

CHEMICAL AND BIOCHEMICAL ASPECTS OF SEED DORMANCY AND
RECALCITRANCE IN HAZELNUTS (*CORYLUS AVELLANA* L)

DISSERTATION

Submitted in Partial Fulfilment of the Requirements for
the Degree of Doctor of Philosophy in the Graduate
Research School of Teesside University, UK

October 2014

by

Shaikh Abdul Hamid

Supervisors: Dr William Thomas O'Hare,

Dr Mosharraf Hossain Sarker,

Dr Komang Ralebitso-Senior

Advisor: Prof Janey Henderson

School of Science and Engineering

Approved by

Director of Studies: Dr William Thomas O'Hare

Dedication

This thesis is dedicated to my
late **Mother** and **Father**, and
Dr Novak (operated on my cranium)

Abstract

Hazelnuts are mostly non-dormant at harvest but develop seed dormancy after a few days of storage. The seeds have been classified as recalcitrant since they cannot be stored for more than one year under ambient conditions. Cryopreservation has not been satisfactory so an alternative protocol is required.

To test for recalcitrance, chilled non-dormant seeds (control) were compared with gibberellic acid (GA₃) treated seeds during 6 weeks storage at 5°C or at ambient temperature. Control seed moisture content (MC) was 14-15% compared with 20% for GA₃ treated seeds. No change in viability was noted until the end of 6 weeks at ambient temperature, when infection proliferated. Reduced germinability, associated with increased leachate conductivity, was noted on all treatments and controls, with ambient temperature storage most harmful for seed viability. This supports classification of hazel seeds as recalcitrant. However, orthodox behaviour could be induced by reducing seed moisture to <6%, showing survival for more than 3 years at -20°C with acceptable germinability and producing healthy seedlings. Pathogen tests show that 6 weeks chilling to break seed dormancy may activate the seeds' internal protective mechanisms, thereby reducing infection and enabling germination and healthy seedling establishment.

The link between seed viability and protection from free radicals and pathogens was examined. Antioxidant activity in hazelnut seed associates (such as endocarp, funiculus and testa) was found to be much higher than in the seed embryo, perhaps indicating that hazel seeds have natural protective mechanisms within the pericarp. Antioxidant activity of seed associates increased during chilling, indicating their role in protecting the seed. Nevertheless, TTC test revealed that seeds acclimatised to

<6% MC and stored at 5°C for 45 weeks showed viability loss due to damage of the embryonic axes, probably caused by free radicals.

Initial tests to stabilise seed moisture content showed that reduction in seed moisture did not impose dormancy and seed moisture content (MC) stabilisation resulted in >80% germination but many abnormal seedlings.

Dormancy reversibility was tested by treatments T1 (one period at 15°C) and T2 (two periods at 15°C), designed following a consideration of the natural environment. Both resulted in reduced germination, delayed seedling emergence, increased abnormal seedlings, reduced seedling height and decreased internode numbers. To test the role of temperature in reduced seed performance, non-dormant hazelnuts were held at either 5°C or at ambient temperature for up to 6 weeks. Seeds from both sets exhibited high viability, but germinability was significantly decreased in the ambient temperature set, associated with increases in leachate conductivity and infection.

Work in this thesis has confirmed that dormancy was broken by chilling, with gradually increasing germination as chilling time was increased. Germination increased with increase in chilling and reduction in infection. No infection was recorded after 6 weeks chilling. It is most likely that protective agents are produced causing suppression of infection.

In these experiments it was observed that not all germinated seeds produced healthy seedlings, suggesting that germination tests without observation of seedlings may give an incomplete assessment of germination success. Assessment using the Tetrazolium test (TTC) was found to be much more dependable and it was also possible to detect damage to specific tissues that might result in unhealthy seedlings.

Acknowledgements

I would like to thank my Director of Studies Dr William Thomas O'Hare for the continuous encouragement, guidance and understanding throughout the course of this study and for his advice with respect to the preparation of this thesis.

I am also grateful to the other members of my supervisory team: Dr Mosharraf Hossain Sarker and Dr Komang Ralebitso-Senior for their suggestions and support throughout the research period and also to my Advisor Prof Janey Henderson for support and understanding.

My gratitude remains for Mrs Shirley O'Hare for assistance with the GC/MS technique.

During the initial stages of this work I received helpful suggestions from Dr David A. Wright, for which I am grateful.

Helpful assistance and suggestions from Mr Nigel Atkinson will be remembered.

As always the never ending helpful co-operation of the laboratory technicians Helen Hodgson, Doug McLellan, and Paul Douglas throughout the experimental period is unforgettable. Thanks to technicians in Bio-laboratory Andy and Karen for their helpful co-operation.

Encouragement, motivation and co-operative support from Dr Qarib Ullah and Mr Osama Askari are also acknowledged.

I would not be able to complete this work without deep sympathy, understanding and support of Mr A. Sattar and family. I remain indebted to them in gratitude.

Thanks also go to all my relatives and friends for their support. My deepest regards for my in-laws for all the support they have given me and my family. My wife Shahida, daughters Sabera, Sophia Sabir and son Mohiuddin for continually encouraging me through their agonies of sufferings but never failed to support me which gave me the zeal. Finally, support from my son-in-law Dr Sabir Bin Muzaffar gave me confidence and grand-children Ihsan and Ayesha provided extra pleasant and delectable environment.

Table of Contents

Abstract	i
Acknowledgements	iii
1 Introduction	1
1.1 Seeds.....	1
1.1.1 Seeds and their Biology	1
1.1.2 Seeds in Agriculture	1
1.2 Seed Storage and Longevity.....	2
1.2.1 Seed Moisture Content and Water Activity	3
1.2.2 Temperature.....	4
1.2.3 Determination of Viability.....	4
1.2.4 Organisms Associated with Seeds in Storage.....	5
1.3 Seed Dormancy	8
1.4 Orthodox and Recalcitrant Seeds	10
1.4.1 Orthodox Seeds	10
1.4.2 Recalcitrant Seeds	10
1.5 Desiccation and Imbibition Injury	15
1.5.1 Physical Injury	15
1.5.2 Chemical injury.....	17
1.6 The Development of Desiccation Tolerance	18
1.7 Seed Banks	20
1.8 Hazelnut.....	21
1.8.1 Hazel Seed Viability and Vigour	21
1.8.2 Hazel Seed Recalcitrance	23
1.8.3 Hazel Seed Dormancy	24
1.9 The Importance of Hazelnuts.....	30
1.9.1 Food.....	30
1.9.2 Taxol Production	31

1.9.3	Biofuel and Other Uses	32
1.10	Hypothesis/Plan.....	32
2	Materials and Methods	35
2.1	Hazelnut Provenance.....	35
2.2	Consumables.....	36
2.3	Nut/Seed Storage	37
2.4	Moisture Content (MC) Determination	37
2.5	Water Activity (a_w) Measurement	38
2.6	Measurement of Leachate Conductivity.....	38
2.7	Antioxidant Measurement	39
2.7.1	Seeds and embryos	39
2.7.2	Seed Associates (endocarp, funiculus or testa)	40
2.8	Thin Layer Chromatography (TLC)	41
2.9	Pathogen Test.....	41
2.10	Chilling Treatment.....	42
2.11	Germination Assessment.....	42
2.12	Seed Viability Test by TTC	43
2.13	Seedling Measurement.....	44
2.14	Seedling Dry Mass Measurement.....	44
2.15	Statistics	45
3	Effects of Selected Treatments to Test Seed Dormancy of Hazelnuts	46
3.1	Stabilisation Treatments of Hazelnuts and Seeds.....	46
3.1.1	Introduction.....	46
3.1.2	Materials and methods	47
3.1.2.1	Nut Provenance	47
3.1.2.2	Experimental Procedure	47
3.1.2.3	Stabilisation Treatment	47
3.1.3	Results	48

3.1.3.1	Seed Moisture Content (MC)	48
3.1.3.2	Seed Germination	49
3.1.3.3	Mean Daily Germination (MDG), Peak Value (PV) and Germination Index (GI).....	50
3.1.3.4	Seedling Growth	51
3.1.3.5	Seedling Measurement.....	53
3.1.3.6	Seedling Dry Mass.....	55
3.1.4	Discussion.....	55
3.1.4.1	Seed Moisture Content and Germination.....	55
3.1.4.2	Seedling Growth	56
3.1.5	Summary.....	59
3.2	Effects of Dormancy Reversal Treatments on Germination and Seedling Performance	61
3.2.1	Introduction	61
3.2.2	Materials and Methods	64
3.2.2.1	Nut Provenance	64
3.2.2.2	Nut Storage.....	64
3.2.2.3	Experimental Procedure	65
3.2.2.4	Seedling Measurement.....	65
3.2.2.5	Dormancy Reversal Treatments	65
3.2.3	Results	66
3.2.3.1	Seed Germination.....	66
3.2.3.3	Mean Daily Germination (MDG), Peak Value (PV) and Germination Index (GI).....	70
3.2.3.4	Seedling Performance.....	72
3.2.4	Discussion.....	73
3.2.5	Summary.....	77
3.3	Effects of Storage of Non-dormant Hazelnuts at 5°C and at Ambient Room Temperature	79

3.3.1	Introduction.....	79
3.3.2	Materials and Methods	82
3.3.2.1	Nut Provenance	82
3.3.2.2	Nut Storage.....	82
3.3.2.3	Experimental Procedure	82
3.3.3	Results	82
3.3.3.1	Storage at 5°C	83
3.3.3.2	Storage at Room Temperature	83
3.3.3.3	Seed Germination	84
3.3.3.4	TTC Test Evaluation	87
3.3.3.5	Total Seed Viability	88
3.3.3.6	Mean Daily Germination (MDG), Peak Value (PV) and Germination Index (GI).....	89
3.3.3.7	Seed Moisture Content (MC)	91
3.3.3.8	Seed Water Activity	93
3.3.3.9	Conductivity of Seed Leachate	95
3.3.3.10	Seed Antioxidant Activities.....	97
3.3.4	Discussion	99
3.3.4.1	Seed Germination	99
3.3.4.2	Seed Viability	99
3.3.4.3	Seed Moisture Content	100
3.3.4.4	Seed Water activity	100
3.3.4.5	Conductivity of Seed Leachate	101
3.3.4.6	Seed Antioxidant Activities.....	102
3.3.5	Summary.....	103
3.4	Dormancy Breaking in Hazel seeds	105
3.4.1	Introduction.....	105
3.4.2	Materials and Methods	107

3.4.2.1	Nut Provenance	107
3.4.2.2	Nut Storage.....	107
3.4.2.3	Experimental Procedure	107
3.4.3	Results	107
3.4.3.1	Seed Germination and Total Viability.....	107
3.4.3.2	Seed Moisture Content and Water Activity	111
3.4.3.3	Seed Antioxidant Activities	113
3.4.4	Discussion	114
3.4.4	Summary.....	116
3.5	Acclimatisation of Hazel Seeds.....	117
3.5.1	Introduction	117
3.5.2	Materials and Methods	117
3.5.2.1	Seed Provenance	117
3.5.2.2	Experimental Procedure	117
3.5.3	Results	118
3.5.3.1	Seed Germination and Total Viability.....	118
3.5.3.2	Seed Moisture Content and Water Activity	120
3.5.3.3	TTC Viability Test	122
3.5.3.4	Conductivity of Seed Leachate	124
3.5.3.5	Seed Antioxidant Activities	125
3.5.4	Discussion.....	126
3.5.5	Summary.....	128
3.6	Study of the Infections in Hazel Seeds	129
3.6.1	Introduction	129
3.6.2	Materials and Methods	130
3.6.2.1	Seed Sources	130
3.6.2.2	Pathological Test	130
3.6.3	Results	131

3.6.3.1	Systemic Pathogen	131
3.6.3.2	Commercial Hazelnut Product	134
3.6.3.3	Physical Observation	134
3.6.3.4	Pathogen Test	135
3.6.3.5	TTC Viability Test	136
3.6.4	Discussion	137
3.6.5	Summary.....	139
3.7	Free Radicals and Antioxidants in Hazelnuts.....	141
3.7.1	Introduction.....	141
3.7.2	Materials and Methods	147
3.7.2.1	Nut Provenance	147
3.7.2.2	Experimental Procedure	147
3.7.3	Results	147
3.7.3.1	Phase 1: Standardisation of Antioxidant Assay Protocol	147
3.7.3.2	Phase 2: Involvement of Antioxidants from Hazelnut Associates in Seed Physiology	161
3.7.3.3	Recommendations.....	167
3.7.4	Discussion	167
3.7.5	Summary.....	170
4	Recalcitrance in Hazelnuts	172
4.1	Test of Recalcitrance in Hazelnuts.....	172
4.1.1	Introduction.....	172
4.1.2	Material and Methods	173
4.1.2.1	Nut Provenance	173
4.1.2.2	Seed Storage.....	173
4.1.2.3	Analytical	173
4.1.2.4	Germination Assessment.....	173
4.1.2.5	Viability Assessment.....	174

4.1.3	Results	174
4.1.3.1	Effects of 6 Weeks Storage of Non-dormant Control and GA Treated Seeds at 5°C.....	174
	Seed Germination and Viability	174
4.1.3.2	Effects of 6 Weeks Storage of Non-dormant Control and GA Treated Seeds at Room Temperature.....	181
4.1.3.3	Overview of Seed Germination and Viability	188
4.1.4	Discussion.....	189
4.1.5	Summary.....	191
4.2	Storage of Hazelnuts at -20°C	193
4.2.1	Introduction	193
4.2.2	Materials and Methods	194
4.2.2.1	Nut Provenance	194
4.2.2.2	Nut Storage.....	194
4.2.2.3	Chilling.....	195
4.2.2.4	Moisture Content Determination	195
4.2.2.5	Seed Germination.....	195
4.2.2.6	Seedling Growth	195
4.2.3	Results	195
4.2.3.1	Seed Germination and Total Viability.....	195
4.2.3.2	Seed Moisture Content and Water Activity	200
4.2.3.3	Conductivity of Seed Leachate	202
4.2.3.4	Seedling Performance	203
4.2.4	Discussion.....	207
4.2.4.1	Storage of Nuts at -20°C.....	207
4.2.4.2	Seedling Performance	208
4.2.5	Summary.....	210
5	General Discussion	212

5.1	Tests of Seed Dormancy	212
5.1.1	Effects of Stabilisation Treatment.....	212
5.1.2	Effects of Dormancy Reversal Treatments	214
5.1.3	Dormancy Breaking in Hazel Seeds.....	215
5.2	Recalcitrance in Hazelnuts	216
5.2.1	Test of Recalcitrance in Hazelnuts	216
5.2.2	Effects of Storage of Non-dormant Hazelnuts at 5°C and at Ambient Temperature.....	217
5.2.3	Acclimatisation of Hazel Seeds	218
5.2.4	Infections in Hazel Seeds	219
5.2.5	Free Radicals and Antioxidants in Hazelnuts	220
5.3	Storage of Hazelnuts at -20°C	221
5.4	Summary	222
6	Conclusions.....	229
7	Future Work	234
8	References	235

List of Tables

Table 1.1: Possible sources of seed losses in hazelnuts due to infection and insect attacks (data taken from (Duke, 1989) and presented as a table)	7
Table 1.2: Examples of some species variously classified as recalcitrant, intermediate and orthodox	13
Table 1.3: Storage conditions and longevity of various species of hazel seeds as reported by researchers (Hong & Ellis, 1998) are listed:.....	24
Table 1.4: List of metabolic research works done related to seed dormancy of hazelnuts.	25
Table 1.5: Dormancy breaking mechanisms of newly harvested, dormant and chilled hazelnuts by various physical, physiological and chemical treatments. Symbol (✓) in Pericarp [(+)(with), -(without) shell], in seed [(+)(with), -(without) testa]	29
Table 2.1: List of growers and suppliers of hazelnuts used during the experimental period.....	36
Table 3.1: Effect of stabilisation treatments on moisture content and final germination of nuts and seeds: Nuts with non-dormant seeds were air-dried at ambient conditions ($20 \pm 2^{\circ}\text{C}$) for 24 h before subjecting to 24 h stabilisation treatment at 5°C and at ambient temperature for 24 h in closed containers.	49
Table 3.2: Effect of dormancy reversal treatments of hazelnuts on seedling performance after Treatment 1.	72
Table 3.3: Effect of dormancy reversal treatments of hazelnuts on seedling performance after Treatment 2.	73
Table 3.4: Summarised list of physiological, chemical and biochemical results obtained during 6 weeks storage of non-dormant hazelnuts at 5°C . Results include \pm SD.....	83
Table 3.5: Summarised list of physiological, chemical and biochemical results obtained during 6 weeks storage of non-dormant hazelnuts at ambient room temperature (RT). Results include \pm SD.	84
Table 3.6: Comparison of the use of different parts of hazelnut and plant parts for antioxidants.....	145
Table 4.1: Seed moisture content and storage period of desiccated hazelnuts at -20°C	195

List of Figures

Figure 1.1: Reduction of white coloured tetrazolium chloride to red colour formazan in living tissue (Grzybowski, et al., 2012).	5
Figure 1.2: Schematic drawing of the effect of dehydration on cellular membranes: Different membranes may become appressed and fused on dehydration; upon rehydration, cellular contents leak out (Walters, et al., 2002).	16
Figure 1.3: Quenching free radical action by antioxidants: A. Stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), B. Structure of free radical quenched DPPH. Adapted from (Pyrzynska & Anna Pękal, 2013).	18
Figure 3.1: Experimental treatments of hazelnuts and seeds stabilisation at 5°C and at ambient temperature (RT).	48
Figure 3.2: Effect of desiccation and stabilisation treatment on seed germination. Non-dormant hazelnuts were desiccated for 24 h at ambient temperature and held in closed containers at 5°C and at room temperature as nuts and seeds. Seeds from treated nuts were used for germination tests:	50
Figure 3.3: Effect of desiccation and stabilisation treatment of hazelnuts and seeds on (a) Mean daily germination, (b) peak value and (c) Germination index of seed germination. Data are obtained from Figure 3.2.	51
Figure 3.4: Effects of desiccation and stabilisation treatments of non-dormant hazelnuts and seeds on normal and abnormal seedling emergence from germinated seeds. Results are based on samples of 25 seedlings.	52
Figure 3.5: Typical seedling features observed from germinated seeds after stabilisation treatments.	53
Figure 3.6: Effects of desiccation and stabilisation treatments on seedling growth. Twenty five germinated seeds from each treatment were sown for seedling growth. (a) Seedling height (mm),	54
Figure 3.7: Effects of desiccation and stabilisation treatments on shoot and root dry mass from normal seedlings: Shoot and root samples were obtained from seedlings grown in Figure 3.6. Error bars represent \pm SD, dry mass is based on average of 25 seedlings.	55
Figure 3.8: Experimental treatments and steps in investigation of physiological and biochemical analysis of non-dormant seeds held at 5°C and at 15°C for 6 weeks in each.	66

- Figure 3.9:** Effects of dormancy reversal treatment on hazel seed germination. Seed germination tests were conducted at 15°C. Error bars represent \pm SD based on samples of 100 seeds calculated as described in section 2.15. 67
- Figure 3.10:** Effects of dormancy reversal treatments on seed moisture content. (a) Treatment 1, (b) Treatment 2. Error bars represent \pm SD of 10 seeds. 69
- Figure 3.11:** Treatment 1: Effects of dormancy reversal treatments on (a) MDG, (b) PV and (c) GI of hazel seed germination Data taken from Figure 3.9 (a). Treatment 2: Effects of dormancy reversal treatments on (d) MDG, (e) PV and (f) GI of hazel seed germination Data taken from Figure 3.9(b). 71
- Figure 3.12:** Moisture loss of seeds from hazelnut held at 5°C and RT. The values are mean of ten replicates \pm SD. 84
- Figure 3.13:** Effect of 6 weeks storage of non-dormant hazelnuts at (a) 5°C and (b) ambient room temperature (RT) on seed germination. No additional water was added during the storage period. 85
- Figure 3.14:** Germination pattern of non-dormant seeds held at 5°C and at RT for up to 6 weeks. Seeds were taken out at 0, 2, 4 and 6 weeks interval from both storage conditions and tested for germination at 15°C up to 28 days. 86
- Figure 3.15:** Effect of TTC test on hazel seed viability conducted on un-germinated seeds after germination test. (a) Unstained embryonic axis (dead) and faintly stained cotyledons (weak); (b) Dead plumule, radicle and cotyledon. 87
- Figure 3.16:** The effects during 6 weeks storage of non-dormant hazelnuts on germination and total seed viability. During storage period no additional water was added. Hazelnuts held at (a) 5°C (Germination details from Figure 3.13 a) and (b) ambient room temperature (Germination details from Figure 3.13 b). 88
- Figure 3.17:** Effects during 6 weeks storage of non-dormant hazelnuts on MDG, PV and GI of hazel seed germination. Storage at i) 5°C (a, b, c) and ii) room temperature (d, e, f). Mean daily germination (a, d), Peak value (b, e) and Germination index (c, f). Germination data obtained from Figure 3.13(a) for a, b, and c; and Figure 3.13 (b) for d, e and f. 90
- Figure 3.18:** Moisture content of non-dormant seeds during 6 weeks storage at (a) 5°C and (b) room temperature. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of ten replicates \pm SD. 92
- Figure 3.19:** Effect during 6 weeks storage of non-dormant hazelnuts on seed water activity. Hazelnuts were removed after 0, 2 4 and 6 weeks and water activity

determined. Hazelnuts were held for 6 weeks at (a) 5°C and (b) room temperature. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD. 94

Figure 3.20: Effects during 6 weeks storage of non-dormant on hazel seed leachate conductivity. Hazelnuts held at (a) 5°C and (b) room temperature. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD. 96

Figure 3.21: Effects during 6 weeks storage of non-dormant hazelnuts on DPPH inhibition with antioxidants from embryo extracts. Hazelnuts held at (a) 5°C. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$) and (b) room temperature (Student's t-test, $p < 0.05$). The values are mean of three replicates \pm SD. 98

Figure 3.22: Germination of hazel seeds from nuts chilled up to 6 weeks. Seeds were incubated at 15°C. 108

Figure 3.23: Effects during 6 weeks chilling on seed viability of dormant hazelnuts at 5°C. Seeds were incubated at 15°C for germination. Un-germinated seeds were subjected to TTC test. Germination data obtained from Figure 3.22. 109

Figure 3.24: Effect of 6 weeks chilling of dormant hazelnuts on MDG, PV and GI of hazel seed germination. Mean daily germination (a) and Peak value (b) and Germination index (c). Germination data obtained from Figure 3.22. 110

Figure 3.25: Effect of 6 weeks chilling on dormant hazelnuts on seed moisture content. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of ten replicates \pm SD. 111

Figure 3.26: Effect of 6 weeks chilling on dormant hazelnuts on seed water activity. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD. 112

Figure 3.27: Effect of 6 weeks chilling on dormant hazelnuts on DPPH inhibition with antioxidant from embryo extracts. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD. 113

Figure 3.28: Hazel seeds after acclimatisation test and 45 weeks storage at 5°C showing seeds that were healthy (normal), partly oily and totally oily in appearance. 119

- Figure 3.29:** Acclimatised hazel seeds after 45 weeks storage at 5°C tested for seed viability using the TTC test. Normal seeds do not show any sign of oiliness whereas the oily seeds are visibly oily as shown in Figure 3.28. 120
- Figure 3.30:** Moisture content and Water activity of acclimatised hazel seeds. (a) Moisture content, (b) Water activity. (Student's t-test $p < 0.05$). Moisture content values are mean of ten replicates \pm SD. Water activity values are mean of three replicates \pm SD. 121
- Figure 3.31:** Acclimatised hazel seeds tested for viability by TTC test. Seeds after three dehy-rehydration treatments were dehydrated for eight days prior to storage. (a) Non-viable seeds, X1, X2 and X3 show dead embryo and most cotyledonary tissues dead but edges still live, (b) viable seeds. 123
- Figure 3.32:** Leachate conductivity of acclimatised hazel seeds post imbibition. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD. 124
- Figure 3.33:** Comparison of DPPH inhibition by extracts of acclimatised normal and control hazel seeds (Student's t-test $p < 0.05$). The values are means of three replicates \pm SD. 125
- Figure 3.34:** A selection of various types of pathogen on hazel seeds observed during experiment setting. Hazelnuts of 2008 were chilled at 5°C for 6 weeks. Arrows indicate a few infected areas which is also present in each of the seed. 131
- Figure 3.35:** Split embryos show infection on the inner side of the cotyledon. Arrows indicate the infected areas within the cotyledon. 132
- Figure 3.36:** Hazel seedling showing pathogen in the inner part of the split cotyledons. 133
- Figure 3.37:** Commercial hazel seeds showing infected zone in the inner side of the cotyledon. Arrows indicate a few infected areas. 134
- Figure 3.38:** After treatment with cryoprotectant raffinose, few desiccated seeds are sound and some are wrinkled. Arrows indicate the wrinkled areas. Inset shows one specimen of wrinkled seed. 135
- Figure 3.39:** Bacterial and fungal colonies grown from buffer washes from hazelnut associates. 136
- Figure 3.40:** TTC test show no staining of the central oval area of the cotyledons and the plumule parts of the embryonic axes. Seeds are taken from experiment in section 3.3 in which seeds were held at 5°C. 136

- Figure 3.41:** Staining pattern in hazel seeds after TTC test show very light (non-viable) to deeper (viable) staining. These seeds are part of experiment in section 3.3 where dehydrated seeds were held at ambient room temperature. 137
- Figure 3.42:** (a) Developing hazelnuts enclosed within the green leafy involucre, (b) hazel seed associates within the nut..... 145
- Figure 3.43:** Antioxidant activity from hazelnut endocarp extracts on DPPH inhibition. Interpolating from the graph the amount of AsA required for 50% DPPH inhibition were estimated as below: 149
- Figure 3.44:** DPPH inhibition by antioxidants from hazel embryo tissues extracted at 5°C, 15°C and RT. Embryo sample of 1.0 g ground and extracted 3 times in 5 ml solvent prior to assay. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD. 150
- Figure 3.45:** DPPH inhibition by antioxidants extracted from hazel embryos by stirring or non-stirring; (a) Normal, (b) de-fatted. Interpolating from these graphs, embryo sample mass (mg) required for 50% DPPH inhibition were estimated as below:..... 151
- Figure 3.46:** Comparison of hazel embryo mass requirement for 50% inhibition of DPPH by antioxidant activities of extracted sample by normal and defatted extraction methods. Data taken from interpolated Table in Figure 3.45..... 152
- Figure 3.47:** Antioxidant activity of Normal and Defatted hazel embryo samples compared with Non Fat Dry Matter estimation. Interpolating from this graph, embryo sample mass (mg) required for 50% DPPH inhibition were estimated as below: 153
- Figure 3.48:** Comparison of antioxidant activity in Funiculus by dH₂O and 60/40 (v/v) (IMS/dH₂O) solvent extraction. Extracted samples were used to test inhibition of standard free radical DPPH. 154
- Figure 3.49:** Comparison of antioxidant extraction from embryo samples in solvent (60/40)(v/v) and buffer (60/40)(v/v). The extracted antioxidant samples were used in inhibition test of standard free radical DPPH. Interpolating from this graph, embryo sample mass (mg) required for 50% DPPH inhibition were estimated as below: 155
- Figure 3.50:** Effect of standard antioxidant BHT on DPPH inhibition, (a) incubation time (b) sample mass..... 156
- Figure 3.51:** DPPH inhibition by standard antioxidants AsA and BHT dissolved in solvent [60/40 (v/v) IMS/dH₂O)]. 157
- Figure 3.52:** Scavenging of DPPH by standard antioxidants; (a) Ascorbate (AsA), and (b) Trolox..... 158

- Figure 3.53:** Both TLC plates are loaded with standard antioxidants AsA, BHT and Trolox. Plate (a) with extracts from Endoderm and Funiculus. Plate (b) loaded with extracts from Embryo and Testa. 159
- Figure 3.54:** Antioxidant activity on DPPH inhibition of extracted samples from hazel seed and associates and stored at (a) 5°C and (b) RT in log scale. 160
- Figure 3.55:** Incubation time of extracted antioxidants from hazelnut embryo and associate to inhibit standard DPPH. (a) Endocarp, (b) Funiculus, (c) Testa and (d) Embryo. 162
- Figure 3.56:** Comparison of standard DPPH inhibition by extracts from hazel seed and embryo tissues. Interpolating from the graph sample mass (mg) needed for 50% DPPH inhibition were estimated as below: 163
- Figure 3.57:** Effect of 6 weeks chilling at 5°C on antioxidant activity of hazel seed Endocarp, Funiculus and Testa on DPPH inhibition. Interpolating from the graph the amount of AsA required for 50% DPPH inhibition were estimated as below:..... 164
- Figure 3.58:** Comparison of Endocarp, Funiculus and Testa sample mass needed for DPPH inhibition during 6 weeks chilling. Interpolating from the graph sample mass (mg) needed for 50% DPPH inhibition were estimated as below: 165
- Figure 3.59:** DPPH inhibition activities of hazel embryo extracts obtained from hazelnuts used in dormant to non-dormant and non-dormant to dormant treatments. Dormant seeds were chilled for 6 weeks at 5°C for non-dormant seeds and then these nuts were held at 5°C for 6 more weeks without any added water. Samples were taken out at 2, 4 and 6 weeks. Error bars indicate \pm 95%CI..... 166
- Figure 4.1:** Effect of storage of non-dormant hazelnuts for 6 weeks at 5°C on seed germination and total viability. (a) Control seeds and (b) seeds imbibed in GA₃.175
- Figure 4.2:** Effect of storage of non-dormant hazelnuts for 6 weeks at 5°C on moisture content. 176
- Figure 4.3:** Effect of 6 weeks storage of non-dormant hazel seeds at 5°C on water activity. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD. 178
- Figure 4.4:** Effect of 6 weeks storage of non-dormant hazel seeds held at 5°C on leachate conductivity. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD..... 180

- Figure 4.5:** Effect of 6 weeks storage of non-dormant hazelnuts at room temperature on seed germination and total viability. (a) Control seeds and (b) seeds imbibed in GA₃. 181
- Figure 4.6:** Effect of 6 weeks storage at room temperature on seed moisture content of non-dormant seeds. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of ten replicates \pm SD. 183
- Figure 4.7:** Effect of 6 weeks storage of non-dormant hazelnuts at room temperature on water activity of seeds. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD. 185
- Figure 4.8:** Effect of 6 weeks storage of non-dormant hazelnuts at room temperature on leachate conductivity of seeds. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD. 187
- Figure 4.9:** The pattern of seed viability loss and increase of infection during 6 weeks at 5°C and room temperature (RT) storage of non-dormant seeds..... 188
- Figure 4.10:** Newly harvested hazelnuts of 2008, 2010 and 2011 crop after desiccation were held in -20°C for 189, 80 and 36 weeks respectively. A batch of 2008 nuts was held at 5°C for 189 weeks. Nuts after storage period were chilled for 6 weeks at 5°C at the end of which the seeds were subjected to germination test in a 15°C incubator. 196
- Figure 4.11:** Average lag phase of first germination of seeds subjected to storage at 5°C and -20°C followed by 6 weeks chilling at 5°C. Storage treatments of hazelnuts are as detailed in Fig. 4.10. 197
- Figure 4.12:** Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seed viability. Nuts held 5°C and -20°C were given 6 weeks chilling at 5°C prior to germination test. Inset showing the final germination of 5°C and -20°C stored seeds. Storage treatments of hazelnuts are as detailed in Fig. 4.10. 198
- Figure 4.13:** Effect of 6 weeks chilling of hazelnuts held at 5°C and -20°C on; (a) mean daily germination, (b) peak value and (c) germination index. Storage treatments of hazelnuts are as detailed in Fig. 4.10..... 199
- Figure 4.14:** Effects of chilling of hazelnuts post storage at 5°C and -20°C on seeds: (a) moisture content; (b) water activity. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). Moisture content values are mean of

- ten replicates \pm SD; water activity values are mean of three replicates \pm SD. Storage treatments of hazelnuts are as detailed in Fig. 4.10.....200
- Figure 4.15:** Effect of 6 weeks chilling of hazelnuts held at 5°C and -20°C on leachate conductivity. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD. Storage treatments of hazelnuts are as detailed in Fig. 4.10.202
- Figure 4.16:** Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seedling performance. Hazelnuts held 5°C and -20°C were given 6 weeks chilling at 5°C prior to germination test. Germinated seeds were sown in ambient conditions and performance recorded after 2 weeks as detailed in Fig. 4.10. Storage treatments of hazelnuts are detailed in Table 4.1.203
- Figure 4.17:** Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seedling performance: (a) Seedling emergence time (days), (b) average seedling height (mm) and (c) average internode numbers recorded after 2 weeks growth period. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of 30 replicates \pm SD. Seed germination of hazelnuts are as detailed in Fig. 4.10.204
- Figure 4.18:** Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seedling performance: (a) seedling height (mm) and (b) internode numbers recorded after 2 and 13 weeks growth period. Seed germination of hazelnuts are as detailed in Fig. 4.10.206
- Figure 4.19:** Photograph showing typical 13 week old seedlings grown from hazelnuts held in -20°C, and 5°C. After appropriate storage nuts were chilled for 6 weeks before germination test. Germinated seeds were sown for seedling performance. Seed germination of hazelnuts are as detailed in Fig. 4.10.207
- Figure 5.1:** Role of chilling on restoration of hazel seed viability. A) Simplified model of free radical initiated viability loss proposed (incorporated from (Kranner, et al., 2008) and B) counter action of antioxidants by chilling hazelnuts from this experiments. Blue lines indicate the modifications tested in this experiment.223
- Figure 5.2:** Viability of hazel seeds after storage for 189 weeks at 5°C and 6 weeks each at 5°C and room temperature. A) TTC test done on un-germinated seeds reveal the following staining patterns which likely correspond to the seedling indicated. B) Germinated seeds after physiological tests result in the varieties of normal and abnormal seedlings shown vertically. Included images are illustrative of

different defects and are not necessarily taken from the specific treatments included in this figure. See also Figure 5.3..... 225

Figure 5.3: Viability of hazel seeds after storage for 189 weeks at 5°C and -20°C. Column A) TTC test done on un-germinated seeds reveal the following staining patterns which likely correspond to the seedling indicated. Column B) Germinated seeds after physiological tests result in the varieties of normal and abnormal seedlings shown vertically. See also Figure 5.2..... 227

Figure 5.4: A model to illustrate the role of antioxidants in the viability of desiccated hazel seeds. Chilling activates antioxidants which counters the free radicals to enable seeds' normal metabolic activities leading to germination..... 228

List of abbreviations

The following table shows symbols and abbreviations used throughout the thesis.

Abbreviation	Meaning
ABA	Absciscic Acid
AsA	Ascorbic Acid
BHT	Butylated Hydroxytoluene
CCC	β - chloroethyltrimethylammonium chloride (chlorocholine chloride)
dH ₂ O	De-ionised Water
DMSO	Dimethyl Sulphoxide
DND	Dormant to Non-Dormant
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DW	Dry Weight
EC	Electrical Conductivity
FR	Free Radical
FW	Fresh Weight
GA	Gibberellic Acid (subscript number represent specific compound)
GI	Germination Index
IBA	Indole Butyric Acid
IMS	Industrial Methylated Spirit
IPGRI	International Plant Genetic Resources Institute
LEAs	Late Embryogenesis Abundant Proteins
MDG	Mean Daily Germination
MC	Moisture Content
NDD	Non Dormant to Dormant
NFDM	Non Fat Dry Matter
PGR	Plant Growth Regulators
PV	Peak Value
ROS	Reactive Oxygen Species
RT	Room Temperature
TLC	Thin Layer Chromatography
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
WW	Wet Weight

1 Introduction

1.1 Seeds

1.1.1 Seeds and their Biology

Since time immemorial man has been dependent on seed plants as provider of food, materials for tools, housing, clothing, medicines etc. Seed is both the start of a new generation and the culmination of the activities of one plant generation. It contains within it the embryo, the miniature plant, with the potential for growth and develop into an adult plant. Plants make possible the efficient organisation of agriculture, horticulture and forestry, and they also help to maintain the environment by providing oxygen and removing carbon dioxide, providing habitat for animals, preserving soil and reducing noise pollution. Storage of seed is therefore, important for plant genetic resource conservation which provides the most effective and efficient *ex situ* conservation strategy (Roberts, 1973; Pritchard, et al., 1995; Hong & Ellis, 1996).

1.1.2 Seeds in Agriculture

Seeds fulfil two very important roles in agriculture and horticulture. Firstly as the basic propagule, seed is the starting point for most crops. Secondly, seeds may also represent the end product, the crop, which the grower will harvest. The ability of seeds to survive storage is important, and is relevant in situations where crop failure may lead to the use of seeds stored for a longer time than usual. Therefore, seed germination is an important aspect of seed biology with particular importance to agriculture. Failure of seeds to germinate leads to gaps in the crop stand, whilst variability in the time taken to germinate leads to variations in the size and maturity of plants within the stand. Seed batches of high viability and vigour need to be used

to avoid this problem. Thus the quality of seeds in terms of their viability and vigour is of critical importance in agriculture and horticulture (Bewley & Black, 1994).

1.2 Seed Storage and Longevity

The ability to withstand loss of cellular water is an unusual feature for survival. It is an adaptive feature of the majority of seed species, which retain their viability when dried; in fact, drying is the normal, final phase of maturation for most seeds growing in temperate climates. For example, the minimum 5% water, often referred to as bound water (Vertucci & Leopold, 1987) is retained in dry hazelnut seeds due to strong hydrogen bonding between water molecules and the humectants such as sugar inside hazelnuts. Hence, it is common for seeds to be stored in a 'dry' state, or more correctly, with low moisture content. These seeds are defined as orthodox seeds (Roberts, 1973). Roberts also described seeds which lost viability on drying as being recalcitrant. Such seeds are not suitable for long-term storage. In the original classification compiled by Roberts, the recalcitrant group included hazelnuts. This is discussed in more detail in section 1.4. Although for storage, most seeds are said to be held in a 'dry' state, the term 'dry' is a relative one. When storage water content is below 5% (fresh mass basis) the term 'ultra-dry' (Zheng & Jing, 1998) is used, while seeds with water content between 5.5-6.8% are referred to as 'dry' (Ellis, et al., 1996).

As most of our current understanding of seed storage relates to orthodox seeds the factors involved in the longevity of orthodox seeds in storage will be considered first. Development of appropriate storage condition is essential as storage of seeds under unfavourable conditions results in the production of 'aged' seeds exhibiting a variety of symptoms ranging from reduced viability or germinability to more or less full viability (i.e. no obvious decline in germinability) but with abnormal development of the seedling (i.e. poor vigour) (Bewley & Black, 1994).

1.2.1 Seed Moisture Content and Water Activity

Seed moisture content is probably the most important factor that may affect the viability of seeds during storage (Farrant, et al., 1985). The metabolic activities of quiescent seeds are dependent on their state of hydration. Moreover it helps to establish the relative humidity surrounding the seeds which in turn determines the extent of microbial growth in the seed batch (section 1.2.4). Water activity in seeds is important as it is the relative availability of water in a substance which also indicates the possibility of pathogen growth. Therefore, measuring water activity will shed light on the possible link to pathogenic proliferation (Magan, et al., 1984; Beuchat & Scouten, 2002; Bruce & Horn, 2005; Igawa, et al., 2007 and Nguyen, et al., 2013).

The optimal seed moisture content varies from species to species and in different varieties of the same species. While some seeds do not survive at very low water contents (section 1.6), seed viability may also decline and deterioration may occur more readily at higher moisture content when metabolism is imbalanced and pose a hazard for long term seed survival (Vashisth & Nagarajan, 2009). The role of water activity and seed moisture content on dormancy release is discussed in section 1.3. Seeds may be hygroscopic and therefore they can pick up moisture from the surrounding air until equilibrium is established between the vapour pressure of seed moisture and atmospheric moisture (Shelar, 2008). Therefore, control of environmental humidity is important. In most cases, if moisture level can be controlled throughout the storage period at lower moisture content, the longer seeds can be stored (Roberts, 1973).

Absorption capacity of a seed depends on its chemical composition. Thus seeds such as peanut, flax, lettuce and pine, which contain large amounts of oil, as do hazelnuts (Shewry, et al., 1972), have low water binding capacities.

1.2.2 Temperature

Another important factor that affects seed viability is the storage temperature. The results of a vast number of investigations summarised (Roberts, 1973) by stating: *'In the vast majority of cases it has been shown that the lower the temperature and the lower the moisture content the longer the period of viability'*.

The best combination of seed moisture content and storage temperature varies from species to species. For most kinds of seeds, below freezing temperatures were superior to above freezing temperatures for storage (Shelar, 2008) but see also section 1.4.2. High temperature increases the rate of the biochemical processes, triggering more rapid deterioration and result in rapid losses in viability of seeds having high moisture content (Shelar, 2008). Another report also supports the sensitivity of seeds to high temperatures being strongly dependent on their water content, the loss of viability accelerating with increased moisture (Kibinza, et al., 2006).

1.2.3 Determination of Viability

Seed germination tests have been the usual method to determine the viability and vigour of the seeds (Roberts, 1973), while an easier and quicker way to assess seed viability using a biochemical test has been by the use of 2,3,5-triphenyltetrazolium chloride (TTC), which differentiates live from dead seeds (Lakon, 1949). The activity of dehydrogenase enzymes increases when dry seeds are hydrated. In living seeds, the action of these enzymes results in the release of hydrogen ions, which in turn reduce colourless tetrazolium salt solution to a red compound (formazan) and this stains respiring plant cells red. Dead cells (not respiring) remain colourless (Fig. 1.1).

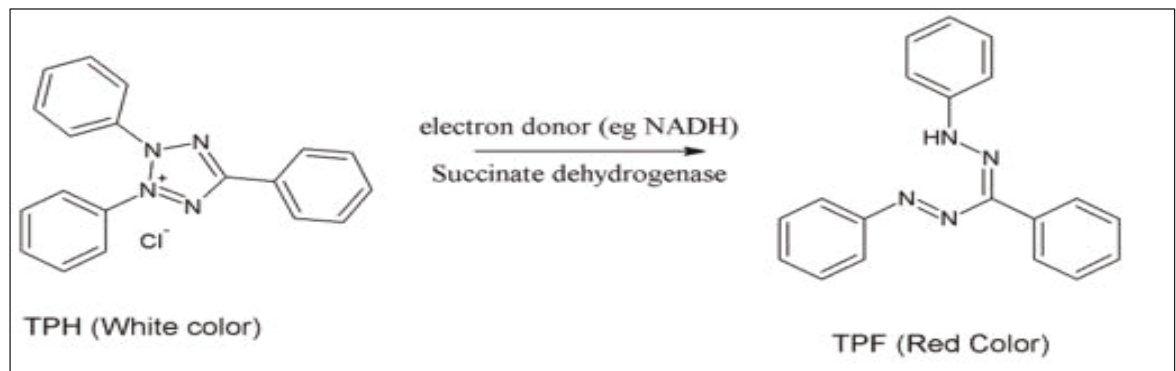


Figure 1.1: Reduction of white coloured tetrazolium chloride to red colour formazan in living tissue (Grzybowski, et al., 2012).

Two types of assay results are used in evaluating the TTC test in various laboratories:

- i) Spectrophotometric assay of the extracted red dye after the test and
- ii) Visual observations of the colouring pattern of the seeds.

Spectrophotometric assay is more quantitative and may be useful to check viability of grains where the seeds are small and it is difficult to assess individual seeds. Spectrophotometric assay of extracted reduced tetrazolium salt was used for indirect analyses of vigour of *Corylus avellana* (Rendon, 1983); moreover, similar experiment had been conducted on *Jatropha curcas* (Zaidman, et al., 2010).

Whole seed assay of *Cattleya* seeds was done by Hosomi, et al. (2011), where staining marked the viable and non-viable areas. The advantage of visual inspection is that it reveals the exact area and level of stress related damages.

1.2.4 Organisms Associated with Seeds in Storage

One of the problems associated with seed storage is to maintain good health countering the adversities in nature like pathogens, insects etc. There are five main types of organisms (bacteria, fungi, insects, mites and rodents) whose activities can lead to damage resulting in the loss of vigour, viability and eventually leading to complete loss of seed. Table 1.1 includes an extensive list of possible sources of

seed loss in hazelnut due to infections and insects which include fungi, bacteria and nematodes.

Problems represented by microbial, particularly fungal, infection of stored seeds have been previously outlined (Swarbrick, 1965; Christensen, 1972). These reports have distinguished between field fungi - those invade seeds during seed development, ripening and harvesting procedures prior to storage, and storage fungi - those grow on products in storage, often at fairly low moisture content. Evidence of the existence of field fungi includes 56 fungal taxa in soybean reported by Miller & Roy (1982). Furthermore, another study also reported 51 species and three varieties from 20 pathogenic genera which were collected from 20 samples of each of hazelnut and walnut seeds (Abdel-Hafez & Saber, 1993). On the other hand, it has been observed that, in hazelnuts, seeds managed to germinate even if they were infected (Rendon, 1983) which indicates the embryonic axis utilises storage food materials and able to germinate, unless pathogen proliferation overcomes the germination metabolism.

Stink bugs (*Halyomorpha halys*, *Melissopus latiferreanus*) perforate the Hazel stem (Aliniasee, 1983; Hedstrom, et al., 2014); whereas other plant bugs (*Acrosternum hilare* and *Nezara viridula*) attack the developing buds and cause these to be bitter when mature (Duke, 2000; McPherson & McPherson, 2000; Tillman, et al., 2010).

