecular Biology,* 41**,** 187-223.

Hachey, J. E. Sharma, K. K. & Moloney, M. M. 1991. Efficient shoot regeneration of *Brassica-campestris* using cotyledon explants cultured invitro. *Plant Cell Reports,* 9**,** 549-554.

Hadi, F. Gilpin, M. & Fuller, M. P. 2011. Identification and expression analysis of CBF/DREB1 and COR15 genes in mutants of *Brassica oleracea* var. botrytis with enhanced proline production and frost resistance. *Plant Physiology and Biochemistry,* 49**,** 1323-1332.

Hadley, P. & Pearson, S. 1998. Effects of environmental factors on progress to crop maturity in selected brassica crops. *In:* THOMAS, G. & MONTEIRO, A. A. (eds.) *Brassica 97 - International Symposium on Brassicas.*

Haldeman, J. H. Thomas, R. L. & Mckamy, D. L. 1987. Use of benomyl and rifampicin for *in vitro* shoot tip culture of *Camellia sinensis* and *C. japonica*. *HortScience,* 22**,** 30-307.

Hale, K. L. Mcgrath, S. P. Lombi, E. Stack, S. M. Terry, N. Pickering, I. J. George, G. N. & Pilon-Smits, E. a. H. 2001. Molybdenum Sequestration in BrassicaSpecies. A Role for Anthocyanins. *Plant Physiology,* 126**,** 1391-1402.

Hamdia, H. M. El-Komy, H. M. Shaddad, M. a. K. & Hetta, A. M. 2005. Effect of molybdenum of nitrogenase activities of wheat inoculated with *Azospirillum brasillense* growth under drought stress. *General and Applied Plant Physiology,* 31**,** 43-54.

Hammerschlag, M. R. 1982. Activity of trimethoprim-sulfamethoxazole against *Chlamydia trachomatis in vitro*. *Rev. Infect. Dis,* 4**,** 500-504.

Han, B. & Kermode, A. R. 1996. Dehydrin-like proteins in castor bean seeds and seedlings are differentially produced in response to ABA and water-deficit-related stresses. *Journal of Experimental Botany,* 47**,** 933-939.

Hand, D. J. & Atherton, J. G. 1987. Curd Initiation in the cauliflower: I. JUVENILITY. *J. Exp. Bot.,* 38**,** 2050-2058.

Hara, M. Terashima, S. Fukaya, T. & Kuboi, T. 2003. Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco. *Planta,* 217**,** 290-298.

Harb, A. Krishnan, A. Ambavaram, M. M. R. & Pereira, A. 2010. Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. *Plant Physiology,* 154**,** 1254-1271.

Hazarika, B., N V.A, P. & Nagaraju, V. 2004. Influence of *in vitro* preconditioning of Citrus sp. microshoots with sucrose on their *ex vitro* establishment. *Indian Journal of Horticulture,* 61**,** 29-31.

Heidarvand, L. & Maali Amiri, R. 2010. What happens in plant molecular responses to cold stress? *Acta Physiologiae Plantarum,* 32**,** 419-431.

Hincha, D. K. Heber, U. & Schmitt, J. M. 1989. Freezing ruptures thylakoid membranes in leaves, and rupture can be prevented *in vitro* by cryoprotective proteins. *Plant Physiol Biochem,* 27**,** 795-801.

Hinchee, M. a. W. & Rost, T. L. 1986. The Control of Lateral Root Development in Cultured Pea Seedlings. I. The Role of Seedling Organs and Plant Growth Regulators. *Botanical Gazette,* 147**,** 137.

Hirai, D. & Sakai, A. 1999. Cryopreservation of *in vitro*-grown meristems of potato (*Solanum tuberosum* L.) by encapsulation-vitrification. *Potato Research,* 42**,** 153-160.

Hirayama, T. & Shinozaki, K. 2007. Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends in Plant Science,* 12**,** 343-351.

Hirose, T. Takano, M. & Terao, T. 2002. Cell Wall Invertase in Developing Rice Caryopsis: Molecular Cloning of OsCIN1 and Analysis of its Expression in Relation to its Role in Grain Filling. *Plant and Cell Physiology,* 43**,** 452-459.

Hoekstra, F. A. Golovina, E. A. & Buitink, J. 2001. Mechanisms of plant desiccation tolerance. *Trends in plant science,* 6**,** 431-438.

Horeau, N. Arona, R. & Bhojwani, S. S. 1988. A comparative study of *in vitro* shoot regeneration from cotyledon and root explants of four varieties of *Brassica oleracea* L. *Current Science,* 57(24)**,,** 1349-1351.

Houde, M. Daniel, C. Lachapelle, M. Allard, F. Laliberté, S. & Sarhan, F. 2003. Immunolocalization of freezing‐tolerance‐associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues. *The Plant Journal,* 8**,** 583-593.

Huang, P. M. Chen, J. Y. & Wang, S. J. 2009. Tissue-specific regulation of rice molybdenum cofactor sulfurase gene in response to salt stress and ABA. *Acta Physiologiae Plantarum,* 31**,** 545-551.

Huda, A. K. M. N. & Bari, M. A. 2007. Production of Synthetic Seed by Encapsulating Asexual Embryo in Eggplant (*Solanum melongena* L.). *International Journal of Agricultural Research* 9.

Hussain, S. S. Kayani, M. A. & Amjad, M. 2011. Transcription factors as tools to engineer enhanced drought stress tolerance in plants. *Biotechnology Progress,* 27**,** 297-306.

Ingram, J. & Bartels, D. 1996. The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Biology,* 47**,** 377-403.

Ivanova, M. Novak, O. Strnad, M. & Van Staden, J. 2006. Endogenous cytokinins in shoots of Aloe polyphylla cultured *in vitro* in relation to hyperhydricity, exogenous cytokinins and gelling agents. *Plant Growth Regulation,* 50**,** 219-230.

Ivanova, M. & Van Staden, J. 2008. Effect of ammonium ions and cytokinins on hyperhydricity and multiplication rate of *in vitro* regenerated shoots of *Aloe polyphylla*. *Plant Cell Tissue and Organ Culture,* 92**,** 227-231.

Jaglo-Ottosen, K. R. Gilmour, S. J. Zarka, D. G. Schabenberger, O. & Thomashow, M. F. 1998a. Arabidopsis CBF1 Overexpression Induces COR Genes and Enhances Freezing Tolerance. *Science,* 280**,** 104-106.

Jaglo-Ottosen, K. R. Gilmour, S. J. Zarka, D. G. Schabenberger, O. & Thomashow, M. F. 1998b. Arabidopsis CBF1over-expression inducesCOR genesand enhances freezing tolerance *Science,* 280**,** 104-106.

Jaglo, K. R. Kleff, S. Amundsen, K. L. Zhang, X. Haake, V. Zhang, J. Z. Deits, T. & Thomashow, M. F. 2001. Components of the Arabidopsis C-Repeat/Dehydration-Responsive Element Binding Factor Cold-Response Pathway Are Conserved in *Brassica napus* and Other Plant Species. *Plant physiology,* 127**,** 910-917.

Jain, S. Gupta, P. Bapat, V. & Mhatre, M. 2005. Bioencapsulation of Somatic Embryos in Woody Plants. *Protocol for Somatic Embryogenesis in Woody Plants.* Springer Netherlands.

Jaleel, C. A. Manivannan, P. Wahid, A. Farooq, M. Al-Juburi, H. J. Somasundaram, R. & Panneerselvam, R. 2009. Drought stress in plants: a review on morphological characteristics and pigments composition. *Int. J. Agric. Biol,* 11**,** 100-105.

Jan, N. Mahboob Ul, H. & Andrabi, K. I. 2009. Cold resistance in plants: A mystery unresolved. *Electronic Journal of Biotechnology,* 12.

Janssens, J. & Sepelie, M. 1989. Rapid multiplication of ferns by homogenization. *Scientia Horticulturae,,* 38**,** 161-164.

Javed, F. & Ikram, S. 2008. Effect of sucrose induced osmotic stress on callus growth and biochemical aspects of two wheat genotypes. *Pakistan Journal of Botany,* 40**,** 1487-1495.

Jenks, M. A. & Wood, A. J. 2010. *Genes for Plant Abiotic Stress,* Iowa, USA, Willy-Blakwell.

Jones, A. & Petolino, J. 1988. Effects of support medium on embryo and plant-production from cultured anther of soft-red winter-wheat (*Triticum-aestivum* L) *Plant Cell, Tissue, and Organ Culture,* 12**,** 253-261.

Jones, H. G. 1974. Asseeement of stomatal control of plant water status. *New Phytologist,* 73**,** 851-859.

Julliard, J. Sossountzov, L. Habricot, Y. & Pelletier, G. 1992. Hormonal requirement and tissue competency for shoot organogenesis in two cultivars of *Brassica napus*. *Physiologia Plantarum,* 84**,** 521-530

Kaiser, B. N. Gridley, K. L. Brady, J. N. Phillips, T. & Tyerman, S. D. 2005. The role of molybdenum in agricultural plant production. *Annals of Botany,* 96**,** 745-754.

Kalemba, E. M. & Pukacka, S. 2008. Changes in late embryogenesis abundant proteins and a small heat shock protein during storage of beech (Fagus sylvatica L.) seeds. *Environmental and Experimental Botany,* 63**,** 274-280.

Kalloo , G. & Bergh, B. O. 1993. *Genetic improvement of vegetable crops* Oxford, Pergamon Press.

Kao, C. Y. Cocciolone, S. M. Vasil, I. K. & Mccarty, D. R. 1996. Localization and interaction of the cis-acting elements for abscisic acid, VIVIPAROUS1, and light activation of the C1 gene of maize. *Plant Cell,* 8**,** 1171-1179.

Kares, C. Prinsen, E. Onckelen, H. & Otten, L. 1990. IAA synthesis and root induction with iaa genes under heat shock promoter control. *Plant Molecular Biology,* 15**,** 225-236.