Table 1.1: Possible sources of seed losses in hazelnuts due to infection and insect attacks (data taken from (Duke, 1989) and presented as a table)

Fungus known to cause diseases on European filbert		
<i>Anthostoma dubium</i>	<i>Gleosporium perexiguum</i>	<i>Nitschkia tristis</i>
<i>Apioportha anomala</i>	<i>Gnomonia amoena</i>	<i>Orbilia crenato-marginata</i>
<i>Armillaria mellea</i>	<i>Gnomonia coryli</i>	<i>Peniophora cinerea</i>
<i>Cercospora coryli</i>	<i>Gnomonia gnomon</i>	<i>Pestalozzia coryli</i>
<i>Chorostate conjuncta</i>	<i>Gnomoniella coryli</i>	<i>Pezicula corylina</i>
<i>Ciboria amentacea</i>	<i>Helminthosporium macrocarpum</i>	<i>Phellinus punctatus</i>
<i>Coriolus hoehnelii</i>	<i>Helmenthosporium velutinum</i>	<i>Phoma suffulta</i>
<i>Cryptospora corylina</i>	<i>Helotim fructigenum</i>	<i>Phyllactinia corylea</i>
<i>Cylindrosporium coryli</i>	<i>Hypoxylon fuscum</i>	<i>Phyllosticta coryli</i>
<i>Cytospora corylicola</i>	<i>Hypoxylon multiforme</i>	<i>Phytophthora cactorum</i>
<i>Cytospora fuckelii</i>	<i>Hypoxylon unitum</i>	<i>Radulum orbiculae</i>
<i>Diaporthe decedens</i>	<i>Labrella coryli</i>	<i>Rhizopus nodosus</i>
<i>Diaporthe eres</i>	<i>Lachnum hedwigiae</i>	<i>Sclerotinia fructigena</i>
<i>Diatrype disciformis</i>	<i>Mamiania coryli</i>	<i>Septoria avellanae</i>
<i>Diatrype stigma</i>	<i>Mamianiella coryli</i>	<i>Sillia ferruginea</i>
<i>Diatrypella favacea</i>	<i>Marasmius foetidus</i>	<i>Stereum hirsutum</i>
<i>D. verrucaeformis</i>	<i>Melconis sulphurea</i>	<i>Stereum rugosum</i>
<i>Cryptosporiopsis grisea</i>	<i>Melanomma pulvis-pyrius</i>	<i>Sphaeropsis coryli</i>
<i>Diplodia sarmentorum</i>	<i>Merulius rufus</i>	<i>Stictis mollis</i>
<i>Diplodia coryli</i>	<i>Merulius serpens</i>	<i>Taphrina coryli</i>
<i>Fenestella princeps</i>	<i>Monostichella coryli</i>	<i>Tyromyces semipileatus</i>
<i>Fomes annosus</i>	<i>Nectria coryli</i>	<i>Valsa corylina</i>
<i>Fumago vagans</i>	<i>Nectria ditissima</i>	<i>Vuilleminia comedens</i>
<i>Gleosporium coryli</i>	<i>Anisogramma anomala (European Filbert Blight)*</i>	<i>Penicillium aurantiogriseum**</i>
<i>Aspergillus parasiticus***</i>		
European filberts attacked by bacteria		
<i>Agrobacterium tumefaciens</i>		<i>Xanthomonas coryli</i>
Nematodes isolated from European filberts		
<i>Caconema radicolica</i>		
<i>Heterodera marioni</i>		
Sting bugs and other plant bugs		
<i>Halyomorpha halys, Melissopus latiferreanus, Acrosternum hilare and Nezara viridula</i>		

* (Molnar, et al., 2010); ** (Yang, et al., 2014); *** (Simsek, et al., 2002)

Not all pathogens are dreadful. For example, Phytoalexins are antimicrobial and possibly antioxidative substances synthesized by plants in response to pathogen infection. They are a heterogeneous group of compounds that show biological activity towards a variety of pathogens and have been considered as molecular markers of disease resistance (Ahuja, et al., 2012). In other examples, endophytic bacteria of *Bacillus* sp. had been found to be antagonistic against several fungal

pathogens of maize (Szilagyi-Zecchin, et al., 2014) while the role of infection in the production of taxol is considered in section 1.9.2.

1.3 Seed Dormancy

In the present investigation, the term 'seed dormancy' is used in the sense used by (Wareing, 1965), *'for instances where the seed of a given species fails to germinate under conditions of moisture, temperature and oxygen supply which are normally favourable for the later stages of germination and growth of that species'*. Several schemes for classifying seed dormancy have been published reflecting the fact that dormancy is determined by both morphological and physiological properties of the seed (Nikolaeva, 1967; Nikolaeva, 2004). A more comprehensive classification system has been proposed by Baskin & Baskin (1998; 2004). This system includes five classes of seed dormancy: physiological (PD), morphological (MD), morphophysiological (MPD), physical (PY) and combinational (PY + PD).

For the germination of dormant seeds, certain conditions have to be met to break the dormancy prior to germination test. Breaking of dormancy has to culminate in the germination of the seed i.e., protrusion of the radicle, thus radicle emergence is the 'visible sign' of germination and the non-emergence indicates either the seed to be dormant or non-viable.

For dormant seeds, failure of the radicle to penetrate the surrounding tissues has been attributed to either an inability of the radicle to develop sufficient thrust or to mechanical restraint by the tissues. Such mechanical restraint must be relieved by limited hydrolysis to weaken the cell walls (Bewley, 1997b). Evidence has been presented for enzymatic action on the constraining tissue which may promote radicle penetration during seed germination (Black, 1996). It has also been suggested that, just prior to radicle emergence, there is a weakening of the

endosperm in the region of the root tip so that the growth thrust of the radicle becomes sufficient to penetrate it (Groot & Karssen, 1987). More recently it was demonstrated that the enzyme endo- β -mannanase, produced by the endosperm itself, acts to hydrolyse galactomannan (which is a major component of the cell walls) (Groot, et al., 1988; Leviator, et al., 1995; Nomaguchi, et al., 1995); and this may be the hydrolytic mechanism that promotes radicle penetration. This is supported by the observation that, in a gibberellin-deficient mutant (*gib-1*) (whose seeds cannot germinate without treatment with gibberellin), the endosperm produces the enzyme only when this hormone is applied (Groot, et al., 1988). It is further supported by the observation that the use of red light to promote germination is associated with the accumulation of endo- β -mannanase in the micropylar cap endosperm, while the use of abscisic acid to suppress germination is associated with its absence (Nomaguchi, et al., 1995).

Also, development of peroxidase activity specifically in the micropylar region of the endosperm of imbibed tomato seeds prior to radicle emergence has been reported by Morohashi (2002). It has also been proposed that, late in the germination process, the hydrolysis of reserves and solutes stored in the radicle cells may cause the osmotic potential of the radicle cells to become more negative relative to the surrounding tissues and this may allow the radicle to break through the surrounding mechanical barriers (endosperm, testa and pericarp, as the case may be) (Bewley, 1997b). It has been shown that dormancy release in sunflower seeds (*Helianthus annuus* L.) is associated with higher water activity and increased molecular mobility within the embryonic axes, but that there was no similar relationship in the cotyledons. The authors proposed that the changes in water binding properties

result from oxidative processes and can, in turn, allow metabolic activities (Bazin, et al., 2011)

1.4 Orthodox and Recalcitrant Seeds

1.4.1 Orthodox Seeds

The vast majority of species have seeds whose period of viability may be extended by lowering their storage temperature and moisture content. In such seeds the moisture content may be reduced to 2-5% before further drying ceases to increase the viability period. Such viability behaviour was termed as orthodox (Roberts, 1973). The great majority of seeds terminate their development by maturation drying, during which there is a metabolic switch from the developmental mode to that of germination (Kermode & Bewley, 1988). Most orthodox seeds can be dried to low moisture content of 5% or less without damage. This group includes majority of the crops and probably all of the weeds of arable land. The majority of the orthodox seeds which contain 5-7% moisture, will, if stored at -18°C or less in sealed containers, survive for a century or more (IPGRI, 1976).

1.4.2 Recalcitrant Seeds

Difficult to store (recalcitrant) seeds had been recognised a century ago. Few examples of such seeds include many of the tropical tree crops e.g. *Hevea brasiliensis* (Kidd, 1914), *Acer saccharinum* (Jones, 1920), *Inga edulis* (Castro & Krug, 1951), temperate tree *Quercus* spp. (Holmes & Buszewicz, 1958), *Mangifera indica* (Koopman, 1963), *Elaeis guineensis* (Rees, 1963), *Theobroma cacao* (Barton, 1965). To name a few seeds after coining the word 'orthodox' and 'recalcitrant'; *Corylus* spp.; *Juglans* spp (Schopmeyer, 1974), *Dipterocarpos* spp. (Yap, 1981), *Avicennia marina* (Farrant, et al., 1986), *Aesculus hippocastanum* (Tompsett & Pritchard, 1993). The term 'recalcitrant' seed was coined in 1973 by Roberts who explained that recalcitrant seeds tend to be much larger than orthodox

seeds and are desiccation sensitive (Roberts, 1973). The large seed size may contribute to the recalcitrant behaviour as there are greater problems in the internal transport of water compared to smaller seeds (King & Roberts, 1979). Although, several recalcitrant seed species have been recorded as undergoing a measure of dehydration during their development (Finch-Savage, 1995; Vertucci & Farrant, 1995), they do not fully undergo maturation drying and are shed at relatively high water contents. For example, seeds of sensitive species of *Galanthus nivalis* L. and *Narcissus pseudonarcissus* L. are shed with > 50% moisture (Newton, et al., 2013); *Avicennia marina* (Farrant, et al., 1986), *Aesculus hippocastanum* (Tompsett & Pritchard, 1993). If these seeds are dried even a little below the fully hydrated condition they may be killed; reduction of moisture levels below relatively high values (even up to 31%, depending on the species) will reduce the period of viability. Recalcitrant seeds are desiccation sensitive both before and after shedding and have very limited post-harvest life spans, even in hydrated conditions. If they are stored under optimal moisture conditions, seeds lose viability over a relatively short time, as short as a few weeks in some cases; *Avicennia marina* (Farrant, et al., 1986), *Aesculus hippocastanum* (Tompsett & Pritchard, 1993), *Acer pseudoplatanus* (Valerie, et al., 2000). Furthermore, because of the high water content and high water activity of the seeds, recalcitrant seeds contain freezable water and so are killed by freezing.

Desiccation sensitivity and storage behaviour of recalcitrant seeds were reported to differ greatly among species (Pammenter, et al., 1994) and it has been claimed that there is a continuum of recalcitrant seed behaviour, with individual species showing highly, moderately or minimally recalcitrance, depending on their degree of desiccation sensitivity, hydrated life span, and also chilling sensitivity (Farrant, et al., 1988; Berjak, et al., 1989). The degree of recalcitrance may depend on the rate of

dehydration (King & Roberts, 1979; Berjak, et al., 1989) and the developmental status of the seeds (Berjak, et al., 1992; Finch-Savage, 1992; Hong & Ellis, 1992; Tompsett & Pritchard, 1993).

A characteristic of recalcitrant seeds is that if they are stored under conditions that prevent water loss, they will ultimately lose viability. A current view of recalcitrant seeds is that in hydrated state they are metabolically active and undergo germination associated changes in storage. Some of the changes include extensive vacuolation and increase in cell size as seen in *Landolphia kirki* (Berjak, et al., 1992), implying a requirement for water additional to that present in the seed on shedding. When water was not supplied at this point, viability declined. Similar trends were observed in *Camellia sinensis* (Berjak, et al., 1993). This shows that, in storage, recalcitrant seeds are exposed to an initially mild, but increasingly severe, water stress. The deleterious events associated with water stress of considerable duration results in tissue death.

Some discrepancies have been noted as experimental results from various research laboratories that included some species in more than one group presented in Table 1.2. The most interesting example being hazelnut, which has been included in all three storage groups. Inter-species variation in storage behaviour is also exhibited in several seeds. Seeds of *Coffea arabica* (arabica coffee) and *C. canephora* (robusta coffee) have intermediate habit but *C. liberica* (liberica coffee) shows recalcitrant habit (Hong & Ellis, 1995). In the Family Aceraceae *Acer pseudoplatanus* and *A. saccharinum* exhibits recalcitrant habit, whereas *A. platanoides* and *A. rubrum* (Dickie, et al., 1991; Hong & Ellis, 1992) are desiccation tolerant (orthodox). As for other examples, *Araucaria augustifolia* (Farrant, et al., 1989), *A. excelsa* (Magini, 1962) and *A. hunsteinii* (Pritchard & Pendergast, 1986)

are recalcitrant, although seeds of *A. columnaris* have been characterised as orthodox (Tompsett, 1984). Inter-specific variation in storage behaviour is also exhibited in *Dipterocarpus* (Tompsett, 1987). In Meliaceae, *Aglaia clarkii* and *Sandoricum koetjape* showed no seed survival at moisture contents of 20% or below and therefore were recalcitrant in storage habit (Hong & Ellis, 1998). On the other hand, *Melia azadirachta* survived desiccation to 3.5% moisture content and showed orthodox seed storage behaviour (Hong & Ellis, 1998).

Table 1.2: Examples of some species variously classified as recalcitrant, intermediate and orthodox

Species	Recalcitrant	Intermediate	Orthodox
<i>Corylus avellana</i> L. (Hazelnut)	(Slate, 1969); (King & Roberts, 1980(a)); (Mehlenbacher, 1991)	(Stanwood, 1985); (Bonner, 1986); (Dickie & Pritchard, 2002)	(Barbour & Brinkman, 2004) (Gosling, 2007) (Michalak, et al., 2013)
<i>Azadirachta indica</i> A. Juss (Neem)	(Ezumah, 1986); (CNSF, 1993) (Gunasena & Marambe, 1995); (Msanga, 1996)	(Berjak & D. Dumet, 1996); (Gamene, et al., 1996); (Sacande, et al., 1996); (Nayal, et al., 2000)	(Roederer & Bellefontaine, 1989); (Dickie & Smith, 1992)
<i>Carica papaya</i> L. (Papaya)	—	(Becwar, et al., 1983); (Ellis, et al., 1991b)	(Bass, 1975); (Perez, et al., 1980)
<i>Fagus sylvatica</i> L.	—	(Bonner, 1990); (Gosling, 1991) (Pedro & Ellis, 2002)	(Poulsen, 1993); (Poulsen & Kundsén, 1999)
<i>Michelia compressa</i> (Max.) Sargent	Huang, 1964, cited in Lin & Wu, 1995	—	(Lin & Wu, 1995); (Chu, Kuo & Tsai, 1993, cited in Lin & Wu, 1995, p.310)
<i>Coffea arabica</i> L.	—	(Ellis, et al., 1990); (Hong & Ellis, 2002)	—
<i>Cinnamomum camphora</i> L. Sieb	(Chin, 1988); (King & Roberts, 1980(a))	(Chien & Lin, 1999)	—

*survived 10 yrs at -20°C and stated to possess an 'exceptional storage habit'

There have been recent challenging question whether recalcitrant seeds exists (Barbedo, et al., 2013). In the review paper it is argued that although the terms orthodox (desiccation and freezing temperature tolerant) and recalcitrant (loss of moisture reduces viability) was coined in 1973, seeds showing storage problems

(recalcitrant) were cited a century ago, *Hevea brasiliensis* (Kidd, 1914), *Acer saccharinum* (Jones, 1920). The argument is based on the observations of:

- a) Orthodox seeds developed desiccation tolerance (Alpert, 2005) whereas recalcitrant seeds developed mechanisms for quick germination; (Barbedo & Bilia, 1998; Manfre, et al., 2009; Obroucheva, et al., 2012).
- b) Desiccation tolerance varies among seeds within the same species and amongst different species, depending on the conditions of development (Pammenter, et al., 1994; Walters, 2000; Bovi, et al., 2004; Daws, et al., 2004; Daws, et al., 2006; Lamarca, et al., 2011; Delgado & Barbedo, 2012; Pereira, et al., 2012; Lamarca, et al., 2013).
- c) Desiccation tolerance acquired progressively during maturation of the species (Manfre, et al., 2009; Hay, et al., 2010; Ellis, 2011) which is also observed in orthodox seeds behaviour during its formation and maturation.
- d) High water content (> 50%) in sensitive seeds of *Galanthus nivalis* L. and *Narcissus pseudonarcissus* L. at dispersal with the ability to germinate prior to acquisition of desiccation tolerance undergo considerable embryo development after shedding (Newton, et al., 2013). The suggestion indicates that seeds of those species are still immature at shedding, as in cases of seeds of *Eugenia* species reported by (Delgado & Barbedo, 2012).

Therefore, the argument resulted from the observations above that biochemical and anatomical aspects of both sensitive and desiccation tolerant seeds behave similarly during the early stages of development and have high metabolic activity. Later on during further maturation, tolerant seeds switch off the metabolism, whereas the sensitive seeds maintain increased metabolism until shedding. Thus, completion of maturation process of the so-called recalcitrant seeds and immature phase of the

so-called orthodox seeds by keeping them linked to the mother plant need to be amplified to elucidate if recalcitrance does exist.

Even orthodox seeds are not able to withstand desiccation at all stages of development. Rather, tolerance to desiccation is acquired at a precise stage of development and is irreversibly lost on germination of the seed; (Bewley & Black, 1982; Leopold, 1990; Hong & Ellis, 1992). In most plant species, tolerance is first observed towards the end of the seed's development on the plant, but there is some variation between species.

1.5 Desiccation and Imbibition Injury

Seeds whether orthodox or recalcitrant are not able to withstand desiccation at all stages of development but acquire at a specific stage of development, mostly achieved towards the end of seed development which is lost on germination (Bewley & Black, 1982; Leopold, 1990; Hong & Ellis, 1992). Not all seeds are germinated immediately after maturation but need to be stored for re-use at a convenient time and need. Orthodox seeds are capable of surviving low moisture and low temperature but recalcitrant seeds are unable to do so (Roberts, 1973). In orthodox seeds, this situation is reversed with the membranes either physically reverting to their most stable configuration, and/or else being repaired by some unidentified mechanism, probably metabolic repair (Simon, 1984; Tilden & West, 1985; Pandey, 1988; Pandey, 1989). Most damages are expected in recalcitrant seeds and are dealt with below.

1.5.1 Physical Injury

During dehydration, the drying protoplast will be subjected to tension due to volume contraction and its adherence to the cell wall. Figure 1.2 shows the effects of dehydration on shrinkage of membranes with possible rupture and deterioration of

the organelles. As seed deterioration progresses, the cell membranes become less rigid and become more water permeable. It allows the cell contents to leach into solution with the water (Bryant, et al., 2001). This provides a rapid indication of seed viability for seed lots as the leachate concentration can be measured by electrical conductance methods and by determining the soluble sugar content of the leachate.

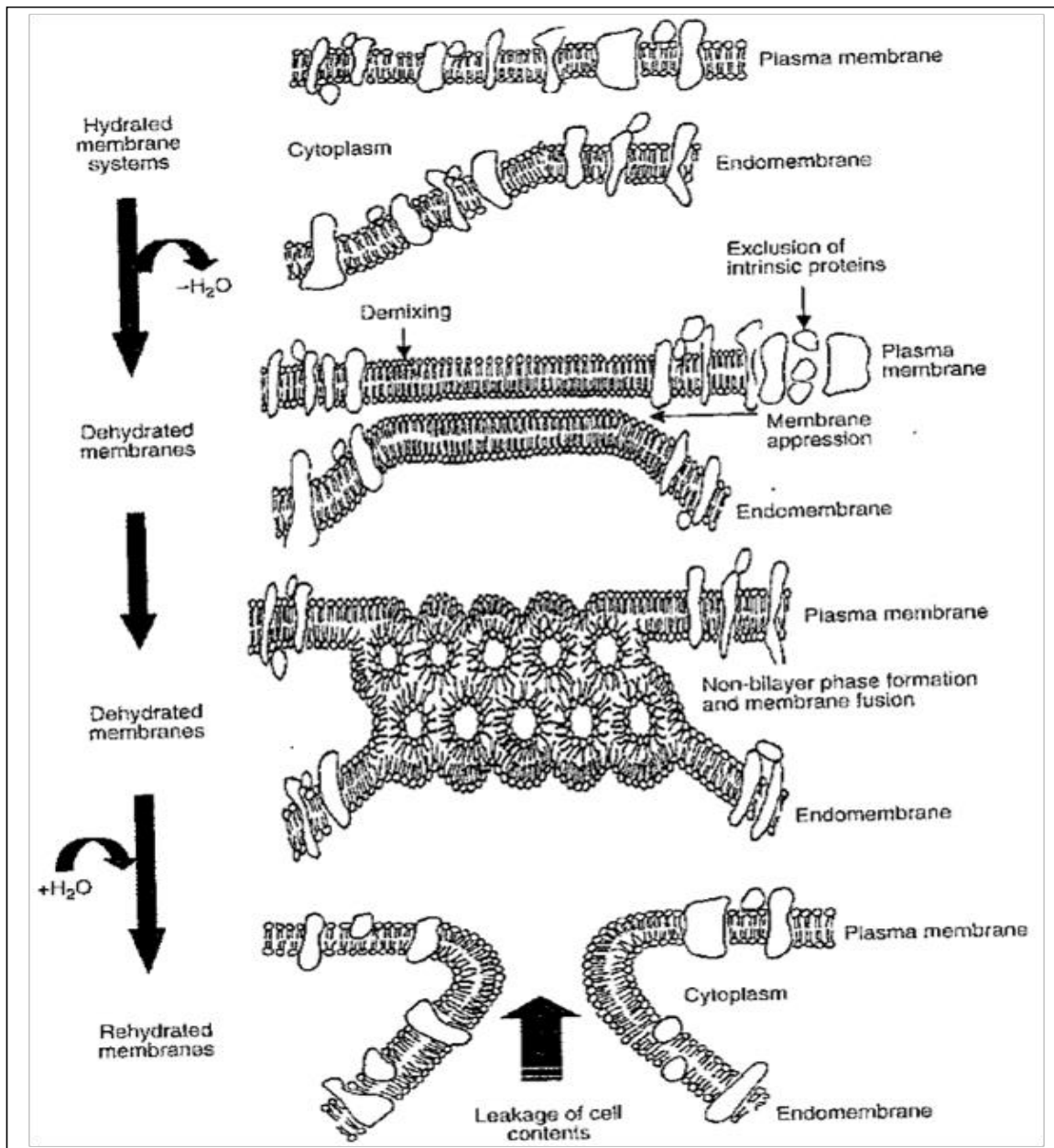


Figure 1.2: Schematic drawing of the effect of dehydration on cellular membranes: Different membranes may become appressed and fused on dehydration; upon rehydration, cellular contents leak out (Walters, et al., 2002)

An excessively rapid inrush of water into the seeds may result in imbibition injury due to cellular rupture (Powell, et al., 1986). On re-imbibition a high osmotic gradient between the desiccated seed and the surrounding imbibing medium will cause a rapid inrush of water and this may lead to the displacements of the membrane components, phospholipids and proteins, thus disrupting membrane reorganization. In the absence of intact membranes, deleterious mixing of cellular constituents and their loss through leakage into the aqueous medium by diffusion into and dispersal by the turbulent flow of water may follow (Simon, 1984). Therefore with a view to the establishment of suitable moisture content for seed storage of recalcitrant hazel seeds, it is important to examine the various effects of desiccation on seeds with particular interest in leachate conductivity.

1.5.2 Chemical injury

Seeds progressively lose their viability during prolonged storage (Roberts, 1973). Among the biochemical processes involved in seed deterioration, oxidative injuries and lipid peroxidations have often been considered to be major events in recalcitrant seeds of *Acer saccharinum* (Pukacka, 1989; Pukacka, 1993; Pukacka, 1998; Kranner & Birtic, 2005; Pukacka & Ratajczak, 2006). Chemical injury in the form of free radical damage has been used to explain desiccation injury and death. Several biological oxidations, both enzymatic and chemical, generate the free superoxide radical ($O_2^{\cdot -}$) and this can in turn react with hydrogen peroxide to produce singlet oxygen and the hydroxyl radical (OH^{\cdot}), all of which are potential cytotoxic oxidants. Hydroxyl radicals are so reactive that they can attack and damage almost every molecule of living cells (Bailly, 2004). Free radical/s once activated or formed (initiation) undergo propagation and termination which results in damaging impact on tissues unless deactivated by antioxidants. It is notable that aerobic metabolism

generally depends on a stringent control of reactive oxygen species (ROS) by antioxidants (Finkel & Holbrook, 2000; Abele, 2002). A comprehensive review of salt stress in plants has identified a wealth of information on soluble sugars, proteins, amino acids & amides, quaternary ammonium compounds, polyamines, polyols, antioxidants and ATPases (Ashraf & Harris, 2004). The review emphasises antioxidants show resistance to salinity and other abiotic stress. Figure 1.3 shows free radical quenching by antioxidants.

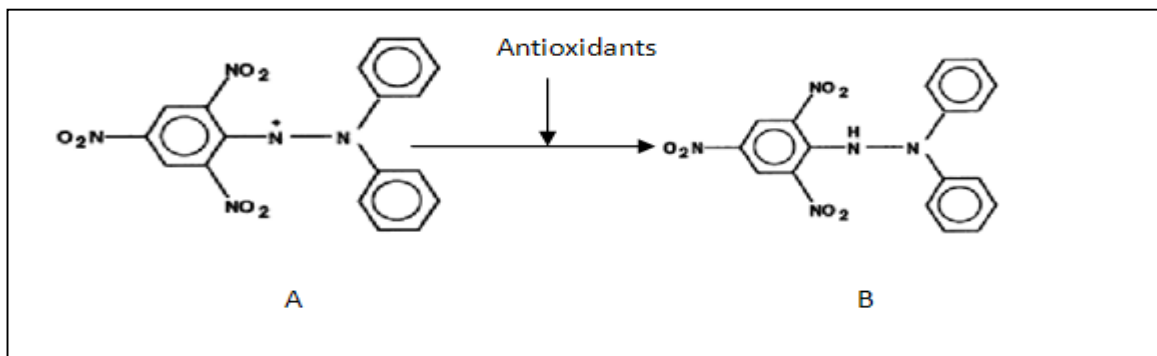


Figure 1.3: Quenching free radical action by antioxidants: A. Stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), B. Structure of free radical quenched DPPH. Adapted from (Pyrzynska & Anna Pękal, 2013).

There seems to be a connection between oxidative damage, on one hand and antioxidant defence mechanisms on the other hand (Abele, 2002; Alpert, 2005). Therefore, it can be concluded that living organisms (seed in this case) are capable to counter free radical attacks provided they are equipped or provided with appropriate mechanisms. In sunflower seeds, loss of viability is associated with an accumulation of malondialdehyde (MDA), which is a major lipid oxidation product, suggesting that seed deterioration is linked with lipid peroxidation and related to a decline in the efficiency of the antioxidant defence system (Kibinza, et al., 2006).

1.6 The Development of Desiccation Tolerance

In most plant species, desiccation tolerance is first observed towards the end of the seed's development on the plant, but there is some variation between species. In order for plant cells to tolerate desiccation they must have the ability to withstand

the mechanical stresses associated with the consequent reduction in volume (Iljin, 1957). Mechanisms exhibited by plants include:

- i. Through reduction of the volume of fluid-filled vacuoles during shrinkage;
- ii. Breaking up of large vacuoles into many smaller ones;
- iii. But the seed becoming filled with insoluble reserve material.

Thus, there appears to be a relationship between the degree of desiccation sensitivity and the extent of vacuolation and of insoluble reserve accumulation (Berjak, et al., 1989; Farrant, et al., 1989). Reduction of moisture levels may be associated with reduced or altered metabolic activity and induce production of protectants for the protection of cell organelles (Bewley, 1979; Kermode, 1990; Bewley & Oliver, 1992). These include ascorbates which acts as an antioxidant (Tommasi, et al., 1999; Tommasi, et al., 2006; Tang, 2012; Jamalomidi & Gholami, 2013). Seeds may contain sugars such as raffinose and stachyose which can prevent the crystallisation of sucrose and upon drying may permit formation of a stable glassy state preventing membrane fusion and acts as a protectant (Caffrey, et al., 1988; Koster, et al., 1994) while polyols may act as scavengers of free radicals (Orthen, et al., 1994).

An important group of protectants are the Late Embryogenesis Abundant (LEA) proteins (Galau, et al., 1986) and LEA homologues (Dehydrins) which were first observed to accumulate during cotton seed development at the point when the seed becomes desiccation tolerant (Dure, et al., 1981) and were later observed in other seeds (Bewley & Oliver, 1992; Vertucci & Farrant, 1995) and vegetative organs (Close, et al., 1989; Close, et al., 1993a; Close, et al., 1993b) under stress conditions such as desiccation, but also low temperature and high salt

concentrations (Tunnacliffe & Wise, 2007; Tunnacliffe, et al., 2010). LEA proteins are heat stable and appear to be intrinsically disordered in their hydrated state, but they seem to become structured and partially fold to form α -helices during drying (Tunnacliffe, et al., 2010; Hand, et al., 2011; Hinch & Thalhammer, 2012). It is not yet clear how these properties may relate to their physiological roles in the dry state, which may include stabilisation of membranes, subcellular structures and enzymes during desiccation (Lane, 1991; Close, et al., 1993a; Close, et al., 1993b; Dure, 1993), water or ion binding, acting as antioxidants, or sugar glass stabilization (Close, et al., 1989; Dure, et al., 1989; Tunnacliffe & Wise, 2007; Tunnacliffe, et al., 2010). Similar proteins also seem to be involved in resistance to freezing. The presence of LEA proteins has also been associated with high contents of abscisic acid (ABA) (Kermode, 1990) and it has been shown that ABA can induce their production (Galau, et al., 1986; Finch-Savage, et al., 1994). Comparative proteomics between orthodox *Spartina pectinata* and recalcitrant *Spartina alterniflora* identified 83 heat-stable proteins in *S. pectinata* which were mostly missing in recalcitrant *S. alterniflora* (Wang, 2013), dehydrin-like proteins have been found in recalcitrant seeds from species such as *Zizania palustris* (Bradford & Chandler, 1992; Still, et al., 1994), *Quercus robur*, *Castanea sativa*, *Aesculus hippocastanum*, *Acer pseudoplatanus* and *Acer saccharinum* (Finch-Savage, et al., 1994).

1.7 Seed Banks

For the majority of plants, Seed Banks are considered to be the best way for the long-term maintenance of crop plant germplasm from the natural environment according to the Report on the State of the World's Plant genetic Resources for Food and Agriculture of the Food and Agriculture Organization of the United Nations (FAOSTAT, 2014). Storage of seeds at low temperature is the main conservation

method employed in genebanks and the recommended preferred conditions for long-term seed storage are 3-7% seed moisture content, depending on the species, at -18°C or cooler (Engles & Engelmann, 1998). Over 95% of the species stored in this way have agricultural or commercial importance and the majority of these are cereals (47%) and pulses (16%) (Villalobos & Engelman, 1995).

This is not so effective for recalcitrant seeds and for example, as a result of an inability to preserve hazel seeds, intact hazel trees are reported to be preserved as *in situ* collections in 20 stations (seed banks) over 16 countries (Battencourt & Konopka, 1989).

1.8 Hazelnut

In this study, hazelnut was chosen as the nuts are consumed all over the world due to its organoleptic characteristics (section 1.9.1). Hazelnuts also known as filberts (*Corylus spp.*) are a perennial tree crop that has been grown for many years in various countries around the world. Over 8.5 million tons of nuts were produced throughout the world per year, 700 thousand tons of which are hazelnuts (*Corylus avellana* L.) (Oliveira, et al., 2008). Turkey is the world's largest hazelnut producer, contributing approximately 70% to the total global production, followed by Italy (12%), the United States (6%), and Spain (2%) (Oliveira, et al., 2008). Studies showed the importance of the food benefits of hazelnuts (Alasalvar, et al., 2003a) and phenolic compounds in human health promotion and disease risk reduction (Alasalvar, et al., 2006). However, it is also reported to cause allergy in some cases as detailed in section 1.9.1.

1.8.1 Hazel Seed Viability and Vigour

Many researches were conducted to develop the safe storage and continuation of the species. Test of storage habit of hazelnut is a problem as it shows both seed

dormancy and recalcitrance like that of *Aesculus hippocastanum* L. (Tompsett & Pritchard, 1993), *Cycas* L. and *Zamia* L. as well as *Encephalartos cycadifolius* (Jacq.) Lehm. (Dehgan & Yuen, 1983), *Zizania palustris* L. (Kovach & Bradford, 1992) and *Acer pseudoplatanus* (Valerie, et al., 2000). Hazel seeds exhibit dormancy, which needs to be discussed to elaborate its significance in viability and longevity and, in the 1980s the problems of recalcitrance in hazel seeds and many other plant species (Table 1.3) encouraged further assessment of its storage habit. Viability of newly harvested hazel seeds has been reported to be between 6 months (Bradbeer, 1968; King & Roberts, 1980) to a year (Mehlenbacher, 1991). Earlier, Pinfield (1965), also observed that hazel seed tended to show a fall in their viability during storage, and the phenomenon was more pronounced at higher temperatures (20-22°C) (ambient laboratory conditions) than when stored in cooler conditions. Decrease in hazel seed germination was observed with increased time of storage at 5, 10 and 20°C, with the most severe decrease at 20°C (Rendon, 1983). In another report, *Corylus* seed maintained viability for 6 months when stored at 1°C in a polythene sack and claimed that drying was responsible for seed damage (Slate, 1969). Two contradicting storage conditions have been suggested for hazelnuts:

i) Storage at 3°C (Tylkowski, 1999), and ii) Storage in Liquid N₂ (Michalak, et al., 2013).

A list of reported hazelnuts' storage life is presented in Table 1.3 which presents storage in hydrated and dehydrated conditions from 5°C, 1°C, -3°C, -18°C and even in LN. All storage practice should be based on producing substantial percentage of healthy seedlings. Hence, any research on seed germination and viability needs to be complemented with satisfactory seedling establishment.

1.8.2 Hazel Seed Recalcitrance

Table 1.2 shows the inclusion of hazelnuts in all three storage groups, (see also Table 1.3) and classification of hazel and some other seeds for storage purpose had caused some confusion. Of the various classifications of hazel, those suggesting orthodox habit have been the latest (Barbour & Brinkman, 2004; Gosling, 2007; Michalak, et al., 2013). Recalcitrance in hazelnuts has been reported (Slate, 1969; King & Roberts, 198; Mehlenbacher, 1991). Whereas, hazelnut referred to as intermediate by (Stanwood, 1985; Bonner, 19; Dickie & Pritchard, 2002).

Nevertheless, since hazelnuts are shed with >40% seed moisture, it features recalcitrant behaviour. But for storage purpose, seed moisture need to be reduced, which will result in free radical attack. Therefore, hazelnuts must have some protective mechanism against free radicals. It is reported that 100 g of hazel seeds contain 2.2 mg of ascorbic acid, and 2 - 5% of sucrose, raffinose and stachyose (Duke, 1989), which are highly likely to be part of their natural defence mechanism.

Besides *Corylus avellana*, another species of hazel (*Corylus americana* Marsh) was allocated to a group for which recalcitrant seed behaviour had been suggested (Harrington, 1972) but for which evidence of viability is lacking (King & Roberts, 1980(a)). Tests on dry hazel seeds stored at 20°C and also at 5°C showed that the expected increase in electrolyte exudates with storage time was not apparent from the data obtained while an assessment of seed viability by means of the tetrazolium test during the period of storage indicated that cotyledon tissue and axis material were viable throughout the course of dry storage (Rendon, 1983). Moreover, glucose test sticks (sensitive to 1 mg/ml of glucose) failed to detect glucose in exudates. None of these parameters showed appreciable evidence for the loss of

seed viability as a result of drying out alone and thus, it appears that the dry seed is not intrinsically non-viable (Rendon, 1983).

Table 1.3: Storage conditions and longevity of various species of hazel seeds as reported by researchers (Hong & Ellis, 1998) are listed:

Species	Storage characteristics	Viability	Reference
<i>Corylus americana</i> Marsh. (American hazel)	Seeds do not tolerate excessive desiccation	Not tested	(Heit, 1967); (Harrington, 1972)
<i>Corulus americana</i> and <i>Corylus avellana</i> L. (European hazel)	i) Cold moist storage ii) Open storage at room temperature	i) 1 year ii) 1 year	(Brinkman, 1974)
	Drying damages seed viability	Not tested	(Slate, 1969); (Harrington, 1972) (Kowalski & Kawecki, 1982)
	Moist storage at 1°C.	More than 6 months	(Slate, 1969)
	Storage at 10°C	6 months	(Bradbeer, 1968); (Jarvis, 1975)
	Desiccation below 15% moisture content	Not tested	(Gordon & Rowe, 1982)
	Desiccation below 13.5% moisture content	Not tested	(Degeyter, 1987, cited in Hong & Ellis, 1996)
	Storage at 2°C or -5°C with 13.5% MC	3.5 months	(Degeyter, 1987, cited in Hong & Ellis, 1996)
	80% survived cryostorage in liquid nitrogen (LN)	Not tested	(Pence, 1990)
<i>Corylus avellana</i> L	i) Seed 13.6% MC in LN ii) Embryonic axes 3% MC in LN	i) No survival ii) Viable	(Normah, et al., 1994)
	Long-term storage of hazel seeds at -18°C	Not tested	(Battencourt & Konopka, 1989)
<i>Corylus cornuta</i> Marsh.	Hermetic storage at 5°C	Not tested	(Brinkman, 1974)
	14 months dry storage (MC not mentioned)	Not tested	(Hartman, 1989)
	Dry (m.c. not reported) seeds killed in LN	Not tested	(Stanwood & Bass, 1981)
<i>Corylus avellana</i> L	12.2% MC at -3°C	6 years	(Tylkowski, 1999)
	5-10% MC in LN	50% viable	(Michalak, et al., 2013)
	3°C	<1 year	(Reed, 1999)

1.8.3 Hazel Seed Dormancy

During the last five decades a number of research works had been carried out on the metabolic aspects on dormancy breaking of hazel seeds (Table 1.4).

Table 1.4: List of metabolic research works done related to seed dormancy of hazelnuts.

Title	Reference
Nucleotide synthesis in hazel seeds during after-ripening.	(Bradbeer & Floyd, 1964)
Studies in seed dormancy 1. The metabolism of (^{14}C) acetate by chilled seeds of <i>Corylus avellana</i> L.	(Bradbeer & Colman, 1967)
Studies in seed dormancy. II. The nucleic acid metabolism of the cotyledons of <i>Corylus avellana</i> L.	(Wood & Bradbeer, 1967)
Studies in seed dormancy. III. The effects of gibberellin on dormant seeds of <i>Corylus avellana</i> L.	(Bradbeer & Pinfield, 1967)
Studies in seed dormancy. IV. The role of inhibitors and gibberellin in the dormancy and germination of <i>Corylus avellana</i> L. seeds.	(Bradbeer, 1968)
Increased nucleic-acid synthesis in relation to the breaking dormancy of hazel seed by gibberellic acid.	(Jarvis, et al., 1968)
Concentrations of gibberellins in chilled hazel seeds.	(Ross & Bradbeer, 1968)
Gibberellin-stimulated nucleic acid metabolism in cotyledons and embryonic axes of <i>Corylus avellana</i> (L) seeds.	(Pinfield & Stobert, 1969)
Glycerol utilization in seeds of <i>Corylus avellana</i> (L)	(Stobart & Pinfield, 1970)
Studies in seed dormancy.V. The content of endogenous gibberellins in seeds of <i>Corylus avellana</i> L.	(Ross & Bradbeer, 1971a)
Studies in seed dormancy. VI. The effects of growth retardants on the gibberellin content and germination of chilled seeds of <i>Corylus avellana</i> L	(Ross & Bradbeer, 1971b)
Studies in seed dormancy. VII. The abscisic acid content of the seeds and fruits of <i>Corylus avellana</i> L.	(Williams, et al., 1973)
Studies in seed dormancy. VIII. The identification and determination of gibberellins A_1 and A_9 in Seeds of <i>Corylus avellana</i> L.	(Williams, et al., 1974)
The role of seed parts in the induction of dormancy of hazel (<i>Corylus avellana</i> L.).	(Jarvis, 1975)
Studies in seed dormancy. IX. The role of gibberellin biosynthesis and the release of bound gibberellin in the post chilling accumulation of gibberellin in seeds of <i>Corylus avellana</i> L.	(Arias, et al., 1976)
The role of chilling in the breaking of seed dormancy in <i>Corylus avellana</i> L.	(Bradbeer, et al., 1978)

Continued on next page

Table 1.4 continued

Title	Reference
Factors Influencing Growth of Embryonic Axes from Dormant Seeds of Hazel (<i>Corylus avellana</i> L.).	(Jarvis & Wilson, 1978)
Growth of isolated axes from dormant seeds of hazel (<i>Coryllus avellana</i> L.),	(Jarvis, et al., 1978)
The influence of cotyledons on embryonic axes during Induction of Dormancy in <i>Corylus avellana</i> .	(Jarvis, 1979)
Pentose phosphate metabolism during dormancy-breakage in <i>Corylus avellana</i> L.	(Gosling & Ross, 1980b)
Peroxidase levels in the cotyledon of hazel seed (<i>Corylus avellana</i> L.),	(Gosling & Ross, 1981)
Messenger RNA in embryonic axes of dormant and non-dormant seeds of hazel (<i>Corylus avellana</i> L.).	(Shannon, et al., 1981)
Lipid mobilisation during dormancy-breakage in oilseed of <i>Corylus avellana</i> .	(Li & Ross, 1990)
Isolation and characterisation of phytase from dormant <i>Corylus avellana</i> seeds.	(Andriotis & Ross, 2003)
Isolation and partial characterisation of acid phosphatase isozymes from dormant oilseed of <i>Corylus avellana</i> L	(Andriotis & Ross, 2004)

The series of investigations on the dormancy breaking methods of hazel seed are detailed in Table 1.5. Research on hazelnuts first concentrated on the physiology and biochemistry of the germination process including the mobilisation of the food reserves of the seeds (Bradbeer, 1988).

Hazelnuts show physiological dormancy due to germination inhibitors present in embryo, testa and seed coat (Bradbeer, et al., 1978). The dormancy mechanism may therefore, reside in the embryo coverings or in the embryo or in both. The embryo coverings include endosperm, testa and pericarp. In the case of hazel, however, endosperm is only one or two cells thick (Vaughan, 1970).

In the 1960s it was found that embryos of freshly harvested hazel seed were not dormant but that embryo dormancy developed during storage, and that testa and pericarp contained germination inhibitors (Bradbeer, 1968). Removal of the inhibitory effects of the testa and pericarp, by either the removal of these tissues or by treatment with GA₃, ethylene or illumination was effective in stimulating the germination of most of the seeds Table 1.4). The addition of GA₃, kinetin, thiourea,

potassium nitrate and ethylene enhanced embryo (without testa) germination whereas, seed (with testa) germination in kinetin, potassium nitrate and ethylene was much reduced. Chilling hazelnuts for various periods ranging from 2 to 20 weeks on the other hand enhanced seed germination (Table 1.4).

Dormancy and germination may be controlled by the embryonic axis. Treatment of seeds with Gibberellic Acid (GA_3) breaks dormancy and GA_3 is known to increase RNA synthesis in the hazel embryonic axis (Jarvis, et al., 1968; Pinfield & Stobert, 1969). Although GA_3 acts directly on the embryonic axis, irrespective of any effect on the cotyledons (Jarvis, 1975) it has also been suggested that, in hazel, embryo dormancy may also be caused by the inherent constraints of the presence of the cotyledons. Several researchers have reported how they have overcome this effect of the cotyledons in dormancy by the use of the so-called 'half-seeds' in which the embryo remains attached to one cotyledon after the excision of the other cotyledon. Amputation of one cotyledon has been reported to result in germination and growth of the embryonic axis of the dormant embryo in *Corylus avellana* L. Jarvis (1975 and 1979). Even removal of the distal halves of the cotyledons brought about germination of dormant hazel seeds (Jarvis, 1975; Arias, 1976).

Treatment of intact seeds with GA_3 results in increased metabolism within the cotyledons (Stobart & Pinfield, 1970), while treatment of isolated cotyledons is reported to stimulate RNA synthesis (Pinfield & Stobert, 1969). It has been reported that cotyledons could clearly regulate the development of their petioles in the absence of the axis, suggesting that there is one or more GA_3 -sensitive sites in the cotyledons capable of initiating petiole development independent of axis control (Gosling & Ross, 1980a). It has also been suggested that the increased metabolism within the cotyledons caused by GA_3 may break dormancy through mobilization of oil reserves in the cotyledons of hazel seeds (Bradbeer & Pinfield, 1967). It is not

clear how this is achieved by removing a cotyledon or parts of cotyledons. Suggestion that inhibitors present in the dormant cotyledon is reduced by removing a whole or part of a cotyledon thereby allowing metabolic activities for germination (Rendon, 1983). Removal of a whole or part of a cotyledon may also reduce food reserves available for the developing embryonic axes. Tests on effects of cotyledons on germination and seedling production had shown that embryonic axes on its own cannot produce a seedling while seedling growth reduced to half when one whole or two half cotyledons was removed (Rendon, 1983). When two half cotyledons were attached with the embryonic axis the seedling height was much bigger compared to one whole cotyledon attached. This indicates the need of reserve food supply from both cotyledons during seedling growth.

Table 1.5: Dormancy breaking mechanisms of newly harvested, dormant and chilled hazelnuts by various physical, physiological and chemical treatments. Symbol (✓) in Pericarp [(+)(with), -(without) shell], in seed [(+)(with), -(without) testa]

Status	Pericarp		Testa		Treatments	% Germination	Reference
	+	-	+	-			
Newly harvested		✓	✓		None	7	(Bradbeer, 1968)
		✓		✓	None	64	
		✓	✓		Ethylene	80	(Arias, et al., 1976)
		✓		✓	Ethylene	98	
		✓	✓		Light	40	
		✓		✓	Light	88	
		✓	✓		GA ₃	80	(Bradbeer, 1968)
Dormant		✓	✓		Leaching	20	(Jarvis, 1966)
		✓	✓		GA ₃	100	(Frankland, 1961)
		✓		✓	GA ₃	100	
		✓	✓		Kinetin	5	
		✓		✓	Kinetin	90	
		✓	✓		Thiourea	95	
		✓		✓	Thiourea	90	
		✓	✓		Potassium nitrate	5	
		✓		✓	Potassium nitrate	30	
		✓	✓		Ethylene	15	(Arias, et al., 1976)
	✓		✓	Ethylene	54		
Chilling		✓	✓		2 Weeks chilling	60	(Frankland & Wareing, 1962)
		✓		✓	2 Weeks chilling	70	
		✓	✓		4 Weeks chilling	64	(Ross, 1970)
		✓	✓		6 Weeks chilling	64	(Arias, 1976)
		✓	✓		6 Weeks chilling + GA	86	
		✓	✓		6 Weeks chilling + Ethylene	94	
		✓	✓		6 Weeks chilling + Light	76	
		✓		✓	6 Weeks	24	
	✓		✓		9 Weeks	62	(Rendon, 1983)
	✓		✓		12 Weeks	70	
	✓		✓		18 Weeks	76	
	✓		✓		20 Weeks	75	

1.9 The Importance of Hazelnuts

1.9.1 Food

Organoleptic, nutritional and nutraceutical properties of hazelnut have resulted in its consumption all over the world as a fruit and in cereals and in manufactured food products including chocolate, pastries, spreads and ice cream (Ozdemir & Akinci, 2004; Amaral, et al., 2006a). It is considered a 'healthy' food because of the high concentration of monounsaturated fatty acids (mainly oleic acid) as well as and polyunsaturated fatty acids which protects against cardiovascular diseases (Oliveira, et al., 2008). It also has high protein and fibre content as well as tocopherol, phytosterols, squalene and other antioxidant and antiradical activities (Alasalvar, et al., 2003a; Alasalvar, et al., 2006), leading to suggestions of the potential in the prevention of cancer, atherosclerosis and diabetes (Alasalvar, et al., 2003a). Antioxidants have been extracted from hazelnut kernel, hazelnut green leafy cover (Alasalvar, et al., 2006) and other hazelnut byproducts such as hazelnut tree leaf (Oliveira, et al., 2007; Shahidi, et al., 2007).

The weakness of hazelnuts being several reports on hazelnut allergy. Researches have been carried out to find the cause and cure to hazelnut allergies. The severity of the symptoms varies from mild to potentially fatal anaphylaxis. Susceptibility appears to vary between age groups and between different locations (De Knop, et al., 2011; Ebo, et al., 2012). While *in vitro* studies suggest differences in IgE profiles of allergic and tolerant individuals across Europe (Hansen, et al., 2009), a lipid transfer protein (LTP, Cor a 8) together with the seed storage globulins (Cor a 9 and Cor a 11) have been identified as major food allergens in hazelnut (Rigby, et al., 2008). A report also suggests development of a novel and safe approach of specific immunotherapy of hazelnut allergy (Lüttkopf, et al., 2002). It is also suggested that

in the absence of a cure, avoidance remains the key measure of effective deterrent, particularly in those patients presenting with a severe form.

1.9.2 Taxol Production

Taxol (paclitaxol) was first identified in yew tree (*Taxus brevifolia*) (Wani, et al., 1971) and is one of the best known drugs for the treatment of breast, ovarian and some other cancers (Ojima, et al., 2002). Works on bark, wood, root, leaf, twig and seedling from *Taxus brevifolia*, *Taxus baccata* L., *Taxus media* Rehder and *Taxus cuspidata* Sieb. et Zucc. resulted that 10, 000 kg bark would produce 1 kg of Taxol (Vidensek, et al., 1990) which will have a serious impact on *Taxus* spp. Currently, time consuming and expensive to manufacture through fermentation or semi-synthesis, or extraction from the tree bark are the only feasible sources of the drug, but yew tree is also endangered and slow growing (Kingston, 1994). The importance of hazelnuts has been increased enormously with the discovery that the hazelnut tree is the master producer of Taxol (Hoffman, et al., 1998). Not only that, hazel Taxol is stated to be more active than manufactured Taxol (Bemani, et al., 2012; Qaderi, et al., 2012; Bemani, et al., 2013). It is stated that taxol found in hazel was actually derived from endophytic fungi living inside the hazel, rather than from hazel itself (Hoffman, et al., 1998; Heinig, et al., 2013). Therefore, it will be very interesting to look at the presence of endophytes in hazelnuts. Three fungal species has been identified to be related to taxol production in *Taxus brevifolia* (*Taxomyces andreanae*) (Stierle, et al., 1993); *Pestalotiopsis microspora* in *Taxus wallichiana* (Strobel, et al., 1996); and *Tubercularia* sp strain TF5, in *Taxus mairei* (Wang, et al., 2000). Although, 160 publications and patents have resulted since the first report of Taxol in *Taxus brevifolia*, a comprehensive research using a combination of phytochemistry, molecular biology and genetic sequence failed to find evidence of independent taxane biosynthesis in any of the endophytes, including *Taxomyces*

andreae (Heinig, et al., 2013). Thereafter, Kusari, et al., (2014), points to the variability of Taxol production (nanogram to milligram per liter media) due to differences in understanding endophyte biology, physiology and molecular and chemo-ecological aspects in relation to their secondary metabolites which led to disagreement in scientific community over the ability of endophytes to produce Taxol (Staniek, et al., 2009; Heinig, et al., 2013). It is therefore, pointing to further works to clarify the involvement of endophytes in Taxol production (Kusari, et al., 2014). Therefore, identification of hazel endophytes will be interesting to further the search for source or sources of Taxol production in hazelnuts.

1.9.3 Biofuel and Other Uses

Renewable energy sources can be a good substitute of the fossil fuels. Hazelnuts had been grown as a tree nut for food, but the seed contains high quality and quantity of oil (Xu & Hanna, 2009; Xu & Hanna, 2010) while another report suggests the physical/chemical characteristics of hazelnuts for biodiesel are substantially superior to soybean oil (Xu, et al., 2007). The other advantage is that hazelnut trees can be planted in less productive soil and successfully adapt, thus, the species may hold the potential as a future biofuel crop (Molnar, 2014). Husk of hazelnut seems to be a promising energy source (Guney, 2013) and the nuts are potentially useful for removal of toxic cations and chromium (Cr VI) (Cimino, et al., 2000).

1.10 Hypothesis/Plan

Hypothesis: Assessment of the chemical and biochemical activities and physiology may help the development of protocols for safer medium or long-term storage of hazel seeds.

Aim: To assess aspects of hazel seed biochemistry, physiology, viability, longevity and storage under experimental storage protocols in order to check and improve the storage capability.

Objectives

1. To study the biochemical properties of seeds associated with seed viability and successful germination of seeds.
2. To investigate classification of hazelnut in recalcitrant, intermediate and orthodox storage groups.
3. To study the interaction of seed dormancy and recalcitrance in hazel seeds.
4. To observe the impact of seed borne pathogens.
5. To investigate the role of the seed's own defence mechanism.
6. To check storage capability.
7. Survival of hazelnuts in soil seed banks
8. The standard test methods used in this research will be validated, optimised and checked.

Plan

1. To assess the impacts of various seed moisture and temperature related tests on hazel seed dormancy
2. Hazelnuts will be stored under different conditions (various combinations of time, temperature and moisture) intended to induce and break dormancy and to preserve them longer than would be expected from their common classification as recalcitrant.
3. The chemical and biochemical conditions considered will be seed moisture content and water activity, cell integrity (as estimated by seed leachate conductivity), the protective role of seed borne antioxidants (estimated by DPPH assay) and seed viability test by TTC during the physiological stages and stress periods.
4. Two dormancy reversal tests, with successive alternation of storage temperature, were designed to compare soil seed bank condition.

5. The effect of lowering seed moisture by acclimatisation to repeated dehydration-rehydration-dehydration treatments will be tested by germination and viability tests.
6. Checking the involvement of seed associates in providing antioxidants at the various physiological stages.
7. A further test of hazel seed recalcitrance will be examined.
8. For medium term storage, hazelnuts will be held at 5°C and -20°C and tested for viability.

2 Materials and Methods

2.1 Hazelnut Provenance

Hazelnut (*Corylus avellana* L.) is a common native shrub distributed widely across the British Isles (Rendon, 1983). The nuts used during the course of this investigation were commercially grown Kent cobnuts obtained, either directly from the growers or from a grover dealing with a specific grower, as soon as possible at the start of each new season as listed in Table 2.1. The fruit is a globose nut, tapering at one end and surrounded by an involucre. The pericarp or shell is hard and woody which contains a single seed covered by a thin brown testa, consisting of two large fleshy cotyledons enclosing an embryonic axis at the tapering end (Rendon, 1983). Table 2.1 illustrates a brief account of the sources of procurement of hazelnuts. On arrival cupules were removed and nuts were air dried on the laboratory bench at ambient conditions for 3 days (Wood & Bradbeer, 1967). Moisture content (MC) (section 2.4) was determined before packing 450 nuts per re-sealable polyethylene bag for storage at 5°C refrigerator until needed.

Hazel seeds from 2010 samples showed 22.8% MC, which is much lower than the previous years (Table 2.1). It might be due to late procurement from the grower during which the seeds possibly lost some water.

Table 2.1: List of growers and suppliers of hazelnuts used during the experimental period.

Date obtained	Grower	Supplier	Seed moisture content (% fresh weight)
04-10-04	Mr. Cannon Roughways Farm, Roughway Lane, Tonbridge, Kent TN11 9SN	Grocer, T.Adamu & Sons, Chiswick, London W4 1PU	38.1
12-01-06	Mr. Cannon Roughways Farm, Roughway Lane, Tonbridge, Kent TN11 9SN.	Grocer, T.Adamu & Sons, Chiswick, London W4 1PU	44.5
29-09-08	Mr. Cannon Roughways Farm, Roughway Lane, Tonbridge, Kent TN11 9SN.	From Grower	42.2
11-09-09	Allens Farm Allens Lane, Plaxtol, Sevenoks, Kent, TN15 0QZ	From Grower	42.5
06-12-10	Allens Farm Allens Lane, Plaxtol, Sevenoks, Kent, TN15 0QZ	From Grower	22.8
07-10-11	Mr. Cannon Roughways Farm, Roughway Lane, Tonbridge, Kent TN11 9SN.	From Grower	36.25

2.2 Consumables

The consumables and their sources are listed as follows: Industrial methylated spirit (IMS), 4.0 ml plastic disposable cuvettes FB55143, Sodium hypochlorite (12% available chlorine), glycerol and Vermiculite were obtained from Fisher Scientific, Loughborough, England.

GA₃ (analytical grade, 90% HPLC purity), streptomycin sulphate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (AsA), 6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid (Trolox), Butylated hydroxytoluene (BHT), 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) and raffinose were obtained from Sigma-Aldrich, Gillingham, England.

Whatman No 1 filter papers 90 mm and 150 mm obtained from Whatman International Ltd. Maidstone, England.

5.0 ml and 1.0 ml Gilson pipettes from Gilson, France.

Thin Layer Chromatography plate, SIL G/UV 254, obtained from Macherey-Nagel Gm bH & Co. Kg, Duren, Germany.

Water activity measuring instrument obtained from Decagon AquaLab LITE, Decagon Devices Inc., Pullman, WA, 99163, USA.

WPA CM35 conductivity meter obtained from WPA Scientific Instruments, Saffon Walden, England. Hand held conductivity meter Primo 5 from Timstar Laboratory Suppliers Limited, Winsford Industrial Estate, Winsford, Cheshire, CW7 3BX, UK.

Seedling growing trays and multipurpose compost obtained from a local grocer.

2.3 Nut/Seed Storage

Batches of 450 nuts each in re-sealable polyethylene bags had been held at 5°C refrigerator and at 15°C incubator for the experimental period. No extra water was added during the entire storage period to nuts/seeds held at 5°C or at 15°C. For some experiments non-dormant nuts were held in cupboards in ambient room temperature at $20 \pm 2^\circ\text{C}$. No water was added to the nuts in the polyethylene bags. Moisture content was measured at each experimental step to compare level of seed hydration. For long term storage after specific experimental treatments batches of 450 nuts were put in re-sealable polyethylene bags and kept in 5°C refrigerator or in -20°C freezer until needed for experiment.

2.4 Moisture Content (MC) Determination

As MC is probably highly critical over a narrow range seeds were weighed immediately after separation from the shell/pericarp following appropriate

treatments (Farrant, et al., 1985). MC was determined gravimetrically using 10 x 1 seed per treatment and held in an oven at 80°C for 24 h (Dhindsa & Bewley, 1976). Seeds were then held over silica gel in a desiccator to adjust to ambient temperature before measuring the seed mass. Moisture percentage was determined by the following:

$$\%MC = (\text{Fresh weight} - \text{dry weight}) \times 100 / \text{Fresh weight}$$

2.5 Water Activity (a_w) Measurement

Water activity (a_w) is the relative availability of water in a substance, also defined as the vapour pressure of water divided by that of pure water at the same temperature. Ten hazel seeds were ground in a grinder at 700 rpm (KRUPS Type F203, Krups, Mexico) and 2.5 g was used to measure water activity (Decagon AquaLab LITE, Decagon Devices Inc., Pullman, WA, 99163, USA). Instrument calibration was made with standard aqueous NaCl solutions (5.25 M = 0.795 a_w , 4.41 M = 0.833 a_w , 3.08 M = 0.891 a_w and 1.54 M = 0.949 a_w) and de-ionised water ($a_w = 1.0 \pm 0.003$) as blank according to manufacturer's instructions. All measurements were made in triplicate.

2.6 Measurement of Leachate Conductivity

Weakening of cell membrane in poor vigour seeds causes leakage of water soluble compounds such as sugars, amino acids and electrolytes when immersed in water. Fresh seeds on the other hand, having intact membranes, leach less of these chemicals. Therefore, assay of electrical conductivity (EC) of the leachates gives an accepted estimation of membrane integrity. Measurement of leachate conductivity of hazel seed was done by modification (Bonner, 1996). It is now known that seeds will leach numerous substances when soaked in water and that the amount of leached substances will increase as the seeds deteriorate (Pasquini, et al., 2011; Ramos, et al., 2012). In the current study, two seeds were weighed and soaked in

20 ml de-ionised water in glass tubes held at ambient temperature ($\sim 20 \pm 2^\circ\text{C}$) for 24 h. Two blanks of 20 ml de-ionised water were also maintained under the same conditions. Leachate was decanted from the tubes and electrical conductivity measured with a WPA CM35 conductivity meter (WPA Scientific Instruments, Saffon Walden, England). The mean of the two blanks was subtracted from the sample reading and divided by seed weight to obtain the conductivity as microSiemens per gram of fresh weight ($\mu\text{S g}^{-1}$).

For measurements of acclimatised seeds (section 3.6) conductivity was measured using a Primo 5 conductivity meter (Timstar laboratory Suppliers Limited, Cheshire, UK).

2.7 Antioxidant Measurement

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a free radical which when dissolved in industrial methylated spirit (IMS) turns blue-violet. On the addition of standard antioxidants or antioxidants extracted from experimental seed samples, DPPH loses colour and indicates radical scavenging activity.

DPPH scavenging by hazelnut extracts was measured by a modified method (Sung-Sook, et al., 2005).

Variations of the method were used to test activity in hazel seeds or embryos (without testa), endocarp, funiculus or testa. The optimisation of this method is described in section 3.7.3.1.

2.7.1 Seeds and embryos

Ten hazel seeds or embryos (without testa) were ground in a blender at 700 rpm for 5 min. The ground seed samples were dried in an oven at 50°C overnight. Defatting of seed was done using 150 ml n-hexane using soxhlet for 4 h at 60°C . The process was repeated three times. Each sample was then air-dried overnight in fume cup-

board at ambient conditions. The ground and dried seed sample (1.0 g) were crushed to very small pieces. Each sample was then extracted in 5 ml solvent (60/40 (v/v) IMS/de-ionised water) for up to 24 h in darkness in ambient conditions. The sample was then stirred for 1 h with magnetic stirrer, centrifuged for 5 min at 2500 rpm and filtered using 9.0 cm Whatman No.1 filter paper. The process was repeated twice more and the filtrate pooled together which formed the extract. Three separate assays were performed from each extract. DPPH (2.25 ml, 60 μ M (w/v) in 60/40 IMS/de-ionised water) was added to hazel sample extracts (0.75 ml) and incubated at ambient temperature for up to 3 h in disposable plastic cuvettes (Fisherbrand, Fisher Scientific, UK) and absorbance measured at 517 nm using JENWAY 6305 uv/vis. spectrophotometer (Genway Limited, Essex, CM 6 3LB, England). In a preliminary study, standard antioxidants ascorbic acid, Butylated hydroxytoluene (BHT) and Trolox had been tested and based on the results of these experiments ascorbic acid was used as the standard antioxidant for all DPPH measurements.

The antioxidant activity was calculated as inhibition (%) of DPPH radical formation.

$$\text{Inhibition (\%)} = (\text{Control absorbance} - \text{extract absorbance}) \times 100 / \text{Control absorbance}$$

2.7.2 Seed Associates (endocarp, funiculus or testa)

The method was as described above except the samples used were two gram endocarp, one gram funiculus or one gram testa. Reflux apparatus was used for defatting of seed associates, each with 20 ml n-hexane at 60°C for 4 hours. Also, the dried seed associates became crispy after defatting and drying treatments and extra care was needed during the crushing stage.

2.8 Thin Layer Chromatography (TLC)

Qualitative screening of the crude extracts of hazel seed and byproduct extracts were conducted by a modified method (Aderogba, et al., 2012). Finely ground (700 rpm, KRUPS Type F203, Krups, Mexico) defatted embryo (1.0 g) and 200 mg each of endocarp, funiculus and testa were extracted overnight at ambient room temperature in 10 ml methanol (MeOH). Each extract was filtered through Whatman No. 1 filter paper and the filtrate concentrated in a rotary evaporator at 40°C. The dried sample was resuspended in 1.0 ml MeOH. An aliquot (10 µl) of each sample were loaded on activated TLC metal plates in lines ca 1.0 cm wide, developed using n-hexane-methanol-ethyl acetate (2:10:2 v/v) and air-dried in fume cupboard. The TLC plate was then sprayed with 0.2% (v/v) DPPH methanolic solution and the plate was stored in the dark (Awah, et al., 2010). The TLC was observed after 30 min where the active components appeared as yellow spots against a purple background.

2.9 Pathogen Test

A test to check for the presence of pathogens and microbes was conducted with the nuts harvested in 2006. Details of the nut provenance are given (2.1). For pathogen detection in hazelnut associates, two types of solid culture media were used: a) agar, b) Sabouraud. Four healthy hazelnuts were surface sterilized with 1% (v/v) sodium hypochlorite solution and washed under running tap water prior to use. Each hazelnut was cracked to separate its various constituent parts or seed associates viz., a) pericarp, b) testa, c) cotyledons and d) embryonic axes. The accumulated pericarp pieces were placed in a flat bottomed flask. The testa was separated carefully from the seed and the embryonic axes detached carefully from the cotyledons. Each seed associate was then placed in a separate 25 ml glass tube containing 10 ml phosphate-buffered saline, PBS (8 g of NaCl, 1.44 g of Na₂HPO₄,

0.24 g of KH_2PO_4 dissolved in 800 ml de-ionised water and pH was adjusted to 7.4 with HCl solution and made up to 1L.) shaken vigorously and filtered. The filtrates constituted the medium dilution and 100 μl from each dilution were inoculated separately on i) nutrient agar plate and ii) Sabouraud plate. Plates were incubated overnight at 37°C and all detectable colonies were counted.

2.10 Chilling Treatment

The natural stratification process by which hazel seed dormancy is broken by overwintering in moist soil/leaf litter was simulated by placing nuts in moist vermiculite in a 5°C refrigerator (Bradbeer, 1968). Throughout this thesis, the term 'chilling' has been used to mean nuts/seeds that have been held at 5°C in moist condition. In the present study, this was done by placing nuts in moist vermiculite in standard horticultural seedling trays with perforated bottoms (56 x 29 x 6 cm). The bottom layer was filled with 2.0 cm vermiculite, with two layers of nuts separated by a 1.0 cm layer of vermiculite and with a 1.0 cm layer of vermiculite on top. The vermiculite was saturated with tap water, the tray allowed to drain excess water before refrigeration at 5°C refrigerator on a non-perforated tray. The seedling trays depending on the number of seeds planted were re-watered weekly.

2.11 Germination Assessment

Unless stated otherwise, 100 seeds were used for the germination test in each treatment. The tests on hazel seeds were carried out after the removal of the pericarp. The nuts were cracked manually with a conventional nut cracker. Intact seeds (with testa) were surface sterilised by soaking in sodium hypochlorite solution (1% (v/v) available chlorine) for 5 min followed by three washings with deionised water. Twenty batches of five seeds were placed in a 9 cm disposable sterile petri-dish, lined with a 90 mm Whatman No.1 filter paper and containing 20 ml of deionised (dH_2O) water. Streptomycin sulphate solution (5 ml, 10 ppm)

was added to the petri-dishes which were then kept in an incubator at 15°C in the dark. Germination, as determined by 2 mm protrusion of the radicle (Michalak, et al., 2013; ISTA, 1999; cited in Liu, et al., 2014), was recorded daily under ambient laboratory light. Suggestions to evade errors in germination assessment was taken into consideration (Baskin, et al., 2006).

As the final percentage of germination may not accurately reflect seed vigour because it does not account for the rate of germination; the germination index (GI) was calculated as described by (Czabator, 1962).

$$GI = MDG \times PV$$

Mean daily germination (MDG) is a measure of the totality of germination achieved in the test divided by the length of the test (28 days in this investigation) (Czabator, 1962). Peak value (PV) was calculated as the maximum value of percentage germination on any one day divided by the number of days taken to achieve that percentage as a measure of the rate of germination (Czabator, 1962).

2.12 Seed Viability Test by TTC

Triphenyl tetrazolium chloride (2,3,5-triphenyl-2H-tetrazolium chloride; TTC) is a redox indicator used in biochemical experiments which differentiates live from dead tissues of seed embryos on the basis of respiratory dehydrogenases activity. In hydrated tissues, dehydrogenase enzyme activity increases resulting in the release of hydrogen ions which reduce the colourless tetrazolium salt solution into a red chemical compound called formazan. Thus, while the dead or inactive areas remain white, metabolically live seeds will stain red to prove viability. Also, the TTC staining and pattern reflect the health of the seed. A 1% (w/v) TTC solution was used in this experiment (Crane, et al., 2003). Viability of seeds which will not be subjected to germination test were imbibed in dH₂O for 24 h at room temperature and seeds

which remain ungerminated at the end of any germination test were taken in TTC solution and kept overnight in the dark at room temperature. Change in staining and uniformity of distribution of the red colour were used to determine seed viability. Level of stain depth (deep, moderate or light or unstained) was also carefully studied and noted (ISTA, 2003).

2.13 Seedling Measurement

Seedling growth and measurement was followed with some modifications of a previous protocol (Zaidman, et al., 2010). Thirty germinated seeds were sown in perforated plastic seedling tray filled with multipurpose compost and placed on a non-perforated tray. The seedling tray was then soaked with tap water and left for seedling emergence on a bench in ambient conditions in the laboratory. Observations continued daily for the emergence of the shoot and the following growth parameters were measured:

- a) Days taken for emergence of plumule from the compost.
- b) Height of the shoot measured in mm at weekly intervals.
- c) Weekly counts of the number of internodes.
- d) Number of healthy seedlings (morphologically complete) expressed as a percentage of the total number of germinated seeds sown
- e) Abnormal seedling recorded at the end of the test period.

2.14 Seedling Dry Mass Measurement

At the end of the 6th week of growth seedlings were withdrawn and the compost washed off the roots very carefully so that the root system was not damaged. The shoots and roots were separated at the axial point and dried in an oven at 40°C for 4 days and the dry mass recorded (Zaidman, et al., 2010).

2.15 Statistics

Where two samples were to be compared a two-tailed Student's t Test routine in Microsoft Excel 2010 was used with an alpha value of 0.05 to indicate rejection or failure to reject the null hypothesis with 95% confidence.

Where three or more results compared results were subjected to one-way ANOVA the single factor routine in Microsoft Excel 2010 was used with an alpha value of 0.05 set to determine the value of F_{crit} and so signal rejection of the null hypothesis with 95% confidence. Where there was a need to compare means the one-way routine in Minitab was applied with the Tukey's family error post-hoc test.

Where error bars are shown on figures, at least three replicates were used for analytical assays. In each case the figure or table legend indicates whether results are expressed as \pm one standard deviation (calculated in Microsoft Excel using the STDEV.S function) or \pm 95% confidence intervals (calculated with the CONFIDENCE.T function and with alpha set to 0.05).

The 95% confidence limits were determined with germination tests, from a table calculated by the use of the χ^2 test with a two by two contingency table (Roberts, 1963).

3 Effects of Selected Treatments to Test Seed Dormancy of Hazelnuts

3.1 Stabilisation Treatments of Hazelnuts and Seeds

3.1.1 Introduction

For proper storage condition of a seed, its physiological, chemical and biochemical nature must be fully assessed. Hazel seeds show both dormancy and recalcitrance (Bradbeer, 1968), therefore, investigating seed dormancy will be helpful to identify a better method to protect the viability of the seed. Differences in seed provenances coupled with conditions of treatments (handling of the crop, storage temperature, moisture status etc.) have been reported (Table 1.2). Seed moisture content is an important factor in determining the storage habit (Roberts, 1973) but experimental results show that moisture content of individual recalcitrant seeds may vary considerably (Chin, 1988; Normah, et al., 1994). On the other hand, storage of hazelnuts in LN and subsequent production of seedling has been reported (Michalak, et al., 2013) which is achievable by reducing seed moisture and is contradicting hazel seed's recalcitrance nature.

In the present investigation, several protocols have been drawn up to examine the various factors involved in hazel seed viability. The first, being to look at the effects of stabilisation treatment of seed moisture on hazel seed viability, tested at 5°C and ambient room temperature (RT) (20°C ± 2°C). The experimental results will indicate whether reduction of seed moisture and short storage at 5°C and at RT will show any effect on dormancy. Twenty five germinated seeds from each treatment were also sown to observe the seedling performance of the treated seeds.

3.1.2 Materials and methods

3.1.2.1 Nut Provenance

Hazelnuts of 2004 harvest were used in this experiment. Details of the provenance are given (section 2.1).

3.1.2.2 Experimental Procedure

All relevant treatments and measurements were performed as stated in, nut storage (section 2.3), moisture content determination (section 2.4), germination assessment (section 2.11), seedling growth assessment (section 2.13) and seedling dry mass measurement (section 2.14).

3.1.2.3 Stabilisation Treatment

Experimental treatments of the nuts and seeds used in this experiment are shown in Figure 3.1. This experiment started with hydrated hazelnuts held in closed containers at 5°C for 31 weeks. The nuts were chilled further for 6 weeks at 5°C to break seed dormancy. The non-dormant hazelnuts were air dried at ambient conditions on the laboratory bench for 24 hours. The desiccated nuts form the basis of the remainder of the experiment as whole nuts and as seeds (obtained by cracking these nuts) and were packed in 500 ml brown glass bottles with screw tops and held for 24 h at: a) 5°C refrigerator and b) in a laboratory cupboard (ambient room temperature) (20°C ± 2°C). At the end of the storage period whole nuts or nuts cracked to get the seed which was used for moisture determination and germination tests.

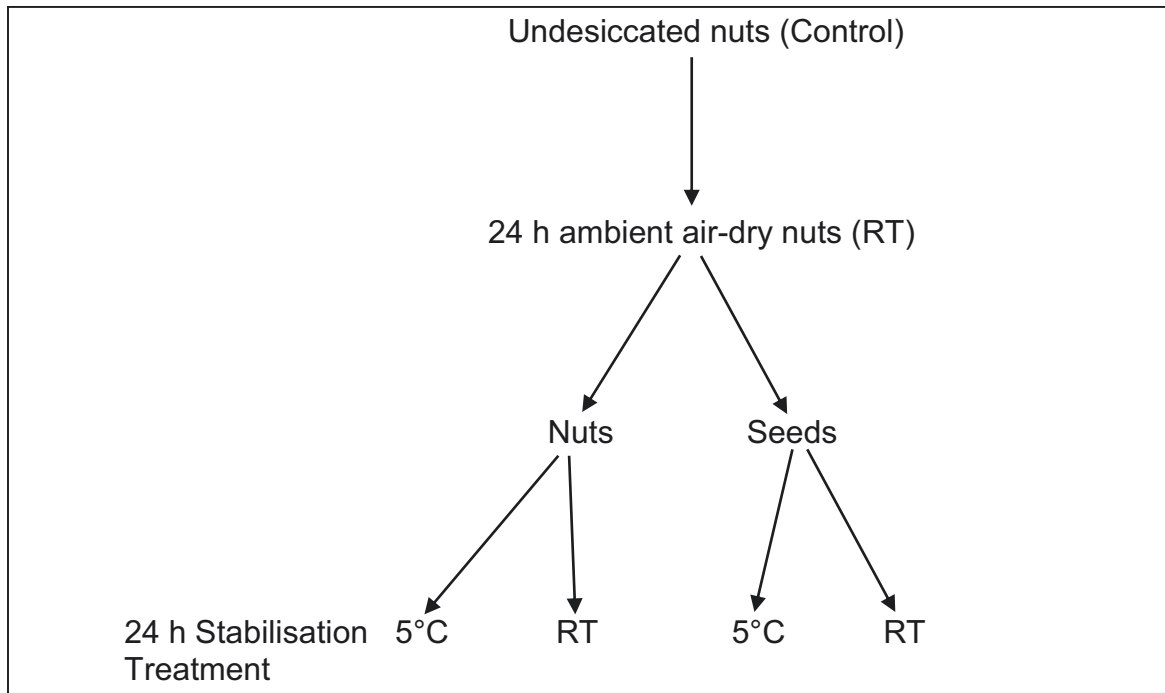


Figure 3.1: Experimental treatments of hazelnuts and seeds stabilisation at 5°C and at ambient temperature (RT).

3.1.3 Results

3.1.3.1 Seed Moisture Content (MC)

Table 3.1 shows a summarised account of achieved moisture content and respective seed germination after stabilisation treatments. Dehydration in ambient temperature resulted in seed MC reduced from 39% to between 25% and 22%. Undesiccated seeds with 39% moisture and stabilised to 21% after the treatments exhibited between 78% to 90% germinability. During the 24 h stabilisation treatment, seeds held at 5°C showed no appreciable water loss compared to desiccated seeds but at ambient temperature lost only about 1% water. Whereas seeds from nuts lost about 2% water when stabilisation treatment was done at 5°C or at room temperature.

Table 3.1: Effect of stabilisation treatments on moisture content and final germination of nuts and seeds: Nuts with non-dormant seeds were air-dried at ambient conditions ($20 \pm 2^\circ\text{C}$) for 24 h before subjecting to 24 h stabilisation treatment at 5°C and at ambient temperature for 24 h in closed containers.

The moisture values are mean of ten replicate seeds \pm SD. The germination results are expressed as per cent from samples of 100 seeds.

Treatments	Seed moisture content (% fresh weight basis)	Final germination (%)
Control (Un-desiccated seed)	38.9 ± 2.1	86
24 h desiccated nut	24.9 ± 1.9	88
24 h desiccated seed	22.1 ± 1.2	83
Desiccated, 24 h stabilisation at 5°C nut	22.6 ± 4.9	78
Desiccated, 24 h stabilisation at 5°C seed	22.3 ± 2.1	89
Desiccated, 24 h stabilisation at room temperature nut	22.4 ± 0.8	90
Desiccated, 24 h stabilisation at room temperature seed	21.0 ± 2.6	83

3.1.3.2 Seed Germination

Figure 3.2 shows seed germination pattern from various treatments listed in Table 3.1. Control seeds with 39% moisture showed first germination on the 8th day whereas seeds from desiccated samples which contained 22% water germinated earlier on the 4th day. Germination rate in control seed was significantly lower than all desiccated seeds but became insignificant on the 11th day. In this experiment lowest germination of 78% was recorded in seeds from nuts stabilized at 5°C . There was an overall increase in vigour in all desiccated seeds as shown by earlier lag phase. Speed of germination was significantly greater in all desiccation treatments but from the 12th day this difference was overcome.

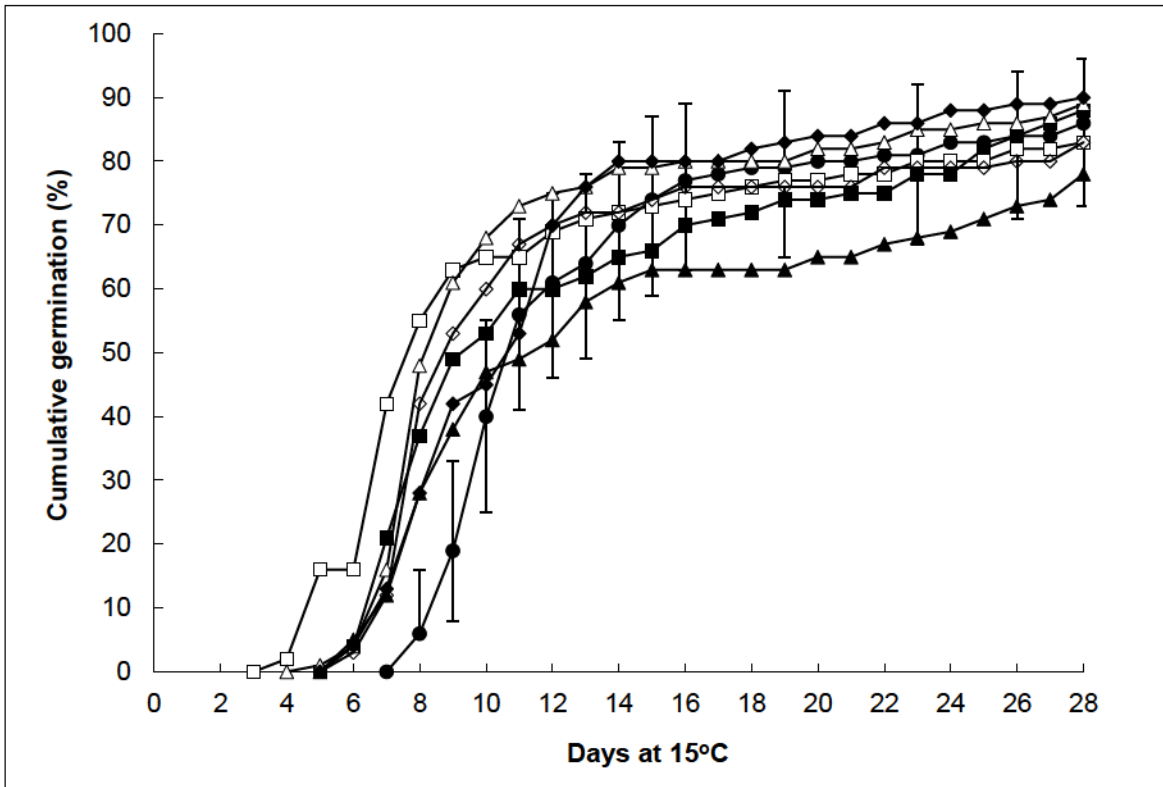


Figure 3.2: Effect of desiccation and stabilisation treatment on seed germination. Non-dormant hazelnuts were desiccated for 24 h at ambient temperature and held in closed containers at 5°C and at room temperature as nuts and seeds. Seeds from treated nuts were used for germination tests:

- Un-desiccated seed (●)
- 24 h desiccated nut (■),
- 24 h desiccated seed (□),
- 24 h desiccated, 24 h 5°C stabilised nut (▲),
- 24 h desiccated, 24 h 5°C stabilised seed (△),
- 24 h desiccated, 24 h ambient temp stabilised nut (◆),
- 24 h desiccated, 24 h ambient temp stabilised seed (◇).

Error bars represent ± SD based on samples of 100 seeds calculated as described in section 2.15.

3.1.3.3 Mean Daily Germination (MDG), Peak Value (PV) and Germination Index (GI)

Figure 3.3 shows the impact of stabilisation treatment on seed germination. Figure 3.3 (a) shows mean daily germination (MDG) which appears to be lower in seeds from nuts held at 5°C and seeds held for 24 h at room temperature. Figure 3.3 (b) on the other hand shows lower peak values in seeds after stabilisation for 24 h at 5°C and also at room temperature. Figure 3.3 (c) shows decrease of germination index in seeds from nuts held at 5°C and at room temperature.

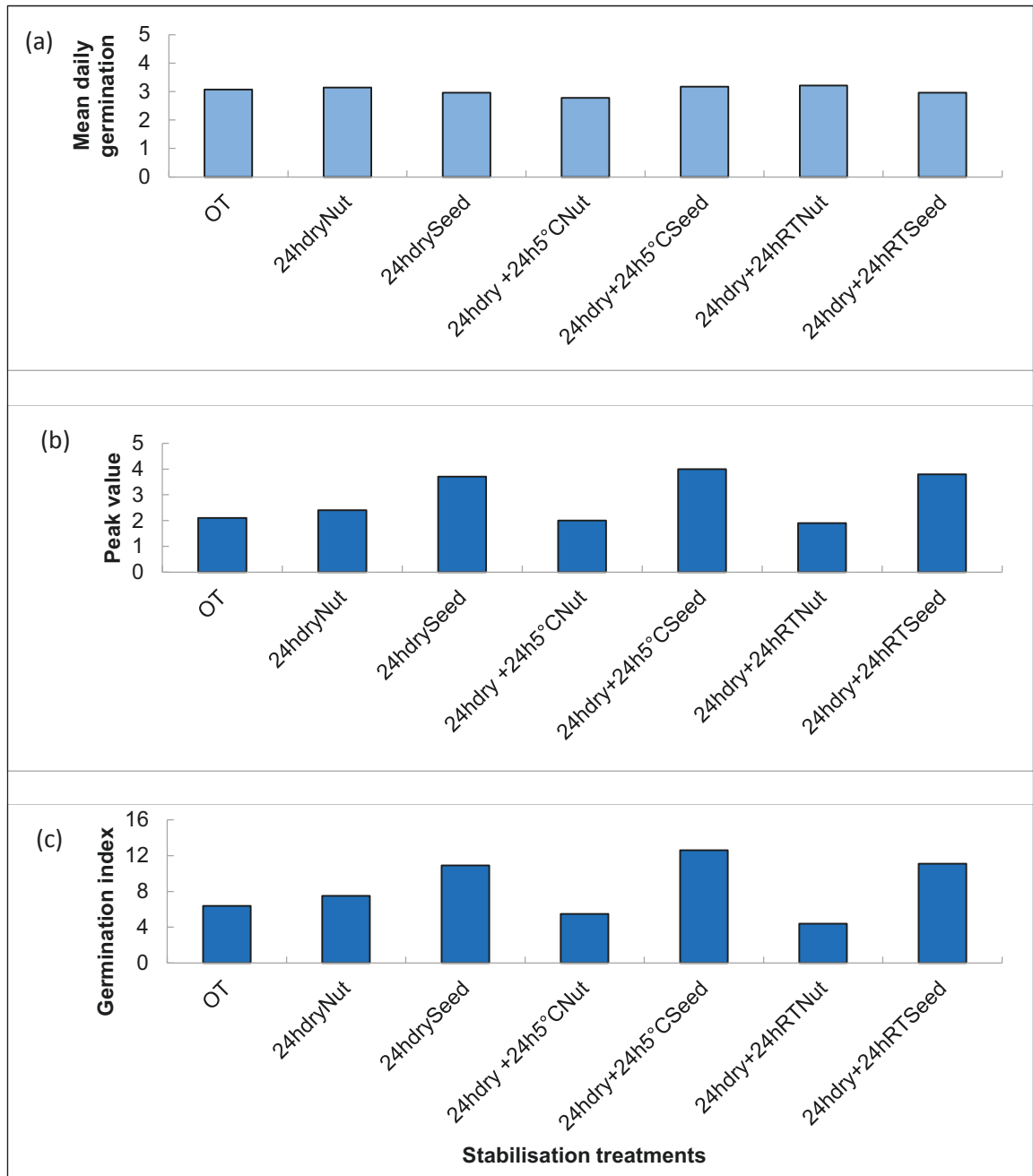


Figure 3.3: Effect of desiccation and stabilisation treatment of hazelnuts and seeds on (a) Mean daily germination, (b) peak value and (c) Germination index of seed germination. Data are obtained from Figure 3.2.

3.1.3.4 Seedling Growth

To examine further of the effects of desiccation and stabilisation, 25 germinated seeds from each treatment were planted in compost pots for seedling growth on the laboratory bench at ambient conditions (section 2.13). Seedling growth was recorded up to 6 weeks.

Figure 3.4 shows the damaging effects of stabilisation treatments on seedling performance as between 32% to 56% seedling showed abnormality in growth. Among the abnormalities dwarf, deformed, stunted and tip dry in seedlings were the common deformities observed (Fig. 3.5). Only the normal and healthy looking seedlings were used for assessments.

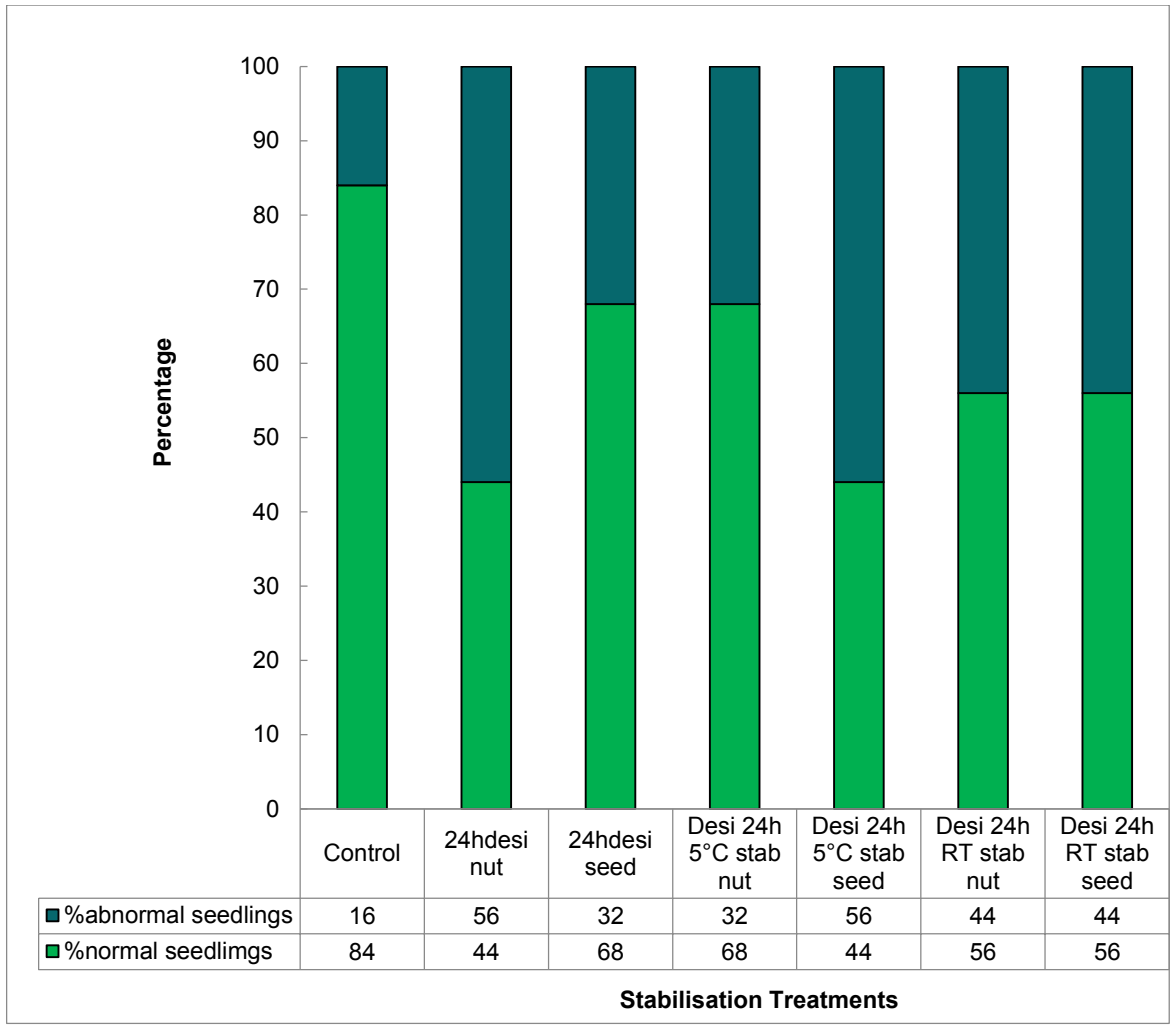


Figure 3.4: Effects of desiccation and stabilisation treatments of non-dormant hazelnuts and seeds on normal and abnormal seedling emergence from germinated seeds. Results are based on samples of 25 seedlings.

Desiccation and stabilisation treatments have reduced both root and shoot growth and resulted in abnormal seedlings. Evidence of abnormalities in developing seedlings are presented in Figure 3.5, which includes polyembryony, stunted growth, deformed shoot and shoot tip dry (where only the roots developed). Presence of almost intact seed after seedling establishment also indicated the

emerging plumules utilised a small amount of seed nutrients until they start synthesising nutrients and become independent with regards to nutrition.



Figure 3.5: Typical seedling features observed from germinated seeds after stabilisation treatments. A. Normal seedling, B. Polyembryony, C- D. Stunted growth and E. Tip dry. B, C, and D are shorter seedlings due to abnormalities seen after seedling emergence.