Karlson, D. & Imai, R. 2003. Conservation of the cold shock protein of *Escherichia coli*. *Plant Physiol* 131**,** 12-15.

Kasuga, M. Liu, Q. Miura, S. Yamaguchi-Shinozaki, K. & Shinozaki, K. 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature Biotechnology,* 17**,** 287-291.

Kataeva, N. V. Alexandrova, I. G. Butenko, R. G. & Dragavtceva, E. V. 1991. Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured *in vitro*. *Plant Cell, Tissue and Organ Culture,* 27**,** 149-154.

Kempa, S. Krasensky, J. Dal Santo, S. Kopka, J. & Jonak, C. 2008. A central role of Abscisic Acid in stress-regulated carbohydrate metabolism. *Plos One,* 3.

Kevers, C. Coumans, M. Coumans-Gillès, M. F. & Caspar, T. 1984. Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. *Physiologia Plantarum,* 61**,** 69-74.

Kieffer, L., M. 1996. *The invitro manipulation of cauliflower (Brassica oleraceae L. convar. botrytis (L.) Alef. var. botrytis L.) meristematic tissue for utilisation in genetic improvement programmes.* Plymouth university.

Kieffer, M. Fuller, M. P. & Jellings, A. J. 1995. Rapid mass-production of cauliflower propagules from fractionated and graded curd. *Plant Science,* 107**,** 229-235.

Kieffer, M. Simkins, N. Fuller, M. P. & Jellings, A. J. 2001. A cost effective protocol for *in vitro* mass propagation of cauliflower. *Plant Science,* 160**,** 1015-1024.

Kishitani, S. Watanabe, K. Yasuda, S. Arakawa, K. & Takabe, T. 1994. Accumulation of glycinebetaine during cold acclimation and freezing tolerance in leaves of winter and spring barley plants. *Plant, Cell & Environment,* 17**,** 89-95.

Kitto, S. L. & Janick, J. 1982. Polyox as an artificial seed coat for a sexual embryos. *Hortscience,* 17**,** 488-488.

Knauss, J. F. 1976. A partial tissue culture method for pathogen-free propagation of selected ferns from spores. *Proceedings of the annual meeting of the Florida State Horticultural Society,* 89**,** 63-365.

Kneifel, W. & Leonhardt, W. 1992. Testing of different antibiotics against gram positive and gram negative bacteria isolated from plant tissue cultures. *Plant Cell Tissue Organ Cult,* 29**,** 139-144.

Knight, H. & Knight, M. R. 2000. Imaging spatial and cellular characteristics of low temperature calcium signature after cold acclimation in Arabidopsis. *Journal of Experimental Botany,* 51**,** 1679-1686.

Knight, H. Zarka, D. G. Okamoto, H. Thomashow, M. E. & Knight, M. R. 2004. Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiology,* 135**,** 1710-1717.

Koch, K. 2004. Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology,* 7**,** 235-246.

Kodym, A. & Zapata-Arias, F. 2001. Low-cost alternatives for the micropropagation of banana. *Plant Cell, Tissue and Organ Culture,* 66**,** 67-71.

Komor, E. Liegl, I. & Schobert, C. 1993. Loading and translocation of various cytokinis in phloem and xylem of the seedlings of *Ricinus-communis* L. *Planta,* 191**,** 252-255.

Koornneef, M. Bentsink, L. & Hilhorst, H. 2002. Seed dormancy and germination. *Current Opinion in Plant Biology,* 5**,** 33-36.

Koornneef, M. Léon-Kloosterziel, K. M. Schwartz, S. H. & Zeevaart, J. a. D. 1998. The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in Arabidopsis. *Plant Physiology and Biochemistry,* 36**,** 83-89.

Koster, K. L. & Lynch, D. V. 1992. Solute acclimation and compartmentation during the cold-acclimation of Puma Rye. *Plant Physiology,* 98**,** 108-113.

Kovalchuk, I. Lyudvikova, Y. Volgina, M. & Reed, B. M. 2009. Medium, container and genotype all influence *in vitro* cold storage of apple germplasm. *Plant Cell, Tissue and Organ Culture,* 96**,** 127-136.

Kraj, W. & Dolnicki, A. 2003. The influence of PPM upon the sterility of the *in vitro* cultures in European beech (*Fagus sylvatica* L.). *Acta Societatis Botanicorum Poloniae,* 72**,** 303-307.

Kreps, J. A. Wu, Y. J. Chang, H. S. Zhu, T. Wang, X. & Harper, J. F. 2002. Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. *Plant Physiology,* 130**,** 2129-2141.

Kučera, V. V, C. Vyvadilová, M. & Klíma, M. 2006. Hybrid breeding of cauliflower using self-incompatibility and cytoplasmic male sterility. *Horticulture Science,* 33**,** 148–152.

Kuiper, D. 1988. Growth Responses of *Plantago major* L. ssp. pleiosperma (Pilger) to Changes in Mineral Supply : Evidence for Regulation by Cytokinins. *Plant Physiol.,* 87**,** 555-557.

Kuiper, D. Kuiper, P. J. C. Lambers, H. Schuit, J. & Staal, M. 1989. Cytokinin concentration in relation to mineral nutrition and benzyladenine treatment in Plantago major ssp. pleiosperma. *Physiologia Plantarum,* 75**,** 511-517.

Kumar, A. Kumar, V. A. & Kumar, J. 1993. Rapid *in vitro* propagation of cauliflower. *Plant Science,* 90**,** 175-178.

Kumar, V. A. Kumar, A. & Kumar, J. 1992. In-vitro plant regeneration of cauliflower (*Brassica oleracea* convar botrytis var botrytis) from mature curd. *Indian Journal of Agricultural Sciences,* 62(7)**,** 429-431.

Kumria, R. Sunnichan, V. G. Das, D. K. Gupta, S. K. Reddy, V. S. Bhatnagar, R. K. & Leelavathi, S. 2003. High-frequency somatic embryo production and maturation into normal plants in cotton (*Gossypium hirsutum*) through metabolic stress. *Plant Cell Reports,* 21**,** 635-639.

Kurkela, S. & Franck, M. 1990. Cloning and characterization of a cold-inducable and ABA-inducable Arabidopsis gene. *Plant Molecular Biology,* 15**,** 137-144.

Kwak, J. M. Mori, I. C. Pei, Z.-M. Leonhardt, N. Torres, M. A. Dangl, J. L. Bloom, R. E. Bodde, S. Jones, J. D. G. & Schroeder, J. I. 2003. NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. *EMBO J,* 22**,** 2623-2633.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* 227**,** 680-685.

Lakshmana Rao, P. V. & Singh, B. 1991. Plantlet regeneration from encapsulated somatic embryos of hybrid *Solanum melongena* L. *Plant Cell Reports,* 10**,** 7-11.

Lang, V. & Palva, E. T. 1992. The expression of a Rab-related gene, RAB18, is induced by Abscisc-acid during the cold acclimation process of *Arabidopsis-thaliana* (L) Heynh. *Plant Molecular Biology,* 20**,** 951-962.

Larkin, M. A. Blackshields, G. Brown, N. P. Chenna, R. Mcgettigan, P. A. Mcwilliam, H. Valentin, F. Wallace, I. M. Wilm, A. Lopez, R. Thompson, J. D. Gibson, T. J. & Higgins, D. G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics,* 23**,** 2947-2948.

Larkin, P. J. Davies, P. A. & Tanner, G. J. 1988. Nurse culture of low numbers of Medicago and Nicotiana protoplasts using calcium alginate beads. *Plant Science,* 58**,** 203-210.

Last, D. I. & Brettell, R. I. S. 1990. Embryo yield in wheat anther culture is influenced by the choice of sugar in the culture medium. *Plant Cell Reports,* 9**,** 14-16.

Lata, H. Chandra, S. Khan, I. & Elsohly, M. 2009. Propagation through alginate encapsulation of axillary buds of *Cannabis sativa* L. an important medicinal plant. *Physiology and Molecular Biology of Plants,* 15**,** 79-86.

Lazzeri, P. A. & Dunwell, J. M. 1984. *In vitro* regeneration from seedling root segments of *Brassica oleracea* and *Brassica napus* cultivars. *Annals of Botany,* 54**,** 341-350.

Lazzeri, P. A. & Dunwell, J. M. 1986. *In vitro* regeneration from seedling organs of *Brassica oleracea* var. italica Plenck cv. Green Comet. Effect of plant growth rwgulators. *Annals of Botany,* 58**,** 689-697.

Lee, B. H. Henderson, D. A. & Zhu, J. K. 2005. The Arabidopsis cold-responsive transcriptome and its regulation by ICE1. *Plant Cell,* 17(11)**,** 3155-3175.

Lee, S. C. & Luan, S. 2012. ABA signal transduction at the crossroad of biotic and abiotic stress responses. *Plant, Cell & Environment*.

Leifert, C. Camotta, H. & Waites, W. M. 1992. Effect of combinations of antibiotics on micropropagated Clematis, Delphinium, Hosta, Iris, and Photinia. *Plant Cell Tiss. Organ Cult,* 29**,** 153-160.

Leifert, C. & Cassells, A. C. 2001. Microbial hazards in plant tissue and cell cultures. *In Vitro Cellular & Developmental Biology-Plant,* 37**,** 133-138.

Leonkloosterziel, K. M. Gil, M. A. Ruijs, G. J. Jacobsen, S. E. Olszewski, N. E. Schwartz, S. H. Zeevaart, J. a. D. & Koornneef, M. 1996. Isolation and characterization of abscisic acid-deficient Arabidopsis mutants at two new loci. *Plant Journal,* 10**,** 655-661.

Leroy, X. J. Leon, K. Charles, G. & Branchard, M. 2000. Cauliflower somatic embryogenesis and analysis of regenerant stability by ISSRs. *Plant Cell Reports,* 19**,** 1102-1107.

Leshem, B. Werker, E. & Shalev, D. P. 1988. The effect of cytokinins on vitrification in melon and carnation. *Annals of Botany,* 62**,** 271-276.

Lessey, E. C. Mo, B. L. Yu, X. Z. & Lessey, B. A. 2005. The Chinese herb, *Prunella vulgaris*, exhibits anti-estrogenic activity and inhibits proliferation of the endometrial cancer cell line, ECC-1. *Journal of the Society for Gynecologic Investigation,* 12**,** 14.

Leung, J. & Giraudat, J. 1998. Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology,* 49**,** 199-222.

Leustek, T. & Kirby, E. 1990. Selection and physiology of cell cultures of Douglas-fir grown under conditions of water stress. *Tree Physiology,* 6**,** 317-327.

Leustek, T. & Kirby, E. G. 1988. The influence of glutamine on growth and viability of cell suspension cultures of Douglas-fir after exposure to polyethylene glycol. *Tree Physiology,* 4**,** 371-380.

Levin, R. Gaba, V. Tal, B. Hirsch, S. Denola, D. & Vasil, I. K. 1988. Automated Plant Tissue Culture for Mass Propagation. *Nat Biotech,* 6**,** 1035-1040.

Levitt, J. 1960. Some problems in drought resistance. *Bulletin of the Research Council of Israel. Section D. Botany,* 8**,** 173-80.

Levitt, J. 1980. *Responses of Plants to Environmental Stress.Chilling, Freezing, and High Temperature Stresses,* New York, Academic Press.

Li, W. Wang, Z. Mi, G. Han, X. & Zhang, F. 2001. Molybdenum deficiency in winter wheat seedlings as enhanced by freezing temperature. *Journal of Plant Nutrition,* 24**,** 1195-1203.

Lichtenthaler, H. K. 2006. The stress concept in plants: an introduction. *Annals of the New York Academy of Sciences,* 851**,** 187-198.

Lin, C. & Thomashow, M. F. 1992. DNA sequence analysis of a complementary DNA for cold-regulated Arabidopsis gene cor15 and characterization of the COR 15 polypeptide. *Plant physiology,* 99**,** 519-525.

Linden, L. 2002. Measuring cold hardiness in woody plants*,* Finland, University of Helsinki.

Lisse, T. Bartels, D. Kalbitzer, H. R. & Jaenicke, R. 1996. The recombinant dehydrin-like desiccation stress protein from the resurrection plant Craterostigma plantagineum displays no defined three-dimensional structure in its native state. *Biological chemistry,* 377**,** 555-562.

Liu, Q. Kasuga, M. Sakuma, Y. Abe, H. Miura, S. Yamaguchi-Shinozaki, K. & Shinozaki, K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in Arabidopsis. *Plant Cell,* 10**,** 1391-1406.

LukoševičiūTė, V. Rugienius, R. Stanienė, G. Stanys, V. & Baniulis, D. 2009. Thermostable protein expression during hardening of sweet and sour cherry microshoots *in vitro*. *Sodininkystė ir Daržininkystė,* 28**,** 35-43.

Lulsdorf, M. M. Tautorus, T. E. Kikcio, S. I. Bethune, T. D. & Dunstan, D. I. 1993. Germenation of encapsulated embryos of interior spruce (*Picea-glauca-engelmann*i complex) and black spruce (*Picea-mariqana* Mill). *Plant Cell Reports,* 12**,** 385-389.

Lunghusen, J. 1998a. An effective biocide for plant tissue culture. *Australian Horticulture***,** 46-48.

Lunghusen, J. 1998b. An effective biocide for plant tissue culture. *Aust Hortic Sci,* 96**,** 45-48.

Lux-Endrich, A. Treutter, D. & Feucht, W. 2000. Influence of nutrients and carbohydrate supply on the phenol composition of apple shoot cultures. *Plant Cell, Tissue and Organ Culture,* 60**,** 15-21.

Lynch, D. V. & Steponkus, P. L. 1987. Plasma-membrane lipid alternation assosiated with cold-acclimation of winter rye seedlings (Secale-Cereale-CV Puma). *Plant Physiology,* 83**,** 761-767.

Machii, H. 1992. *In vitro* growth of encapsulated adventitious buds in Mulberry, *Morus alba* L. *Jap. J. Breed,* 42**,** 553-559.

Machii, H. & Yamanouchi, H. 1993. Growth of mulberry synthetic seeds on vermiculite, sand and soil media. *J. Seric.Sci. Jpn,* 62**,** 85-87.

Mahajan, S. & Tuteja, N. 2005. Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics,* 444**,** 139-158.

Mahmood, R. 2002. *In vitro* effect of salt on the vigor of potato (*Solanum tuberosum* L.) plantlets. *International Journal of Biotechnology,* 1.

Mandal, J. Pattnaik, S. & Chand, P. 2000. Alginate encapsulation of axillary buds of *Ocimum americanum* L. (hoary basil), *Basilicum* L. (sweet basil), *Gratissimum* L. (shrubby basil), and Sanctum L. *In Vitro Cellular; Developmental Biology - Plant,* 36**,** 287-292.

Mansfield, S. & Briarty, L. 1992. Cotyledon cell development in *Arabidopsis thaliana* during reserve deposition. *Canadian journal of botany,* 70**,** 151-164.

Mante, S. & Tepper, H. B. 1983. Production of *Musa textiles* cv. Nee plants from apical meristem slices *in vitro*. *Plant Cell, Tissue and Organ Culture,* 2**,** 151-159.

Mantyla, E. Lang, V. & Palva, E. T. 1995. Role of Abscisc acid in drought-induced freezing tolerance, cold acclimation and accumulation of LT178 AND RAB18 proteins in *Arabidopsis thaliana*. *Plant Physiology,* 107**,** 141-148.

Marc, R. K. Alexander, W. S. Helen, J. R. & Paul, M. L. Calcium-regulation of cold gene expression Society for Experimental Biology Annual Meeting, 2010 Prague. Portland Press, 306.

Margara, J. 1969. Etude des facteurs de la neoformation de bourgeons en culture *in vitro* chez le choufleur (*Brassica oleracea* L., var. botrytis). *Annals de Physiologie vegetale,* 11**,** 95-112.

Mariani, C. De Beuckeleer, M. Truettner, J. Leemans, J. & Goldberg, R. B. 1990. induction of male sterility in plants by a chimaeric ribonuclease gene. *Nature,* 347**,** 737-741.

Maruyama, K. Sakuma, Y. Kasuga, M. Ito, Y. Seki, M. Goda, H. Shimada, Y. Yoshida, S. Shinozaki, K. & Yamaguchi-Shinozaki, K. 2004. Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. *The Plant Journal,* 38**,** 982-993.

Mathur, J. Ahuja, P. S. Lal, N. & Mathur, A. K. 1989. Propagation of *Valeriana wallichii* DC. using encapsulated apical and axial shoot buds. *Plant Science,* 60**,** 111-116.

Matthew, A. J. 2005. *Plant Abiotic stress,* Indiana-USA, Blackwell.

Mckersie, B. D. & Bowley, S. R. 1997. Active oxygen and freezing tolerance in transgenic plants. *Plant Cold Hardiness. Plenum Press, New York***,** 203-214.

Mckhann, H. Gery, C. Berard, A. Leveque, S. Zuther, E. Hincha, D. De Mita, S. Brunel, D. & Teoule, E. 2008. Natural variation in CBF gene sequence, gene expression and freezing tolerance in the Versailles core collection of *Arabidopsis thaliana*. *BMC Plant Biology,* 8**,** 105.

Mediavilla, S. & Escudero, A. 2004. Stomatal responses to drought of mature trees and seedlings of two co-occurring *Mediterranean oaks*. *Forest Ecology and Management,* 187**,** 281-294.

Medina, J. Bargues, M. Terol, J. Pérez-Alonso, M. & Salinas, J. 1999. The Arabidopsis CBF gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiology,* 119**,** 463-470.

Mendel, R. R. & Hansch, R. 2002. Molybdoenzymes and molybdenum cofactor in plants. *Journal of Experimental Botany,* 53**,** 1689-1698.

Mendoza, M. & Kaeppler, H. 2002. Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat (*Triticum aestivum* L.). *In Vitro Cellular and Development Biology - Plant,* 38**,** 39-45.

Metwali Gharieb, E. M. R. 2006. *Improving abiotic stress resistance in cauliflower (Brassica oleracea var. botrytis L.) by mutagenesis and agrobacterium transformation.* PhD, University of Plymouth.

Meza-Basso, L. Alberdi, M. Raynal, M. Ferrero-Cadinanos, M. L. & Delseny, M. 1986. Changes in protein synthesis in rapeseed (*Brassica napus*) seedlings during a low temperature treatment. *Plant Physiology,* 82**,** 733.

Michel, Z. Hilaire, K. T. Mongomake, K. Georges, A. N. & Justin, K. Y. 2008. Effect of genotype, explants, growth regulators and sugars on callus induction in cotton (*Gossypium hirsutum* L.). *Australian Journal of Crop Science,* 2**,** 1-9.

Micheli, M. Hafiz, I. A. & Standardi, A. 2007. Encapsulation of *in vitro*-derived explants of olive (*Olea europaea* L. cv. Moraiolo): II. Effects of storage on capsule and derived shoots performance. *Scientia Horticulturae,* 113**,** 286-292.

Mohamed, M. Rahman, R. A. Parklanathan, L. Slang, T., K & Sharuddin, N., A. 2009. *http://www.scribd.com/doc/22585470/Application-of-Synthetic-Seeds-Present-and-the-Future* [Online]. [Accessed 28/12/2009 2009].

Mohamed, M. a. H. & Alsadon, A. A. 2010. Influence of ventilation and sucrose on growth and leaf anatomy of micropropagated potato plantlets. *Scientia Horticulturae,* 123**,** 295-300.

Mori, I. C. & Muto, S. 1997. Abscisic acid activates a 48-kilodalton protein kinase in guard cell protoplasts. *Plant Physiology,* 113**,** 833-839.