3.1.3.5 Seedling Measurement

To check the development of the normal seedlings, height and internode numbers of seedlings from all treatments were recorded every week up to 6 weeks. Figure 3.6 (a) and (b) shows no appreciable difference in seedling height or internode numbers, respectively, but show a tendency of difference at 6th week. A longer test might have given a better understanding.

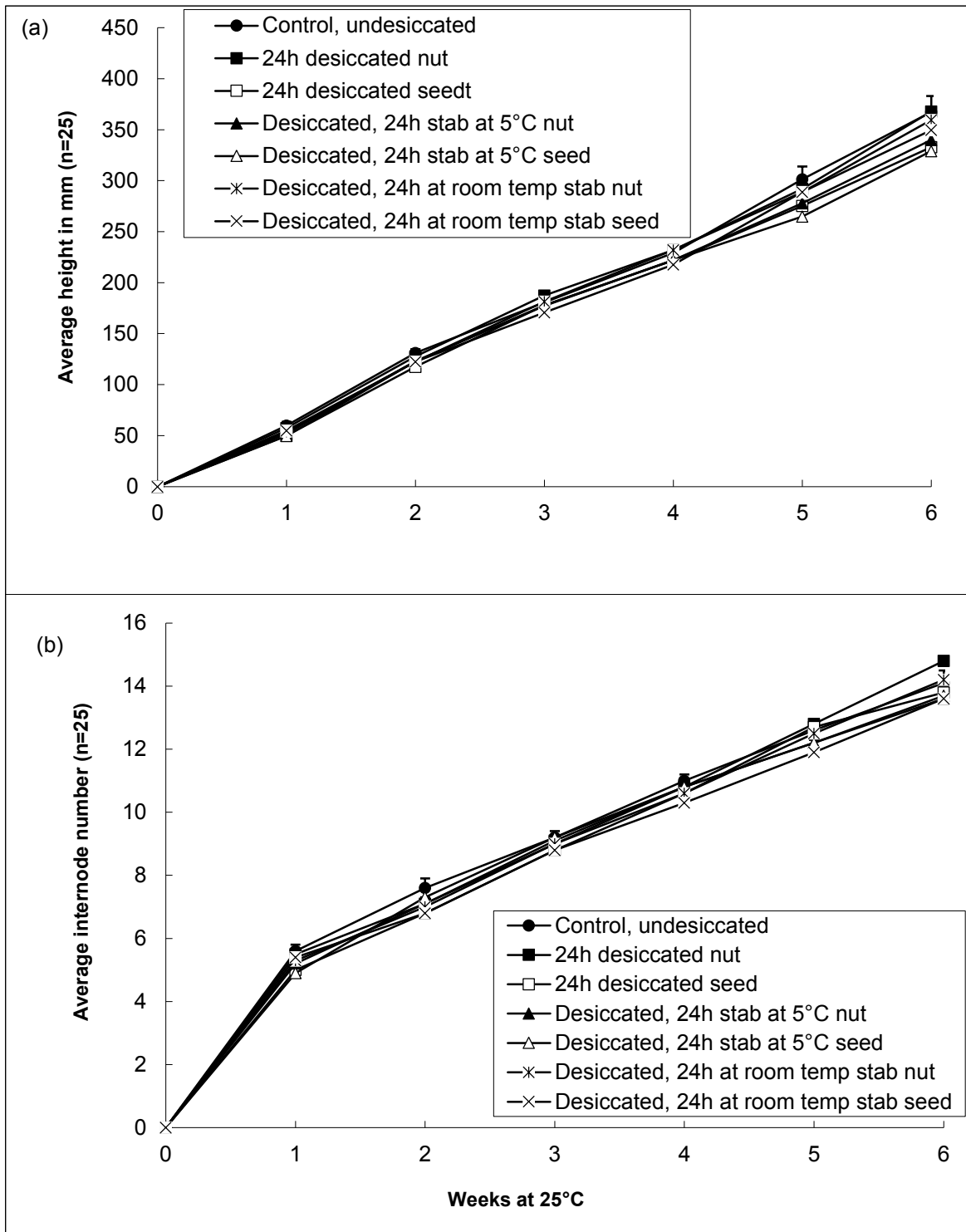


Figure 3.6: Effects of desiccation and stabilisation treatments on seedling growth. Twenty five germinated seeds from each treatment were sown for seedling growth. (a) Seedling height (mm), (b) Internode numbers. Error bars represent \pm SD based on samples of 25 seedlings.

3.1.3.6 Seedling Dry Mass

Seedlings from normal control and stabilised seeds did not indicate any significant difference in shoot or root dry mass (Fig. 3.7). Shoot and root recorded lower dry mass when nuts and seeds were stabilised at 5°C but no noticeable difference observed when stabilisation treatment was given at ambient conditions.

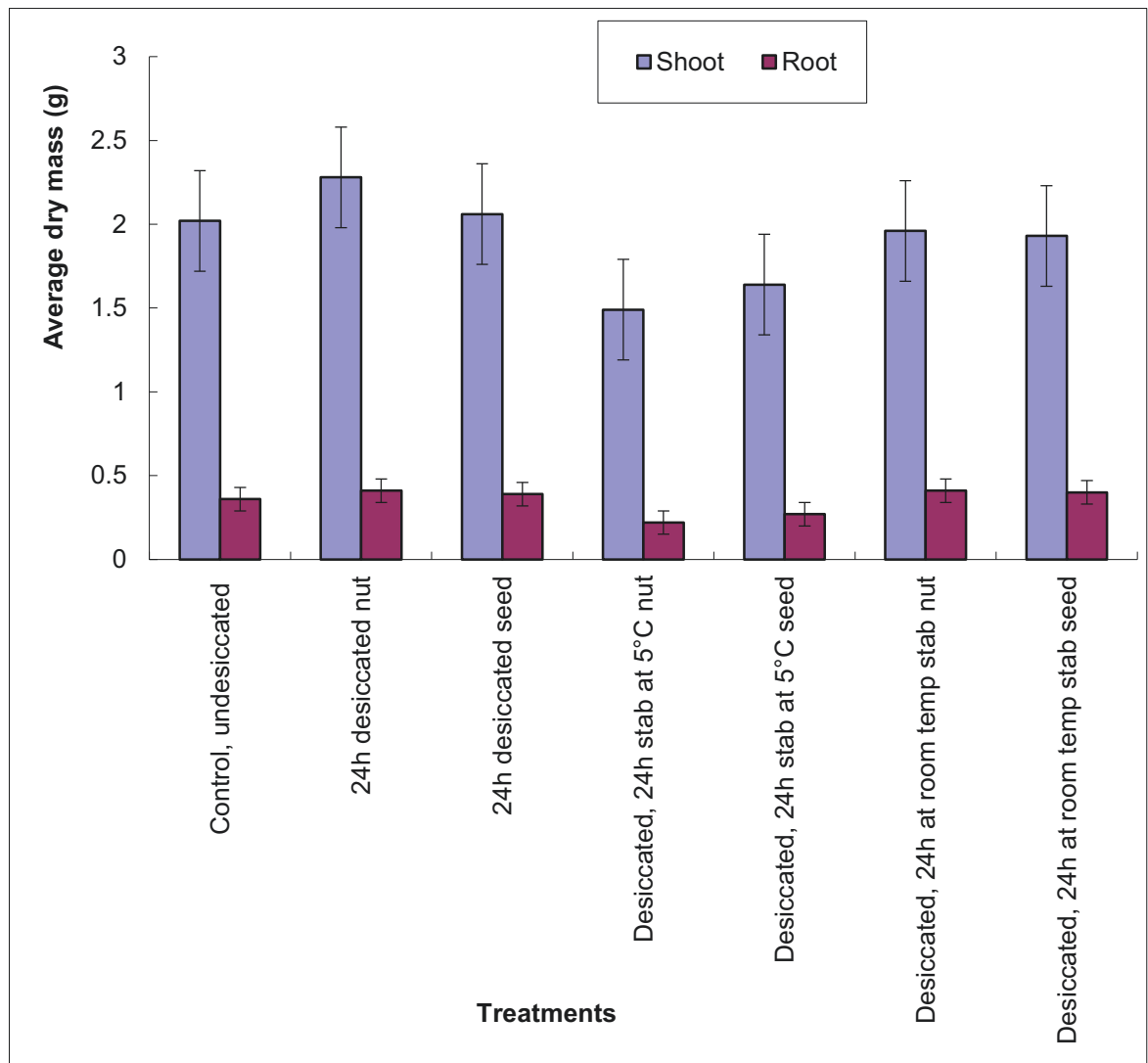


Figure 3.7: Effects of desiccation and stabilisation treatments on shoot and root dry mass from normal seedlings: Shoot and root samples were obtained from seedlings grown in Figure 3.6. Error bars represent \pm SD, dry mass is based on average of 25 seedlings.

3.1.4 Discussion

3.1.4.1 Seed Moisture Content and Germination

Variability in seed moisture content is observed in *Avicennia* (Berjak, et al., 1984), *Hevea brasiliensis* (Normah, 1987), *Nephelium lappaceum* & *Artocarpus*

heterophyllus (Chin, et al., 1989, cited in Normah, et al., 1994, p.317). *Hevea* seeds also show higher moisture content in the embryonic axis than other seed parts (Normah, 1987). High coefficient of variation for moisture contents in hazel seeds has also been reported (Normah, et al., 1994).

Therefore, stabilisation treatment was tried to balance an equal distribution of moisture within the seed. Stabilisation treatment did not however, show any impact on dormancy in hazel seeds as between 78 to 88% seed germination was recorded. Besides, desiccated seeds recorded earlier germination and showed a higher rate of germination compared to control seeds. Reports to the contrary, suggest desiccation could be harmful particularly in recalcitrant seed. For example, the involvement of volatile gases acetaldehyde, methanol and ethanol in the deterioration of recalcitrant seeds of *Ligustrum japonicum*, *Quercus serrata*, *Quercus myrsinaefolia* and *Camilla japonica* has been described (Akomito, et al., 2004). Released volatile gas from hazel seeds during desiccation and stabilisation treatment are trapped within the container some of which may be responsible for the deleterious effects for seed metabolic activities and hence poor performance. In the present experiment seed within a shell was enveloped by a small environment within the shell which, in turn prevents a quick escape of the volatiles and were exposed to higher concentration of volatiles. On the other hand, seeds had a wider environment within the container and thus exposed to lesser concentration of the volatile components. Hence the seeds were less affected by volatiles gases and show higher speed and rate of germination compared to seeds from nuts (Fig. 3.3 c).

3.1.4.2 Seedling Growth

Observation of dry shoot tips indicated direct damage to the plumular part of some embryonic axes as a consequence of desiccation (Fig. 3.5 d). Stunted growth may

be related to the impact of water loss (stress) which significantly reduced normal metabolic activities in the germination process and hence reduced growth (Figs. 3.5 b, c and d).

Stabilisation treatment resulted in seeds maintaining between 21-24% MC falling within the range classified as 'Type 2 water' which predicts death of 'intermediate seeds' (Vertucci & Farrant, 1995) as this water level is not protective of the organelles since protein structure destabilises and free radical production degrades enzymes. Survival of some hazel seed indicates the activities of some inherent protective molecules which counters the adversities of the damaging molecules. Report of ascorbic acid, sucrose, raffinose and stachyose in hazel seeds (Duke, 1989) might be linked to protective mechanism by antioxidant activities.

Results obtained also suggest seeds with ca. 22% moisture and held at 5°C or at ambient temperature could result in 80% or more seed germination (Table 3.1). But this does not necessarily mean the treatment had maintained full storability as seedling growth experiment revealed the damages done during the desiccation and stabilisation treatments.

Reduced growth in some seedlings indicated the damaging effects incurred on the embryonic axes due to stabilisation treatments. A longer seedling growing period would have given a clearer understanding of the effects of desiccation and stabilisation on overall seedling performance (Fig. 3.6). Although seed germination had not indicated any tell-tale effects but seedling growth experiments had shown some indications of the adverse actions on the germplasm due to desiccation and stabilisation treatments (Fig. 3.4).

It is indicative that stabilisation treatments even for a short period of 24 h at 5°C affects metabolic activities as reflected in the seedlings overall performance. At room temperature seeds metabolic activities being more active results in increased root-shoot dry matter. It is most likely that during stabilisation treatments seeds were exposed to a larger space within the container whereas, seed within the nut was exposed to a very small space between the seed and shell and at 5°C the volatiles were less penetrative and slow to escape the protective shell. This subjected the seeds within nut to greater concentration of volatiles than seeds alone and it could not be however inferred whether the abnormalities in about 50% (Fig. 3.4) of desiccated seeds were due to difference in MC of the individual seed or its physiological health due to volatiles. The other possibility is that desiccation depleted the seed of some vital chemicals (for example, reduction in GA, ethylene, increase in ABA etc.) or increase in production of free radicals which could have damaged membranes hence slower growth.

On the other hand, desiccation has clearly demonstrated an increase in the rate of germination in hazel seeds (Fig. 3.2). Similar germination enhancement by limited desiccation has been reported in recalcitrant *Telfaria occidentalis* (Nkang, et al., 2000). In a further investigation with the same species, (Nkang, et al., 2003) reported that seeds desiccated for 6 days at either 5°C or 25°C showed increased germination but a decline thereafter when moisture content dropped below 30% on the 9th day. Increase in germination of recalcitrant *Aesculus hippocastanum* L. by desiccation is also reported (Tompsett & Pritchard, 1998). It is also observed that desiccation enhances germination in recalcitrant *Quercus robur* L. (Finch-Savage & Blake, 1994).

Results from this experiment emphasises three aspects of seed biology: i) Seed dormancy is not imposed if germination is tested immediately after dehydration; ii) all germinated seeds may not produce normal/healthy seedlings, and iii) a variety of abnormalities in seedling performance is expected from desiccation of seeds which need to be addressed prior to germination test and seedling establishment.

3.1.5 Summary

Results indicate stabilisation treatments of seeds did not affect seed moisture content and final seed germination compared to control seeds. Therefore, losing seed moisture and short storage for 24 h at 5°C or room temperature did not impose dormancy. But stabilisation treatments had impact on seeds from treated hazelnuts as peak value and germination index was lower than control seeds. All treated seeds and seeds from nuts showed increase in abnormal seedlings. It may be due to seeds were exposed to seed volatiles within the container. Seedling growth recorded up to 6 weeks show a trend in gradual differentiation in seedling height and internode numbers compared to control seedlings. Dry mass of root and shoot from normal seedlings of 5°C stabilised seeds and nuts were lower than those of room temperature at the end of 6 weeks seedling growth test. The dry mass difference may be due to reduced metabolic activities at 5°C than at room temperature. There was no significant difference in dry mass between treated and control seedlings indicating normal seedling could recover from stress. Presence of entire healthy looking seed connected to the seedling indicates growing seedling used a little amount of storage food materials prior to its own synthesis (Fig. 3.5 A). Photographic images show a selection of various abnormalities seen among seedlings grown from the treated seeds (Fig. 3.5). Thus, it is evident that all germinated seeds may not result in normal seedlings.

The experimental results show the survival of seeds under reduced moisture content strongly suggesting that hazelnuts can be dehydrated to lower moisture content than expected from recalcitrant seeds. This may be important when designing storage strategies for seeds and also supports classification of hazel as exhibiting some properties usually associated with orthodox seed habit.

Observation of abnormal seedlings after stabilisation treatment indicates impact of some stress factors which requires further tests to look for the causes of damages.

3.2 Effects of Dormancy Reversal Treatments on Germination and Seedling Performance

3.2.1 Introduction

Another aspect to check in hazel seeds' physiological status is that, in nature, dormancy in hazel seed is broken by stratification. Temperature fluctuations may occur in nature. Therefore, this experiment was aimed at investigating whether temperature fluctuation affects the physiological response of hazel seeds.

In vitro stratification is simulated by storage of hazelnuts at 5°C in moist conditions (chilling) to break seed dormancy and then germination tested at higher temperature (Bradbeer, 1968). Although chilling is helpful in dormancy breaking in many species, numerous plant growth regulators (PGR), chemicals, gases and high temperature treatments etc. has also been used on their own or in combination to break dormancy in various seeds including hazelnuts (Bewley & Black, 1994). *Aesculus hippocastanum* which is a recalcitrant seed and shows seed dormancy has been stored for 3 years in moist conditions (Tompsett & Pritchard, 1998). *Fagus sylvatica* which also has seed dormancy has been held in moist condition at 4°C for medium term storage and suggested moistening of these nuts to about 30% seed water content by sprinkling exogenous water was necessary to achieve this (Suszka & Tylkowski, 1980). Storage of recalcitrant seeds at 4°C has also been suggested but moisture content should be about 28% and if the MC drops below this level additional water has to be sprinkled on top to raise the seed moisture to the required level (Finch-Savage, 1998). He suggested this would enable longer storage of dormant recalcitrant seeds.

In laboratory storage conditions hazelnuts have maintained germinability for a period of 6 months to a year (Bradbeer, 1988). In nature, hazelnuts, those escaped predators are buried in the top soil, over-winters and with the rising temperature in

spring, germinate. Variations in dormancy level in hazelnuts from year to year have been reported due to seasonal temperature variations (Rendon, 1983).

What happens to hazelnuts in nature?

Possible fate of hazelnuts shed and held in soil seed bank:

- i) Those survived predators, may be buried under leaf litter in the top soil.
- ii) Smaller nuts may be buried deeper than the larger nuts.
- iii) Buried nuts (small or large) may be prevented from germinating in the first year due to fluctuation of soil water and become dormant during the summer.
- iv) This might lead to dormancy reversal in the next winter (dormancy release) to a limited number of nuts depending on soil and seed water content. Thus all buried nuts may or may not germinate.
- v) The resultant seedling may or may not show normal features.

Seasonal variations in hazel seed dormancy has been reported by (Rendon, 1983) as observed from works done by previous researchers. Degree of dormancy is dependent on the environmental factors and since hazelnuts are shed with > 40% moisture it is not clear whether fluctuation in atmospheric temperature during seed development is responsible for the seasonal differences in the level of dormancy. Fluctuation in soil water content affected dormancy status of buried seeds of *Polygonum aviculare* L. stored at dormancy breaking temperature and suggested that fluctuations in soil water could be an additional factor affecting dormancy and weed emergence patterns under field conditions (Batlla & Benech-Arnold, 2006). In another experiment on hydration and dehydration cycles on *Aster kantoensis* Kitamura (Compositae), time required to attain 50% germination increased when desiccation time was higher than hydration time (Kagaya, et al., 2005). Experiments

of hydration-dehydration cycles with seven species of *Calligonium* resulted in the observation of delay in minimum germination after 3 hydration-dehydration cycles and five out of seven species recorded decrease in complete germination (Ren & Tao, 2003). Wetting and drying cycles were applied on bull thistle (*Cirsium vulgare* (savi) Ten.) on petri-dishes and pots of soil (Downs & Cavers, 2000) and observed total percentage germination was reduced after eight cycles and germination rate was reduced after two or more exposures. Seedling emergence patterns in pots that experienced any wetting-drying treatment were bimodal, with a second pulse of emergence several weeks after the termination of the cycles. This suggests that some seeds were induced to a dormant state through exposure to the cycles of wetting and drying which might have prevented seed germination in the autumn, promoting an attenuated and intermittent pattern of germination. It has been reported that low atmospheric temperature during grain development in barley results in low dormancy and higher temperature results in higher dormancy (Reiner & Loch, 1975).

Another problem may occur in nature where some hazelnuts may go deeper under the leaf litter and may not be able to germinate the same year but become dormant as soon as mild spring temperature turns into hotter summer. These nuts may remain dormant and passes the next winter (stratification) and in the following spring might sprout. This also gives a possibility of dormancy reversal in nature. The method of sprinkling exogenous water as suggested also has a similar sequence in *Quercus rubra* (Suszka & Tylkowski, 1980; Finch-Savage, 1998). The time elapsed between losing moisture below the desired level, its detection, re-supply by sprinkling and actual absorption into the cellular level may leave some seeds below the critical water content needed to remain non-dormant and may become dormant. These seeds may break dormancy again when sufficiently moistened and held at

low temperature for appropriate period. Some seeds therefore, may undergo a dormancy reversal treatment during the post sprinkling period. Therefore, the question is: Can dormancy be released and reversed back in the same seed by alternating storage temperature *in vitro*? In the present experiment we tried to look at the dormancy reversibility in hazel seeds by alternating the hydrated nuts at 5°C (dormancy breaking) and 15°C (dormancy imposing) storage temperature (Finch-Savage, 1998). Seed dormancy in *Taxus chinensis* var. *mairei* seeds is reported primarily due to the presence of germination inhibitory substances in different parts of the seed which inhibits embryo growth and seed germination. Cold + warm + cold stratification was effective for releasing morphophysiological dormancy in this species (Liu, et al., 2011). The present investigation also looks at the performance of seed germination after two dormancy reversal treatments and its effect on seedlings.

3.2.2 Materials and Methods

3.2.2.1 Nut Provenance

Hazelnuts of 2004 harvest were used in this experiment. Details of the provenance are given (section 2.1).

3.2.2.2 Nut Storage

Hazelnut storage protocol had been followed as described (section 2.3). Appropriate batches of 450 hydrated hazelnuts in re-sealable polyethylene bags had been held at 5°C for 6 weeks and were then transferred to a 15°C incubator and held for 6 weeks for the next experiment. At the end of that reversed back to 5°C and held for 6 weeks storage. During the entire storage period no extra water was added. MC was recorded at each experimental step to compare level of seed hydration.

3.2.2.3 Experimental Procedure

All relevant treatments and measurements were performed as stated in, moisture content determination (section 2.4), germination assessment (section 2.11).

3.2.2.4 Seedling Measurement

Thirty germinated seeds were sown in perforated plastic trays filled with multipurpose compost and placed on a tray filled with tap water and left for seedling emergence on laboratory bench in ambient conditions ($20^{\circ}\text{C} \pm 2^{\circ}\text{C}$). Seedling performance test were conducted as described (section 2.13). Seedling height, internode number and abnormal seedlings were recorded after 2 weeks growth period.

3.2.2.5 Dormancy Reversal Treatments

Experiment started with non-dormant hazelnuts stored for 38 weeks (initial storage) in moist vermiculite held in trays described in section 2.10. Nuts at this stage were stored in re-sealable polyethylene bags at 5°C (Treatment 1, step 1) for 6 weeks. At the end of 6 weeks one set was taken out from each batch for MC determination and germination test and the storage temperature of the remaining nuts were changed from 5°C to 15°C (Treatment 1, step 2) and held for 6 weeks. At the end of 6 weeks storage, all tests done with the Step 1 seeds were repeated on this batch. Thereafter, nuts from Step 2 were held at 5°C for the next 6 weeks (Step 3) at the end of which, the same tests as done in Step 1 and 2 were conducted with the seeds of this batch. So, at the end of the experiment, storage temperatures of each set of nuts were reversed twice. For Treatment 2, the first 6 weeks storage were at 15°C (Step 1), followed by 6 weeks at 5°C (Step 2) and the final storage at 15°C (Step 3). Determination of seed moisture content and germination were conducted in the same method as in each step in Treatment 1.

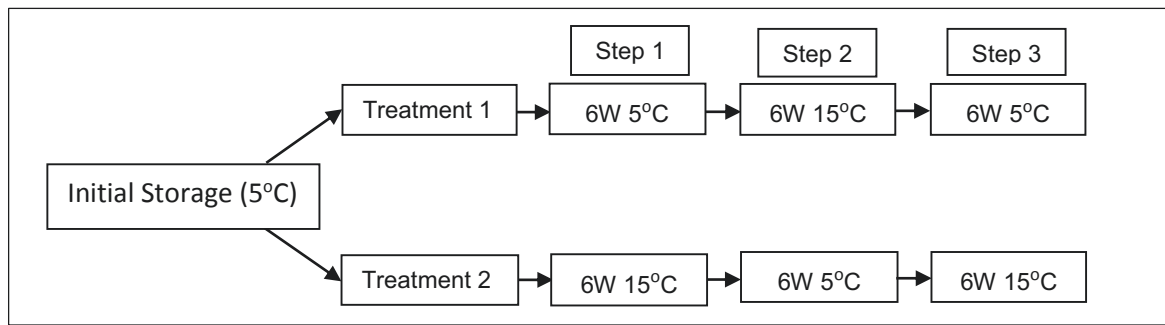


Figure 3.8: Experimental treatments and steps in investigation of physiological and biochemical analysis of non-dormant seeds held at 5°C and at 15°C for 6 weeks in each.

3.2.3 Results

3.2.3.1 Seed Germination

In Treatment 1, first dormancy reversal experiment was set on the 38th week storage at 5°C (seed MC 36.1%), the initial storage set scored 80% germination seen in Figure 3.9 (a). In the first experiment, the control nuts were subjected to a further 6 weeks at 5°C (Treatment 1, step1) showed an increased rate of germination which was significantly different from initial set up to 11 days. The first temperature reversal was done by transferring nuts to 15°C (Treatment 1, step 2) for the next 6 weeks. At the end of this storage period this batch showed reduced rate of germination up to 20 days, then the difference became insignificant. The lag phase of the first germination was delayed by 2 days from control seeds and started first germination on the 8th day. In the second reversal of the remaining nuts held in this temperature regime (15°C) for 6 weeks was transferred to 5°C (Treatment 1, Step 3) storage for the final 6 weeks. At the end of this storage period when germination test conducted it showed the reversal of reduced germination rate to a significantly higher rate of germination which maintained up to 10 days.

The second set (Treatment 2) of dormancy reversal experiments showed in Figure 3.9 (b) started with the same control of Treatment 1, which recorded 80% final germination. In the first experiment of Treatment 2, step 1, non-dormant seeds

stored for 6 weeks at 15°C resulted in reduction of final germination to 67%. Then after the first reversal, nuts from this batch were transferred back to 5°C for 6 weeks (step 2) at the end of which final germination increased to 85%. In the last reversal treatment (step 3) when the remaining nuts were transferred from 5°C to 15°C, a sharp fall in final germination to 28% was observed.

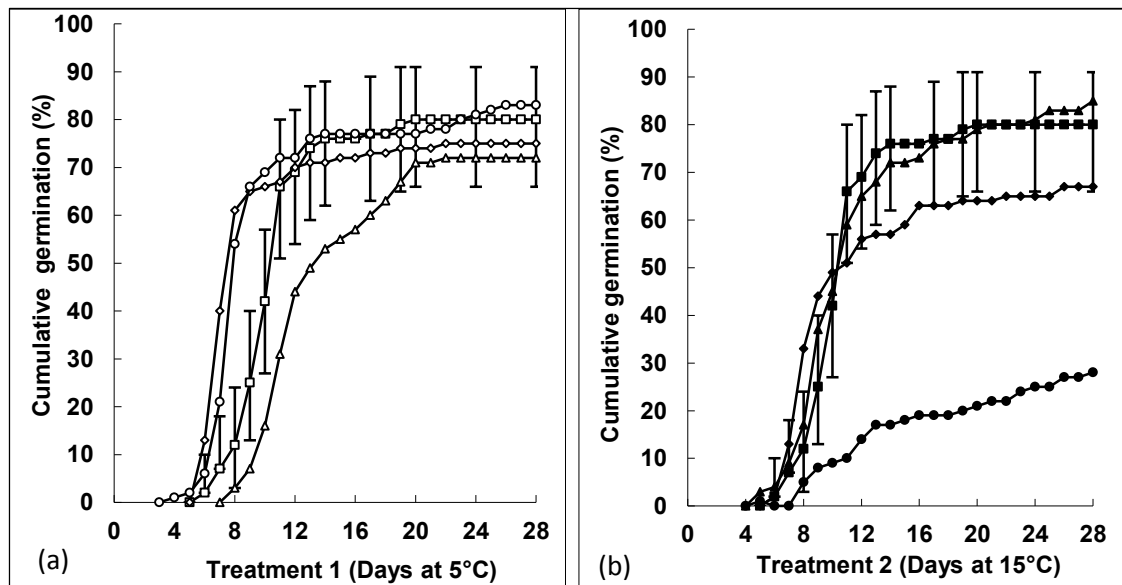


Figure 3.9: Effects of dormancy reversal treatment on hazel seed germination. Seed germination tests were conducted at 15°C. Error bars represent \pm SD based on samples of 100 seeds calculated as described in section 2.15.

(a) Treatment 1: Nuts were stored at 5°C or 15°C in re-sealable polyethylene bags for appropriate time and at the end of this period moisture content and germination was recorded.

Control (□),

6 weeks at 5°C (◇) (Step 1),

6 weeks at 5°C + 6 weeks at 15°C (△) (Step 2),

6 weeks at 5°C + 6 weeks at 15°C + 6 weeks at 5°C (○) (Step 3)

(b) Treatment 2: Nuts were stored at 15 or 5°C in re-sealable polyethylene bags for appropriate time and at the end of this period moisture content and germination was recorded.

Control (■),

6 weeks at 15°C (◆) (Step 1),

6 weeks at 15°C + 6 weeks at 5°C (▲) (Step 2) and

6 weeks at 15°C + 6 weeks at 5°C + 6 weeks at 15°C (●) (Step 3)

3.2.3.2. Moisture Content

Treatment 1 shows in Figure 3.10 (a) the reduction of seed moisture content during first 6 weeks storage at 5°C in Step 1 dropped from 36% to 31% while germination reduced from 80% to 75% (Fig. 3.9 a). In Step 2, when transferred to 15°C for the next 6 weeks, the seeds at the end of storage period resulted in moisture content reduced to 28% and final germination recorded as 72% Figure 3.9 a. In the final reversal of the seeds to another 6 weeks at 5°C resulted in seed moisture remaining at 28% and final germination increased to 83% Figure 3.9 a.

Figure 3.10 (b) shows seed moisture content during first 6 weeks at 15°C in step 1 of Treatment 2, reduced from 36 to 30% which showed a reduction in final germination from 80 to 67% (Fig. 3.9 b). After the reversal of storage temperature from 15°C to 5°C for the next 6 weeks in step 2, increase in seed moisture to 36% and final germination to 85% was observed (Fig. 3.9 b). In step 3 after third reversal and second time at 15°C, seed moisture content reduced to 31% but final germination dropped drastically to 28% (Fig. 3.9 b).

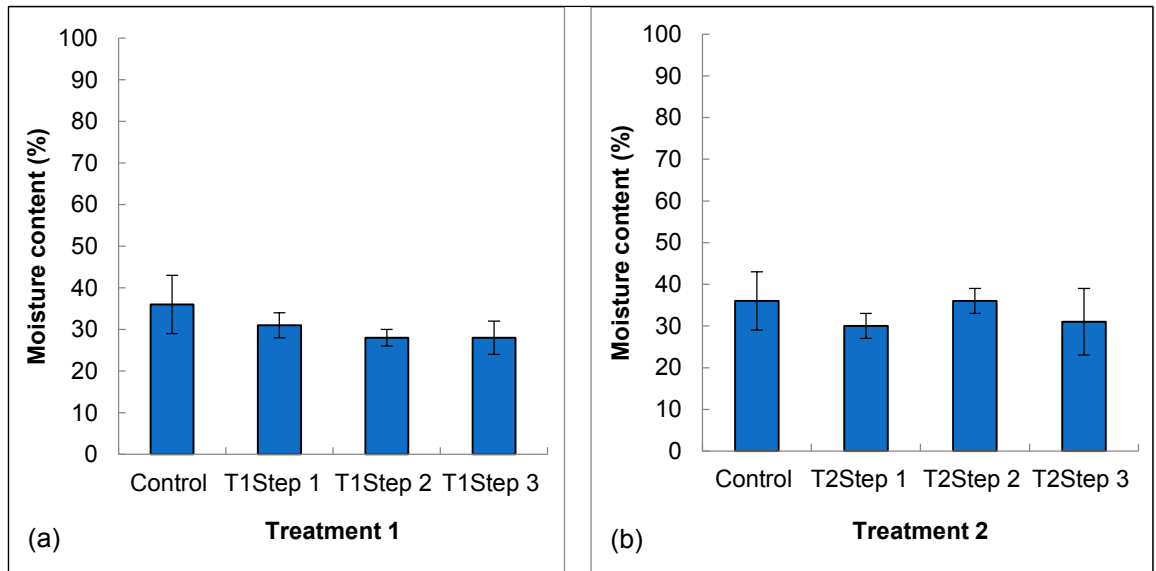


Figure 3.10: Effects of dormancy reversal treatments on seed moisture content. (a) Treatment 1, (b) Treatment 2. Error bars represent \pm SD of 10 seeds.

Treatment 1

Control 5°C
 T1Step 1 5°C + 5°C
 T1Step 2 5°C + 5°C + 15°C
 T1Step 3 5°C + 5°C + 15°C + 5°C

Treatment 2

Control 5°C
 T2Step 1 5°C + 15°C
 T2Step 2 5°C + 15°C + 5°C
 T2Step 3 5°C + 15°C + 5°C + 15°C

3.2.3.3 Mean Daily Germination (MDG), Peak Value (PV) and Germination Index (GI)

Figure 3.11 shows the impact of Treatment 1 and Treatment 2 on MDG, PV and GI of the seeds tested. Figure 3.11 (a) shows very little difference in the various steps of temperature changes on MDG, only in step 3, was reduced after 6 weeks storage at 15°C while seeds in Treatment 2 shows fluctuations due to change in storage temperature. MDG reduced in Step 1 when seeds were held at 15°C for 6 weeks (Fig. 11 d). The reversal to 5°C for the next 6 weeks in Step 2 increased the MDG but on transfer to 15°C for the next 6 weeks for Step 3 showed further reduction in MDG (Fig. 11 d). Figure 3.11 (b) shows PV significantly increased after Step 1 where seeds which had another 6 weeks at 5°C but was reduced in Step 2 when the seeds were subjected to 6 weeks at 15°C. In Step 3 however, when the seeds were reversed to another 6 weeks 5°C period, PV increased again. Seeds in Treatment 2 (Fig. 3.11 e), show slightly different result as the PV value was higher than the control passing through 6 weeks at 15°C then 6 weeks at 5°C but a second 6 weeks reversal to 15°C resulted in reduction in PV. Compared to the control seeds, Figure 3.11 (c) shows GI increased after Step 1 when the seeds were subjected to 6 weeks at 5°C but reduced sharply after Step 2 when kept at 15°C for a 6 weeks period. GI recovered after Step 3 when the seeds were reversed back to 6 weeks at 5°C. For seeds in Treatment 2, GI peaked up after Step 1 and 2 but reduced sharply when subjected to 6 weeks at 15°C (Fig. 3.11 f).

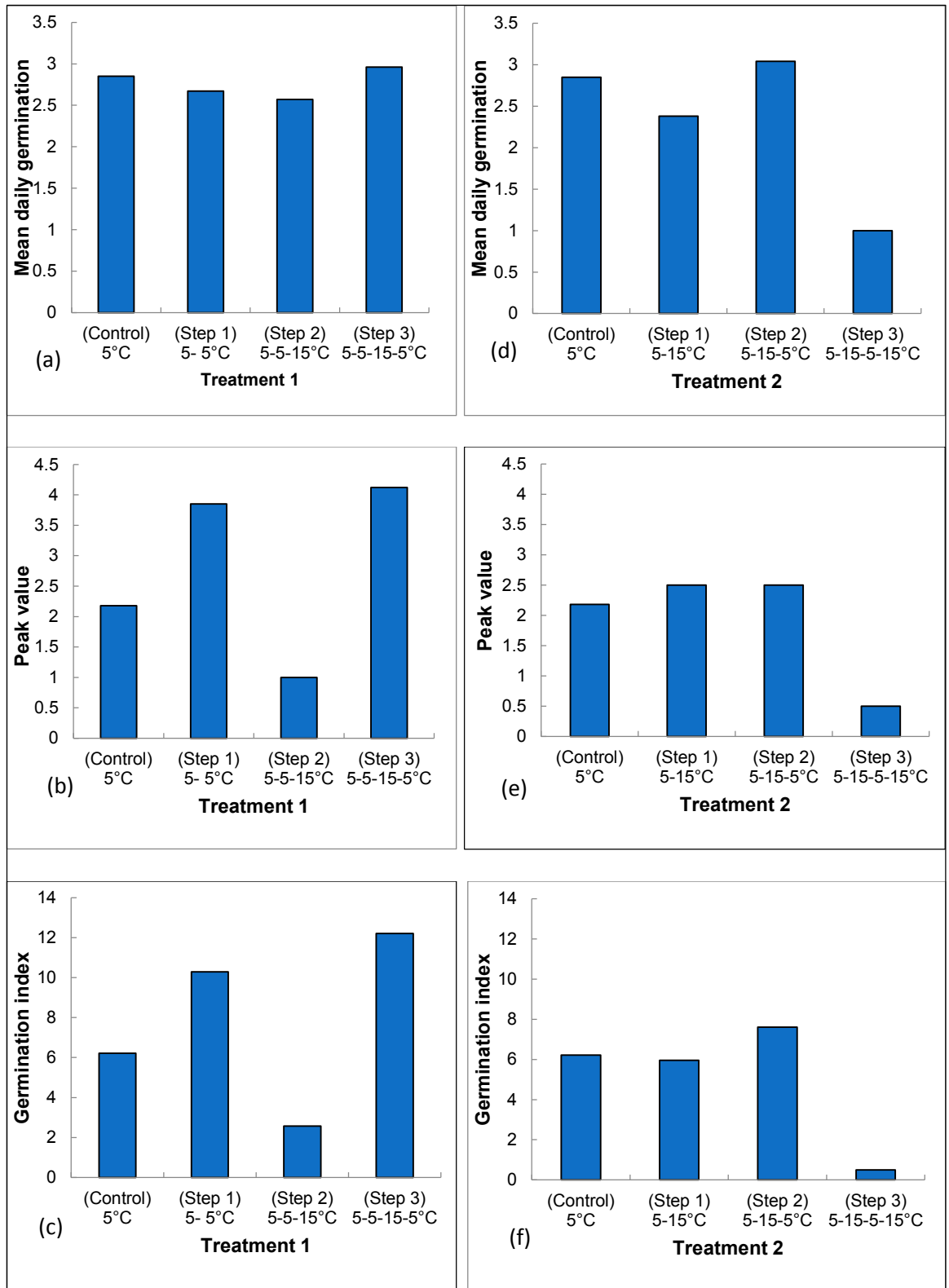


Figure 3.11: Treatment 1: Effects of dormancy reversal treatments on (a) MDG, (b) PV and (c) GI of hazel seed germination. Data taken from Figure 3.9 (a). Treatment 2: Effects of dormancy reversal treatments on (d) MDG, (e) PV and (f) GI of hazel seed germination. Data taken from Figure 3.9(b).

3.2.3.4 Seedling Performance

Treatment 1

Dormancy reversal tests were followed with seedling performance test planting 30 germinated seeds in compost pots. Table 3.2 contains a summarised account of seedling response from seeds after Treatment 1. Compared to 6 weeks at 5°C in Step 1, 6 weeks at 15°C in Step 2 shows reduced MC, delayed seedling emergence time from 7 to 10 days and abnormal seedling increased from 13% to 37%. In Step 3, reversing seed storage from 15°C to 5°C for 6 weeks showed similar MC but increase in germination from 72% to 83%. Seedling emergence time increased further from 10 to 13 days and abnormal seedlings recorded at 30%. Seedling height dropped from 140 mm in Step 1 to 120 mm in Step 2, but recorded a drastic reduction to 67 mm after Step 3. Internode numbers were also reduced in Step 1, 2 and 3 to 8, 7 and 5 respectively.

Table 3.2: Effect of dormancy reversal treatments of hazelnuts on seedling performance after Treatment 1.

	Seed m. c.	Final seed germination	Emergence time(days)	% abnormal seedling	Average seedling height (mm)	Average internode numbers
Treatment 1						
Control	36.1±6.9	80	ND	ND	ND	ND
Step 1: (6W 5°C)	30.9±2.9	75	6.7±0.3	13	139.7±8.7	8.3±0.3
Step 2: (6W5°C)+ (6W 15°C)	27.5±2.4	72	10.4±0.4	37	120.2±6.2	6.7±0.6
Step3:(6W5°C)+ (6W15°C)+ (6W5°C)	27.7±3.9	83	13.4±0.7	30	67.4±7.4	5.2±0.2

ND= not done

Treatment 2

Table 3.3 contains a summarised detail of effects on seedling performance after each step in Treatment 2. In Step 1, 6 weeks storage at 15°C reduced seed moisture from 36.1% to 29.7% and resulted in reduced final germination from 80 to 67%. In Step 2 seeds held at 5°C for 6 weeks after 6 weeks at 15°C shows final germination increased from 67 to 85% but seedling emergence time delayed further from 8 to

11 days and abnormal seedling increased from 23% to 37%. Compared to Step 1, seedling height showed reduction from 138 to 126 mm while internode numbers decreased from 8 to 7. A final period of 6 weeks storage at 15°C in Step 3 compared to Step 2 showed a sharp reduction of final germination from 85 to 28%, seedling height reduced from 126 to 83 mm and internode number reduced from 7 to 6.

Table 3.3: Effect of dormancy reversal treatments of hazelnuts on seedling performance after Treatment 2.

Treatment 2	Seed m. c.	Final seed germination	Emergence time(days)	% abnormal seedling	Average seedling height (mm)	Average internode numbers
Control	36.1±6.9	80	ND	ND	ND	ND
Step 1: (6W 15°C)	29.7±3.4	67	7.8±0.2	23	137.7±8.3	7.7±0.2
Step 2:(6W 15°C)+ (6W 5°C)	36.4±2.7	85	11.0±1.4	37	125.7±1.5	6.6±0.1
Step 3: (6W 15°C)+ (6W5°C)+(6W15°C)	31.0±7.7	28	14.8±2.7	38	82.7±7.3	5.6±0.3

ND= not done

3.2.4 Discussion

Nuts at the end of Treatment 1, Step 1 actually passed through 44 weeks at 5°C and showed drop in MC from 36% to 31% as a result of 6 weeks storage in closed polyethylene bags. This could be due to utilisation of moisture because of continued metabolic activities in the seed tissues. Seed germination of 75% after 44 weeks at 5°C agrees with (Bradbeer, 1988) who reported hazel seeds can survive between 6 months to a year in storage. Although MC was reduced from 36% to 31% the germination speed was higher in Step 1 up to 11 days (Fig. 3.9 a). The increased germination rate can be attributed to the longer time available at 5°C (chilling) to pursue more synthesis of growth hormones at low temperature (Ross & Bradbeer, 1971a).

Delayed germination (Fig. 3.9 a), reduced MDG, PV and GI (Figs. 3.11 a, b and c) at 15°C shows an Impact of water stress but the final germination shows no

significant difference between the treatments (Fig. 9 a). The reversal back to 5°C (Step 3) and storage for 6 weeks allowed further GA synthesis (Williams, et al., 1974), therefore lag phase reduced to 3 days and significant higher rate of germination over control (Fig. 3.9 a). At 15°C, the decreased germination may be due to physiological and metabolic changes during 6 weeks storage when the seeds may have started germination related metabolic activities but was not able to get sufficient solvent required to continue further metabolism. This might have resulted in water stress, but as the storage lasted for six weeks only, a low temperature reversal (5°C) allowed recovery from some effects of the stress. Decreased performance may be attributed to reductions in seed moisture and increase in storage temperature which might have reduced the growth promoters and increased growth retardants ABA for example, reported in hazel seeds (Williams, et al., 1973). PV increased after reversing the storage temperature to 5°C for the second reversal for 6 weeks (Treatment 1, Step 3). During this period the trapped water vapour may have condensed increasing seed moisture triggering enzyme activities (type 2 water) (Vertucci & Farrant, 1995) and resulted in higher germination.

In Treatment 2, which started with 6 weeks storage at 15°C, initiates germination related metabolism but were unable to get external water supply which may have caused water stress and resulted in reduced final germination as seen in Figure 3.9 b. Although significantly higher rate of germination was recorded up to the 9th day but the final germination was lower than control. Reversing to 5°C storage in Step 2, although the final germination was significantly higher than those with 6 weeks at 15°C but the rate of germination was significantly lower up to 9 days. The delay could be attributed to the time taken for re-condensation of water vapour within the container to a higher level and facilitating the metabolic process which enabled higher germination after bulk water was added in germination test. Inability to obtain

more free water for metabolic activities during 6 week storage and increased temperature resulted in water stress at the end of the next 6 weeks at 15°C (Fig. 3.9 b, Step 3) and culminated in reduced germination. Moreover, this set of nuts were subjected to two periods of 15°C for 6 weeks each when the free gibberellins were most likely to be used for germination metabolism (Williams, et al., 1974) but lack of required water to assist in metabolic activities caused water stress and might have damaged some seed tissues. Seed germination in treatment 2, step 3, although much reduced still shows a slight but gradual increase in germination indicating possible synthesis or release of bound gibberellins or both (Fig. 3.9 b). The process was assisted with exogenous water (solvent) available on the germination plate. Reversing storage temperature from 15°C to 5°C favoured synthesis of growth promoting substances as shown by the recovery of germination from significantly reduced to increased values, while reversing from 5°C to 15°C storage showed a reverse trend where seed germination showed significant reduction from higher values. This could be due to lack of production/release of required growth promoters while held for 6 weeks at 15°C or a result of water stress during storage period.

Figure 3.11 (d, e and f) shows a sharp fall in MDG, PV and GI in seeds after the second 6 week storage at 15°C in Step 3. It is anticipated that the final 6 week at 15°C after the first 15°C storage alternated with a 6 week at 5°C could not synthesize sufficient GA to enhance rate and final germination or water content between 29-36% was not sufficient for the process and resulted in water stress. This observation leads to compare the response of seedling emergence after each dormancy reversal test.