Mukhopadhyay, A. Arumugam, N. Nadakumar, P. B. A. Pradhan, A. K. Gupata, V. & Pental, D. 1992. Agrobacterium-mediated genetic transformation of oilseed *Brassica campestris*: Transformation frequency is strongly influenced by the mode of shoot regeneration. *Plant cell reports,* 11**,** 506-513.

Murashige, T. & Skoog, F. 1962. A revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum,* 15**,** 473-497.

Murata, M. & Orton, T. J. 1987. Callus initiation and regeneration capacities in *Brassica* species. *Plant Cell, Tissue and Organ Culture,* 11**,** 111-123.

Murelli, C. Rizza, F. Albini, F. M. Dulio, A. Terzi, V. & Cattivelli, L. 1995. Metabolic changes assosiated with cold-acclimation in contrasting cultivars pf barley. *Physiologia Plantarum,* 94**,** 87-93.

Muthalif, M. M. & Rowland, L. J. 1994. Identification of dehydrin-like proteins responsive to chilling in floral buds of blueberry (Vaccinium, section Cyanococcus). *Plant Physiology,* 104**,** 1439-1447.

Naik, S. K. & Chand, P. K. 2006. Nutrient-alginate encapsulation of *in vitro* nodal segments of pomegranate (*Punica granatum* L.) for germplasm distribution and exchange. *Scientia Horticulturae,* 108**,** 247-252.

Nakaminami, K. Karlson, D. T. & Imai, R. 2006. Functional conservation of cold shock domains in bacteria and higher plants. *Proc Natl Acad Sci U S A,* 103**,** 10122-10127.

Nakashimada, Y. Uozumi, N. & Kobayashi, T. 1995. Production of plantlets for use as artificial seeds from horseradish hairy roots fragmented in a blender. *Journal of Fermentation and Bioengineering,* 79**,** 458-464.

Narasimhulu, S. B. & Chopra, V. L. 1987. Plant regeneration from callus cultures of *Brassica carinata* A. Br. and its implications to improvement of oil seed Brassicas. *Plant Breeding,* 99**,** 9-55.

Narasimhulu, S. B. & Chopra, V. L. 1988. Species-specific shoot regeneration response of cotyledonary explants of brassicas. *Plant Cell Reports,* 7**,** 104-106.

Narusaka, Y. Nakashima, K. Shinwari, Z. K. Sakuma, Y. Furihata, T. Abe, H. Narusaka, M. Shinozaki, K. & Yamaguchi-Shinozaki, K. 2003. Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent

expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. *The Plant Journal,* 34**,** 137-148.

Nelson, D. Salamini, F. & Bartels, D. 1994. Abscisc acid promotes novel DNA-binding activity to a dessication-related promoter of *Craterostigma-plantagineum*. *Plant Journal,* 5**,** 451-458.

Ni, M. Dehesh, K. Tepperman, J. M. & Quail, P. H. 1996. GT-2: *in vivo* transcriptional activation activity and definition of novel twin DNA binding domains with reciprocal target sequence selectivity. *The Plant Cell Online,* 8**,** 1041-1059.

Niedz, R. 1998. Using isothiazolone biocides to control microbial and fungial contaminants in plant tissue cultures. *Hort Technol,* 8**,** 598-601.

Nishizawa, A. Yabuta, Y. & Shigeoka, S. 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. *Plant physiology,* 147**,** 1251-1263.

Nkang, A. 2002. Carbohydrate composition during seed development and germination in two sub-tropical rainforest tree species (*Erythrina caffra* and *Guilfoylia monostylis*). *Journal of plant physiology,* 159**,** 473-483.

Nomura, M. Muramoto, Y. Yasuda, S. Takabe, T. & Kishitani, S. 1995. The acclimation of glycinebetaine during cold-acclimation early and late cultivars of barley. *Euphytica,* 83**,** 247-250.

Norton, G. & Harris, J. F. 1975. Compositional changes in developing rape seed (*Brassica napus* L.). *Planta,* 123**,** 163-174.

Nowak, B. Miczy´Nski, K. & Hudy, L. 2004. Sugar uptake and utilisation during adventitious bud differentiation on *in vitro* leaf explants of plum (*Prunus domestica*). *Plant Cell Tissue and Organ Culture Organ. Culture,* 76**,** 255-260.

Nyende, A. Schittenhelm, S. Mix-Wagner, G. & Greef, J.-M. 2003. Production, storability, and regeneration of shoot tips of potato (*Solanum tuberosum* L.) encapsulated in calcuim alginate hollow beads. *In Vitro Cellular; Developmental Biology - Plant,* 39**,** 540-544.

Nyende, A. B. Schittenhelm, S. Mix-Wagner, G. & Greef, J. M. 2005. Yield and canopy development of field grown potato plants derived from synthetic seeds. *European Journal of Agronomy,* 22**,** 175-184.

Nylander, M. Svensson, J. Palva, E. T. & Welin, B. V. 2001. Stress-induced accumulation and tissue-specific localization of dehydrins in *Arabidopsis thaliana*. *Plant molecular biology,* 45**,** 263-279.

Olvera-Carrillo, Y. Campos, F. Reyes, J. L. Garciarrubio, A. & Covarrubias, A. A. 2010. Functional analysis of the group 4 late embryogenesis abundant proteins reveals their relevance in the adaptive response during water deficit in Arabidopsis. *Plant physiology,* 154**,** 373-390.

Onay, A. Jeffree, C. E. & Yeoman, M. M. 1996. Plant regeneration from encapsulated embryoids and an embryogenic mass of pistachio, *Pistacia vera* L. *Plant Cell Reports,* 15**,** 723-726.

Onishi, N. Sakamoto, Y. & Hirosawa, T. 1994. Synthetic seeds as an applicationof mass-production of somatic embryos. *Plant Cell Tissue and Organ Culture,* 39**,** 137-145.

Ovesná, J. Ptáček, L. & Opatrný, Z. 1993. Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments. *Biologia Plantarum,* 35**,** 107-112.

Ovesna, L. Ptacek, L. & Opatrny, Z. 1993. Factors influencing the regeneration capacity of oilseed rape and cauliflower in transformation experiments. *Biologia Plantarum,* 35**,** 107-112.

Owens, C. L. Thomashow, M. F. Hancock, J. F. & Iezzoni, A. F. 2002. CBF1 orthologs in sour cherry and strawberry and the heterologous expression of CBF1 in strawberry. *Journal of the American Society for Horticultural Science,* 127**,** 489-494.

Ozyigit, I. I. 2008. Phenolic changes during *in vitro* organogenesis of cotton (*Gossypium hirsutum* L.) shoot tips. *Afr. J. Biotechnol,* 7**,** 1145-1150.

PaˆQues, M. & Boxus, P. 1987. A model to learn 'vitrification', the rootstock apple M.26 — present results. *Acta Hort,* 212**,** 193–210.

Paiva, R. & Kriz, A. L. 1994. Effect of abscesic-acid on embryo-specific gene-exoression during normal and precocious germination in normal and vivoarous maize (*Zea mays*) embryos. *Planta,* 192**,** 332-339.

Pande, H. & Bhojwani, S. S. 1999. Promotion of Androgenesis in Rice Anther Cultures by Substitution of Sucrose with Maltose and Mannitol. *Biologia Plantarum,* 42**,** 125-128.

Pandey, V. P. Cherian, E. & Patani, G. 2010. Effect of Growth Regulators and Culture Conditions on Direct Root Induction of *Rauwolfia serpentina* L. (Apocynaceae) Benth by Leaf Explants. *Tropical Journal of Pharmaceutical Research,* 9**,** 27-34.

Panta, G. R. Rieger, M. W. & Rowland, L. J. 2001. Effect of cold and drought stress on blueberry dehydrin accumulation. *Journal of Horticultural Science and Biotechnology,* 76**,** 549-556.

Pareek, L. K. & Chandra, N. 1978. Somatic embryogensis in leaf callus from cauliflower (*Brassica-oleracea* var-botrytis). *Plant Science Letters,* 11**,** 311-316.

Parmentier-Line, C. M. Panta, G. R. & Rowland, L. J. 2002. Changes in dehydrin expression associated with cold, ABA and PEG treatments in blueberry cell cultures. *Plant science,* 162**,** 273-282.

Pattison, D. Silman, A. Goodson, N. Lunt, M. Bunn, D. Luben, R. Welch, A. Bingham, S. Khaw, K. & Day, N. 2004. Vitamin C and the risk of developing inflammatory polyarthritis: prospective nested case-control study. *Annals of the Rheumatic Diseases,* 63**,** 843-847.

Pattnaik, S. K. Sahoo, Y. & Chand, P. K. 1995. Efficient plant retrieval from alginate-encapsulated vegetative buds of mature mulberry trees. *Scientia Horticulturae,* 61**,** 227-239.

Patton, A. J. Cunningham, S. M. Volenec, J. J. & Reicher, Z. J. 2007. Differences in freeze tolerance of zoysiagrasses: II. Carbohydrate and proline accumulation. *Crop Science,* 47**,** 2170-2181.

Paul, H. Daigny, G. & Sangwan-Norreel, B. S. 2000. Cryopreservation of apple (*Malus domestica* Borkh.) shoot tips following encapsulation-dehydration or encapsulation-vitrification. *Plant Cell Reports,* 19**,** 768-774.

Pearce, R. S. 2001. Plant Freezing and Damage. *Annals of Botany,* 87**,** 417-424.

Pearce, R. S. & Fuller, M. P. 2001. Freezing of barley studied by infrared video thermography. *Plant Physiology,* 125**,** 227-240.

Pelletier, G. Primard, C. Vedel, F. Chetrit, P. Remy, R. Rousselle & Renard, M. 1983. Intergeneric cytoplasmic hybridization in cruciferae by protoplast fusion. *Molecular and General Genetics MGG,* 191**,** 244-250.

Pennycooke, J. C. & Towill, L. E. 2001. Medium alterations improve regrowth of sweet potato (*Lpomoea batatas* (L.) Lam.) shoot tips cryopreserved by vitrification and encapsulation-dehydration. *Cryoletters,* 22**,** 381-389.

Pérez-Pérez, J. G. Robles, J. M. Tovar, J. C. & Botía, P. 2009. Response to drought and salt stress of lemon 'Fino 49' under field conditions: Water relations, osmotic adjustment and gas exchange. *Scientia Horticulturae,* 122**,** 83-90.

Phillips, R. Arnott, S. M. & Kaplan, S. E. 1981. Antibiotics in plant tissue culture: Rifampicin effectively controls bacterial contaminants without affecting the growth of short-term explant cultures of *Helianthus tuberosus*. *Plant Science Letters,* 21**,** 235-240.

Piccioni, E. & Standardi, A. 1995. Encapsulation of micropropagated buds of 6 woody species. *Plant Cell Tissue and Organ Culture,* 42**,** 221-226.

Picoli, E. a. T. Otoni, W. C. Figueira, M. L. Carolino, S. M. B. Almeida, R. S. Silva, E. a. M. Carvalho, C. R. & Fontes, E. P. B. 2001. Hyperhydricity in *in vitro* eggplant regenerated plants: structural characteristics and involvement of BiP (Binding Protein). *Plant Science,* 160**,** 857-868.

Pla, M. Goday, A. Vilardell, J. Gómez, J. & Pagès, M. 1989. Differential regulation of ABA-induced 23–25 kDa proteins in embryo and vegetative tissues of the viviparous mutants of maize. *Plant Molecular Biology,* 13**,** 385-394.

Pla, M. Vilardell, J. Guiltinan, M. J. Marcotte, W. R. Niogret, M. F. Quatrano, R. S. & Pages, M. 1993.The Cis-regulatory element CCACGTGG is involved in ABA and water-stress responses of the maiz gene RAB28. *Plant Molecular Biology,* 21**,** 259-266.

Porra, R. J. 1997. Recent progress in porphyrin and chlorophyll biosynthesis. *Photochemistry and Photobiology,* 65**,** 492-516.

Pow, J. J. 1969. Clonal propagation *in vitro* from cauliflower curd. *Hortic Sci,* 9**,** 151–152.

Prasil, I. T. Prasilova, P. & Pankova, K. 2004. Relationships among vernalization, shoot apex development and frost tolerance in wheat. *Annals of Botany,* 94**,** 413-418.

Prewein, C. & Wilhelm, E. 2003. Plant regeneration from encapsulated somatic embryos of pedunculate oak (*Quercus Robur* L.). *In Vitro Cellular & Developmental Biology-Plant,* 39**,** 613-617.

Price, A. H. Cairns, J. E. Horton, P. Jones, H. G. & Griffiths, H. 2002. Linking drought‐resistance mechanisms to drought avoidance in upland rice using a QTL approach: progress and new opportunities to integrate stomatal and mesophyll responses. *Journal of Experimental Botany,* 53**,** 989-1004.

Pruski, K. Astatkie, T. Mirza, M. & Nowak, J. 2002. Photoautotrophic micropropagation of Russet Burbank Potato. *Plant Cell, Tissue and Organ Culture,* 69**,** 197-200.

Pua, E.-C. Trinh, T. H. & Chua, N.-H. 1989. High frequency plant regeneration from stem explants of *Brassica alboglabra* Bailay *in vitro*. *Plant Cell, Tissue and Organ Culture,* 17**,** 143-152.

Rady, M. R. & Hanafy, M. S. 2004. Synthetic seed technology for encapsulation and regrowth of *in vitro*-derived *Gypsophila paniculata* L. shoot-tips. *Arab J. Biotech,* 7**,** 251-264.

Rai, M. K. Asthana, P. Singh, S. K. Jaiswal, V. S. & Jaiswal, U. 2009. The encapsulation technology in fruit plants--A review. *Biotechnology Advances,* 27**,** 671-679.

Rai, M. K. & Jaiswa, V. S. 2008. Alginate-encapsulation of nodal segments of guava (*Psidium guajava* L.) for germplasm exchange and distribution. *J Hortic Sci Biotechnol,* 83**,** 569–573.

Rai, M. K. Jaiswal, V. S. & Jaiswal, U. 2008a. Effect of ABA and sucrose on germination of encapsulated somatic embryos of guava (*Psidium guajava* L.). *Scientia Horticulturae,* 117**,** 302-305.

Rai, M. K. Jaiswal, V. S. & Jaiswal, U. 2008b. Encapsulation of shoot tips of guava (*Psidium guajava* L.) for short-term storage and germplasm exchange. *Scientia Horticulturae,* 118**,** 33-38.

Rajashekar, C. B. & Lafta, A. 1996. Cell-Wall Changes and Cell Tension in Response to Cold Acclimation and Exogenous Abscisic Acid in Leaves and Cell Cultures. *Plant Physiology,* 111**,** 605-612.

Rani Debi, B. Taketa, S. & Ichii, M. 2005. Cytokinin inhibits lateral root initiation but stimulates lateral root elongation in rice (*Oryza sativa*). *Journal of Plant Physiology,* 162**,** 507-515.

Rao, A. H. Karunasree, B. & Reddy, A. R. 1993. Water Stress-Responsive 23 kDa Polypeptide from Rice Seedlings is Boiling stable and is Related to the RAB16 Family of Proteins. *Journal of plant physiology,* 142**,** 88-93.

Rao, K. V. M. Raghavendra, A. S. & Reddy, K. J. 2006. *Physiology and Molecular Biology of Stress Tolerance in Plants,* Printed in the Netherlands, Springer.

Rawsthorne, S. 2002. Carbon flux and fatty acid synthesis in plants. *Progress in Lipid Research,* 41**,** 182-196.

Ray, A. & Bhattacharya, S. 2008. Storage and plant regeneration from encapsulated shoot tips of *Rauvolfia serpentina* -- An effective way of conservation and mass propagation. *South African Journal of Botany,* 74**,** 776-779.

Redenbaugh, K. Nichol, J. Kossler, M. E. & Paasch, B. 1984. Encapsulation of somatic embryos for artificial seed production. *In Vitro-Journal of the Tissue Culture Association,* 20**,** 256-257.

Redenbaugh, K. Paasch, B. D. Nichol, J. W. Kossler, M. E. Viss, P. R. & Walker, K. A. 1986. Somatic seeds-encapsulation of asexual plant embryos. *Bio-Technology,* 4**,** 797-801.

Redenbaugh, K. Slade, D. Viss, P. R. & Fujii, J. A. 1987. Encapsulation of somatic embryos in synthetic seed coats. *Hortic Sci* 22**,** 803–809.

Reed, B. M. & Tanprasert, P. 1995. Detection and control of bacterial contaminants of plant tissue cultures: a review of recent literature. *Plant Tiss. Cult. Biotechnology Advances,* 1**,** 137-142.

Reed, R. C. Brady, S. R. & Muday, G. K. 1998. Inhibition of Auxin Movement from the Shoot into the Root Inhibits Lateral Root Development in Arabidopsis. *Plant Physiol.,* 118**,** 1369-1378.

Ren, J.-P. Wang, X.-G. & Yin, J. 2010. Dicamba and Sugar Effects on Callus Induction and Plant Regeneration from Mature Embryo Culture of Wheat. *Agricultural Sciences in China,* 9**,** 31-37.

Riechmann, J. Heard, J. Martin, G. Reuber, L. Keddie, J. Adam, L. Pineda, O. Ratcliffe, O. Samaha, R. & Creelman, R. 2000. Arabidopsis transcription factors: genome-wide comparative analysis among eukaryotes. *Science,* 290**,** 2105-2110.

Riechmann, J. L. & Meyerowitz, E. M. 1998. The AP2/EREBP family of plant transcription factors. *Biological chemistry,* 379**,** 633-646.

Rihan, H. Al-Issawi, M. Burchett, S. & M, P., Fuller 2011. The encapsulation of cauliflower microshoots as artificial seeds and the investigation of their capacity for conversion and growth in commercial substrates (Compost, Vermiculite, Perlite and Sand). *plant cell, tissue and organ culture.* .

Roberts, J. K. Desimone, N. A. Lingle, W. L. & Dure, L. 1993. Cellular Concentrations and Uniformity of Cell-Type Accumulation of Two Lea Proteins in Cotton Embryos. *The Plant Cell Online,* 5**,** 769-780.

Romano, A. Noronha, C. & Martins-Loução, M. A. 1995. Role of carbohydrates in micropropagation of cork oak. *Plant Cell, Tissue and Organ Culture,* 40**,** 159-167.

Rorat, T. 2006. Plant dehydrins—tissue location, structure and function. *Cellular & Molecular Biology letters,* 11**,** 536-556.

Rosegrant, M. W. & Cline, S. A. 2003. Global food security: challenges and policies. *Science,* 302**,** 1917-1919.

Rudd, J. J. & Franklin-Tong, V. E. 2001. Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. *New Phytologist,* 151**,** 7-33.

Rugienius, R. Siksnianas, T. Gelvonauskiene, D. Staniene, G. Sasnauskas, A. Zalunskaite, I. Stanys, V. Zhivondov, A. Gercheva, P. & Koumanov, K. 2009. Evaluation of genetic resources of fruit crops as donors of cold and disease resistance in Lithuania. *Acta Horticulturae,* 825**,** 117-124.

Rurek, M. 2010. Diverse accumulation of several dehydrin-like proteins in cauliflower (*Brassica oleracea* var. botrytis), *Arabidopsis thaliana* and yellow lupin (Lupinus luteus) mitochondria under cold and heat stress. *BMC plant biology,* 10**,** 181.

Sadik, S. 1962. Morphology of the curd of cauliflower. *American Journal of Botany,* 49**,** 290-297.

Sadik, S. & Ozbun, J. 1968. Development of Vegetative and Reproductive Apices of Cauliflower, *Brassica oleraceae* var. Botrytis. *Botanical Gazette***,** 365-370.