Table 3.2 shows delay in seedling emergence time, increase in number of abnormal seedlings, reduction in seedling height and internode numbers irrespective of seed moisture content. It is assumed that one treatment of 15°C in Treatment 1, step 2 or two times 6 weeks at 15°C in Treatment 2, step 1 & 3 may have contributed to the reduced performance of the seedlings. At 15°C seeds undergo metabolic activities when the available water content was lower than needed resulted in water stress. It is stated that although, several recalcitrant seed species have been recorded as undergoing a measure of dehydration during their development, they do not undergo maturation drying *per se*, and are shed at relatively high water contents (Finch-Savage, 1995; Vertucci & Farrant, 1995). Recalcitrant seeds are desiccation sensitive both before and after shedding and have very limited post-harvest life spans, even in hydrated conditions. Hazelnuts can survive up to a year (Bradbeer, 1988) but the germination results in this experiment show some seeds undergo stress.

The phenotypic expressions in the seedlings observed in this experiment points to the fact that even the seeds that managed to germinate carried some effects of changes in moisture content relative to storage temperature (stress).

Although storage temperature reversal was tested for 6 weeks in each treatment, in nature, the length is longer whether at lower (5°C) or at higher ($\geq 15^\circ\text{C}$) temperature regime. The experiment was a test to simulate a similar condition and check the impact on dormancy levels in hazelnuts. Results obtained in this experiment shows that in Treatment 1, two terms at 6 weeks storage at 5°C alternated with one term at 15°C does not impose seed dormancy if the seed moisture content was between 28-36% but in Treatment 2 which had two terms at 15°C alternated with one term at 5°C resulted in reduced germination even when seed moisture was between 36-

30%. The reduction in germination could be due to imposing of dormancy or damaging impact of water stress at 15°C. The results had shown that seed moisture and temperature needed to be carefully considered in maintaining a healthy seed in storage. The main aim would be to protect the seed against the detrimental actions of water stress. Therefore, it is necessary to investigate the impact of stress on the role of water activity in relation to free radicals and leachate both at 5°C and 15°C storage.

3.2.5 Summary

Dormancy reversal test was set to investigate the impact of storage temperature alteration on the level of seed dormancy and seed viability. Treatment 1, Step 2 show hazelnuts held at 15°C for 6 weeks has significant reduction in rate of germination up to 20 days. Seeds recover from this deficiency when stored at 5°C for 6 weeks in step 3 when the germination rate increased over the control. Final germination in Treatment 1 showed no observable difference but germination index (GI) was significantly reduced in Step 2 when the seeds were subjected to 6 weeks at 15°C. Although final germination did not differ much, but delay in seedling emergence, increase in abnormal seedlings, reduction in seedling height and internode numbers was noticeable.

In Treatment 2 Step 1, final germination after 6 weeks at 15°C did not differ much from seeds after Step 2 which received 6 weeks at 5°C. But on transfer to 15°C in Step 3 much reduced germination resulted. The impact of the stress is clearly expressed in significantly low germination index in Step 3 which twice encountered 15°C storage period. During this test, seed moisture content did not vary much but the germination response was due to storage temperature. In all steps compared to control, seedling emergence was delayed, increase in abnormal seedlings,

reduction in height and internode numbers were noticed. Although storage at 5°C recovered final germination but delay in emergence, increase in abnormal seedlings, decrease in height and internode numbers indicated the impact of water stress irrespective to temperature on seedling features.

Considering final germination responses, a single interruption of 6 weeks at 15°C did not affect seed dormancy but if interrupted by 2 terms at 15°C show deleterious effects. Results show the ability of some hazel seeds to survive temperature fluctuation. As with the moisture results discussed in section 3.1, this may have implications for designing of storage protocols. On the other hand, seedlings did exhibit stress impact such as development of abnormal and decapitated seedlings, which suggests that large temperature variations should be avoided. Extension of storage time of both temperature regimes might provide more information related to dormancy reversal. Biochemical aspects like role of water activity, leachate conductivity, antioxidant activities and seed viability test by TTC would provide further information on the role of dormancy reversal tests in seed viability.

3.3 Effects of Storage of Non-dormant Hazelnuts at 5°C and at Ambient Room Temperature

3.3.1 Introduction

In section 3.2, seed dormancy was tested by seeds exposure to one or two times for 6 weeks at 5°C and 15°C storage temperature and the results showed detrimental for both seed germination and seedling performance. The poor performance could be stress related due to the level of seed water content and storage temperature. Hence, another experiment was planned to check some chemical and biochemical aspects during 6 weeks storage at 5°C and ambient room temperature (RT) ($20 \pm 2^\circ\text{C}$).

It is known that chilling breaks seed dormancy in hazelnuts (Bradbeer, 1968). In the present investigation, the fate of seeds from non-dormant nuts are examined after holding these nuts at two contrasting temperatures, 5°C (favourable temperature for synthesis of growth hormones) and at RT (ambient temperature favourable for germination related metabolic activities) in relation to water activity, leachate conductivity, antioxidant activities and tetrazolium (TTC) test to assess total seed viability.

Test of seed viability cannot always be conclusive with germination test alone as some seeds may remain un-germinated due to dormancy or stress related factors. The TTC test had been used to check the viability of various seed during storage, as for example on endemic recalcitrant *Myristica malabarica* Lam. (Krishnan, et al., 2002) and used for assessing freshness of hazelnuts (Schafer, et al., 2002). TTC test had been used for assessment of deep dormant and non-dormant seeds of *Acer caesium* and *Ulmus wallichiana* respectively (Phartyal, et al., 2003). Linear relationship between tetrazolium reduction as seed weight increased and electrical conductivity decreased was reported in *Jatropha curcas* (Zaidman, et al., 2010).

Seed quality of *Amomum villosum* has been assessed by TTC test (Zhang, et al., 2011). Viability testing of black walnut has been well documented (Flores, et al., 2011). Preconditioning seeds of *Cattleya* to improve viability has also been tested by TTC test (Hosomi, et al., 2011). Dehydration of embryonic axes of desiccation-sensitive (recalcitrant) seeds of *Quercus robur* L resulted in viability loss as verified by TTC test (Ntuli, et al., 2011).

Leachate conductivity also shows an important role in seeds biochemical status. Reductions of seed water content in recalcitrant seeds of *Shorea robusta* Gaertn. f. resulted in increased electrolyte leakage (Naithani & Chaitanya, 1994). Desiccated seeds of recalcitrant silver maple (*Acer saccharinum*) seeds lost their germination capacity with strongly correlated increase in electrolyte leakage from seeds (Pukacka & Ratajczak, 2006). Acorns of recalcitrant holm oak (*Quercus ilex* L.) stored in peat can failed to maintain germination vigour and recorded higher electrolyte leakage than those stored in polyethylene bags (Pasquini, et al., 2011). Electrical conductivity test also provided rapid and reliable results of seed viability in *Kielmeyera coriacea* Mart. seeds (Ramos, et al., 2012). Electrical conductivity (EC) measurements of seed soak water from radish seeds showed increased levels of electrolyte leakage which were associated with slower and lower emergence and with poorer storage potential (Mavi, et al., 2014).

The few examples mentioned above point to electrolyte leakage from seeds during stress. It is also reported that stress results in free radical production and in response it had been stated that seeds are capable of countering the damage by its own arsenal of antioxidants (Bailly, 2004), as reported in *Mimusops elengi* seeds (Luo, et al., 2012).

Plants possess a number of antioxidants that protect against the potentially cytotoxic species of activated oxygen. Antioxidants can be divided into three general classes including:

- (i) lipid soluble and membrane-associated α -tocopherol and β -carotene,
- (ii) water soluble reductants, ascorbate (AsA) and glutathione,
- (iii) enzymes such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase.

Examples of antioxidant activities in seeds include a decline in axis viability of *Theobroma* (cocoa) seeds below the critical water content correlated with sharp increases in lipid peroxidation and cellular leakage. Cotyledon tissues on the other hand were more desiccation-tolerant than axes. Desiccation sensitivity was also correlated with decrease in superoxide dismutase and increase in lipid peroxidation products (Li & Sun, 1999). On the other hand, cold stratification broke dormancy of pear (*Pyrus betulaefolia* Bge. and *Pyrus calleryana* Dcne.) seeds during which enzyme peroxidase (POD) and catalase (CAT) activities increased (Bao & Zhanj, 2011). Six species of *Caragana* adapted to arid environment subjected to drought stress showed an elevation of superoxide dismutase (SOD), glutathione peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and reduced glutathione (GSH) with progressive drought stress (Kang, et al., 2012). In the present investigation control seeds which acquired 68% germinability but showed 100% viability after 6 weeks chilling at 5°C were used to check effect on dormancy by holding the non-dormant nuts at 5°C and at RT (ambient laboratory temperature) for 6 weeks each.

3.3.2 Materials and Methods

3.3.2.1 Nut Provenance

Hazelnuts of 2011 crop were used in the following experimental treatments. Details of the nut provenance are given (section 2.1).

3.3.2.2 Nut Storage

Hazelnuts which were chilled for 6 weeks at 5°C were used for this experiment (section 2.3). The experiment had been focussed on nuts held for 6 weeks at:

i) 5°C and ii) room temperature (RT).

Usually 450 nuts were stored in re-sealable polyethylene bags at 5°C or at RT. Nuts required for tests were removed after 0, 2, 4 and 6 weeks storage for physiological and analytical tests.

3.3.2.3 Experimental Procedure

Analyses were conducted as described in, moisture content determination (section 2.4), water activity measurement (section 2.5), leachate conductivity (section 2.6), antioxidant measurement (section 2.7), germination assessment of seeds at the end of each treatment was done as described (section 2.11) and seed viability by TTC test (section 2.12).

3.3.3 Results

Hazelnuts chilled for 6 weeks for dormancy breaking experiment were used as control and remainder of dormancy inducing tests conducted from this set of nuts. During the storage period no exogenous (additional) water was added as nuts were held with inherent seed moisture acquired during previous chilling period. These nuts were held at two temperature regimes: i) 5°C, and at ii) RT in re-sealable polyethylene bags to reduce water loss.

3.3.3.1 Storage at 5°C

Chilled nuts were given further 5°C storage treatments. After 0, 2, 4 and 6 weeks nuts were cracked to obtain seeds and the following physiological, chemical and biochemical tests were conducted.

Table 3.4 summarises the physiological and biochemical activities during 6 weeks storage at 5°C. Seed MC did not reduce much during the first 4 weeks but reduced to about 10% at 6 weeks indicating the possibility of low metabolic activities at 5°C but some activities took place during 6 weeks storage (Fig. 3.12). Lag phase to first germination remained at 3 days up to 4 weeks storage but delayed to 6 days after 6 weeks storage. No seed infection was observed during the first 4 weeks but the first infection of 2% observed after 6 weeks storage.

The un-germinated seeds were tested for viability using TTC test. Control seeds recorded 32% TTC positive, assumed the seeds did not germinate as were yet to break dormancy. There was gradual increase in TTC dependency up to 6 weeks which showed a rise from 16% to 26%.

Table 3.4: Summarised list of physiological, chemical and biochemical results obtained during 6 weeks storage of non-dormant hazelnuts at 5°C. Results include \pm SD.

Treatment: weeks at 5°C	MC	aw	Days to first germination	% germination (infection)	TTC positive	Total viability	Conductivity $\mu\text{S g}^{-1}$	Embryo (mg) for 50% inhibition of DPPH
Control (0)	40.5 \pm 5.4	0.967 \pm 0.003	3	68(0)	32	100	9.1 \pm 2.5	13.8
2	36.1 \pm 7.6	0.965 \pm 0.001	3	84(0)	16	100	15.8 \pm 2.5	15.8
4	38.5 \pm 6.1	0.965 \pm 0.002	3	87(0)	13	100	13.3 \pm 1.9	6.1
6	10.3 \pm 3.7	0.854 \pm 0.003	6	72(2)	26	98	17.0 \pm 2.9	4.1

3.3.3.2 Storage at Room Temperature

Table 3.5 summarises the physiological and biochemical activities of the same batch of nuts held at room temperature for 6 weeks which resulted in significant water loss from 40.5% in control to 21.1% after 4 weeks but reduced to only 4.1% after 6

weeks. Water loss may be attributed to higher metabolic activities at room temperature (Fig. 3.12). Lag phase to first germination increased from 3 days to 13 days after 6 weeks storage. Although control seeds showed no seed infection but at the end of 6 weeks 12% seeds were infected and discarded.

Table 3.5: Summarised list of physiological, chemical and biochemical results obtained during 6 weeks storage of non-dormant hazelnuts at ambient room temperature (RT). Results include \pm SD.

Treatment: Weeks at RT	MC	aw	Days to first germination	% germination (infection)	TTC +ve	Total viability	Conductivity $\mu\text{S g}^{-1}$	Embryo (mg) for 50% inhibition of DPPH
Control (0)	40.5 \pm 5.4	0.967 \pm 0.003	3	68(0)	32	100	9.1 \pm 2.5	13.8
2	39.3 \pm 8.3	0.965 \pm 0.003	2	20(0)	80	100	10.4 \pm 1.2	9.2
4	21.1 \pm 7.7	0.920 \pm 0.002	4	14(1)	85	99	11.1 \pm 2.3	ND
6	4.1 \pm 0.8	0.540 \pm 0.001	13	8(12)	80	88	19.2 \pm 1.1	ND

ND = Not done.

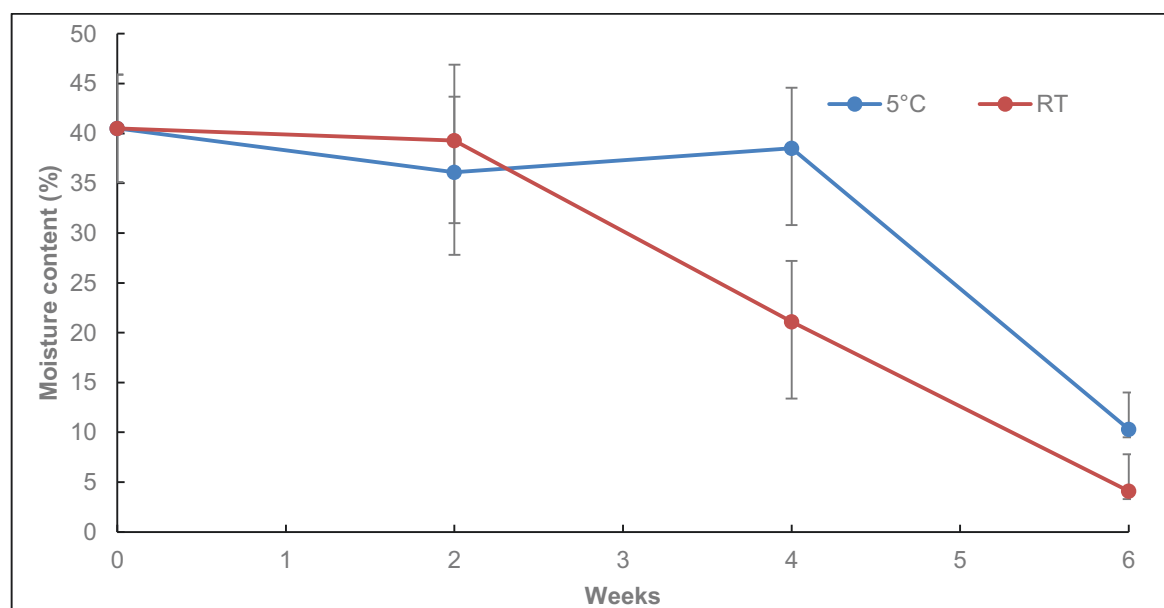


Figure 3.12: Moisture loss of seeds from hazelnut held at 5°C and RT. The values are mean of ten replicates \pm SD.

3.3.3.3 Seed Germination

During storage, hazelnuts lose water and would become dormant. Hence, a batch of hazelnuts was made non-dormant by 6 weeks chilling prior to this test. Figure 3.13 (a) shows germination results of these non-dormant seeds held at 5°C for up to 6 weeks. During storage at 5°C in the first 2 weeks final germination increased

from 68% in control to 84% but after 4 weeks final germination increased 87%. Slight decrease to 72% in final germination was noted after 6 weeks storage. Lag phase to first germination remain at 3 days in control up to 4 weeks then, increased to 6 days after 6 weeks storage. It is evident from Figure 3.13 (a) that some seeds failed to germinate during the 28 days experimental period. Whereas, Figure 3.13 (b) shows germination response of the same batch of non-dormant hazelnuts held at room temperature show a gradual reduction of germination from 68% in control to 20% after 2 weeks storage which reduced further to 14% after 4 weeks and dropped to only 8% after 6 weeks. Gradual delay in germination lag phase was evident as after 4 weeks first germination was recorded on the 4th day whereas the first germination increased to 13th day after 6 weeks storage. Compared to control the room temperature treatment resulted in 80 seeds were unable to germinate.

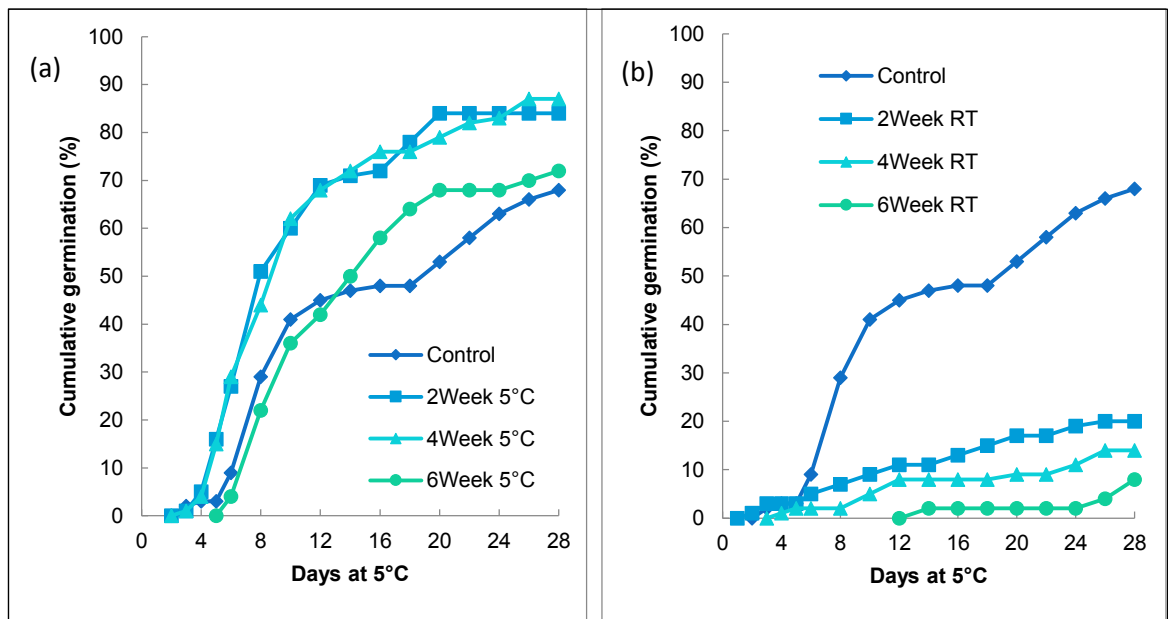


Figure 3.13: Effect of 6 weeks storage of non-dormant hazelnuts at (a) 5°C and (b) ambient room temperature (RT) on seed germination. No additional water was added during the storage period.

Figure 3.14 compares the final germination recorded after 0, 2, 4 and 6 weeks at 5°C and RT. Seeds stored at 5°C showed increased germination from control, which

reduced after 4 weeks. On the other hand, seeds held at RT showed reduced germination from control after 2 weeks and continued to reduce up to 6 weeks.

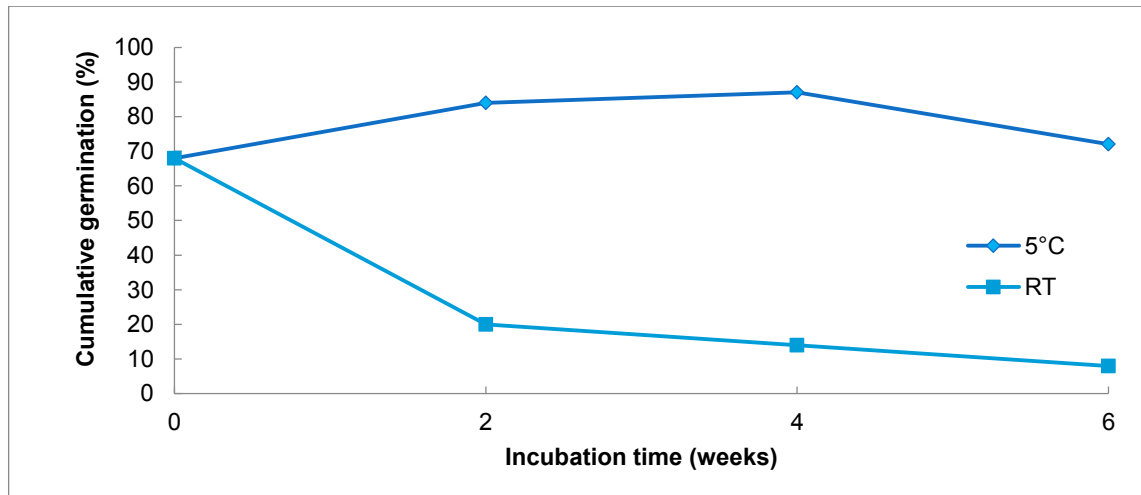


Figure 3.14: Germination pattern of non-dormant seeds held at 5°C and at RT for up to 6 weeks. Seeds were taken out at 0, 2, 4 and 6 weeks interval from both storage conditions and tested for germination at 15°C up to 28 days.

3.3.3.4 TTC Test Evaluation

TTC test on un-germinated treated seeds gives an elaborate picture of the biological activity of the seed tissues. Figure 3.15 show damaged part of the cotyledon and embryonic axis after the treatments. Figure 3.15 (a) shows dead embryonic axis and the faintly stained cotyledon parts indicating dead cells whereas the deeply stained parts indicate biologically active tissue areas. On the other hand, Figure 3.15 (b) clearly shows unstained areas indicating dead plumule and dead radicle.

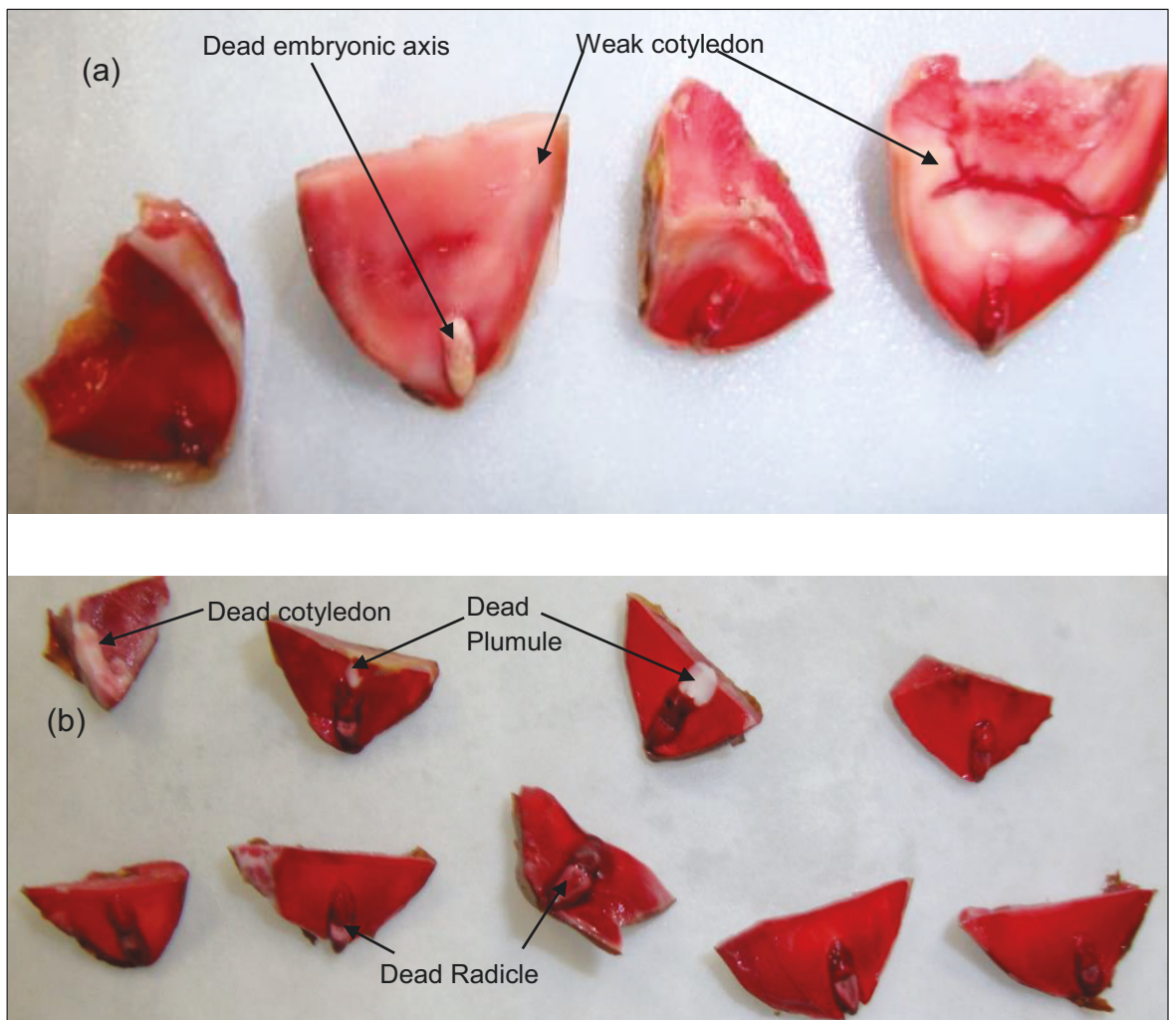


Figure 3.15: Effect of TTC test on hazel seed viability conducted on un-germinated seeds after germination test. (a) Unstained embryonic axis (dead) and faintly stained cotyledons (weak); (b) Dead plumule, radicle and cotyledon.

3.3.3.5 Total Seed Viability

Seeds which failed to germinate but remain healthy after 28 days test period were subjected to TTC viability test to test for total viability. Figure 3.16 (a) shows 32% control seeds held at 5°C were un-germinated and not infected but viable. The un-germinated seeds after 2 and 4 weeks i.e., 16% and 13% respectively were also viable by TTC test. After 6 weeks storage final germination dropped to 72%, but 26% un-germinated seeds proved to be viable and only 2 % seeds at this stage showed infection. Figure 3.16 (b) shows a gradual decrease in germination and increase in un-germinated seeds when seeds were held at room temperature. A total of 80% seeds remained un-germinated after 6 weeks storage at room temperature. Figure 3.16 (b) show un-germinated seeds from this batch were TTC positive but recorded 1% infection after 4 weeks which increased to 12% after 6 weeks storage.

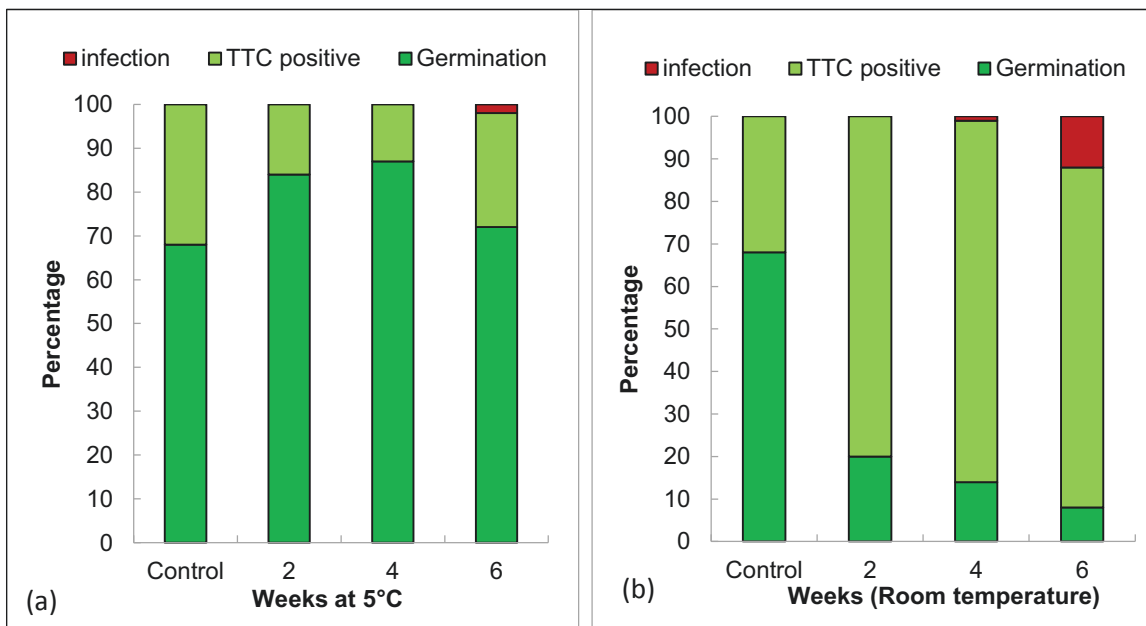


Figure 3.16: The effects during 6 weeks storage of non-dormant hazelnuts on germination and total seed viability. During storage period no additional water was added. Hazelnuts held at (a) 5°C (Germination details from Figure 3.13 a) and (b) ambient room temperature (Germination details from Figure 3.13 b).

3.3.3.6 Mean Daily Germination (MDG), Peak Value (PV) and Germination Index (GI)

Figure 3.17 shows a comparative break down of germination responses of seeds held at 5°C and room temperature on mean daily germination, peak value and germination index.

Mean Daily Germination (MDG):

Storage of hydrated non-dormant hazelnuts at 5°C had slight advantage with increase in MDG over control during increase in storage time as seen in Figure 3.17 (a). Whereas in Figure 3.17 (d) storage at room temperature resulted in significant reduction in MDG from second week and continued reduction up to 6 weeks.

Peak Value (PV):

Figure 3.17 (b) shows slight variation in PV in seeds held at 5°C. There was increase over control after 2nd week but showed downward trend afterwards. Non-dormant seeds held at room temperature showed significant reduction in PV starting from second week and continued up to 6 weeks as exhibited in Figure 3.17 (e).

Germination Index (GI):

Although Figure 3.17 (c) show increase in GI over control after 2 weeks at 5°C but the number reduced during subsequent storage period. Figure 3.17 (f) shows drastic reduction in GI from second week when seeds were held at room temperature.

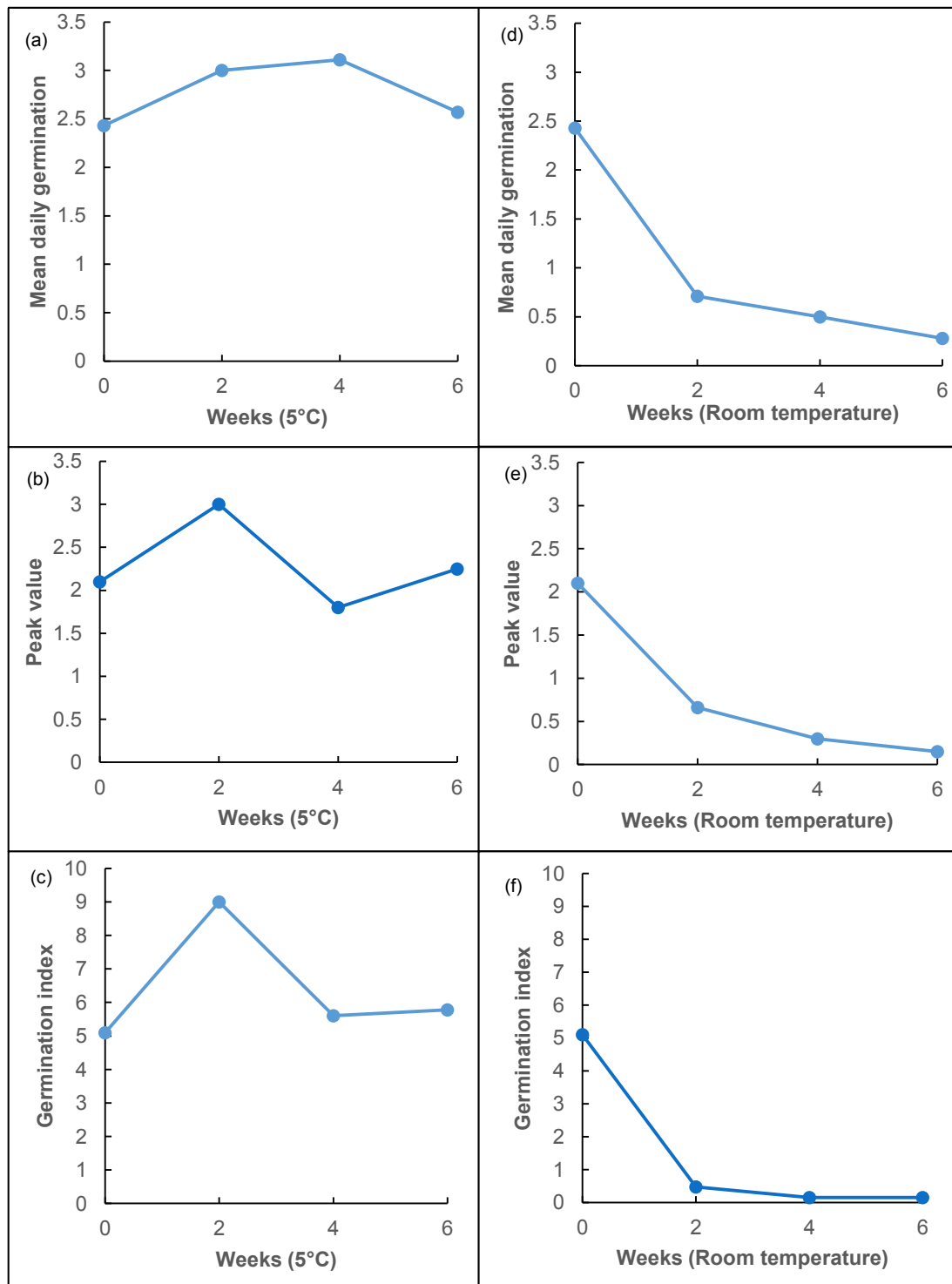


Figure 3.17: Effects during 6 weeks storage of non-dormant hazelnuts on MDG, PV and GI of hazel seed germination. Storage at i) 5°C (a, b, c) and ii) room temperature (d, e, f). Mean daily germination (a, d), Peak value (b, e) and Germination index (c, f). Germination data obtained from Figure 3.13(a) for a, b, and c; and Figure 3.13 (b) for d, e and f.

3.3.3.7 Seed Moisture Content (MC)

Figure 3.18 (a) shows moisture content of seeds (fresh weight basis) during 6 weeks storage of the non-dormant nuts held at 5°C. Control seed moisture was recorded as 40.5% which remained 38.5% after 4 weeks storage but was reduced to 10.3% after 6 weeks. On the other hand, non-dormant nuts held at room temperature shows in Figure 3.18 (b) the gradual loss of MC from 40.5% in control seeds to 39.3% after 2 weeks. Then, after 4 weeks storage, reduced to 21.1% but, after 6 weeks reduced further to 4.1%.

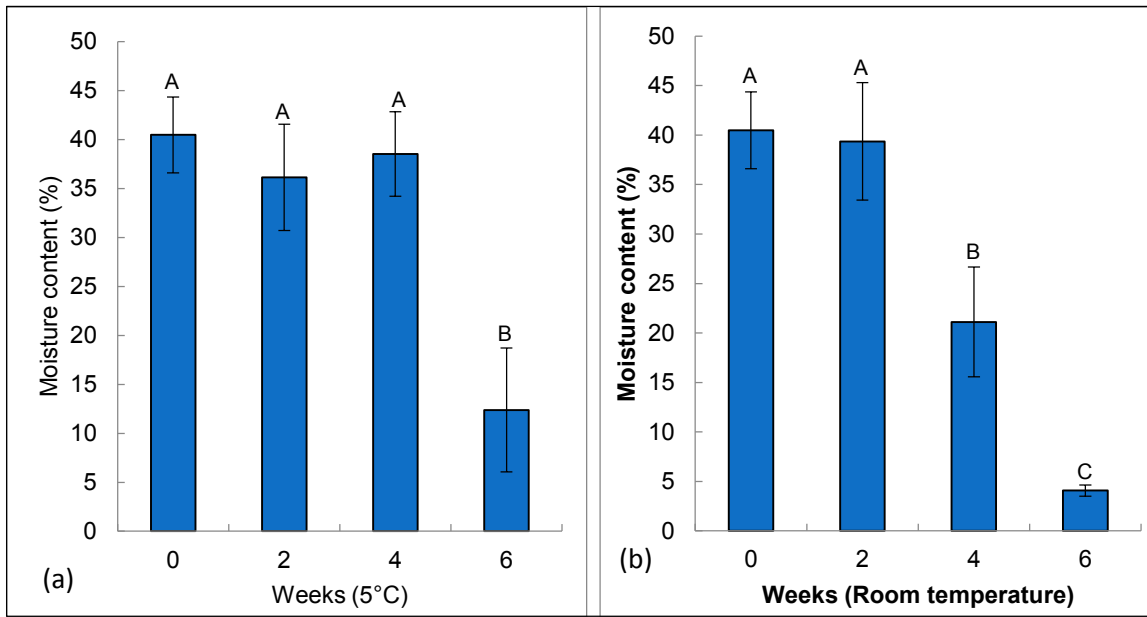


Figure 3.18: Moisture content of non-dormant seeds during 6 weeks storage at (a) 5°C and (b) room temperature. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of ten replicates \pm SD.

(a) One-way ANOVA: Moisture (%) versus Weeks (5°C).

Source	DF	SS	MS	F	P
Weeks	3	5648.7	1882.9	56.07	0.000
Error	35	1175.3	33.6		
Total	38	6824.0			

S = 5.795 R-Sq = 82.78% R-Sq (adj) = 81.30%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
Control (0)	10	40.980	A
4	10	38.530	A
2	10	36.140	A
6	9	10.283	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Moisture (%) versus Weeks room temperature.

Source	DF	SS	MS	F	P
Weeks	3	9066.0	3022.0	78.08	0.000
Error	36	1393.3	38.7		
Total	39	10459.3			

S = 6.221 R-Sq = 86.68% R-Sq (adj) = 85.57%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
Control (0)	10	40.980	A
2	10	39.360	A
4	10	21.120	B
6	10	4.080	C

Means that do not share a letter are significantly different.

3.3.3.8 Seed Water Activity

At the same time, seed water activity as shown in Figure 3.19 (a) also reduced from control value of 0.967 to 0.854 after 6 weeks at 5°C corresponding to moisture content of the respective seeds in Figure 3.18 (a). In Figure 3.19 (b), the water activity in control seeds of 0.967 decreased to 0.920 after 4 weeks at RT and finally reduced further to 0.540 after 6 weeks storage showing similarity with reduction in MC as shown in Figure 3.18 (b).

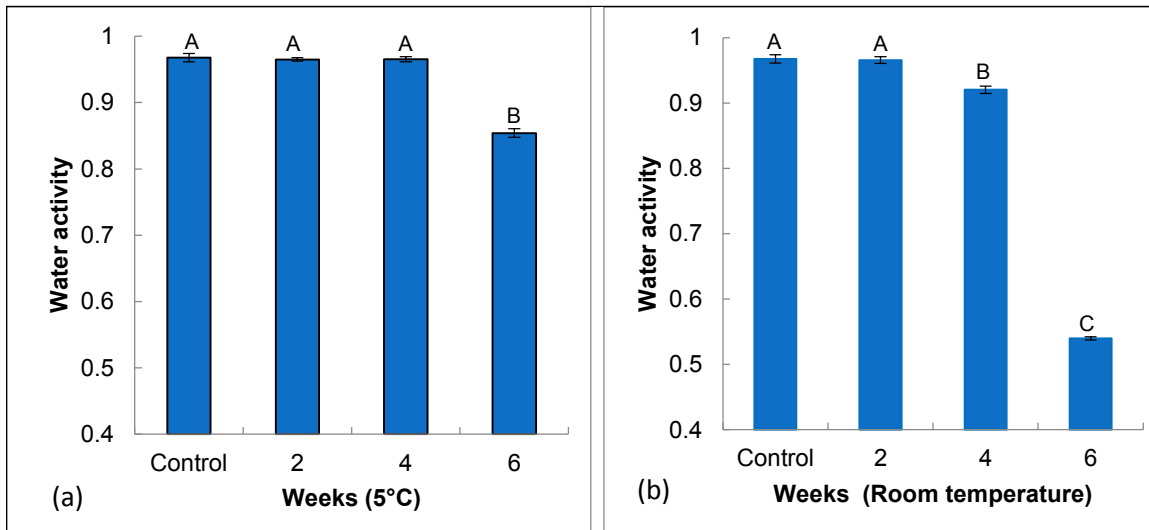


Figure 3.19: Effect during 6 weeks storage of non-dormant hazelnuts on seed water activity. Hazelnuts were removed after 0, 2 4 and 6 weeks and water activity determined. Hazelnuts were held for 6 weeks at (a) 5°C and (b) room temperature. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD.

(a) One-way ANOVA: Water activity versus Weeks at 5°C.

Source	DF	SS	MS	F	P
Weeks	3	0.0282367	0.0094122	2258.93	0.000
Error	8	0.0000333	0.0000042		
Total	11	0.0282700			

S = 0.002041 R-Sq = 99.88% R-Sq (adj) = 99.84%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
Control	3	0.96767	A
4	3	0.96533	A
2	3	0.96500	A
6	3	0.85400	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Water activity versus weeks at room temperature.

Source	DF	SS	MS	F	P
Weeks	3	0.3847829	0.1282610	30179.05	0.000
Error	8	0.0000340	0.0000042		
Total	11	0.3848169			

S = 0.002062 R-Sq = 99.99% R-Sq (adj) = 99.99%
Pooled StDev = 0.00206

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
Control	3	0.96767	A
2	3	0.96567	A
4	3	0.92033	B
6	3	0.54000	C

Means that do not share a letter are significantly different.

3.3.3.9 Conductivity of Seed Leachate

Figure 3.20 (a) shows leachate conductivity from whole non-dormant seeds stored at 5°C. Control seeds show leachate conductivity of 9 $\mu\text{S g}^{-1}$ but with increase in storage time even though stored at 5°C after 6 weeks it recorded 17 $\mu\text{S g}^{-1}$. While leachate conductivity of seeds stored at room temperature show gradual increase in leachate released during storage of non-dormant hazelnuts. Figure 3.20 (b) shows that leachate conductivity increased from a value of 9 $\mu\text{S g}^{-1}$ in the control seeds to 11 $\mu\text{S g}^{-1}$ after 4 weeks and then increasing to 19 $\mu\text{S g}^{-1}$ after 6 weeks.

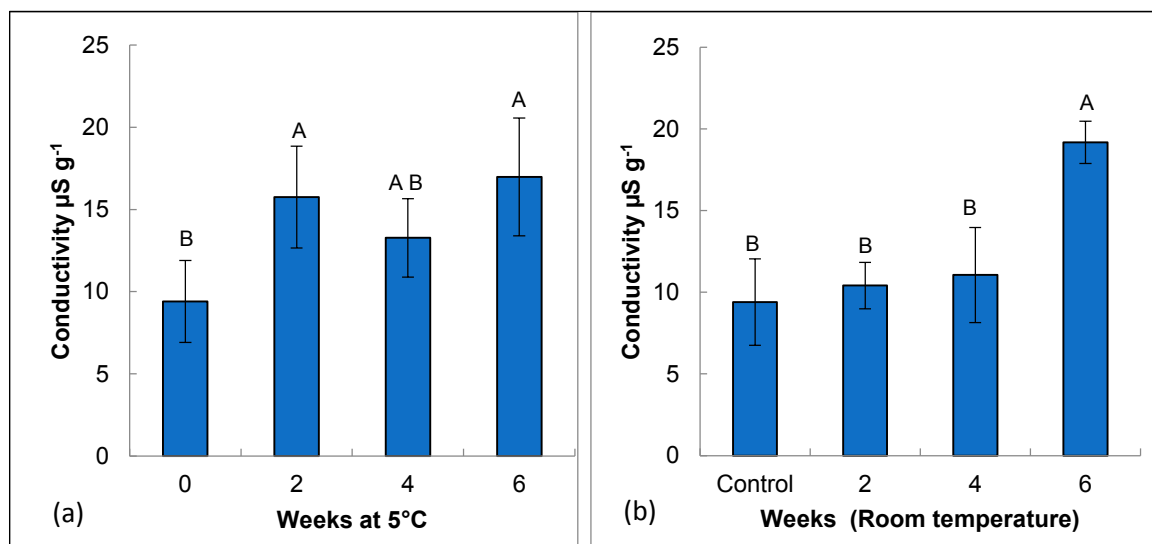


Figure 3.20: Effects during 6 weeks storage of non-dormant on hazel seed leachate conductivity. Hazelnuts held at (a) 5°C and (b) room temperature. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD.

(a) One-way ANOVA: Leachate conductivity versus Weeks at 5°C.

Source	DF	SS	MS	F	P
Weeks	3	179.39	59.80	9.72	0.001
Error	16	98.44	6.15		
Total	19	277.82			

S = 2.480 R-Sq = 64.57% R-Sq (adj) = 57.92%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
6	5	16.976	A
2	5	15.748	A
4	5	13.270	A B
Control (0)	5	9.140	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Leachate conductivity versus Weeks at room temperature.

Source	DF	SS	MS	F	P
Weeks	3	311.25	103.75	29.18	0.000
Error	16	56.88	3.56		
Total	19	368.13			

S = 1.886 R-Sq = 84.55% R-Sq (adj) = 81.65%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
6	5	19.172	A
4	5	11.052	B
2	5	10.412	B
Control (0)	5	9.140	B

Means that do not share a letter are significantly different.