Sagi, M. Scazzocchio, C. & Fluhr, R. 2002. The absence of molybdenum cofactor sulfuration is the primary cause of the flacca phenotype in tomato plants. *The Plant Journal,* 31**,** 305-317.

Saiprasad, G. 2001. Artificial seeds and their applications. *Resonance,* 6**,** 39-47.

Sakai, A. & Larcher, W. 1987. *Frost Survival of Plants,* Berlin, Springer.

Sakuma, Y. Liu, Q. Dubouzet, J. G. Abe, H. Shinozaki, K. & Yamaguchi-Shinozaki, K. 2002. DNA-Binding Specificity of the ERF/AP2 Domain of Arabidopsis DREBs, Transcription Factors Involved in Dehydration- and Cold-Inducible Gene Expression. *Biochemical and Biophysical Research Communications,* 290**,** 998-1009.

Salter, P. 1960. The growth and development of early summer cauliflower in relation to environmental factors. *Journal of Horticultural Science,* 35**,** 21-33.

Salvi, N. D. George, L. & Eapen, S. 2002. Micropropagation and field evaluation of micropropagated plants of turmeric. *Plant Cell, Tissue and Organ Culture,* 68**,** 143-151.

Sarkar, D. & Naik, P. S. 1998. Synseeds in potato: an investigation using nutrient-encapsulated *in vitro* nodal segments. *Scientia Horticulturae,* 73**,** 179-184.

Sato, S. Hagimori, M. & Iwai, S. 1993. Recovering vitrified carnation (*Dianthus caryophyllus* L.) shoots using Bacto-Peptone and its subfractions. *Plant Cell Reports,* 12**,** 370-374.

Sattler, U. Calsou, P. Boiteux, S. & Salles, B. 2000. Detection of oxidative base DNA damage by a new biochemical assay. *Archives of biochemistry and biophysics,* 376**,** 26-33.

Sauter, A. Davies, W. J. & Hartung, W. 2001. The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *Journal of Experimental Botany,* 52**,** 1991-1997.

Schroeder, J. I. Kwak, J. M. & Allen, G. J. 2001. Guard cell abscisic acid signalling and engineering drought hardiness in plants. *nature,* 410**,** 327-330.

Schroeder, J. I. Raschke, K. & Neher, E. 1987. Voltage dependence of K^+ channals in guard-cell protoplasts. *Proceedings of the National Academy of Sciences of the United States of America,* 84**,** 4108-4112.

Schwartz, S. H. Qin, X. & Zeevaart, J. a. D. 2003. Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology,* 131**,** 1591-1601.

Seckinger, G. 1995. The use of antibiotics in plant tissue culture. *In Vitro Cell. Dev. Biol. Plant,* 31**,** 25-28.

Seki, M. Kamei, A. Yamaguchi-Shinozaki, K. & Shinozaki, K. 2003. Molecular responses to drought, salinity and frost: common and different paths for plant protection. *Current Opinion in Biotechnology,* 14**,** 194-199.

Seki, M. Narusaka, M. Abe, H. Kasuga, M. Yamaguchi-Shinozaki, K. Carninci, P. Hayashizaki, Y. & Shinozaki, K. 2001. Monitoring the expression pattern of 1300 Arabidopsis genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell,* 13**,** 61-72.

Seki, M. Narusaka, M. Ishida, J. Nanjo, T. Fujita, M. Oono, Y. Kamiya, A. Nakajima, M. Enju, A. Sakurai, T. Satou, M. Akiyama, K. Taji, T. Yamaguchi-Shinozaki, K. Carninci, P. Kawai, J. Hayashizaki, Y. & Shinozaki, K. 2002. Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. *The Plant Journal,* 31**,** 279-292.

Shahnewaz, S. & Bari, A., M 2004. Effect of Concentration of Sucrose on the Frequency of Callus Induction and Plant Regeneration in Anther Culture of Rice (*Oryza sativa* L.). *Plant Tissue Cult,* 14(1)**,** 37-43.

Shanker, A. K. & Venkateswarlu, B. 2011. Abiotic stress response in plants-physiological, biochemical and genetic perspectives*,* Printed in Croatia, INTECH open access Publisher.

Sharma, K. K. Bhojwani, S. S. & Thorpe, T. A. 1990. Factors affecting high frequency differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Science,* 66**,** 247-253.

Sharma, K. K. Bhojwani, S. S. & Thorpe, T. A. 1991. The role of cotyledonary tissue in the differentiation of shoots and roots from cotyledon explants of *Brassica juncea* (L.) Czern. *Plant Cell, tissue and organ culture,* 24.

Sharma, P. Sharma, N. & Deswal, R. 2005. The molecular biology of the low-temperature response in plants. *Bioessays,* 27**,** 1048-1059.

Sharma, T. R. Singh, B. M. & Chauhan, R. S. 1994. Production of disease free encapsulated buds of *Zingiber officinale* Rosc. *Plant Cell Rep,* 13**,** 300–302.

Shinozaki, K. & Yamaguchi-Shinozaki, K. 1996. Molecular responses to drought and cold stress. *Current Opinion in Biotechnology,* 7**,** 161-167.

Shinozaki, K. & Yamaguchi-Shinozaki, K. 2000. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current Opinion in Plant Biology,* 3**,** 217-223.

Shinozaki, K. Yamaguchi-Shinozaki, K. & Seki, M. 2003. Regulatory network of gene expression in the drought and cold stress responses. *Current Opinion in Plant Biology,* 6**,** 410-417.

Shinwari, Z. K. Nakashima, K. Miura, S. Kasuga, M. Seki, M. Yamaguchi-Shinozaki, K. & Shinozaki, K. 1998. An Arabidopsis gene family encoding DRE/CRT binding proteins involved in low-temperature-responsive gene expression. *Biochemical and Biophysical Research Communications,* 250**,** 161-170.

Siloto, R. M. P. Findlay, K. Lopez-Villalobos, A. Yeung, E. C. Nykiforuk, C. L. & Moloney, M. M. 2006. The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. *The Plant Cell Online,* 18**,** 1961-1974.

Singh, A. K. Varshney, R. Sharma, M. Agarwal, S. S. & Bansal, K. C. 2006. Regeneration of plants from alginate-encapsulated shoot tips of *Withania somnifera* (L.) Dunal, a medicinally important plant species. *Journal of Plant Physiology,* 163**,** 220-223.

Singh, S. 1988. *In vitro* plantlet from internode and inflorescence axis of *Brassica oleracea* var. botrytis. *Current Science,,* 57(13)**,** 730-732.

Singh, S. Rai, M. Asthana, P. Pandey, S. Jaiswal, V. & Jaiswal, U. 2009. Plant regeneration from alginate-encapsulated shoot tips of *Spilanthes acmella* L. Murr. a medicinally important and herbal pesticidal plant species. *Acta Physiologiae Plantarum,* 31**,** 649-653.

Skoog, F. & Miller, C. O. 1957 Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symp Soc Exp Biol* 11**,** 118–131.

Ślesak, H. Skoczowski, A. & Przywara, L. 2004. Exogenous Carbohydrate Utilisation by Explants of *Brassica napus* cultured *in vitro*. *Plant Cell, Tissue and Organ Culture,* 79**,** 45-51.

Smalle, J. & Vierstra, R. D. 2004. The ubiquitin 26S proteasome proteolytic pathway. *Annual Review of Plant Biology,* 0**,** null.

Smallwood, M. & Bowles, D. J. 2002. Plants in a cold climate. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 357 (1423)**,** 831-847.

Soneji, J. R. Rao, P. S. & Mhatre, M. 2002. Germination of synthetic seeds of pineapple (Ananas comosus L. Merr.). *Plant Cell Rep,* 20**,** 891-894.

Song, K. Osborn, T. C. & Williams, P. H. 1990. *Brassica* taxonomy based on nuclear restriction fragment length polymorphisms (RFLPs). *TAG Theoretical and Applied Genetics,* 79**,** 497-506.

Standardi, A. & Piccioni, E. 1998. Recent Perspectives on Synthetic Seed Technology Using Nonembryogenic *In vitro*-Derived Explants. *International Journal of Plant Sciences,* 159**,** 968-978.

Steponkus, P. L. Rains, D. Valentine, R. & Hollaender, A. 1980. A unified concept of stress in plants? Genetic engineering of osmoregulation*. Impact on plant productivity for food, chemicals, and energy.***,** 235-255.

Steponkus, P. L. Uemura, M. Joseph, R. A. Gilmour, S. J. & Thomashow, M. F. 1998. Mode of action of the COR15a gene on the freezing tolerance *of Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 14570-14575.

Steponkus, P. L. Uemura, M. & Webb, M. 1993. Membrane destabilization during freeze-induced dehydration. *Current Topics in Plant Physiology,* 10**,** 37.

Stitt, M. & Hurry, V. 2002. A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. *Current Opinion in Plant Biology,* 5**,** 199-206.

Stockinger, E. J. Gilmour, S. J. & Thomashow, M. F. 1997. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates

transcription in response to low temperature and water deficit. *Proceedings of the National Academy of Sciences,* 94**,** 1035-1040.

Sujatha, G. & Kumari, B. D. R. 2008. Micropropagation, encapsulation and growth of *Artemisia vulgaris* node explants for germplasm preservation. *South African Journal of Botany,* 74**,** 93-100.

Sul, I.-W. & Korban, S., S 1998. Effects of media, carbon sources and cytokinins on shoot organogenesis in the Christmas tree Scots pines (*Pinus sylvestris* L.). *J Hort. Sci. Biotech,* 73**,** 822–827.

Sun, X. Hu, C. & Tan, Q. 2006a. Effects of molybdenum on antioxidative defense system and membrane lipid peroxidation in winter wheat under low temperature stress. *Journal of Plant Physiology and Molecular Biology,* 32**,** 175.