3.3.3.10 Seed Antioxidant Activities

Figure 3.21 (a) shows the effect of storage of seed's antioxidant activities on standard free radical DPPH inhibition. Embryo samples from seeds held at 5°C show a decrease in sample quantity required for 50% DPPH inhibition as control samples required 13.8 mg which decreased to 4.1 mg after 6 weeks indicating the antioxidant system had been very active during 5°C storage. Figure 3.21 (b) shows antioxidant activities from seeds from hazelnuts held at room temperature needed 13.8 mg embryo samples from control seeds for 50% inhibition of standard free radical DPPH. After 2 weeks at room temperature storage it required lesser amount of 9.0 mg embryo sample to inhibit 50% DPPH which indicates antioxidant system had been activated due to water stress. Accidental waste of some samples prevented to present the antioxidant response of seeds held for 4 and 6 weeks at room temperature.

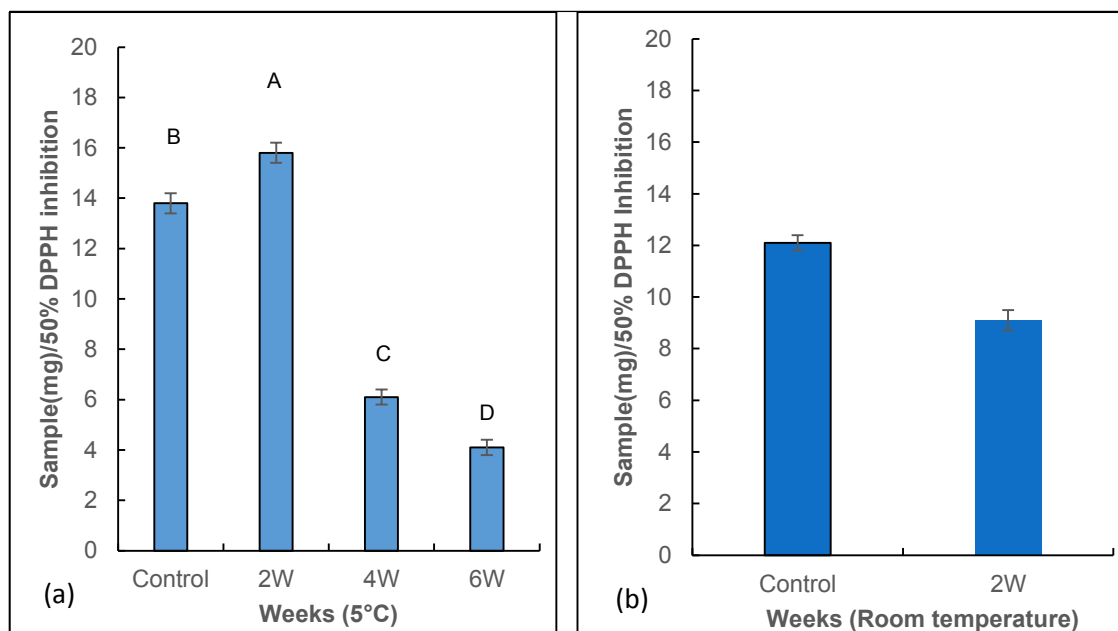


Figure 3.21: Effects during 6 weeks storage of non-dormant hazelnuts on DPPH inhibition with antioxidants from embryo extracts. Hazelnuts held at (a) 5°C. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$) and (b) room temperature (Student's t-test, $p < 0.05$). The values are mean of three replicates \pm SD.

(a) One-way ANOVA: Sample mass versus Weeks

Source	DF	SS	MS	F	P
Weeks	3	291.569	97.190	724.40	0.000
Error	8	1.073	0.134		
Total	11	292.642			

S = 0.3663 R-Sq = 99.63% R-Sq (adj) = 99.50%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
2W	3	15.800	A
Control	3	13.800	B
4W	3	6.167	C
6W	3	4.133	D

Means that do not share a letter are significantly different.

(b) t-Test: Two-Sample Assuming Equal Variances

	12.2	8.7
Mean	9.175	7
Variance	35.08917	19.38
Observations	4	4
Pooled Variance	27.23458	
df	6	
t Stat	0.589405	
p(T<=t) two-tail	0.577093	
t Critical two-tail	2.446912	

3.3.4 Discussion

In this test non-dormant hazelnuts were held at 5°C and RT to investigate the effect on seed dormancy. The relevant chemical and biochemical test results below indicate the impact of water stress on seed tissues resulting in damaging actions and thereby showing the limitations of storage of hazelnuts in hydrated state. Observations from various test results are discussed below.

3.3.4.1 Seed Germination

Figure 3.13 (a) showed seeds held at 5°C initially recorded increase in germination indicating the benefit of 5°C storage (thought to be related to the synthesis of growth regulators (Ross and Bradbeer, 1971a) but on continued storage showed reduction, most likely indicating water stress. Increase in lag phase for germination and signs of infection are indications of stress impact. The reduced germination may also be related to drop in seed moisture indicating water stress.

Effect of water stress was more prominent when seeds were held at room temperature. The RT set had a sharp reduction in germination and indication of water stress was noticeable in the increased lag phase in germination as it increased from control (Table 3.4) but in RT stored seeds it increased further to 13 days after 6 weeks (Table 3.5). The reduction in germination indicates the imposition of seed dormancy. These results indicate that, although non-dormant, hydrated hazel seeds are subject to water stress damages and thus this is not preferable for storage.

3.3.4.2 Seed Viability

Non-dormant seeds stored at 5°C and at room temperature show a gradual reduction in germination and gradual increase in un-germinated seeds but nevertheless proved viable by TTC test. Storage in ambient conditions even though started with 40% moisture was not helpful for seed storage as metabolic activities

failed to get extra water needed and resulted in stress related damages and reduced germination (Fig. 3.16 b). Reduction in metabolic capability to germination and increase in viable seeds indicate seeds increasingly adapting to dormancy.

MDG/PV/GI values did not show much difference indicating that germination is not affected as a result of 5°C storage but sharp reduction in germination following RT storage indicated the damaging effects of hydrated storage at RT.

Loss of viability of recalcitrant seeds in this way has been attributed to initially mild increasing to severe water stress (Pammenter et al., 1994).

3.3.4.3 Seed Moisture Content

Seed moisture is important in seed viability. Thus, gradual reduction of seed moisture from 40 to 10% in 6 weeks of hydrated hazelnuts at 5°C and the lack of extra water, to supplement requirement for metabolic activities, caused stress. Although not severe, this was depicted by reduced germination and signs of infection (Fig. 3.18 a). Ambient storage of the same batch showed much faster reduction in seed water which is an indication of increased metabolic activities at higher temperature ($20 \pm 2^\circ\text{C}$). Reduction of seed moisture to 4% after 6 weeks in ambient conditions was an indicator of continued metabolic activities and lack of supply of extra water during storage, which resulted in low germination (Berjak and Pammenter, 2000). Reduction in seed moisture leads to reduced metabolic activities and imposition of dormancy.

3.3.4.4 Seed Water activity

Water activity results show a close relation to seed moisture content. Higher water activity was indicated by higher seed moisture which reduced with storage time. Seeds at 5°C compared to room temperature storage retained seed water longer as at low temperature metabolic activities are reduced. A maximum reduction of seed water was recorded after 6 weeks at room temperature, which showed lower water

activity (Fig. 3.19 b) and reduced germination (Fig. 3.13 b), and, hence, increased dormancy. Reduction in water activity not only correspond to moisture content but also indicate dormancy setting as metabolism reduced at this (0.540) water activity.

3.3.4.5 Conductivity of Seed Leachate

Storage of hydrated (40% seed moisture) non-dormant seeds at 5°C revealed that even though some seeds had been germinating, seed's biochemical health was affected during storage. Increases of leachate conductivity from control values (Fig. 3.20 a) are indicative of the result of water stress. Similar trends of increases in leachate conductivity were recorded in seeds stored at room temperature. In this batch, the increased conductivity correlated to a rise in infection and delay in the germination lag phase (Table 3.5) indicating membrane damage. It may be interpreted as water stress during storage may have inflicted some membrane damage showing increased leachate conductivity and reduced metabolism depriving the seeds of necessary protection which is indirectly suggesting onset of seed dormancy. Reductions of seed water content in recalcitrant seeds of *Shorea robusta* Gaertn.f. have been linked to increased electrolyte leakage (Naithani & Chaitanya, 1994). Recalcitrant silver maple (*Acer saccharinum*) seeds also lost their germination capacity in desiccated seeds which was strongly correlated with increased electrolyte leakage from seeds (Pukacka & Ratajczak, 2006). Acorns of recalcitrant holm oak (*Quercus ilex* L.) stored in peat containers failed to maintain germination vigour and recorded higher electrolyte leakage (Pasquini, et al., 2011). Rapid and reliable results of seed viability in *Kielmeyera coriacea* Mart. seeds were observed following conductivity tests (Ramos, et al., 2012). Seed soak water from radish seeds showed increased levels of electrolyte leakage which was associated with slower and lower emergence and with poorer storage potential (Mavi, et al.,

2014). These observations suggested leachate conductivity test was useful for seed vigour and viability in many species including hazelnuts.

3.3.4.6 Seed Antioxidant Activities

Antioxidant activities from embryo extracts of non-dormant seeds held at 5°C showed a gradual decrease in sample mass required for DPPH inhibition. This may be due to the tissues being initially hydrated (40% moisture content) and enclosed within the shell having the benefit of enzymic and non-enzymic antioxidants from the seed associates. Therefore, when faced with water stress at RT the activated seed associates probably contribute to the total antioxidant activities tested in this investigation. Thus, during dormancy imposition from hydrated to dehydrated state the antioxidant system was still functioning. Water stress induced the antioxidant mechanism and resulted in more enzyme activities as also reported in *Acer saccharinum* (Pukacka & Ratajczak, 2006). Figure 3.21 (b) shows that storage of the non-dormant hydrated hazelnuts at room temperature also result in similar antioxidant response which also started with 40% (fresh weight) seed water. Only one DPPH assay was possible as the samples were lost during the experiment. The result obtained clearly indicates the stressed seed has benefited from seed associates, presence of ABA (Williams, et al., 1973) thereby protecting the seed when seed loses water and become dormant.

Although a large number of seeds from both 5°C and room temperature storage show viable by TTC test, but not germinating indicating imposition of dormancy. It is not known whether all of them would have germinated with an extended germination test or whether the seeds would succumb to free radical related injury resulting in increased infection. Therefore, TTC viability test alone may not be enough to assume seeds' ability to germinate. Figures 3.15 a, b) represents examples of distinct staining patterns for some un-germinated seeds. The staining pattern clearly

shows the biologically active tissues and less active areas as a result of the treatments that resulted from water stress. Some faintly stained cotyledons indicated weak dehydrogenases reaction while white areas identified the total dead tissue affected by free radicals. Therefore, it is assumed that free radical activity may result in damages which will be expressed in the seedlings, including abnormal plants (developed from faintly stained cotyledons and embryonic axis), reduced growth (developed from faintly stained cotyledons) and dead shoot (developed from dead plumule). Seedling photographs (Fig. 3.5) show the types of abnormalities observed when seeds were subjected to moisture stabilisation treatments. Overall observations from various experiments suggested that TTC test results must be scrutinised to assess the level of seedling damage. It has been observed that TTC staining pattern of hazel seeds under stress show inactivity or dead parts within the cotyledon (Schafer, et al., 2002) or even reveal the unstained dead embryonic axis as reported in *Acer caesium* and *Ulmus wallichiana* (Phartyal, et al., 2003).

3.3.5 Summary

This test was divided into two storage conditions, i.e. 5°C and room temperature, testing seeds for germination and various chemical and biochemical assessments. Storage of hazelnuts at 5°C did not affect the seed moisture content and final germination was increased over control indicating synthesis and impact of growth hormones. But after 6 weeks storage, seed moisture reduced with reduction in final germination and increase in lag phase showing an impact of stress on the seeds. Although, the TTC test showed the un-germinated seeds were viable indicating seed dormancy but increase in leakage conductivity indicated the onset of stress and damages to seed tissues. Results from DPPH inhibition test show the seed tissues being very active in trying to counter the free radical actions as the seed mass needed to deactivate DPPH action was gradually reduced.

Ambient storage on the other hand, resulted in decrease in seed moisture content and delay in lag phase for first germination and increase in number of seed infection after 6 weeks storage. Final germination dropped with increase in storage time related to reduction in seed moisture content. The TTC test proved the ungerminated seeds were still viable suggesting seed dormancy. Lack of germination is reflected in increase in leachate conductivity. Decrease in seed mass needed for quenching DPPH action indicated the positive activity of the antioxidant system in the stressed seeds but with increase in storage time stress increased and caused membrane damages resulting in reduced germination.

Observations from this experiment shows storage at high moisture content ($\pm 40\%$) whether at 5°C or at ambient room temperature results in gradual loss of moisture content over storage time and exhibit stress and reduced germination. This experiment further added to evidence of hazelnuts recalcitrant nature as germination reduced during storage period even though seeds showed viability.

In general, decreases in seed moisture content and water activity and increases in seed viability by TTC test resulted in seed dormancy.

3.4 Dormancy Breaking in Hazel seeds

3.4.1 Introduction

Earlier experiments with non-dormant hazelnuts in sections 3.1, 3.2 and 3.3 have shown the stress related damages to seed expressed in germination and seedling performance. During these tests, nuts were held at room temperature (RT) and alternating temperature of 5°C and 15°C for 6 weeks. Section 3.3 have shown storage of non-dormant hazelnuts for 6 weeks at 5°C and room temperature reduces seed moisture content, increases leachate conductivity and activates antioxidant activities. The following experiment was focussed on dormancy breaking of hazelnuts during the course of 6 weeks chilling and examining some biochemical effects. Hazelnuts show physiological dormancy linked to the presence of germination inhibitors in the embryo and embryo coverings which are overcome by chilling or application of dormancy breaking treatments (Bradbeer, 1988).

Hazelnuts are shed with non-dormant seeds but become dormant within a few days of post-harvest storage (Bradbeer, 1968). Hence, this experiment begins with dormancy breaking treatment by keeping dormant hazelnuts for 6 weeks at 5°C in imbibed condition (chilling). Breaking of hazel seed dormancy by chilling for 6 weeks had been used as a supplement to stratification of hazelnuts in nature (Bradbeer, 1968; 1988). Breaking seed dormancy in black mulberry (*Morus nigra* L.) by cold stratification and exogenous application of gibberellic acid has been reported (Koyuncu, 2005). Similar dormancy breaking treatment has been reported by prechilling at 4°C of common alder (*Alnus glutinosa* L.) which results in early germination (Gosling, et al., 2009). Exogenous gibberellic acid and ethylene have been used to break dormancy in *Brassica napus* (Zhang & Gusta, 2010). On the other hand, complex dormancy breaking by cold-warm-cold stratification combined with concentrated H₂SO₄ scarification and exogenous application of gibberellic acid

(GA₃) at 300 mg l⁻¹ was much more effective than a single or combination of two dormancy release treatments for releasing morphophysiological dormancy of *Taxus chinensis* var. *mairei* seeds (Liu, et al., 2011). The hard to break dormancy of seeds of *Cercis canadensis* was broken by a combined method of immersion in sulphuric acid followed by 4°C stratification and then soaking in GA₃ solution (Li, et al., 2013). Removal of seed coat improved germination in *Mormordica cochinchinensis* (Pandey, et al., 2013). Deep dormancy of Chinese dogwood (*Cornus kousa* var. *chinensis*) seeds was broken by removal of testa and endosperm and also by chilling and addition of GA₃ (Fu, et al., 2013). Thermo-priming of white spruce (*Picea galuca*) post chilling improved germination (Liu, et al., 2013). Combined scarification and chilling treatment resulted in higher germination than applied separately in *Vicia sativa* subsp. *nigra* and *Vicia sativa* subsp. *Macrocarpa* (Uzun, et al., 2013). Pretreatments including GA₃, warm stratification and removing the episperm did not facilitate dormancy release. However, after four months at 4°C seeds germinated, suggesting that the desiccation-sensitive seeds of *Osmanthus fragrans* (Oleaceae) have deep physiological dormancy (PD) (Tang, et al., 2013). Scarification for 30 minutes with 95% sulphuric acid was most effective in breaking the physical dormancy of seeds of *Sida hermaphrodita* seeds (Packa, et al., 2014).

The various methods applied for breaking seed dormancy varies among seeds as seen in some examples mentioned above. Chilling and several chemical treatments had been applied for breaking hazel seed dormancy as listed in (Table 1.2). Chilling (in moist conditions) *in vitro* is complementary to stratification in nature and is the most natural method for breaking seed dormancy which does not require any exogenous growth promoters thereby avoiding the impact of the added substance in post dormancy released period. The present experiment focused on the natural mechanism of dormancy breaking by chilling (Bradbeer, 1968). In this experiment a

single course of 6 weeks chilling on dormant seeds and its effects on germination, viability and protective role of antioxidants were examined.

3.4.2 Materials and Methods

3.4.2.1 Nut Provenance

Hazelnuts of 2011 crop were used in the following experimental treatments. Details of the provenance are given (section 2.1).

3.4.2.2 Nut Storage

In this experiment, nuts were kept in trays covered with moist vermiculite as described (section 2.3) and subjected to 6 weeks chilling at 5°C. After 2, 4 and 6 weeks chilling (section 2.10) appropriate number of nuts was taken out, cleared of the vermiculite and used in the experimental treatments.

3.4.2.3 Experimental Procedure

Analysis were conducted as described in, moisture content determination (section 2.4), water activity measurement (section 2.5), antioxidant measurement (section 2.7) and germination assessment of seeds at the end of each treatment were done as described (section 2.11). Seed viability test by TTC has been conducted on all un-germinated seeds (section 2.12).

3.4.3 Results

Dormant nuts were taken out as control (OT) and then after 2, 4 and 6 weeks nuts were taken out from the chilling set and tested for germinability and other chemical analysis.

3.4.3.1 Seed Germination and Total Viability

Germination assays were carried out at 15°C as described (section 2.11). The effect of chilling on germination is shown in Figure 3.22. Control seeds showing 22% germination by 28 days. Delayed germination in 2 weeks seeds probably due to

deep dormancy among the seeds. Germination of seeds chilled for 2 and 4 weeks continued to increase and went up to 68% after 6 weeks. A significant increase in germination observed after 7 days incubation between control and 6 weeks chilled seeds. Seeds chilled for 6 weeks resulted in 68% germination. Total viability was estimated as the number of seeds that germinated within 6 weeks plus those seeds which did not germinate but were nonetheless TTC positive as determined by the TTC test (section 2.12).

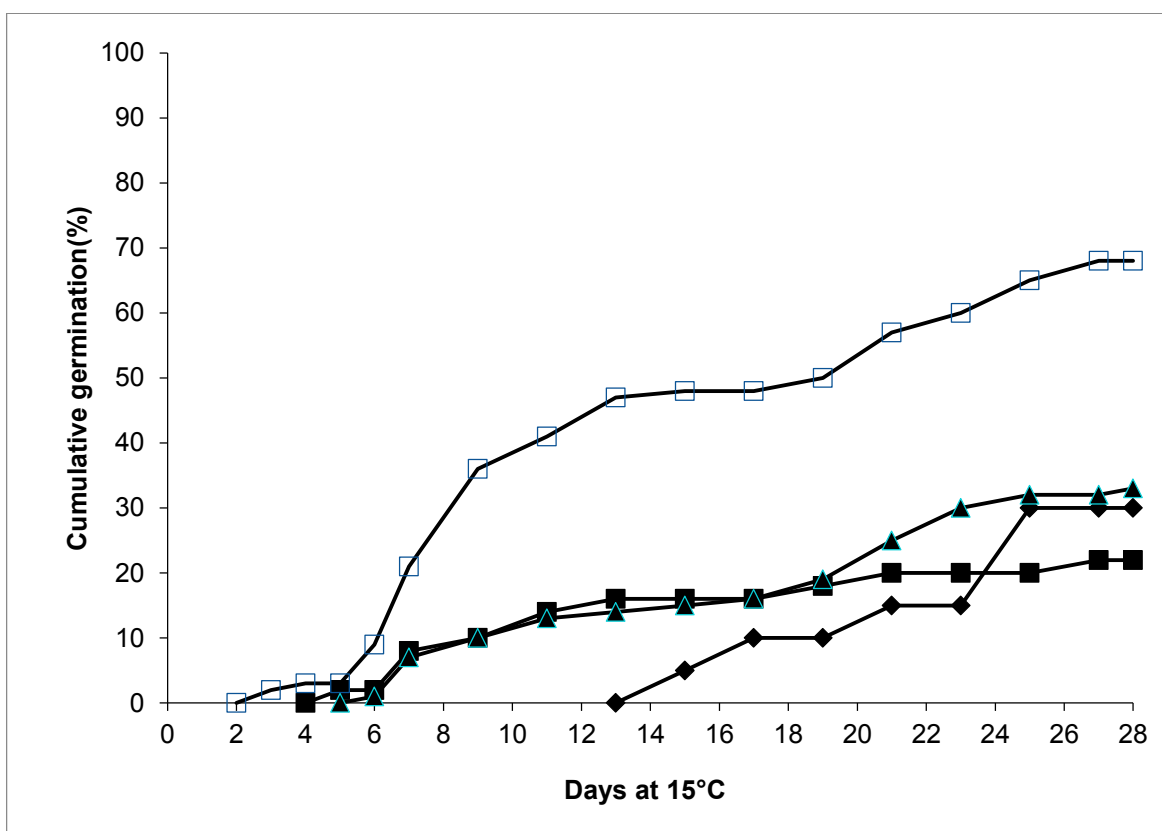


Figure 3.22: Germination of hazel seeds from nuts chilled up to 6 weeks. Seeds were incubated at 15°C.

Control (■),
 2 weeks chilled (◆),
 4 weeks chilled (▲) and
 6 weeks chilled (□).

Figure 3.23 shows the total seed viability during 6 weeks chilling at 5°C. By 28 days, 22% of control seeds had germinated, but on further chilling germination gradually increased to 68% after 6 weeks. The un-germinated healthy seeds proved to be viable by TTC test, giving a total of 100% viability after 6 weeks chilling. Infection of

seeds decreased from 12% in control to no infection after 6 weeks chilling. Seeds showing softness and fungal growth was recorded as infected and discarded.

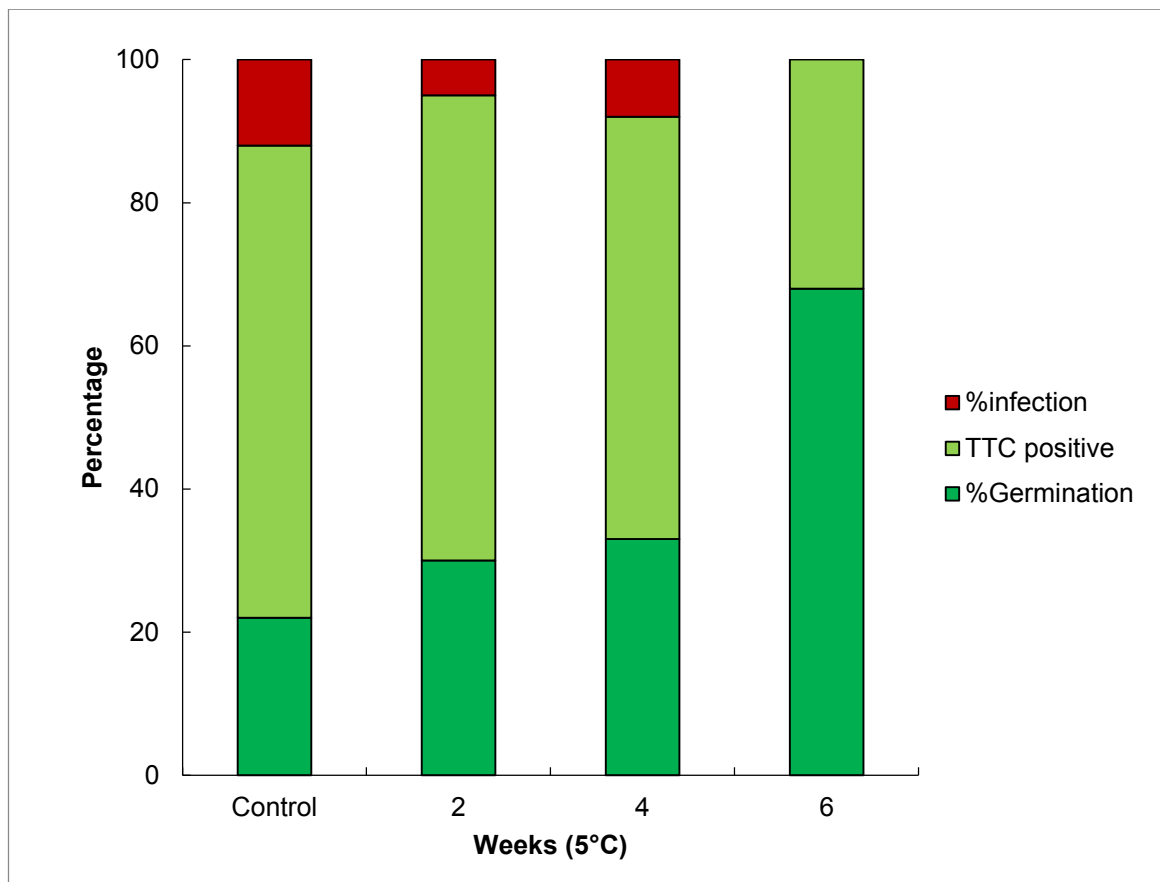


Figure 3.23: Effects during 6 weeks chilling on seed viability of dormant hazelnuts at 5°C. Seeds were incubated at 15°C for germination. Un-germinated seeds were subjected to TTC test. Germination data obtained from Figure 3.22.

Figure 3.24 shows effects of chilling of dormant hazelnuts on mean daily germination, peak value and germination index. Increase in chilling period increases MDG, PV and GI over the control dormant seeds and 6 weeks chilling resulted in higher responses in all observations.

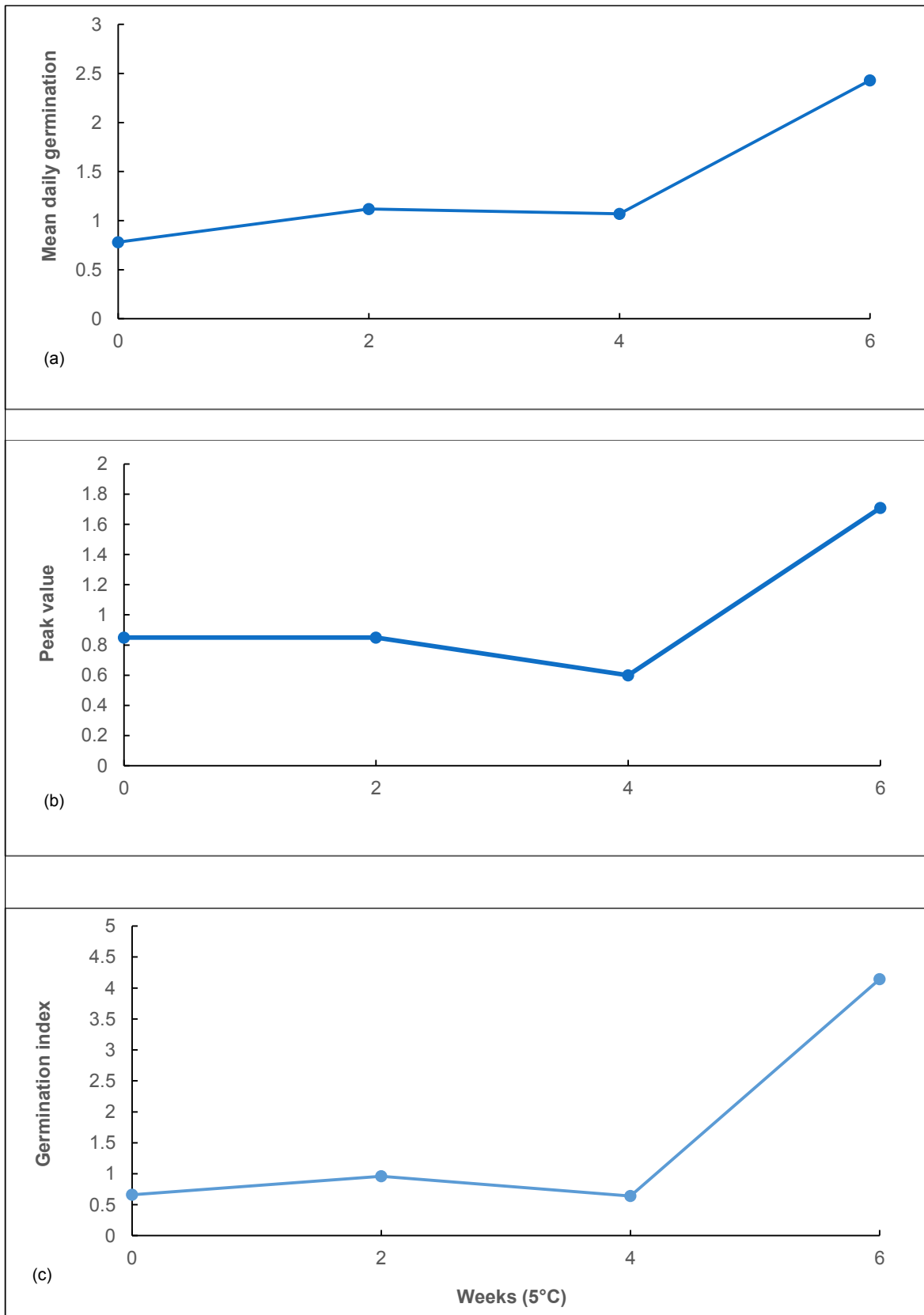


Figure 3.24: Effect of 6 weeks chilling of dormant hazelnuts on MDG, PV and GI of hazel seed germination. Mean daily germination (a) and Peak value (b) and Germination index (c). Germination data obtained from Figure 3.22.

3.4.3.2 Seed Moisture Content and Water Activity

Figure 3.25 shows that during 6 weeks chilling, seed MC increased from an initial 22.8% in control to 42.8% after 2 weeks then to 42.3% after 4 weeks which at the end of 6 weeks contained 40.5%. At the same time, seed water activity value also increased from 0.923 in control to 0.970 after 2 weeks, then finally 0.967 after 6 weeks chilling as shown in Figure 3.26.

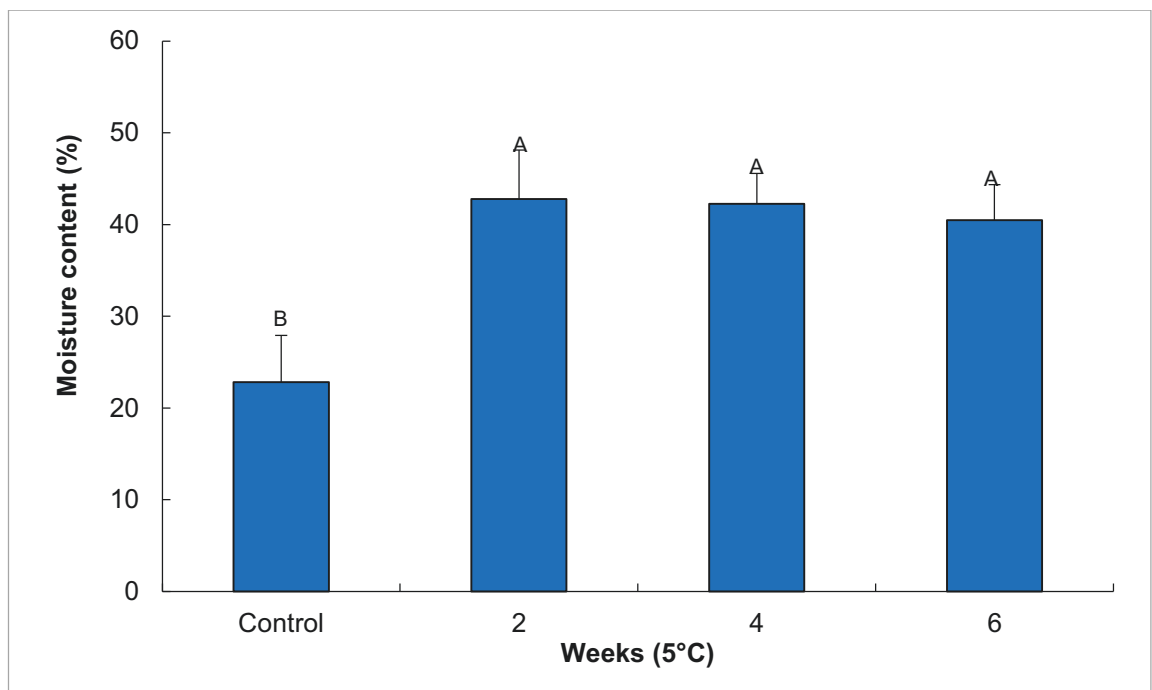


Figure 3.25: Effect of 6 weeks chilling on dormant hazelnuts on seed moisture content. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of ten replicates \pm SD.

One-way ANOVA: Moisture content versus Weeks

Source	DF	SS	MS	F	P
Weeks	3	2745.2	915.1	23.17	0.000
Error	36	1421.7	39.5		
Total	39	4166.9			

S = 6.284 R-Sq = 65.88% R-Sq (adj) = 63.04%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
2	10	42.780	A
4	10	42.260	A
6	10	40.480	A
Control	10	22.810	B

Means that do not share a letter are significantly different.

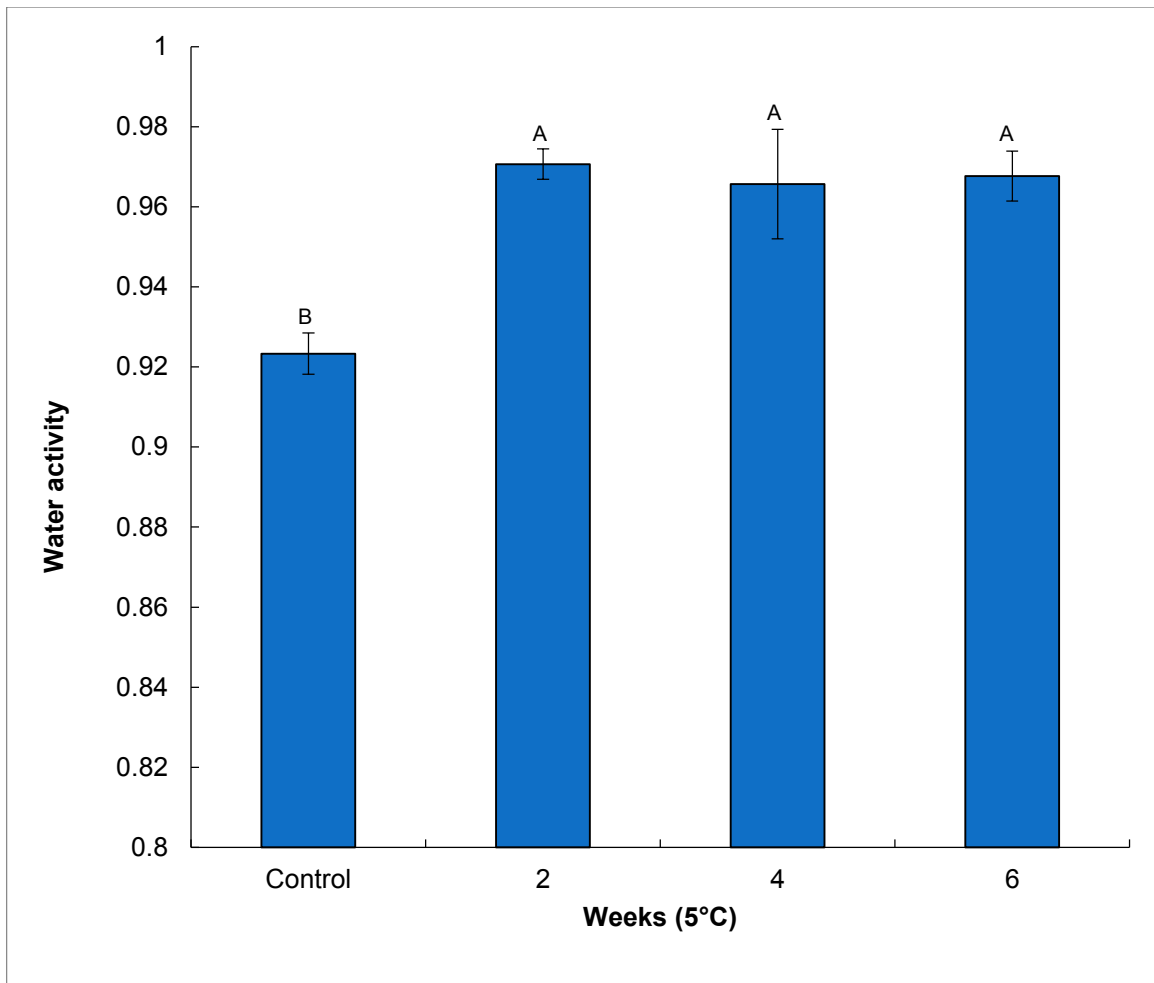


Figure 3.26: Effect of 6 weeks chilling on dormant hazelnuts on seed water activity. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD.

One-way ANOVA: Water activity versus Weeks

Source	DF	SS	MS	F	P
Weeks	3	0.0045270	0.0015090	139.29	0.000
Error	8	0.0000867	0.0000108		
Total	11	0.0046137			

S = 0.003291 R-Sq = 98.12% R-Sq (adj) = 97.42%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
2	3	0.970667	A
6	3	0.967667	A
4	3	0.965667	A
Control	3	0.923333	B

Means that do not share a letter are significantly different.

3.4.3.3 Seed Antioxidant Activities

Treated hazelnuts after each appropriate test were cracked to obtain the seed, the brown skin (testa) of which was removed to give the embryos. Extraction of antioxidants from ground embryo samples were then performed as described (section 2.7). Figure 3.27 show a gradual reduction in the sample mass required for 50% inhibition of standard free radical (DPPH). Control seeds needed 16 mg tissue sample which was reduced to 12 mg after 6 weeks chilling indicating an increase in antioxidant activity with increase in chilling time.

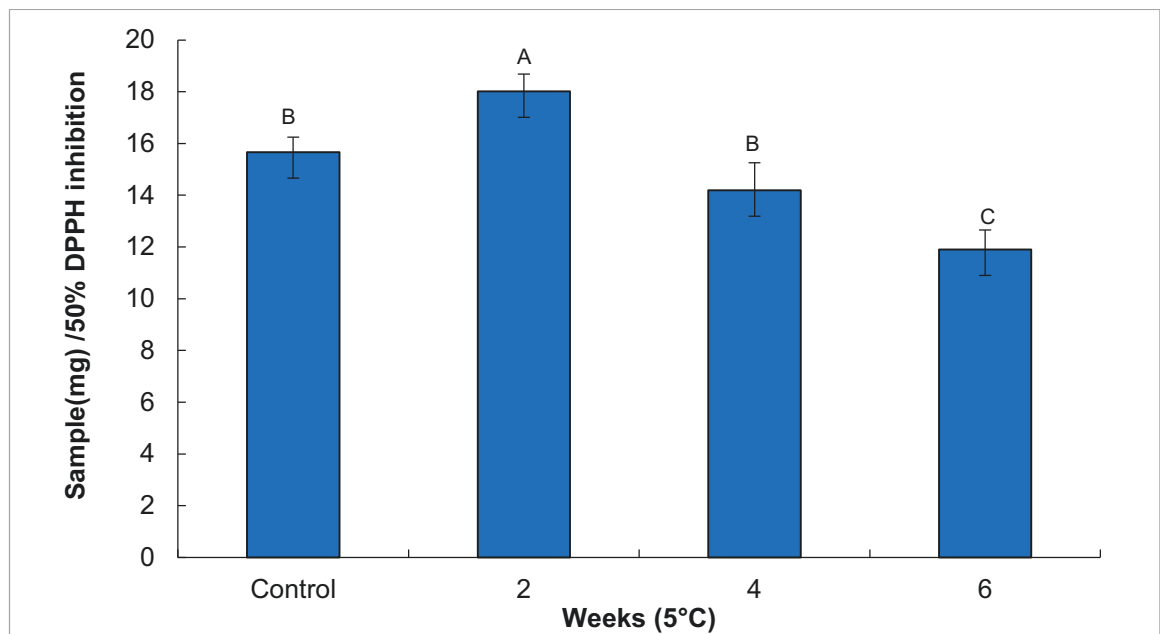


Figure 3.27: Effect of 6 weeks chilling on dormant hazelnuts on DPPH inhibition with antioxidant from embryo extracts. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD.

One-way ANOVA: Sample mass versus Weeks

Source	DF	SS	MS	F	P
Weeks	3	47.149	15.716	46.34	0.000
Error	8	2.713	0.339		
Total	11	49.863			

S = 0.5824 R-Sq = 94.56% R-Sq (adj) = 92.52%

Grouping Information Using Tukey's Method

Weeks	N	Mean	Grouping
2WC	3	17.6667	A
Control	3	15.4000	B
4WC	3	14.5000	B
6WC	3	12.1333	C

Means that do not share a letter are significantly different.

3.4.4 Discussion

After 6 weeks chilling at 5°C, dormancy was broken as seed germination increased from 22 to 68% which also showed increase in total seed viability. Breaking of hazel seed dormancy by chilling has been reported in many reports (Frankland & Wareing, 1966; Bradbeer, 1968; Arias, 1976; Rendon, 1983). Dormancy breaking has been reported by chilling of Norway maple (*Acer platanoides* L.) seeds (Pawlowski, 2009). Cold-warm-cold treatment had been used to break dormancy in *Taxus chinensis* var. *mairei* seeds (Huang, et al., 2006). Chilling breaks physiological and morphological dormancy in European *Chaerophyllum temulum* (Vandelook, et al., 2007). Role of GA and ABA has been involved in dormancy breaking in Norway maple (*Acer platanoides* L.) (Pawlowski, 2009). Chilling had been used to break dormancy in *Cercis canadensis* (Pandey, et al., 2013) and deep dormancy of Chinese dogwood (*Cornus kousa* var. *chinensis*) seeds (Fu, et al., 2013). Chilling had been used to break dormancy in desiccation-sensitive seeds of *Osmanthus fragrans* (Oleaceae) which shows deep physiological dormancy (PD) (Tang, et al., 2013). In case of *Vicia sativa* subsp. *nigra* and *Vicia sativa* subsp. *Macrocarpa* combined scarification and chilling treatment resulted in higher germination than applied separately (Uzun, et al., 2013). The few examples mentioned above shows the involvement of chilling in dormancy breaking. The test results during dormancy breaking in hazelnuts are discussed below.

No significant difference in seed MC between 2 weeks and 6 weeks chilling (Fig. 3.25) has been observed. This indicates the amount of water imbibed in by the seeds in 2 weeks remain nearly constant up to 6 weeks and is sufficient for germination related metabolic activities.

Final germination increased significantly after 6 weeks at 5°C and 100% viability was recorded. Chilling activated germination related metabolic activities as shown

by gradual reduction in TTC positive seeds as more seeds became non-dormant and were able to germinate (Fig. 3.23). Due to increased metabolic activities infection was reduced which was clearly indicated as no infection recorded after 6 weeks chilling, and the 32% seeds which remain un-germinated indicated deep dormancy of this seed lot. The reduction of infection is assumed to be linked to phytoalexins produced in pathogen infected areas as part of plants defensive arsenal (Ahuja, et al., 2012).

The biochemical beneficial aspect of chilling has been demonstrated in the activity of antioxidants produced or released in the seed. Figure 3.27 shows a gradual reduction of embryonic tissue sample was needed to inhibit harmful DPPH free radical actions. During chilling hazel seed is protected within the hard shell which is cracked to get the seed prior to germination test. Hazelnut leafy structures, shell and skin contain a lot of polyphenolic antioxidants (Shahidi, et al., 2007). This may also suggest the increase in antioxidant in controlling pathogen actions by reducing free radical damages or the antimicrobial actions of phytoalexins (Ahuja, et al., 2012) as observed in total elimination of infection in hazel seeds after 6 weeks chilling. Another aspect related to dormancy breaking in seeds has been the activities of proteins. Comparative proteomics between dormant and non-dormant *A. thaliana* Cvi (Cape Verde Island) seeds identified a number of proteins that were associated with dormancy release during cold stratification (Arc, et al., 2012). A similar proteomic approach was used to examine dormancy breaking associated proteins in tree seeds of beech, Norway maple and sycamore, and proteins involved with energy metabolism, protein degradation and protein synthesis may be associated with dormancy release (Pawlowski, 2010).

3.4.4 Summary

Chilling hazelnuts at 5°C for 6 weeks has been the usual method of breaking hazel seed dormancy by countering inhibitor activities. In this experiment, chilling hazelnuts at 5°C for 6 weeks show the seed moisture content increased from 22.8% in control to 42% which show a similarity to recordings of seed water activity. This chilling was enough to break hazel seed dormancy which recorded 68% seed germination. Although a number of seeds did not germinate but TTC test revealed the seeds to be viable but were still dormant. Chilling treatment had also reduced seed infection as no infection noticed after 6 weeks chilling. Decrease in infection may be linked to phytoalexin production by the endophytes. Reduction in seed mass needed for quenching DPPH activity was monitored with increase in chilling time. This indicates chilling may have released more antioxidants which show a gradual increase in activity with increase in chilling time. Dormancy breaking by chilling of recalcitrant hazelnuts results in germination recovery and reduction in infection of the seeds. This may have implications for storage regimes.

3.5 Acclimatisation of Hazel Seeds

3.5.1 Introduction

Since hazel seeds are reported to survive nearly a year in storage (Bradbeer, 1988), a test to check whether acclimatisation test to induce the seeds to a lower (<6%) moisture content would be suitable to maintain viability during storage. Although hazelnut had been classified as recalcitrant (Hong & Ellis, 1996) seed as shed with >40% water content, but other researchers had also placed hazelnuts in intermediate (Ellis, et al., 1990) and orthodox group (Gosling, 2007; Michalak, et al., 2013). As such, storage and seed viability of hazelnuts remain a subject for further investigation. Therefore, this experiment of acclimatisation by repeated rehydration-dehydration cycles was aimed at reducing hazel seed moisture content to <6% and to check whether this will result in seed dormancy and also enable the seeds to behave like an orthodox seed with the capability to maintain seed viability. This will also suggest the way for more storage tests favourable for hazelnuts.

3.5.2 Materials and Methods

3.5.2.1 Seed Provenance

Hazelnuts of 2011 crop were used in the following experimental treatments. Details of the provenance are given (section 2.1).

3.5.2.2 Experimental Procedure

Analyses were conducted as described in, moisture content determination (section 2.4), water activity measurement (section 2.5), leachate conductivity (section 2.6), antioxidant measurement (section 2.7) and germination assessment of seeds at the end of each treatment were done as described (section 2.11).

Hazel seeds were subjected to acclimatisation to low water content by gradual loss of seed moisture using dehydration and rehydration cycles. A batch of 450 seeds

was washed with 1% sodium hypochlorite solution for surface disinfection as mentioned in section 2.11. For hydration twenty five seeds were taken per 9 cm petri-dish with 20 ml deionised water (dH₂O) and streptomycin sulphate (5 ml 10 ppm) solution. For dehydration the imbibition solution was decanted and the seeds were allowed to dehydrate on a fresh petri-dish lined with filter paper on the laboratory bench at ambient conditions. At the end of the dehydration period seeds were placed in a fresh petri-dish with the imbibition solution stated above for the next rehydration in similar conditions. The dehydration/rehydration cycles were as follows:

Seeds were subjected to 24 h dehydration and then given 24 h rehydration. At the end of 24 h rehydration seeds were dehydrated for 48 h and rehydrated again for 48 h. Thereafter, seeds were the dehydrated for 72 h. At the end of 72 h seeds were rehydrated again for 72 h. These seeds were then dried for 192 h when the seed moisture content was recorded to be 4.6% (fresh weight basis). The seeds at this moisture content were then held in polyethylene bags and stored at 5°C until needed for experimental analysis.

3.5.3 Results

3.5.3.1 Seed Germination and Total Viability

Acclimatised seeds were held at 5°C in polyethylene bags for 45 weeks before analytical tests. Figure 3.28 shows after the storage period acclimatised seeds showed 3 types in external appearance:

- i) Healthy,
- ii) Partly oily and
- iii) Totally oily.



Figure 3.28: Hazel seeds after acclimatisation test and 45 weeks storage at 5°C showing seeds that were healthy (normal), partly oily and totally oily in appearance.

Only the apparently neat and healthy seeds were used in physiological and biochemical tests and were compared with the oily seeds. Two batches of 100 normal and oily seeds were set for standard germination test after 45 weeks storage at 5°C. Germination test was carried out as described (section 2.11). Both sets showed infections hence the germination test was discarded and viability was checked with 100 normal and oily seeds each by TTC test. Figure 3.29 shows oily seeds were not viable but 40% normal seeds were viable by TTC test. On further germination test on partly oily seeds after 60 weeks storage resulted in all seeds showing to be non-viable by TTC test.

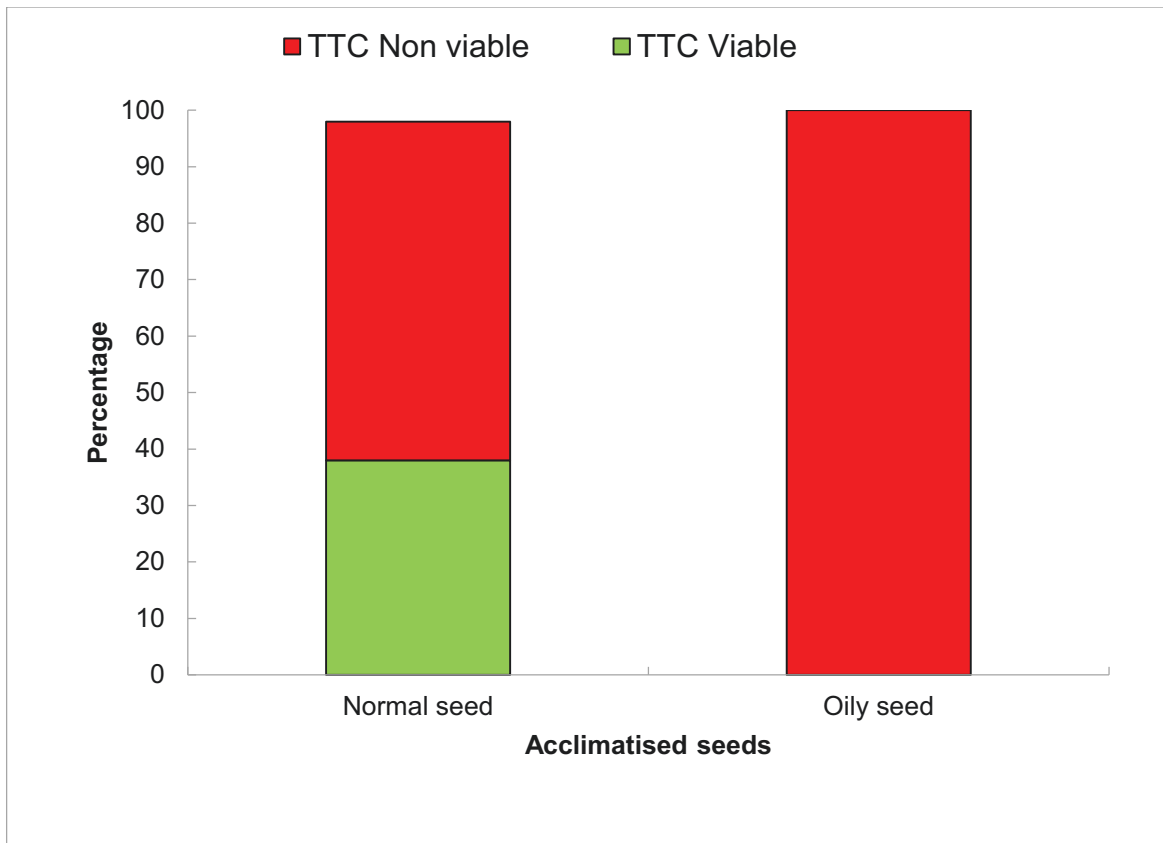


Figure 3.29: Acclimatised hazel seeds after 45 weeks storage at 5°C tested for seed viability using the TTC test. Normal seeds do not show any sign of oiliness whereas the oily seeds are visibly oily as shown in Figure 3.28.

3.5.3.2 Seed Moisture Content and Water Activity

Figure 3.30 (a) shows that moisture content of normal seed is shown to be 4.48% before imbibition and 25.1% after 24 h imbibition at room temperature. Whereas, water activity measurements shown in Figure 3.30 (b) before imbibition was 0.560 but increased to 0.966 after imbibition which reflects increase in moisture content at the same conditions.

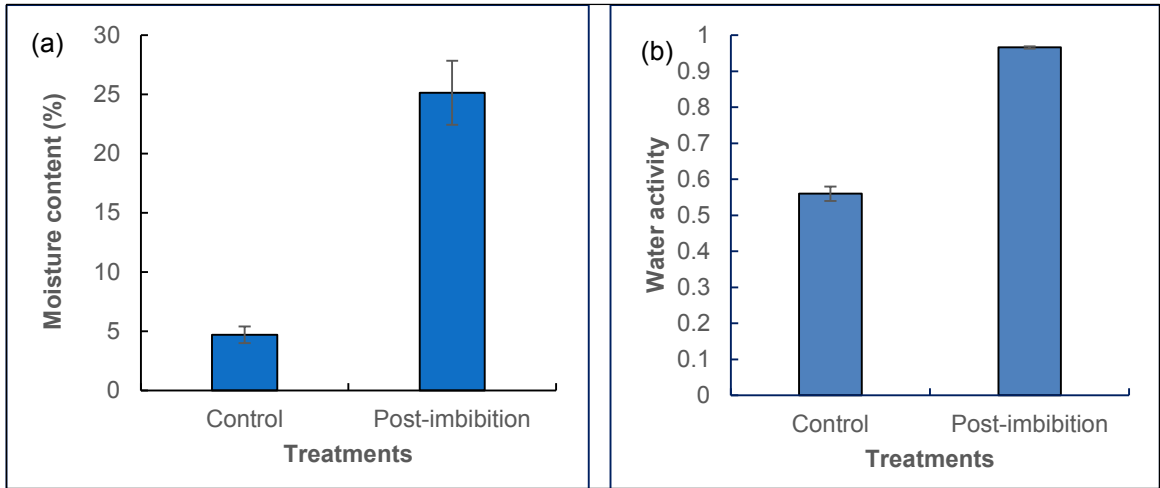


Figure 3.30: Moisture content and Water activity of acclimatised hazel seeds. (a) Moisture content, (b) Water activity. (Student's t-test $p < 0.05$). Moisture content values are mean of ten replicates \pm SD. Water activity values are mean of three replicates \pm SD.

(a) Moisture content of acclimatised seeds.

t-Test: Two-Sample Assuming Equal Variances

	4	24
Mean	4.795556	25.2
Variance	0.470403	8.4225
Observations	9	9
Pooled Variance	4.446451	
df	16	
t Stat	-20.5269	
p (T<=t) two-tail	<0.0001	
t Critical two-tail	2.119905	

(b) Water activity of acclimatised seeds.

t-Test: Two-Sample Assuming Equal Variances

	0.543	0.964
Mean	0.5685	0.967
Variance	2.45E-05	0.000018
Observations	2	2
Pooled Variance	2.13E-05	
df	2	
t Stat	-86.4468	
p (T<=t) two-tail	0.000134	
t Critical two-tail	4.302653	

3.5.3.3 TTC Viability Test

Seeds subjected to germination test were discarded due to infection. Therefore, acclimatised healthy seeds were subjected to viability test by TTC staining method. At the end of the test, seeds showing unstained and stained embryonic axes were pulled together and counted. Figure 3.31 (a) exhibits non-staining of embryonic axes and also some adjacent tissues indicating those areas to be dead and would not have germinated. The non-staining of tissues indicated the effects of free radical attacks. On the other hand, stained embryonic axes as well as adjacent cotyledon tissues as seen in Figure 3.31 (b) represents viable seeds. Although these are biologically viable but failed to germinate. In Figure 3.31 (X1, X2 and X3) shows outer edges of cotyledon parts show staining indicating these areas to be viable.

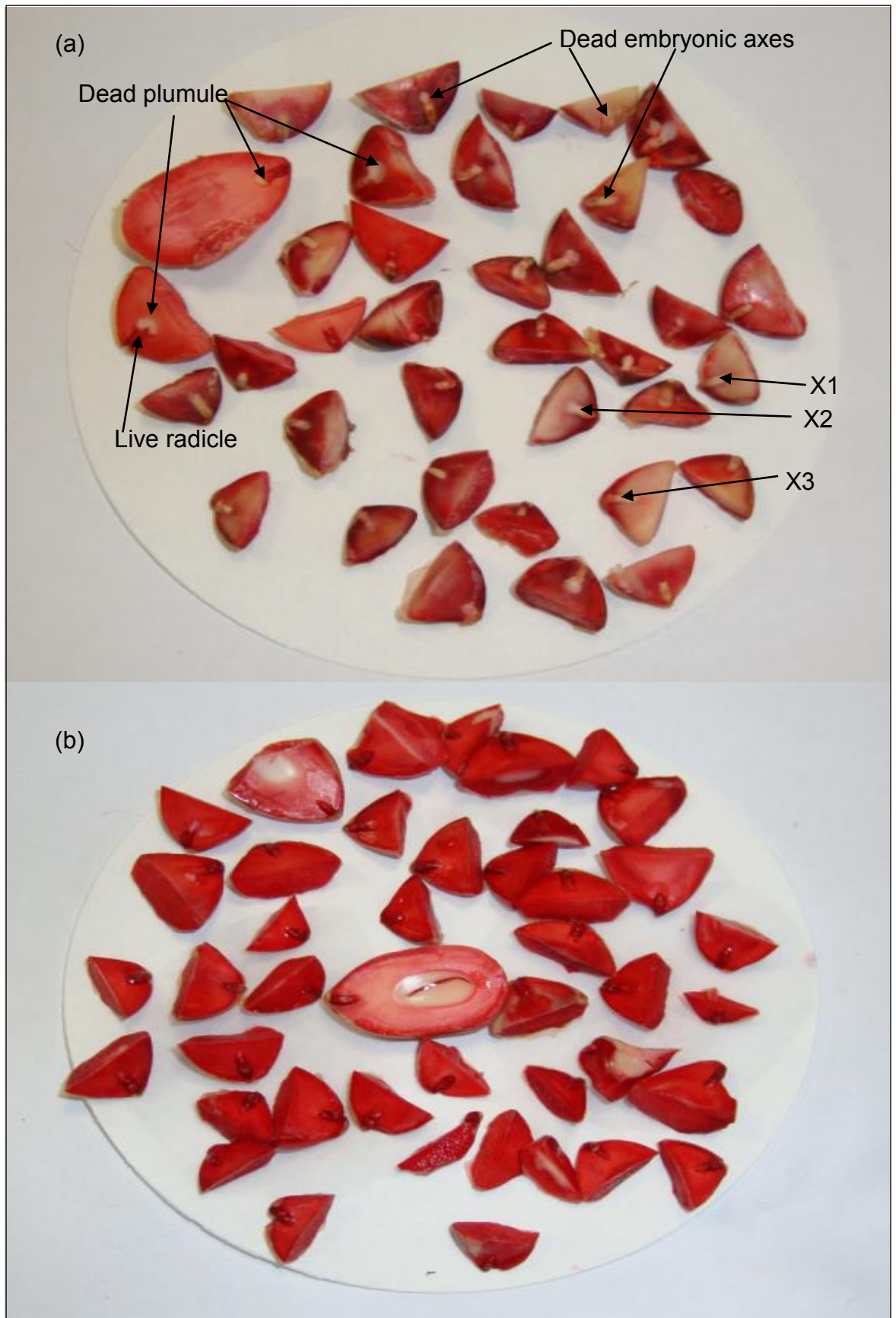


Figure 3.31: Acclimatised hazel seeds tested for viability by TTC test. Seeds after three dehy-
 rehydration treatments were dehydrated for eight days prior to storage. (a) Non-viable seeds, X1, X2
 and X3 show dead embryo and most cotyledonary tissues dead but edges still live, (b) viable seeds.

3.5.3.4 Conductivity of Seed Leachate

Figure 3.32 shows leachate conductivity of control seeds was $48 \mu\text{S g}^{-1}$ whereas the acclimatised normal seed recorded an increase to $220 \mu\text{S g}^{-1}$ but the oily seeds recorded much higher leachate at $343 \mu\text{S g}^{-1}$ clearly indicating membrane damage.

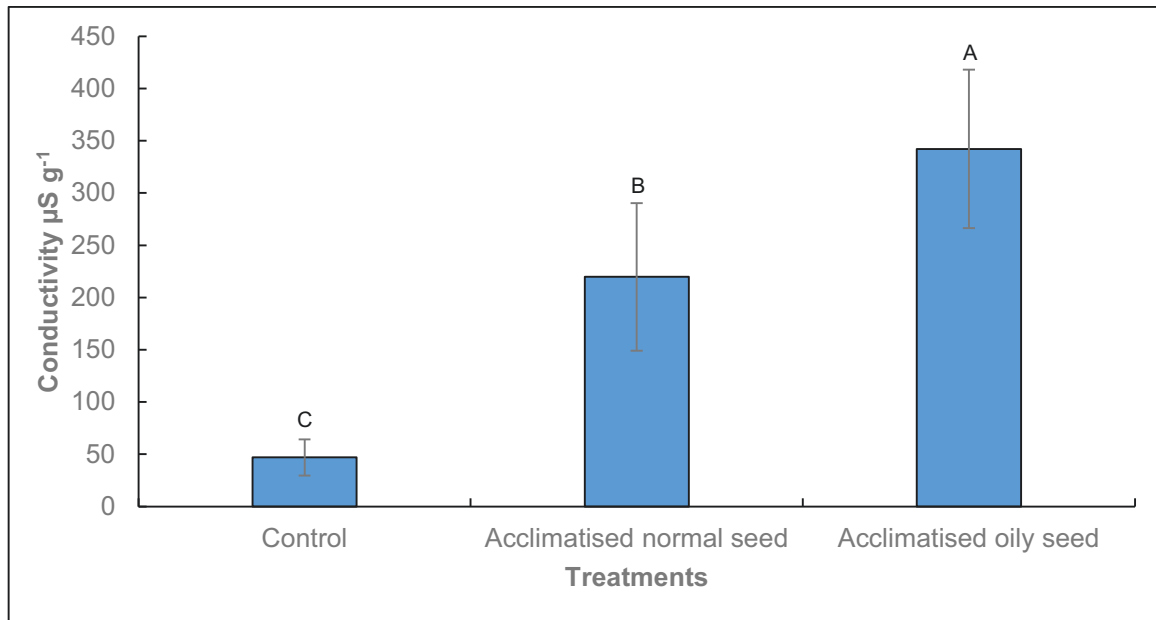


Figure 3.32: Leachate conductivity of acclimatised hazel seeds post imbibition. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD.

One-way ANOVA: Leachate conductivity versus Treatment

Source	DF	SS	MS	F	P
Treatment	2	220145	110072	29.93	0.000
Error	12	44125	3677		
Total	14	264270			

S = 60.64 R-Sq = 83.30% R-Sq (adj) = 80.52%

Grouping Information Using Tukey's Method

Treatment	N	Mean	Grouping
Acclimatised oily	5	342.22	A
Acclimatised normal	5	219.72	B
Control	5	46.90	C

Means that do not share a letter are significantly different.

3.5.3.5 Seed Antioxidant Activities

Figure 3.33 shows acclimatised normal seed tissues required 3.5 mg AsA equivalent per gram tissue for 50% inhibition of standard DPPH in contrast to 7.6 mg for the imbibed normal seed tissues.

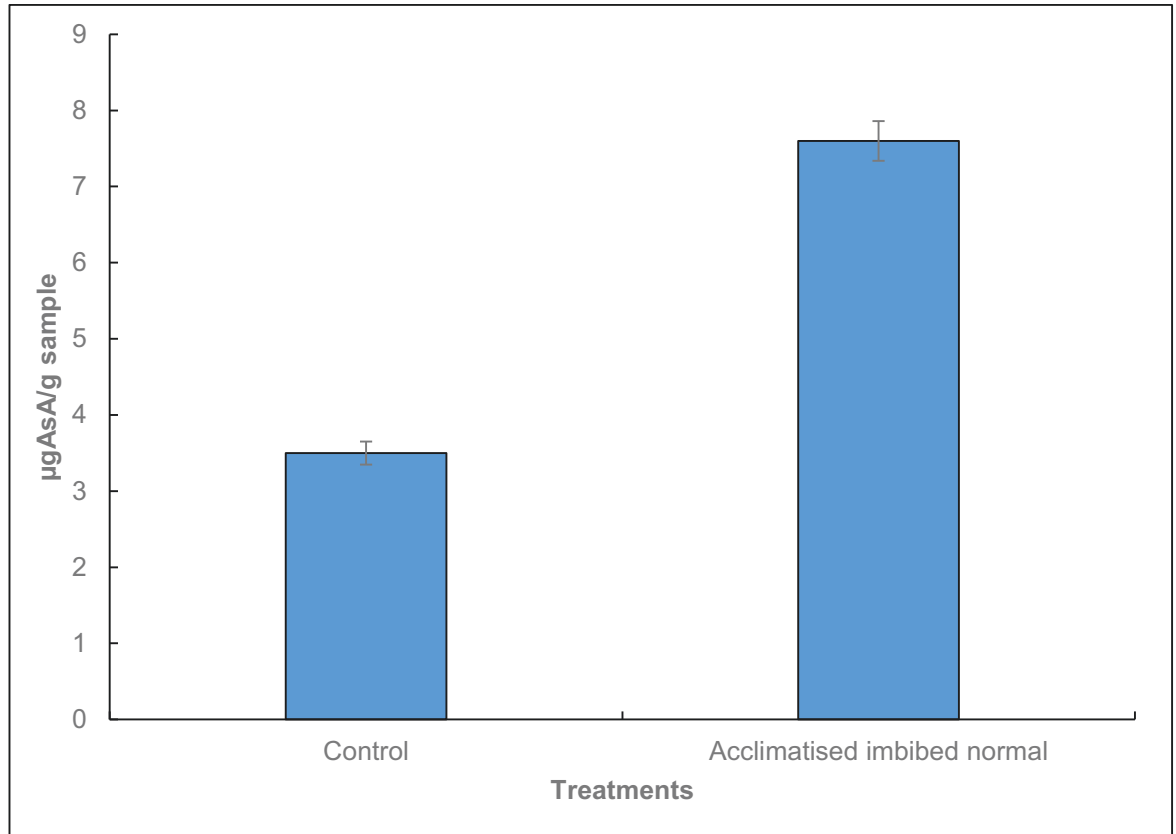


Figure 3.33: Comparison of DPPH inhibition by extracts of acclimatised normal and control hazel seeds (Student’s t-test $p < 0.05$). The values are means of three replicates \pm SD.

t-Test: Two-Sample Assuming Equal Variances

	3.4	7.5
Mean	3.6	7.65
Variance	0.02	0.125
Observations	2	2
Pooled Variance	0.0725	
df	2	
t Stat	-15.0413	
p(T<=t) two-tail	0.004391	
t Critical two-tail	4.302653	

3.5.4 Discussion

Bradbeer (1988), reported that hazel seeds can maintain viability up to a year in storage. Since seeds can maintain viability for a long time if seed MC is low (5-10%) (Roberts, 1973), acclimatisation of hazel seeds to low water content by repeated dehydration, rehydration and dehydration and tested after 45 weeks storage at 5°C was not beneficial. Various observations are described below.

The TTC test shows that the damage caused to the seed is probably due to the attack of free radicals on the vulnerable embryonic axes which must have happened during the dehydration-rehydration-dehydration treatment. Non-stained embryonic axes in Figure 3.31 (a) and surrounding cotyledon tissue indicate free radical impact. In Figure 3.31 (a, X 1, 2 & 3), cotyledon parts showing mostly dead areas and very little viable tissue at the edges where the distal part of the radical shows viability may result in roots only. The seed marked unstained plumule would have produced decapitated seedling as seen in Fig. 3.5. The seed showing unstained radicle would not be able to produce a seedling due to dead tissues. This observation is further proven by the complete staining of embryonic axis and cotyledon in some seeds from the same treatment seen in Figure 3.31 (b) which would have resulted into a healthy seedling provided the seeds were not damaged during germination related imbibition. Viability and vigour test on recalcitrant *Syzygium cumini* L. Skeels) showed only the embryonic axes were stained by tetrazolium, not the cotyledons. The lack of cotyledon staining was probably due to the presence of an impermeable layer (Sivasubramaniam & Selvarani, 2012).

Increased leachate conductivity of the normal seeds also support the fact that storage at 5°C at <5% MC was detrimental for seed storage (Fig. 3.32). This could be due to membrane damage at low water content of a non-orthodox seed or damage due to abrupt imbibition exerting pressure on the membrane. The other

possibility may be that the seeds with low seed water content became dormant during storage and immediate germination test after 45 weeks storage may have exerted pressure on the membranes which is revealed in increased leachate conductivity. Unfortunately, a comparative demonstration of dehydrated but not stored seed is lacking and thus could not be presented here. Although not strictly applicable as a genuine control treatment, other results obtained under similar conditions, but at different times, suggest that the conclusion is reasonably secure. Role of antioxidant activity has also been observed. Increase in tissue sample required for 50% inhibition of DPPH from 3.7 mg in control to 7.3 mg in imbibed normal acclimatised seeds. This indicates acclimatisation procedure might have resulted in water stress and 45 weeks storage resulted in free radicals accumulation as observed in reduced viability and therefore antioxidants needed to counter the free radicals released from imbibed tissues required more tissue sample (Fig. 3.33). This finding has been supported by the increased leachate conductivity observed in (Fig. 3.32) which suggests membrane damages. In mature and immature axes of *Theobroma cacao* (cocoa), cotyledon tissues were more desiccation-tolerant than axes with a low critical water content of 0.24 g^{-1} (DW) (Li & Sun, 1999). Desiccation sensitivity of recalcitrant cocoa axes did not appear to be due to the lack of sugar-related protective mechanisms during desiccation, and it was more likely related to the decrease of enzymatic protection against desiccation induced oxidative stresses. Embryonic axes (Li & Sun, 1999) of hazel seed accounts for only about 0.2% of the seed mass which is attached to the cotyledon on each side through a tiny cotyledonary petiole on each side (Gosling & Ross, 1980a). Therefore, it can be predicted that free radicals target the embryonic axes which depends on metabolites and antioxidant supply from the adjacent cotyledons and thus become vulnerable. Figure 3.31 (a) clearly exhibited the pattern of free radical attack on the cotyledon

extending to the embryonic axes. In some cases the axes were damaged but the cotyledons were not affected showing a similarity with cocoa seeds (Li & Sun, 1999). These observations indicate why 60% embryonic axes were damaged by the free radical attacks. Failure to germinate but remain viable clearly indicates deep dormancy of the acclimatised seeds. Evidence of 40% viability maintenance of acclimatised seeds pushes for more carefully designed experiments to transform viability to established germinability. It would be interesting to imbibe acclimatised seeds in humid atmosphere to reduce imbibitions injury. In this experiment since seed MC was reduced to <5% but showed viability by TTC, therefore, further viability test should be extended to -20°C storage.

3.5.5 Summary

Reduction of seed moisture content by acclimatisation to repeated dehydration-rehydration-dehydration resulted in <5% seed moisture content. The treatment resulted in seeds showing three types of external appearances viz., healthy, partly oily, fully oily. The acclimatised seeds were held at 5°C for 45 weeks but none of the seed germinated when the healthy seeds were tested as profuse fungal growth led to abandonment of the germination test. Therefore, seeds were subjected to the TTC test which showed 40% viability. This test clearly demarcated the faintly stained embryonic axes and adjacent cotyledonary tissues as non-viable whereas fairly stained embryonic axes and adjacent tissues indicated viable areas. The dead areas were most likely affected by free radical attack during dehydration of dehydration-rehydration treatments or during storage at 5°C. Viability of 40% seeds after acclimatisation to <6% MC suggests the possibility that such acclimatisation treatments may enable hazelnut storage at low MC and low temperature.

3.6 Study of the Infections in Hazel Seeds

3.6.1 Introduction

Investigations of hazel seeds performance in physiological, chemical and biochemical experiments require healthy seeds. At the preparatory stage of the experiment all visibly infected, deformed and insect damaged nuts were removed and discarded. The status of the seed within the nut cannot be detected until the shell is cracked and the seed is exposed. Only the sound and healthy looking seeds were used in the experiments. Even then, some seeds exhibited pathogenic proliferations during germination test which was not detectable at the beginning of the test. Hence the microbial or pathogenic state of the seed was needed to be examined. Throughout this investigation, nuts or seeds had been put to various dehydration/rehydration cycles as well as by storage of hydrated and desiccated samples at 5°C or ambient room temperature for 6 weeks each. Therefore, it was important to determine the seeds physiological health during the experimental period.

A comprehensive list of possible sources of seed losses in hazelnuts due to infections and insects are listed in Table 1.1 (Duke, 1989); which included fungi, bacteria and nematodes. Furthermore, 51 species and three varieties from 20 pathogenic genera were collected from 20 samples of each of hazelnut and walnut seeds (Abdel-Hafez & Saber, 1993). In hazelnuts, it has been observed that seeds managed to germinate even if was infected (Rendon, 1983). Usually, germination tests have been used to determine the viability of seeds. Protrusion of the radicle (future root) is considered as sign of germination but it is not guaranteed to result into a healthy seedling as had been observed (Fig. 3.5) which shows abnormalities in seedlings. Therefore, this experiment examined key aspects of hazel seed health

with particular focus on pathogens and effects of water or temperature stress on free radical activity.

In view of the fact that another aspect of hazel seed research has come to light which relates to the involvement hazel seed endophytes in Taxol production. Taxol, one of the bestselling anti-cancer drugs worldwide was first isolated from *Taxus brevifolia* (Wani, et al., 1971) but this is one of the slowest growing trees in the world. In 1998, the exciting discovery was announced that taxol could be extracted from *Corylus avellana* (Hazelnut) (Hoffman, et al., 1998); and later by other groups (Boone, et al., 2000) brought more interest in hazelnuts. It is stated that, taxol found in hazel was actually derived from endophytic fungi living inside the hazel, rather than from hazel itself (Hoffman, et al., 1998). It will be very interesting to look at the presence of endophytes in hazelnuts. Three species has been identified to be related to taxol production in *Taxus brevifolia*, *Taxomyces andreanae* (Stierle, et al., 1993); *Pestalotiopsis microspora* (Strobel, et al., 1996) and *Tubercularia* sp in *Taxus mairei* (Wang, et al., 2000).

3.6.2 Materials and Methods

3.6.2.1 Seed Sources

Seeds were tested from various stages of seed preparations during chilling and storage treatments throughout the experimental process.

3.6.2.2 Pathological Test

Usually after collection, hazelnuts undergo air drying in ambient conditions on the laboratory bench before storage and conducting the relevant tests (section 2.1). Nut/seed losses due to insect attacks as well as pathogen infections were also observed and sorted. Physical observations from different sources were followed with pathological tests designed to check the presence of pathogens and microbes using the nuts of 2006 harvest (Table 2.1).

3.6.3 Results

3.6.3.1 Systemic Pathogen

Additional analyses were made to complement observations of pathogen growth on nutrient and Sabouraud agar plates. Thus visual evidence for seed-borne systemic pathogens were observed and recorded. Below are some of the various observations made during the experimental period.

Figure 3.34 shows examples of seed borne systemic pathogens in hazel seeds visible when the shell of the nut was cracked to collect the seed. These were examples from the 2008 harvested hazelnuts which were chilled at 5°C for 6 weeks. These pathogens may have been present in the seeds during the inactive state.



Figure 3.34: A selection of various types of pathogen on hazel seeds observed during experiment setting. Hazelnuts of 2008 were chilled at 5°C for 6 weeks. Arrows indicate a few infected areas which is also present in each of the seed.

Some healthy seeds may contain pathogen within the seed. Figure 3.35 shows opened embryos with the infected inner part of the cotyledons. The different degrees of infections among the various seeds may, potentially, affect seed germination and the health of the developing seedling.

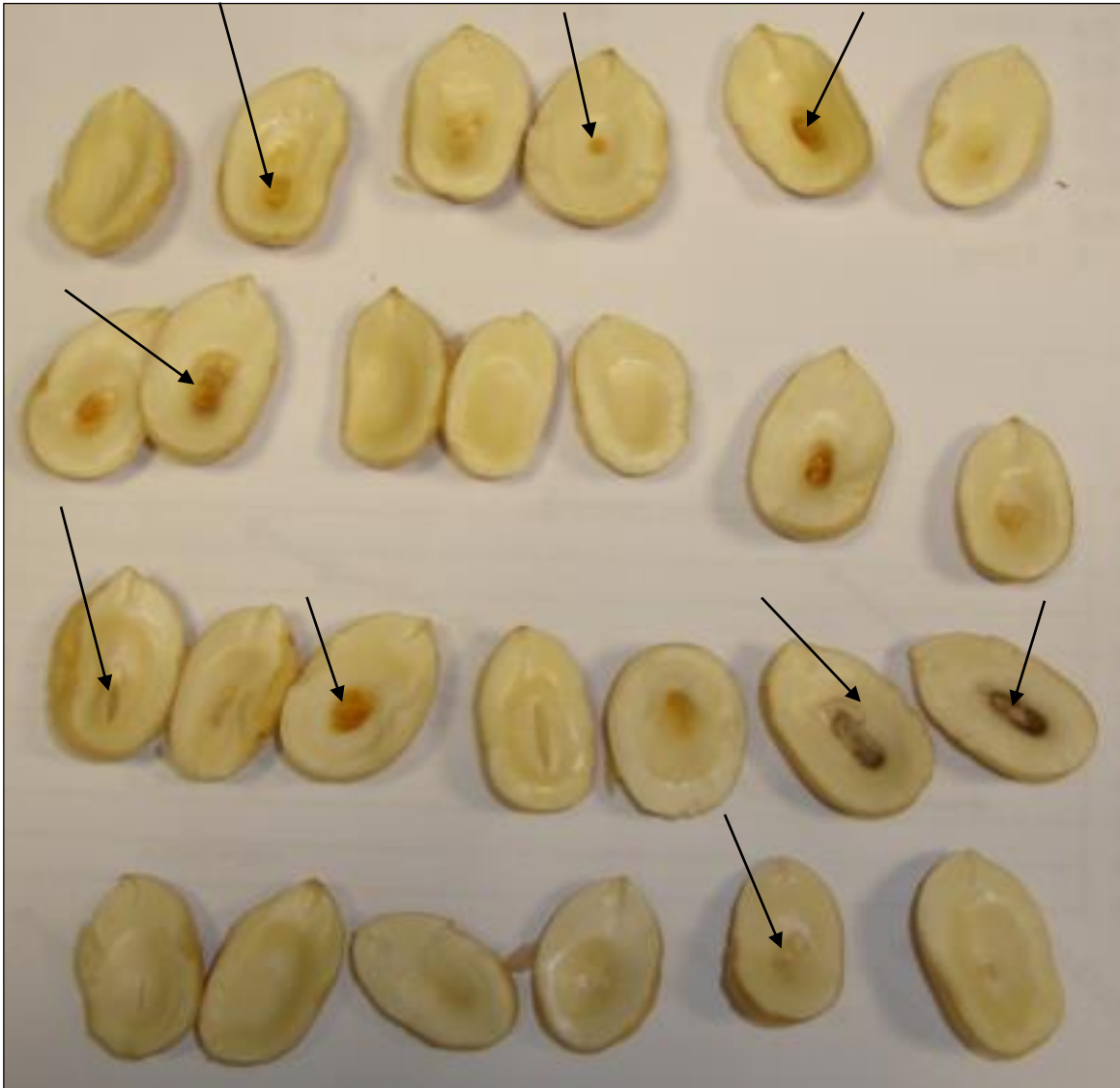


Figure 3.35: Split embryos show infection on the inner side of the cotyledon. Arrows indicate the infected areas within the cotyledon.

In some experiments seeds are planted after germination for seedling establishment. For example, Figure 3.36 shows presence of systemic fungus within the seed of a developing seedling which did not deter seed germination leading to an apparently healthy seedling.

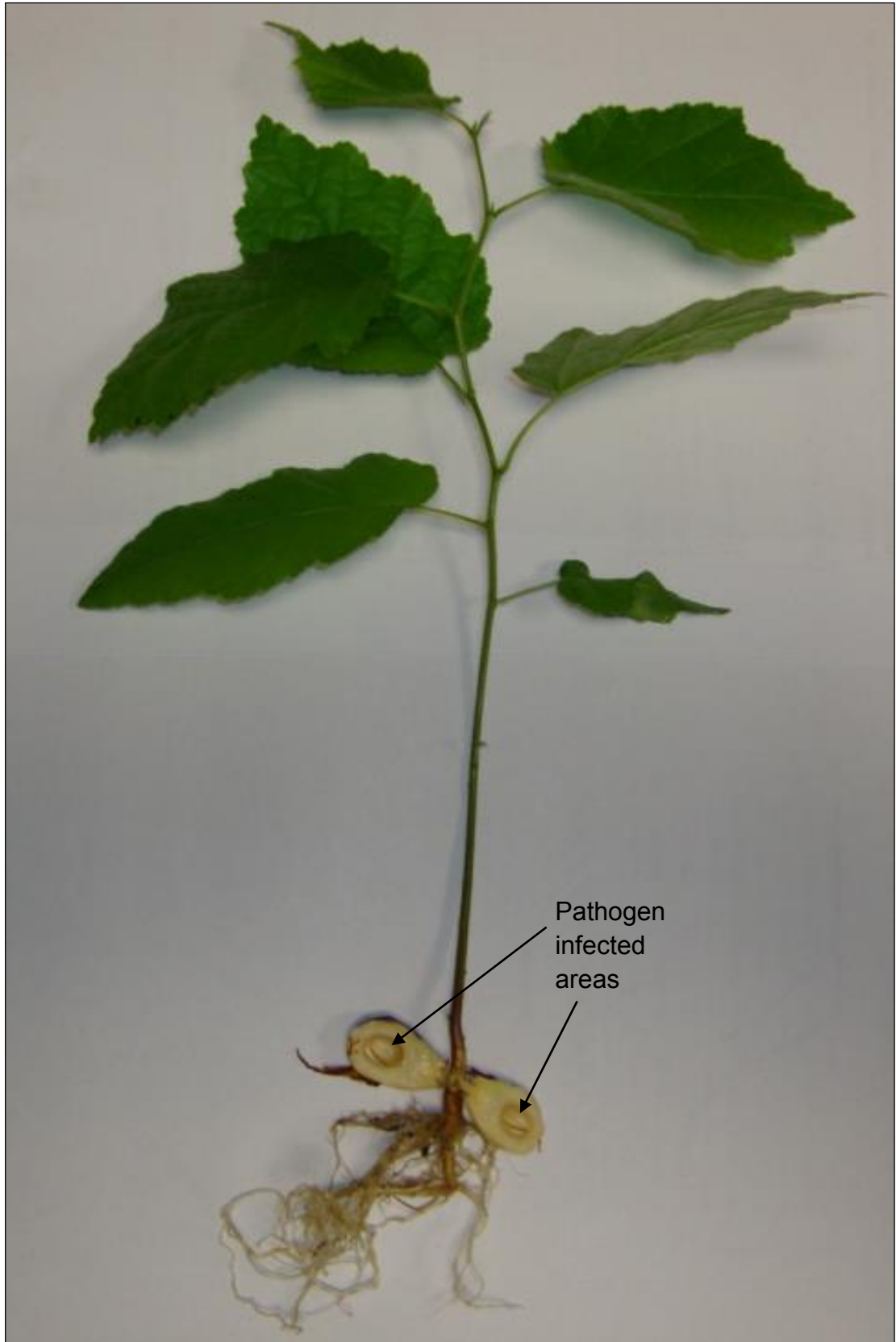


Figure 3.36: Hazel seedling showing pathogen in the inner part of the split cotyledons.

3.6.3.2 Commercial Hazelnut Product

Another observation being, commercial hazelnuts sold as seeds to be consumed also indicate the presence of various types of systemic pathogens. Figure 3.37 show the different levels of infection within the seeds from a bag of hazel seeds bought from the market.



Figure 3.37: Commercial hazel seeds showing infected zone in the inner side of the cotyledon. Arrows indicate a few infected areas.

3.6.3.3 Physical Observation

During another preliminary experiment a batch of seeds were imbibed in various compatible solutes e.g., sucrose, raffinose, trehalose etc. Figure 3.38 below show seeds soaked in raffinose solution, a cryoprotectant and dehydrated at the end of imbibitions, few seeds were wrinkled which on further imbibitions during germination test (results not shown) showed soft areas due to pathogen growth. Externally these seeds look healthy but internally these are highly damaged by pathogens which were rejected.

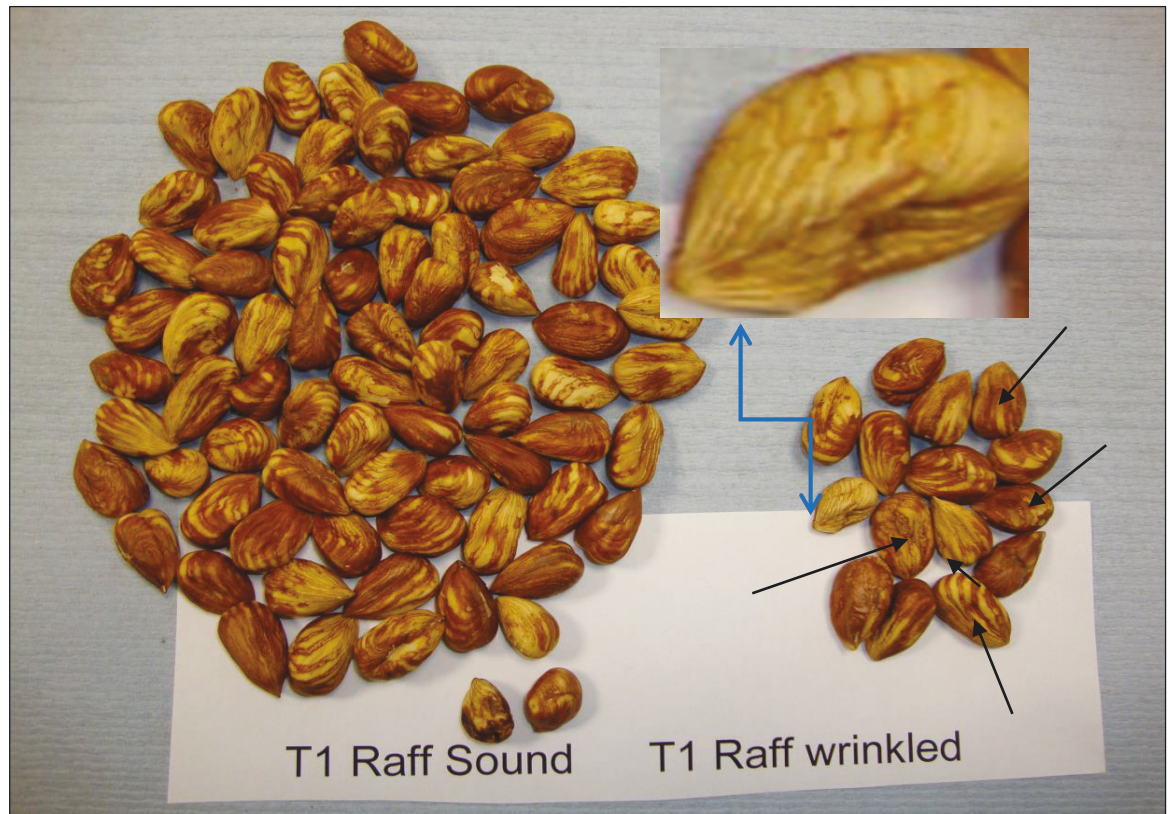


Figure 3.38: After treatment with cryoprotectant raffinose, few desiccated seeds are sound and some are wrinkled. Arrows indicate the wrinkled areas. Inset shows one specimen of wrinkled seed.

3.6.3.4 Pathogen Test

Figure 3.39 shows the spread of pathogenic contamination in hazel pericarp, testa, cotyledon and embryonic axes based on the numbers of colonies on nutrient and Sabouraud agar plates. Pericarp (the outer shell), even after sodium hypochlorite washes show pathogens of both categories which shows fungal colonies are more than the bacterial ones. Testa located below the pericarp and closely wrapped around the cotyledons recorded nearly twice the number of bacterial colonies than fungal ones. Cotyledons, the major constituent of the seed mass, showed almost similar numbers of bacterial and fungal pathogen whereas embryonic axes, the future plant, contained twice the number of fungal than bacterial colonies.

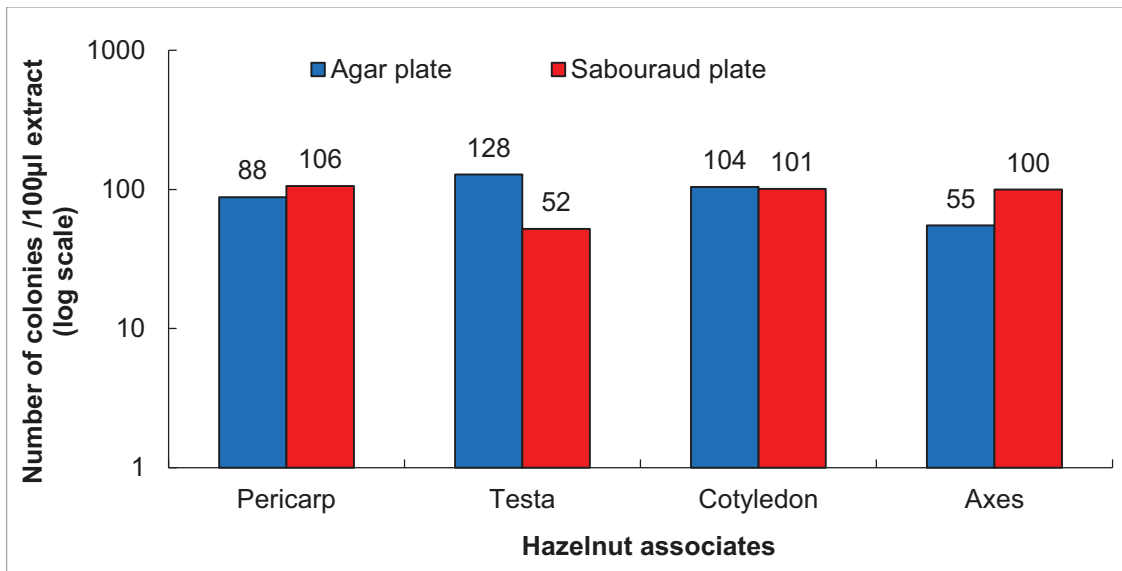


Figure 3.39: Bacterial and fungal colonies grown from buffer washes from hazelnut associates.

3.6.3.5 TTC Viability Test

The TTC test (section 2.12) revealed some interesting aspects of seed viability (section 3.3). Figure 3.40 shows that the inner central parts of the cotyledons are not stained as expected from the conversion of TTC to a red coloured formazan product by respiratory enzyme dehydrogenases in living tissues. The unstained parts such as plumule of the axis and the central oval area may be dead due to previous pathogenic infection or free radical attack.