Sun, X. Hu, C. Tan, Q. & Gan, Q. 2006b. Effects of molybdenum on photosynthetic characteristics in winter wheat under low temperature stress. *Zuo wu xue bao,* 32**,** 1418-1422.

Sun, X. Hu, C. Tan, Q. Liu, J. & Liu, H. 2009. Effects of molybdenum on expression of cold-responsive genes in abscisic acid (ABA)-dependent and ABA-independent pathways in winter wheat under low-temperature stress. *Annals of Botany*.

Sundararaj, S. G. Agrawal, A. & Tyagi, R. K. 2010. Encapsulation for *in vitro* short-term storage and exchange of ginger (*Zingiber officinale* Rosc.) germplasm. *Scientia Horticulturae,* 125**,** 761-766.

Sung, D.-Y. Kaplan, F. Lee, K.-J. & Guy, C. L. 2003. Acquired tolerance to temperature extremes. *Trends in Plant Science,* 8**,** 179-187.

Sutter, E. & Langhans, R. W. 1979. Epicuticular wax formation on carnation plantlets regenerated from shoot tip culture. *J. Am. Soc. Hort. Sci,* 104**,** 493–496.

Swamy, P., M & Smith, B. 1999. Role of abscisic acid in plant stress tolerance. *Current Science,* 76**,** 1220–1227.

Takahata, Y. Brown, D. C. W. Keller, W. A. & Kaizuma, N. 1993. Dry artificial seeds and desiccation tolerance induction in microspore-derived embryos of broccoli. *Plant Cell, Tissue and Organ Culture,* 35**,** 121-129.

Tal, M. 1983. Selection for stress tolerance. *Handbook of plant cell culture,* 1.

Tao, D.-L. Öquist, G. & Wingsle, G. 1998. Active Oxygen Scavengers during Cold Acclimation of Scots Pine Seedlings in Relation to Freezing Tolerance. *Cryobiology,* 37**,** 38-45.

Teige, M. Scheikl, E. Eulgem, T. Dóczi, R. Ichimura, K. Shinozaki, K. Dangl, J. L. & Hirt, H. 2004. The MKK2 Pathway Mediates Cold and Salt Stress Signaling in Arabidopsis. *Molecular Cell,* 15**,** 141-152.

Teng, W. L. & Ngai, Y. W. 1999. Regeneration of *Oxalis triangularis* ssp. triangularis from suspension cells cultured in three different systems (solid, liquid-flask and bioreactor cultures). *Plant Cell Reports,* 18**,** 701-706.

Teng, W. L. & Teng, M. C. 1997. *In vitro* regeneration patterns of *Platycerium bifurcatum* leaf cell suspension culture. *Plant Cell Reports,* 16**,** 820-824.

Thapa, R. Dhakal, D. & Gauchan, P., Dhurva 2007. Effect of different sugars on shoot induction in CV.Basmati. *Journal of Science and Technology,* 1.

Thomas, P. Mythili, J. B. & Shivashankara, K. S. 2000. Explant, medium and vessel aeration affect the incidence of hyperhydricity and recovery of normal plantlets in triploid watermelon. *Journal of Horticultural Science & Biotechnology,* 75**,** 19-25.

Thomas, T. H. 1999. The Physiology of Vegetable Crops. Edited by H.C. Wien. 1997. *Plant Growth Regulation,* 27**,** 137-138.

Thomas, T. H. & Lester, J. N. 1972. Hormonal changes in the stem apex of the cauliflower plant in relation to curd development. *Journal of Horticulture science,* 47**,** 449-455.

Thomashow, M. F. 1990. Molecular Genetics of Cold Acclimation in Higher Plants. *In:* JOHN, G. S. (ed.) *Advances in Genetics.* Academic Press.

Thomashow, M. F. 1999. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology,* 50**,** 571-599.

Thomashow, M. F. 2001. So what's new in the field of plant cold acclimation? Lots! *Plant Physiology,* 125**,** 89-93.

Thomashow, M. F. 2010. Molecular Basis of Plant Cold Acclimation: Insights Gained from Studying the *CBF* Cold Response Pathway. *Plant Physiology,* 154**,** 571-577.

Titov, S. Bhowmik, S. K. Mandal, A. Alam, M. S. & Uddin, S. N. 2006. Control of phenolic compound secretion and effect of growth regulators for organ formation from *Musa spp*. cv. 'Kanthali' floral bud explants. *American Journal of Biochemistry and Biotechnology,* 2**,** 97-104.

Tomlinson, K. L. Mchugh, S. Labbe, H. Grainger, J. L. James, L. E. Pomeroy, K. M. Mullin, J. W. Miller, S. S. Dennis, D. T. & Miki, B. L. A. 2004. Evidence that the hexose-to-sucrose ratio does not control the switch to storage product accumulation in oilseeds: analysis of tobacco seed development and effects of overexpressing apoplastic invertase. *Journal of Experimental Botany,* 55**,** 2291-2303.

Ton, J. Flors, V. & Mauch-Mani, B. 2009. The multifaceted role of ABA in disease resistance. *Trends in Plant Science,* 14**,** 310-317.

Torres, A. Paviani, T. Caldas, L. & Della Vecchia, P. 1980. Anatomy of shoot production *in vitro* from explants of cauliflower curd (*Brassica oleracea* L. var. botrytis subvar. cauliflora DC.). *Pesquisa Agropecuária Brasileira,* 15**,** 435-440.

Towbin, H. Staehelin, T. & Gordon, J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences,* 76**,** 4350.

Trick, M. & Heizmann , P. 1992. Sporophytic self-incompatibility systems: Brassica S gene family. *International review of cytology,* 140**,** 485–524. .

Tsvetkov, I. & Hausman, J.-F. 2005. *In vitro* regeneration from alginate-encapsulated microcuttings of *Quercus sp*. *Scientia Horticulturae,* 103**,** 503-507.

Turton, S. & Fuller, M. P. 1989. Petal culture of winter-heading cauliflower. *Proceedings of the ISTS Conference,.* Cesena Italy,.

Tuteja, N. 2007. Abscisic Acid and Abiotic Stress Signaling. *Plant Signaling & Behavior,* 2**,** 135-138.

Uemura, M. Joseph, R. A. & Steponkus, P. L. 1995. Cold-acclimation of *Arabidopsis-thaliana* - effects on plasma-membrane lipid-composition and freez-induced lesions. *Plant Physiology,* 109**,** 15-30.

Uemura, M. & Steponkus, P. L. 1994. A contrast of the plasma-membrane lipid composition of Oat and Rye leaves in relation to freezing tolerance. *Plant Physiology,* 104**,** 479-496.

Uma, S. Prasad, T. & Kumar, M. U. 1995. Genetic variability in recovery growth and synthesis of stress proteins in response to polyethylene glycol and salt stress in finger millet. *Annals of Botany,* 76**,** 43-49.

Uno, Y. Furihata, T. Abe, H. Yoshida, R. Shinozaki, K. & Yamaguchi-Shinozaki, K. 2000. Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences of the United States of America,* 97**,** 11632-11637.

Vágújfalvi, A. Galiba, G. Cattivelli, L. & Dubcovsky, J. 2003. The cold-regulated transcriptional activator is linked to the frost-tolerance locus on wheat chromosome 5A. *Molecular Genetics and Genomics,* 269**,** 60-67.

Vandemoortele, J.-L. Kevers, C. Billard, J.-P. & Gaspar, T. 2001. Osmotic pretreatment promotes axillary shooting from cauliflower curd pieces by acting through internal cytokinin level modifications. *Journal of Plant Physiology,* 158**,** 221-225.

Vandemoortele, J. Billard, J. Boucaud, J. & Gaspar, T. 1993. Effet de l'osmolarité et de la composition du milieu sur la callogénèse, la caulogénèse et la rhizogénèse de fragments d'hypocotyles de *Brassica oleracea* L. var. botrytis. *Biologia Plantarum,* 35**,** 17-24.

Vankova, R. Hsiao, K.-C. Bornman, C. & Gaudinova, A. 1991. Effects of synthetic cytokinins on levels of endogenous cytokinins and respiration patterns *Beta vulgaris* cells in suspension. *Journal of Plant Growth Regulation,* 10**,** 197-199.

Verleysen, H. Van Bockstaele, E. & Debergh, P. 2005. An encapsulation-dehydration protocol for cryopreservation of the azalea cultivar Nordlicht' (*Rhododendron simsii* Planch.). *Scientia Horticulturae,* 106**,** 402-414.

Vigeolas, H. & Geigenberger, P. 2004. Increased levels of glycerol-3-phosphate lead to a stimulation of flux into triacylglycerol synthesis after supplying glycerol to developing seeds of *Brassica napus* L. in planta. *Planta,* 219**,** 827-835.

Vij, S. & Tyagi, A. K. 2007. Emerging trends in the functional genomics of the abiotic stress response in crop plants. *Plant Biotechnology Journal,* 5**,** 361-380.

Vogel, J. T. Zarka, D. G. Van Buskirk, H. A. Fowler, S. G. & Thomashow, M. F. 2005. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis. *The Plant Journal,* 41**,** 195-211.

Volger, H. G. & Heber, U. 1975. Cryoprotective leaf proteins. *Biochim Biophys Acta,* 412**,** 335-349.

Wakui, K. Takahata , Y. & Kaizuma, N. 1994. Effect of abscisic acid and high osmoticum concentration on the induction of desiccation tolerance in microspore-derived embryos of Chinese cabbage (*Brassica campestris* L.). *Breeding science,* 44**,** 29–34.

Walkey, D. G. A. & Woolfitt, J. M. G. 1970. Rapid clonal multiplication of cauliflower by shake culture. *Journal of Horticultural Science* 45**,** 205-206.

Wang, Q. Batuman, Ö. Li, P. Bar-Joseph, M. & Gafny, R. 2002. A simple and efficient cryopreservation of *in vitro*-grown shoot tips of `Troyer' citrange [*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osbeck.] by encapsulation-vitrification. *Euphytica,* 128**,** 135-142.

Wang, Q. C. Tanne, E. Arav, A. & Gafny, R. 2000. Cryopreservation of *in vitro*-grown shoot tips of grapevine by encapsulation–dehydration. *Plant Cell Tissue Organ Culture,* 63**,** 41–46.

Wang, W.-G. Wang, S.-H. Wu, X.-A. Jin, X.-Y. & Chen, F. 2007. High frequency plantlet regeneration from callus and artificial seed production of rock plant *Pogonatherum paniceum* (Lam.) Hack. (Poaceae). *Scientia Horticulturae,* 113**,** 196-201.

Wang, W. X. Vinocur, B. & Altman, A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta,* 218**,** 1-14.

Wanner, L. A. & Junttila, O. 1999. Cold-induced freezing tolerance in Arabidopsis. *Plant Physiology,* 120**,** 391-400.

Warburg, O. & Christian, W. 1942. Isolation and crystallization of enolase. *Biochem. Z,* 310**,** 384-421.

Wasilewska, A. Vlad, F. Sirichandra, C. Redko, Y. Jammes, F. Valon, C. & Leung, J. 2008. An update on abscisic acid signaling in plants and more. *Molecular Plant,* 1**,** 198-217.

Wei, W. H. Chen, B. Yan, X. H. Wang, L. J. Zhang, H. F. Cheng, J. P. Zhou, X. A. Sha, A. H. & Shen, H. 2008. Identification of differentially expressed genes in soybean seeds differing in oil content. *Plant Science,* 175**,** 663-673.

Welin, B. V. Olson, Å. Nylander, M. & Palva, E. T. 1994. Characterization and differential expression of dhn/lea/rab-like genes during cold acclimation and drought stress in *Arabidopsis thaliana*. *Plant Molecular Biology,* 26**,** 131-144.

Welling, A. Rinne, P. Viherä‐Aarnio, A. Kontunen‐Soppela, S. Heino, P. & Palva, E. T. 2004. Photoperiod and temperature differentially regulate the expression of two dehydrin genes during overwintering of birch (*Betula pubescens* Ehrh.). *Journal of Experimental Botany,* 55**,** 507-516.

Weschke, W. Panitz, R. Gubatz, S. Wang, Q. Radchuk, R. Weber, H. & Wobus, U. 2003. The role of invertases and hexose transporters in controlling sugar ratios in maternal and filial tissues of barley caryopses during early development. *The Plant Journal,* 33**,** 395-411.

Weschke, W. Panitz, R. Sauer, N. Wang, Q. Neubohn, B. Weber, H. & Wobus, U. 2000. Sucrose transport into barley seeds: molecular characterization of two transporters and implications for seed development and starch accumulation. *The Plant Journal,* 21**,** 455-467.

West, T. P. Ravindra, M. B. & Preece, J. E. 2006. Encapsulation, cold storage, and growth of *Hibiscus moscheutos* nodal segments. *Plant Cell Tissue and Organ Culture,* 87**,** 223-231.

Wiebe, H. J. 1975. Effect of temperature on the variability and maturity date of cauliflower. *Acta Hort,* 52**,** 69-76.

Wilkinson, S. & Davies, W. J. 2010. Drought, ozone, ABA and ethylene: new insights from cell to plant to community. *Plant, Cell & Environment,* 33**,** 510-525.

Williams, C. A. & Atherton, J. G. 1990. A role for young leaves in vernalization of cauliflower: I. Analysis of leaf development during curd induction. *Physiologia Plantarum,* 78**,** 61-66.

Williams, R. & Frausto Da Silva, J. 2002. The involvement of molybdenum in life. *Biochemical and biophysical research communications,* 292**,** 293-299.

Winter, H. & Huber, S. C. 2000. Regulation of Sucrose Metabolism in Higher Plants: Localization and Regulation of Activity of Key Enzymes. *Critical Reviews in Biochemistry and Molecular Biology,* 35**,** 253-289.

Wise, M. 2003. LEAping to conclusions: a computational reanalysis of late embryogenesis abundant proteins and their possible roles. *Bmc Bioinformatics,* 4**,** 52.

Wisniewski, M. & Fuller, M. 1999. Ice nucleation and deep supercooling in plants: new insights using infrared thermography. *Cold-Adapted Organisms: Ecology, Physiology, Enzymology and Molecular Biology. Springer-Verlag, Berlin***,** 105-118.

Wisniewski, M. E. Bassett, C. L. Renaut, J. Farrell, R. Tworkoski, T. & Artlip, T. S. 2006. Differential regulation of two dehydrin genes from peach (*Prunus persica*) by photoperiod, low temperature and water deficit. *Tree Physiology,* 26**,** 575-584.

Wolkers, W. F. Mccready, S. Brandt, W. F. Lindsey, G. G. & Hoekstra, F. A. 2001. Isolation and characterization of a D-7 LEA protein from pollen that stabilizes glasses *in vitro*. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology,* 1544**,** 196-206.

Wurr, D. Fellows, J. & Hiron, R. 1990. The influence of field environmental conditions on the growth and development of four cauliflower cultivars. *Journal of Horticultural Science,* 65**,** 565-572.

Xin, Z. & Browse, J. 2000. Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant Cell and Environment,* 23**,** 893-902.

Xiong, L. Ishitani, M. Lee, H. & Zhu, J. K. 2001. The Arabidopsis LOS5/ABA3 locus encodes a molybdenum cofactor sulfurase and modulates cold stress–and osmotic stress–responsive gene expression. *The Plant Cell Online,* 13**,** 2063-2083.

Xiong, L. M. Schumaker, K. S. & Zhu, J. K. 2002. Cell signaling during cold, drought, and salt stress. *Plant Cell,* 14**,** S165-S183.

Yamaguchi-Shinozaki, K. & Shinozaki, K. 1994. A novel cisacting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *Plant Cell,* 6**,** 251–264.

Yamaguchi-Shinozaki, K. & Shinozaki, K. 2005. Organization of cis acting regulatory elements in osmotic-and cold-stress-responsive promoters. *Trends in plant science,* 10**,** 88-94.

Yang, Q. Chauvin, J. E. & Hervé, Y. 1992. A study of factors affecting anther culture of cauliflower (*Brassica oleracea* var. botrytis). *Plant Cell, Tissue and Organ Culture,* 28**,** 289-296.

Yang, Z.-N. Xu, Z.-H. & Wei, Z.-M. 1994. Cauliflower inflorescence protoplast culture and plant regeneration. *Plant Cell, Tissue and Organ Culture,* 36**,** 191-195.

Yanmaz, R. Gulsen, Y. & Abak, K. 1986. *In vitro* propagation of cauliflower (*Brassica oleracea* var. botrytis cv. Winner Osenia) by shoot-tip and curd tissue culture: influence of some auxins and BAP on shoot proliferation. *Cruciferae Newsletter,* 11**,** 8-89.

Yu, M. Hu, C. & Wang, Y. 1999. Influences of seed molybdenum and molybdenum application on nitrate reductase activity, shoot dry matter, and grain yields of winter wheat cultivars. *Journal of Plant Nutrition,* 22**,** 1433-1441.

Yu, M. Hu, C. X. & Wang, Y. H. 2006. Effects of molybdenum on the intermediates of chlorophyll biosynthesis in winter wheat cultivars under low temperature. *Agricultural Sciences in China,* 5**,** 670-677.

Zarka, D. G. Vogel, J. T. Cook, D. & Thomashow, M. F. 2003. Cold induction of Arabidopsis CBF genes involves multiple ICE (inducer of *CBF* expression) promoter elements and a cold-regulatory circuit that is desensitized by low temperature. *Plant Physiology,* 133**,** 910-918.

Zarske, A. 1988. Use of flower stalks for micropropagation of cauliflower (*Brassica oleracea* L. var botrytis). *Archiv für Zuchtungsforschung,* 18(4)**,** 201-211.

Zdunek-Zastocka, E. 2008. Molecular cloning, characterization and expression analysis of three aldehyde oxidase genes from *Pisum sativum* L. *Plant Physiology and Biochemistry,* 46**,** 19-28.

Zhang, J. Jia, W. Yang, J. & Ismail, A. M. 2006a. Role of ABA in integrating plant responses to drought and salt stresses. *Field Crops Research,* 97**,** 111-119.

Zhang, Y. Yang, T.-W. Zhang, L.-J. Zhang, T.-G. Di, C.-X. Xu, S.-J. & An, L.-Z. 2006b. Isolation and Expression Analysis of Two Cold-Inducible Genes Encoding Putative *CBF* Transcription Factors from Chinese Cabbage (*Brassica pekinensis* Rupr.). *Journal of Integrative Plant Biology,* 48**,** 848-856.

Zhao, T.-J. Sun, S. Liu, Y. Liu, J.-M. Liu, Q. Yan, Y.-B. & Zhou, H.-M. 2006. Regulating the Drought-responsive Element (DRE)-mediated Signaling Pathway by Synergic Functions of Trans-active and Trans-inactive DRE Binding Factors in *Brassica napus*. *J. Biol. Chem.,* 281**,** 10752-10759.

Zimmer, W. & Mendel, R. 1999. Molybdenum metabolism in plants. *Plant biology,* 1**,** 160-168.

Zimmerman, T. W. & Cobb, B. G. 1989. Vitrification and soluble carbohydrate levels of Petunia leaves as influenced by media gelrite and sucrose concentrations. *Plant Cell Reports,* 8**,** 358-360.

Ziv, M. & Ariel, T. 1991. Bud proliferation and plant regeneration in liquid-cultured philodendron treated with ancymidol and paclobutrazol. *Journal of Plant Growth Regulation,* 10**,** 53-57.

Ziv, M. Ronen, G. & Raviv, M. 1998. Proliferation of meristematic clusters in disposable presterilized plastic bioreactors for the large-scale micropropagation of plants. *In vitro cellular and Developmental Biology-plant,* 34**,** 152-158.