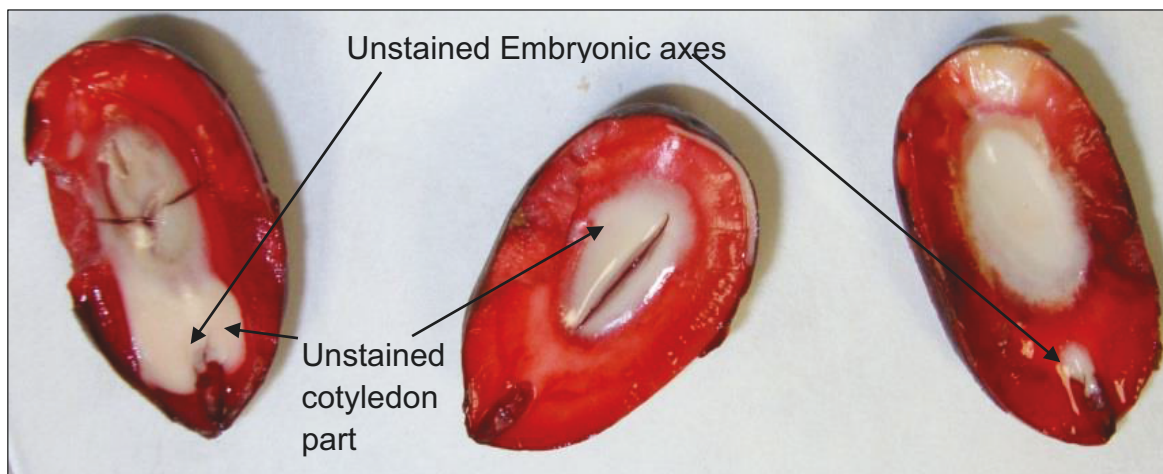


Figure 3.40: TTC test show no staining of the central oval area of the cotyledons and the plumule parts of the embryonic axes. Seeds are taken from experiment in section 3.3 in which seeds were held at 5°C.

Figure 3.41 on the other hand shows some very faintly stained sections of the cotyledon while the embryonic axes are relatively deeply stained. These seeds had

been dehydrated and stored at ambient room temperature for 6 weeks prior to the TTC test. Unstained or lightly stained parts of the cotyledons indicate that the tissues are not live as no respiratory activity could be proven. Further analyses would be required to determine whether this was due to pathogen action or free radical attack during stress related tests.

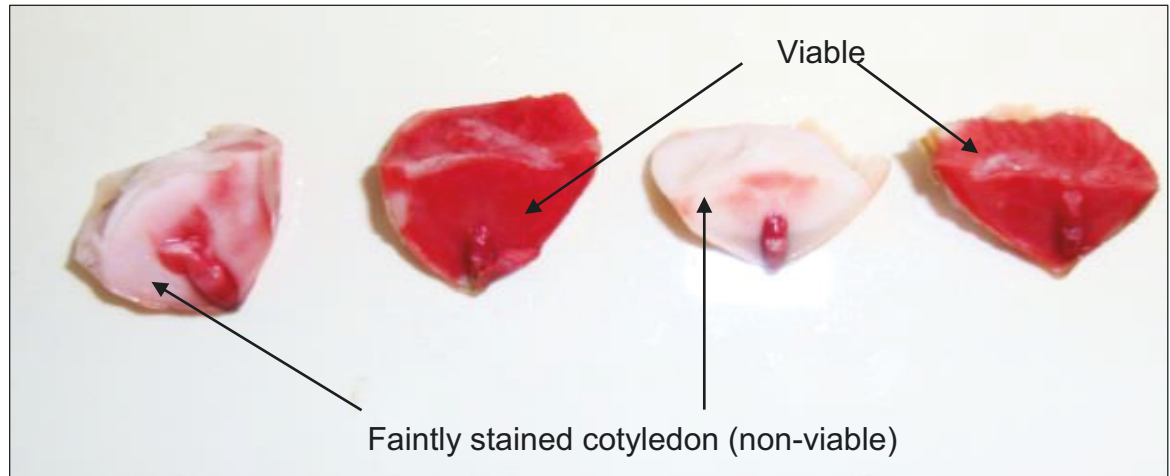


Figure 3.41: Staining pattern in hazel seeds after TTC test show very light (non-viable) to deeper (viable) staining. These seeds are part of experiment in section 3.3 where dehydrated seeds were held at ambient room temperature.

3.6.4 Discussion

Several germination tests were carried out throughout this investigation (sections 3.1 to 3.5) and protrusion of the radicle was the criterion for determining seed germination. Generally, infected hazel seeds continued to germinate, indicating that infection does not necessarily inhibit germination (Rendon, 1983). Although all seeds and nuts were washed with sodium hypochlorite to disinfect from pathogens before testing, the results in Figure 3.34 showed the presence of a large number of pathogens in every part of the nut particularly the seed (devoid of the pericarp). Visibly infected seeds as seen in Figure 3.34 were usually discarded but infections as exhibited in Figure 3.35 evaded detection and removal which might continue to be part of the systemic fungus as shown in Figure 3.36. Seeds which had passed through a period of stress may also show inability to germinate or produce a full

grown seedling (Fig. 3.5). Figure 3.40 and Figure 3.41 show the impact of stress on live tissues as some areas or parts of the seeds were affected by free radical actions (Fig. 3.15). Hence, seed death or dysfunction may not be all related to pathogens only but free radicals might have a role too. Although the pericarp had the highest level of pathogens shown in Figure 3.39, chilling hazelnuts for 6 weeks activated seeds' internal protective mechanisms (increase in antioxidant activities) and GA synthesis which enabled seed germination (Fig. 3.22). Pathogenic infections (Figs. 3.34, 3.35 and 3.37) and the unstained central area of the cotyledons in Figure 3.40 are complementary in location. The unstained parts in Figure 3.40 could have been the result of free radical attacks on the pathogens during stress period or impact of phytoalexins produced by the tissues in response to pathogens (Ahuja, et al., 2012). An increase of antioxidant functions in hazelnut associates during chilling was observed (section 3.7). This suggested that appropriate treatment of seeds (*in vitro* chilling in laboratory or *in situ* stratification of nuts in nature) may overcome the stress related damages and emerge into a healthy seedling unless the attack had been severe (Fig. 3.5). In experiment (Fig. 3.16 a) which required seeds to be held at 5°C without any added water resulted in reduction of seed moisture from 40.5 to 10.3% and the water stress exhibited in 2% infection. Whereas, seeds from the same batch held at room temperature without any added water resulted in moisture content reduction from 40.5 to 4.1% and the inflicting water stress resulted in 12% infection (Fig. 3.16 b). This indicated that systemic flora proliferated more at ambient room temperature than at 5°C when seed moisture was reduced. To the contrary, when dormant seeds were chilled for 6 weeks to break dormancy, seed infection which was recorded as 12% at the onset of the test, showed no infection at all after 6 weeks chilling but also resulted in increase in final germination (Fig. 3.23). Therefore, it is possible that chilling which activated germination related metabolic

activities also reduced or countered pathogenic actions by some unknown/untested mechanism. This is worthy of further study.

In conclusion, it can be suggested that the usual practice of radicle emergence to end a germination test may not be the successful culmination of a test but it required to be complemented with healthy seedling establishment. The TTC test had proved to be useful in assessing the seed health after experimental treatment of the seed/nut.

The presence of endophytes in the central cotyledonary part of hazel seeds seen in Figure 3.35 is observed again in a similar position in the cotyledonary part from which a healthy seedling has emerged (Fig. 3.36). In Figure 3.40, the central necrotic part in cotyledons which remained unstained after TTC test points to the location of the endophytes as also observed in the commercial nuts in Figure 3.37. Reports of Phytoalexins in seeds which accumulate rapidly in pathogen infected areas as part of plants defensive arsenal (Ahuja, et al., 2012), looks identical to locations and TTC staining response in hazel seeds. Phytoalexins are antimicrobial and often antioxidative substances synthesized *de novo* by plants that accumulate rapidly at pathogen infected areas. Phytoalexins are a heterogeneous group of compounds that show biological activity towards a variety of pathogens and are considered as molecular markers of disease resistance (Ahuja, et al., 2012). Hence, identification of hazel endophytes will be interesting to further the search for source or sources of Taxol production.

3.6.5 Summary

Some hazelnuts show various insect and pathogen proliferations. Usually all visibly deformed and diseased nuts are removed before setting up any experiment. In later stages, during experimental procedures infections had been noticed within the seed which is also further supported by the observation of infections in commercial 'ready

to eat hazel seeds'. Based on these observations, the pathogen test was set in this experiment. The test results show the presence of both bacterial and fungal colonies in every part of the hazelnut (associates). Besides, biochemical tests using TTC method for assessing viability of seeds held under stress revealed the damages caused to embryonic axes and/or cotyledon parts by free radical actions. The TTC test indicates that germination by the protrusion of the radicle (future root) may not result in the production of a healthy seedling as a number of abnormal or deformed seedlings had been observed as the probable consequence of stress. Chilling in moist conditions at low temperature (5°C) allowed gradual increase in water content within the seed allowing distribution of solutes which initiates metabolic activities. Observation that healthy seedlings can develop even when the cotyledons showed infected areas suggests that the seeds have some protective mechanisms, such as antimicrobial and antioxidative substances accumulated in response to pathogen attack, including the possibility of phytoalexins. It is also encouraging to note that chilling increased antioxidant activities in all parts of hazel associates with a view to protect the health of the future seedling from damages.

3.7 Free Radicals and Antioxidants in Hazelnuts

3.7.1 Introduction

A significant cause of seed deterioration may be due to free radicals which are produced or liberated by seed tissues when they are faced with stress due to a deficit in seed moisture content or due to temperature extremes, or a combination of both (Bailly, 2004). There is evidence that free radicals increase with the decreasing moisture content of seeds (Priestley, et al., 1985; Hendry, 1993).

Besides, Reactive Oxygen Species (ROS) such as (O_2^-), H_2O_2 or (OH^-) , which are continuously produced during seed development to germination as well as during seed storage play a double role in seed physiology: they function as signalling molecules in plants, acting as regulators of growth and development, programmed cell death, hormone signalling and as toxic products that accumulate under stress conditions in response to biotic and abiotic stresses (Bailly, 2004). But balance of the amount of ROS production and scavenging, being beneficial for germination, and in particular act as a positive signal for seed dormancy release. Therefore, balancing act of antioxidants is important for seed survival (Bailly, et al., 2008).

ROS have been involved in dormancy release in sunflower (*Helianthus annuus* L.), a mechanism that involves ROS production and targeted changes in protein carbonylation patterns (Oracz, et al., 2007; 2009). Loss of seed viability was reported in relation to changes in antioxidant enzymes in ageing onion seeds (*Allium cepa* L.) (Demirkaya, et al., 2010). Germination in Arabidopsis was associated with an accumulation of superoxide and hydrogen peroxide in the radicle (Leymarie, et al., 2012). Exogenous antioxidant ascorbic acid can reduce germination inhibition by ABA in rice seeds (Ye, et al., 2012). Skin, pulp and seeds of miracle fruit (*Synsepalum dulificum*) contain antioxidants (both free and bound) of which seed contains more than other parts (Inglett & Chen, 2011) and whole grain rice also

contain both free and bound antioxidants (Min, et al., 2012). Tocopherols (vitamin E), lipophilic antioxidants as a natural protective mechanism had been reported in *Arabidopsis thaliana* leaves and seeds (Sattler, et al., 2004). These antioxidants are not only reported as possessing antiradical activities which serve to protect the seed, but also have been identified as nutraceuticals because of the many health benefits they offer to consumers of the seeds (Droge, 2002; Lee, et al., 2004; Valko, et al., 2007). Food rich in antioxidants has been reported to play an essential role as anti-cancer and prevention of cardiovascular diseases (Kris-Etherton, et al., 2002). Antioxidant nutrients vitamin E, vitamin C and β -carotene may play a beneficial role in the prevention of several chronic disorders (Diplock, et al., 1998). Antioxidants are also reported to be potentially useful in prevention of neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Di Matteo & Esposito, 2003). Even inflammation and problems caused by cell and cutaneous ageing can be prevented by antioxidants (Ames, et al., 1993). Hazel cell extract has been reported to be a source of human anticancer treatment (Bemani, et al., 2013).

Antioxidant phytochemicals have been extracted from hazelnut kernel, hazelnut skin, hard shell, green leafy cover and tree leaf (Shahidi, et al., 2007) and concluded that these hazelnut byproducts could be an excellent source of natural antioxidants with importance in curative applications in many incurable diseases. A few more works referred here have also confirmed hazelnut antioxidant phytochemicals (Alasalvar, et al., 2009a; Alasalvar, et al., 2009b; Alasalvar, et al., 2010; Jakopic, et al., 2011).

Previously, two types of protection mechanisms have been reported for hazel seed byproducts. Firstly, it has been reported that growth retardant abscisic acid (ABA) present in the byproducts like shell, testa and even in the cotyledons induces seed

dormancy (Bradbeer, 1968; Williams, et al., 1973). Usually, for the germination of dormant seeds special sets of conditions have to be met. Dormancy, the block to germination has evolved differently across species through adaptation to the prevailing environment, so that germination commences when the conditions for establishing a new plant are likely to be suitable (Hilhorst, 1995; Vleeshouwers, et al., 1995; Bewley, 1997a; Li & Foley, 1997; Baskin & Baskin, 2004; Fenner & Thompson, 2005). Besides, using growth regulators, appropriate chilling breaks hazel seed dormancy (Bradbeer, 1968). Secondly, the hard shell of hazelnut physically protects the seed and also helps to reduce excessive loss of moisture after harvest, provides protection by slow imbibition during moistening of the seed during chilling which prevents membrane rupture leading to damages.

In this section the involvement of a third mechanism of hazel seed associates [testa, funiculus and endocarp (inner lining of the shell)] for biochemical protection and retention/restoration of the viability of hazel seed is explored. Effective antioxidant systems against free radical attacks will enable the protection and development of the embryonic axis into a healthy plant. It has been observed that, during unfavourable periods, cotyledons and embryonic axes encounter stresses but may recover given the right conditions. For example, hazelnuts have been reported to survive 6 months to a year in storage but require appropriate chilling to break hazel seed dormancy and restore full germinability (Frankland & Wareing, 1966; Bradbeer & Pinfield, 1967; Ross & Bradbeer, 1971a).

Shahidi, et al., (2007), termed hazelnut tree leaf, green leafy cover, hard shell, hazel seed skin as hazelnut byproducts which were used for evaluating for total antioxidant activity and free radical scavenging activity tests. Results from that investigation suggested that hazelnut byproducts could potentially be considered as

an excellent and readily available source of natural antioxidant. The importance of the seed borne antioxidants for the survival of the embryonic axis has not been explored.

In the present investigation related to seed dormancy and recalcitrance, hazelnuts were subjected to various chilling, hydrated and dehydrated storage tests. During storage, seeds may be under stress related to storage temperature and moisture content, eventually activating free radical production. To counter the damaging actions of free radicals, seeds' own protection agents the antioxidants in hazelnut byproducts might become activated to provide physiological protection by inactivating free radicals. It is apparent from the presence of antioxidants in hazelnut byproducts (Shahidi, et al., 2007). Therefore, survival of hazel seed may depend on the antioxidant protection role of the structures in and around the seed. Figure 3.42 (a) shows part of a hazel tree branch and hazelnuts enclosed by a green leafy involucre. Figure 3.42 (b) shows the position of the mature hazel seed within the shell/pericarp. The spongy endocarp inside the pericarp is closely associated with the seed. A threadlike funiculus connects the base of the shell to the tip of the seed near the location of the embryonic axis. The brown testa is wrapped very closely around the embryo as skin. The close associations and placements of these tissues within the shell form a core of physical protective units for the seed embryo and the embryonic axis (the future plant). This investigation will explore the possibility of all of these seed associates (Table 3.6) contributing antioxidants at the time of need for the protection of the embryonic axis.

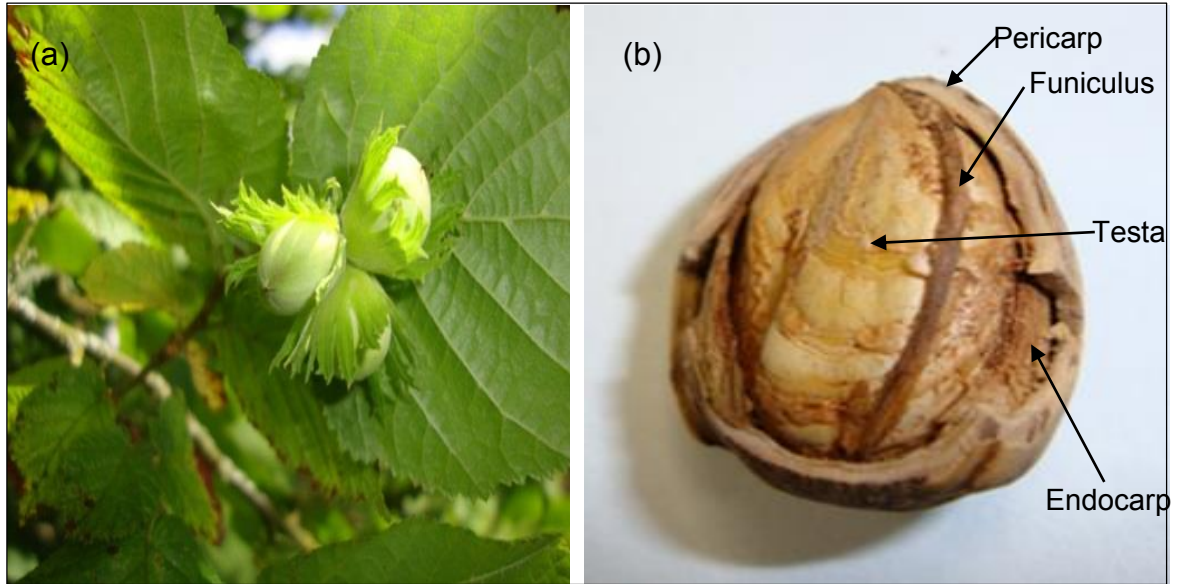


Figure 3.42: (a) Developing hazelnuts enclosed within the green leafy involucre, (b) hazel seed associates within the nut.

Table 3.6: Comparison of the use of different parts of hazelnut and plant parts for antioxidants.

Term used	Leaf	Leafy cover	Shell	Kernel (Seed with testa)	Testa	Funiculus	Endocarp	Use
By-products*	✓	✓	✓	✓	✓			Natural antioxidants
Seed Associates				✓	✓	✓	✓	Antioxidants for seed viability

* (Shahidi, et al., 2007)

However, the mechanism by which this occurs is not clear. Thus a series of assays to elucidate the role of seed associates in the protection of the seed will be carried out. This will include a comprehensive investigation intended to outline a detailed insight on the status of antioxidants in the seed and associates to find a safer storage condition for hazelnuts as well.

Assay Methods

Each hazel seed has been covered by seed associates (pericarp, endocarp, funiculus and testa) and hence it is possible that antioxidants from these structures may be involved in inactivating the free radicals. It can be noted that throughout this

investigation, hazel seeds had undergone various tests which subjected the seeds through a period of stress either water or temperature related or both (sections 3.1, 3.2 and 3.3). As stress is related to free radical formation (Hendry, et al., 1993), and the observation that dormant seeds having lesser seed water, became non-dormant after appropriate chilling indicating the possibility of seed associates as the source of antioxidants to the free radical quenching process.

Therefore, a thorough investigation of standard free radical scavenging by antioxidants from these associates was conducted.

One very popular assay of antioxidant activity is the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay first reported by (Blois, 1958). Since then, to mention a few, it has been very widely applied in other researches (Brand-Williams, et al., 1995; Kim, et al., 2002; Zhu, et al., 2002). But difference in the assay protocol had been observed in various laboratories as listed (Sharma & Bhat, 2009) and pulled together a range of methodologies that have been adopted. The main observations in this review were differences in:

- i) DPPH concentrations used (22.5-250 μ M),
- ii) Incubation time (5 min-1 h),
- iii) Reaction solvent and
- iv) pH of the reaction solvent.

These variations were found to be potentially significant, therefore, the following investigations were carried out in order to establish a standard protocol for free radical inhibition by antioxidant assay which will be used in the present investigation.

3.7.2 Materials and Methods

3.7.2.1 Nut Provenance

Hazelnuts of 2011 crop were used for the various extraction processes tested in this investigation (section 2.1).

3.7.2.2 Experimental Procedure

Analyses were conducted as described in, moisture content determination (section 2.4), water activity measurement (section 2.5), antioxidant measurement (section 2.7), thin layer chromatography (section 2.8), chilling (section 2.10) and germination assessment of seeds at the end of each treatment were done as described (section 2.11). Ground embryo samples were used in all tests. As tissues contain widely differing moisture contents, it is necessary to express the weight of tissue on a dry weight basis to allow reliable comparison of activity between different seed parts or when the treatment applied to seeds has resulted in change in moisture content. In cases where moisture content does not vary significantly it is probably satisfactory to express the results on a wet weight basis. In many cases samples were dried before analysis so re-calculation on a dry weight basis was not necessary. In each case the mass is indicated as wet weight (WW) or dry weight (DW) when results are discussed. An average of three test results was used in each assay.

Where shown, 95% confidence intervals were calculated using the t distribution (Miller & Miller, 1988). ANOVA (in Minitab) was used to test for significant differences between assay results.

3.7.3 Results

3.7.3.1 Phase 1: Standardisation of Antioxidant Assay Protocol

The following investigations were carried out in order to establish a standard protocol for free radical inhibition by antioxidant assay of extracted samples.

In the following investigations standard antioxidants ascorbic acid (AsA), Butylated hydroxytoluene (BHT), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were used to inactivate standard free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). The optimised assay was then as described in section 2.7.

(a) Assay End Point

Previous authors have used various endpoints to evaluate antioxidant activity by DPPH assay. (Shahidi, et al., 2007), reported inhibition activities of fixed quantity of seed extracts against standard free radicals: hydrogen peroxide, superoxide radical and DPPH radicals. One of the most attractive options is to record the amount of tissue (in mg or μg) required to achieve 50% inhibition of standard free radical DPPH. This approach had previously been used in evaluating hazelnut skin (Locatelli, et al., 2010). Besides, a number of researchers (Alma, et al., 2003; Kim, et al., 2004) have even used up to 250 μM DPPH concentration which is far beyond the spectrophotometric accuracy. Differences in reaction conditions show varied results in IC_{50} values of standard antioxidant like ascorbic acid; 56 μM (Kano, et al., 2005) whereas (Ricci, et al., 2005) reported 629 μM needed to achieve similar results. Variations in IC_{50} values for standard antioxidant butylated hydroxytoluene (BHT) has also been observed (Mimica-Dukic, et al., 2004; Sökmen, et al., 2004; Ricci, et al., 2005). Because of the differences in methods used, a carefully tested assay procedure need to be developed.

Figure 3.43 shows the 50% inhibition of DPPH by hazelnut endocarp tissue extracts. It shows a stepwise increase in antioxidant activity with the increase in sample mass resulted from increase in total antioxidant concentration.

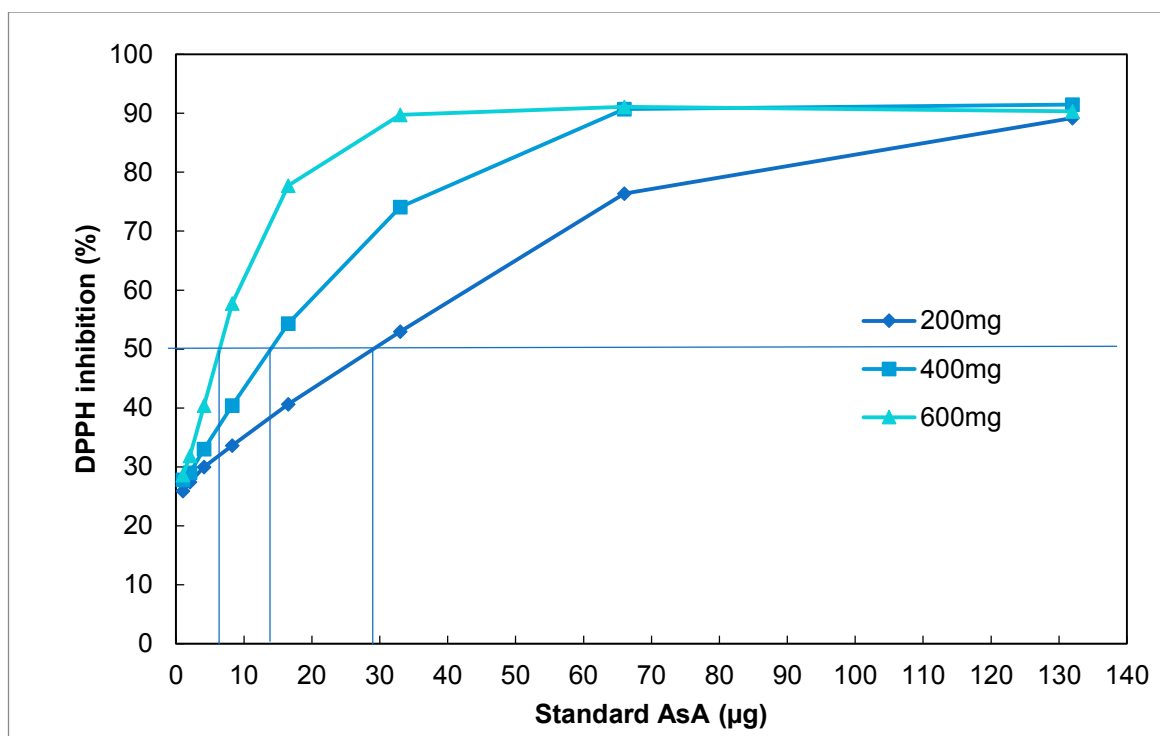


Figure 3.43: Antioxidant activity from hazelnut endocarp extracts on DPPH inhibition. Interpolating from the graph the amount of AsA required for 50% DPPH inhibition were estimated as below:

Endocarp sample mass (mg)	AsA (µg)
200	28
400	14
600	6.2

Samples were dried before analysis so re-calculation on a dry weight basis was not necessary. In each case, the mass is indicated as wet weight (w. w.) or dry weight (d. w.) when results are discussed.

(b) Determination of Suitable Temperature for Antioxidant Extraction from Hazel Embryo

This experiment was done in order to find the appropriate extraction temperature.

The extractions were carried out at 3 temperature regimes:

- i) 5°C, ii) 15°C or iii) ambient laboratory temperature (RT),

Comparisons were based on sample mass required to obtain 50% inhibition of free radical DPPH by antioxidants extracted from hazel embryos. Figure 3.44 shows sample extracted at 5°C required 11.0 mg in comparison to 9.0 mg at 15°C and 8.5

mg at RT. This indicates that extraction of antioxidants at ambient conditions is more suitable. The difference observed raises the question, should the sample be better extracted by stirring or non-stirring together with the impact of temperature on extraction method needs to be tested as well.

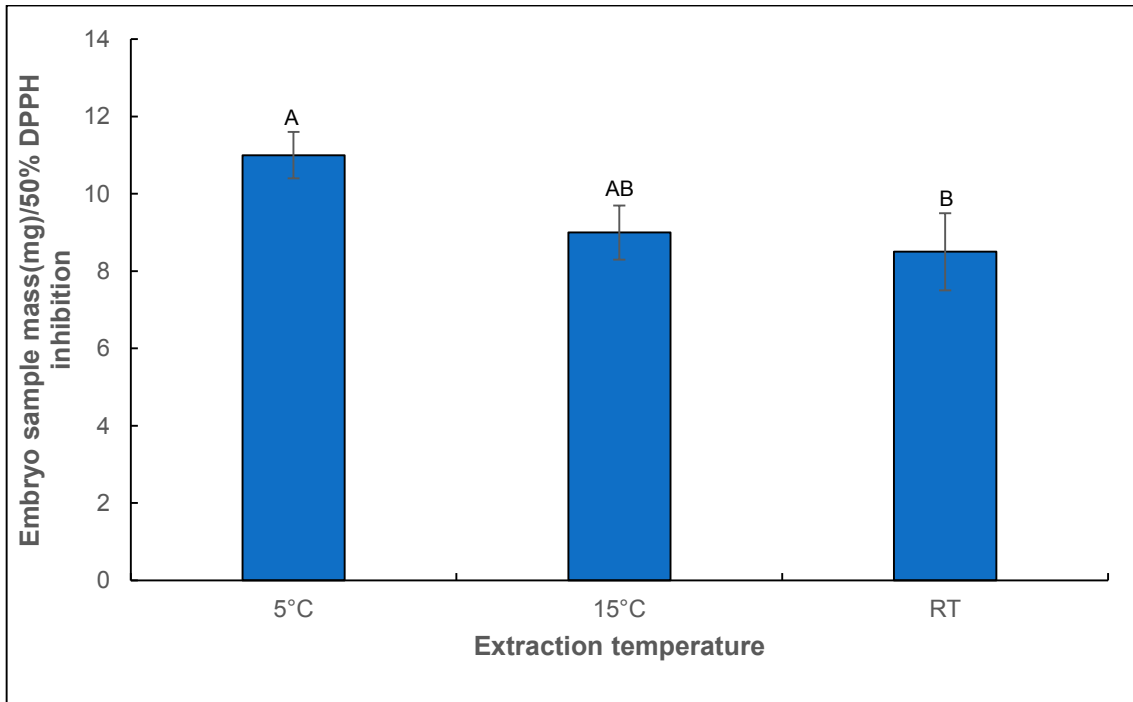


Figure 3.44: DPPH inhibition by antioxidants from hazel embryo tissues extracted at 5°C, 15°C and RT. Embryo sample of 1.0 g ground and extracted 3 times in 5 ml solvent prior to assay. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of three replicates \pm SD.

One-way ANOVA: Sample mass versus Treatments

Source	DF	SS	MS	F	P
Treatments	2	9.842	4.921	7.76	0.022
Error	6	3.807	0.634		
Total	8	13.649			

S = 0.7965 R-Sq = 72.11% R-Sq (adj) = 62.81%

Grouping Information Using Tukey's Method

Treatments	N	Mean	Grouping
5°C	3	11.0000	A
15°C	3	9.2667	A B
RT	3	8.5000	B

Means that do not share a letter are significantly different.

(c) Extraction by Stirring or Non-stirring at 5°C, 15°C and RT

The next experiment was tested with OT (no stirring), stirring (1 h and 3 h at RT), non-stirring (24 h at RT, 15°C and 5°C). In this experiment normal and defatted embryo samples were assayed. Figure 3.45 (a) shows in normal (non-defatted) embryo extraction after 3 h stirring at RT gave similar activities compared to non-stirring at 15°C and 5°C. On the other hand, in defatted embryo samples in Figure 3.45 (b) showed differences in 1 h or 3 h stirring were quite close to 24 h RT non-stirring sample, and lesser amount of defatted sample was required for achieving 50% DPPH inhibition. Hence impact of defatted samples need to be further examined. Table attached to Figure 3.45 presents the sample mass needed for DPPH inhibition in hazel embryo tissue.

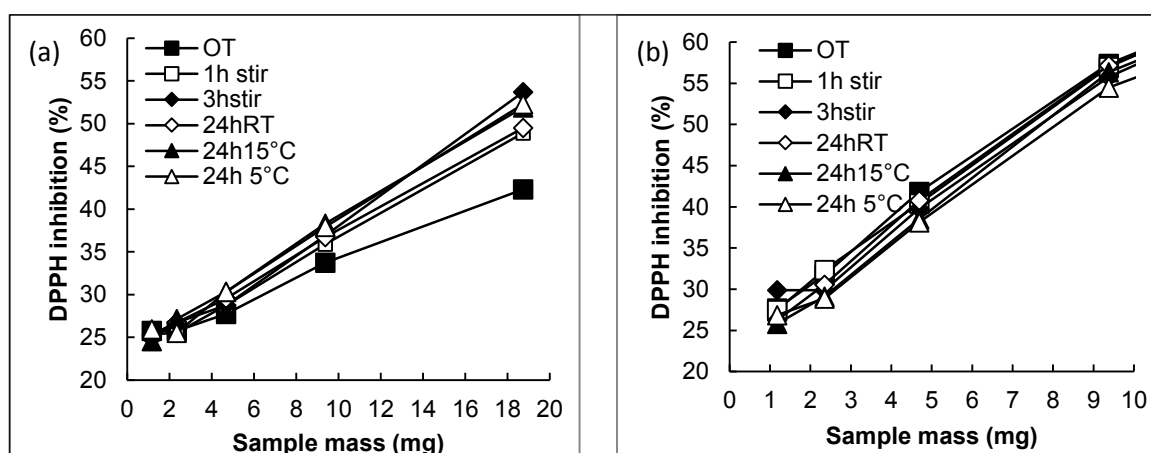


Figure 3.45: DPPH inhibition by antioxidants extracted from hazel embryos by stirring or non-stirring; (a) Normal, (b) de-fatted. Interpolating from these graphs, embryo sample mass (mg) required for 50% DPPH inhibition were estimated as below:

Treatment	Normal	Defatted
OT (■)	26.0	7.0
1 h stir (□)	19.0	7.5
3 h stir (◆)	16.5	7.6
24 h RT (◇)	19.0	7.5
24 h 15°C (▲)	17.5	7.8
24 h 5°C (△)	17.0	8.0

As the defatting process has changed the total weight of tissue it is necessary to express the results on a dry weight/non-fat basis (non-fat dry matter, or NFDM).

(d) Antioxidant Activity of Non-defatted, Defatted Extracts Compared with Non-fat Dry Matter (NFDM) Estimation

Plotting sample mass in interpolated figures from Figure 3.45 shows 1 h stirring or 24 h RT (non-stirring) results in similar antioxidant extraction shown in Figure 3.46. Therefore, either 1 h stirring or 24 h RT non-stirring can be used for extraction.

In Figure 3.46, normal (non-defatted) embryo sample shows variations in the sample mass needed to achieve 50% inhibition, whereas defatted sample exhibit fairly close response irrespective of the treatments. Samples can therefore be defatted before any of the extraction procedures.

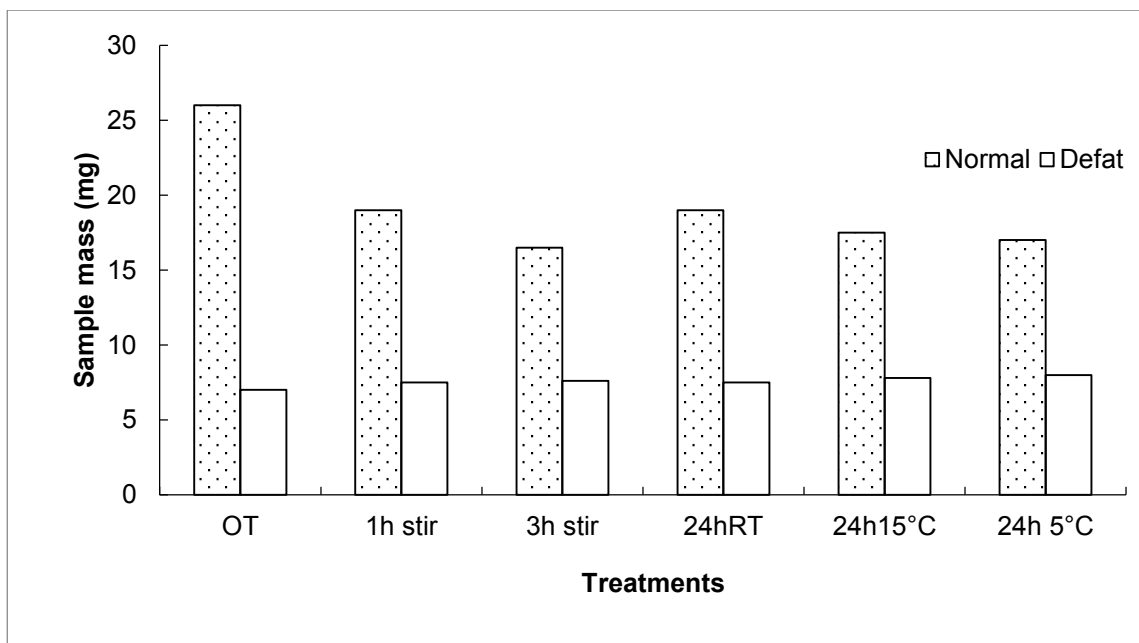


Figure 3.46: Comparison of hazel embryo mass requirement for 50% inhibition of DPPH by antioxidant activities of extracted sample by normal and defatted extraction methods. Data taken from interpolated Table in Figure 3.45.

Extracted samples which were not defatted prior to extraction can also be estimated by non-fat dry matter (NFDM) evaluation. This was achieved by allowing an original fat content of 60% by weight (Alasalvar, et al., 2003a) and a moisture content of

20% (section 4.1). Thus, for each seed, the NFDM content was estimated to be 0.2 x wet weight unless otherwise indicated, for example where specific fat content or moisture content values were available for particular seeds or batches of seeds. In these cases the actual values used are indicated.

The evaluation method used as follows:

NFDM (Non Fat Dry Matter)

Using arbitrary units the calculation as follows:-

(Sample fat 60% + sample MC 20% = 80%)

$100 - 80 = 20/100 = 0.2$

Multiply Normal extract value $\times 0.2 =$ NFDM

Hence de-fatting step can be avoided provided sample MC is known.

Figure 3.47 shows, compared to normal sample assays, defatted or NFDM procedure gives more accurate estimations.

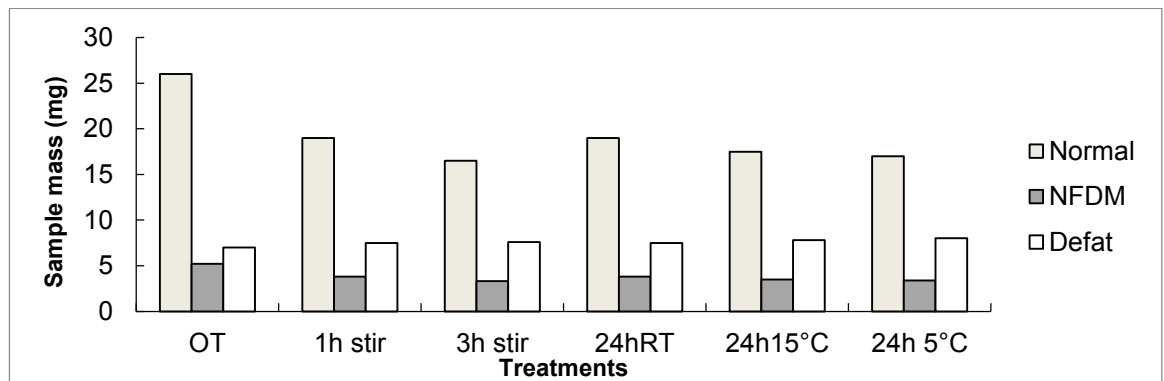


Figure 3.47: Antioxidant activity of Normal and Defatted hazel embryo samples compared with Non Fat Dry Matter estimation. Interpolating from this graph, embryo sample mass (mg) required for 50% DPPH inhibition were estimated as below:

Treatment	Normal	NFDM	Defatted
OT	26.0	5.2	7.0
1 h stir	19.0	3.8	7.5
3 h stir	16.5	3.3	7.6
24 h RT	19.0	3.8	7.5
24 h 15°C	17.5	3.5	7.8
24 h 5°C	17.0	3.4	8.0

(e) Comparison of Antioxidant Activity in Funiculus by dH₂O and Solvent Extraction

The next test was aimed to check the extraction by dH₂O compared to solvent (60/40 (v/v): Ethanol (IMS)/dH₂O).

Funiculus (20 mg) samples were extracted 3X5 ml dH₂O and another lot extracted with 3x5 ml solvent. Figure 3.48 shows extraction in solvent is better than extraction in de-ionized water (dH₂O). Therefore, solvent extraction with IMS (Industrial Methylated Spirit) will be used in all extraction steps.

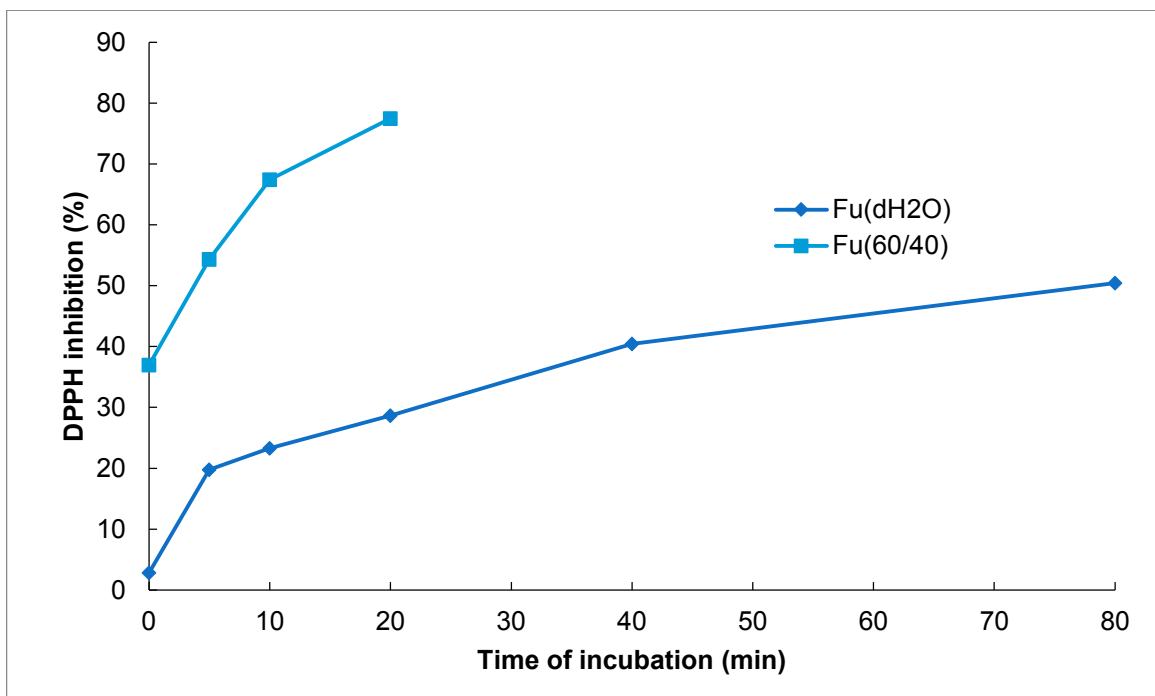


Figure 3.48: Comparison of antioxidant activity in Funiculus by dH₂O and 60/40 (v/v) (IMS/dH₂O) solvent extraction. Extracted samples were used to test inhibition of standard free radical DPPH.

(f) Comparison of Solvent (60/40) (v/v) (IMS/dH₂O) and 60/40(v/v) (IMS/Buffer) Extraction of AO from Embryo Samples

Since, extraction in solvent proved better than extraction in dH₂O, another test to check the impact of buffer added with solvent (IMS) was done and Figure 3.49 shows not much significant difference between the two methods. Hence either of the method can be used.

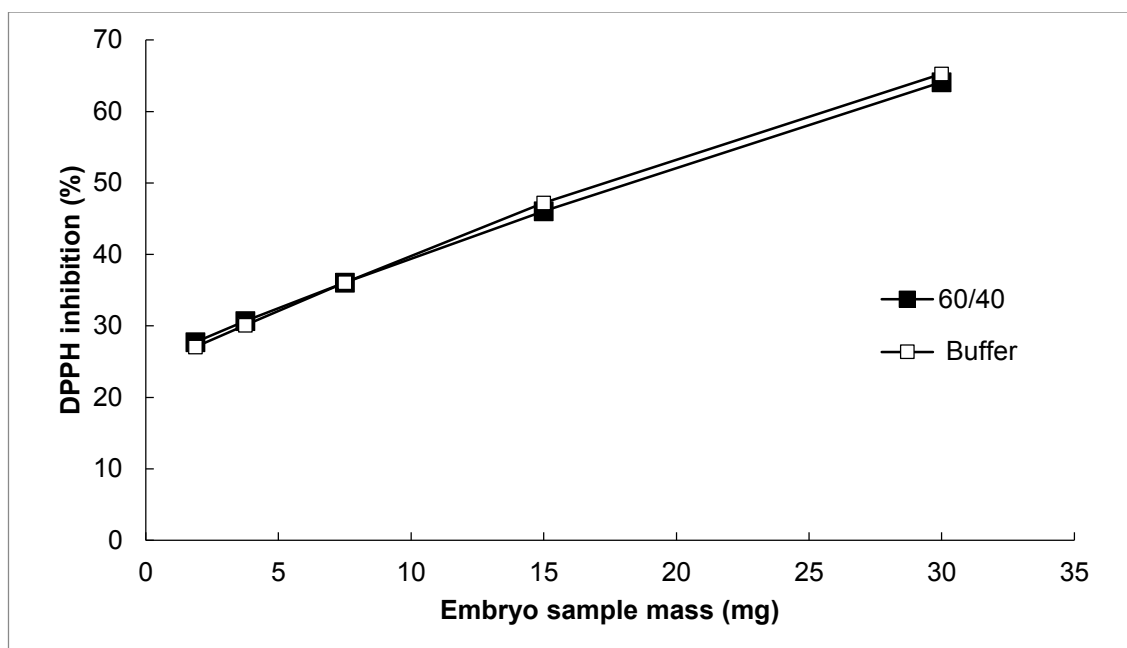


Figure 3.49: Comparison of antioxidant extraction from embryo samples in solvent (60/40)(v/v) and buffer (60/40)(v/v). The extracted antioxidant samples were used in inhibition test of standard free radical DPPH. Interpolating from this graph, embryo sample mass (mg) required for 50% DPPH inhibition were estimated as below:

Extraction solvent	Embryo mass (mg)	NFDM
Buffer in solvent extraction [60/40 (v/v IMS/buffer)]	17	5.9
Solvent [60/40 (v/v IMS/dH ₂ O)] extraction	18.2	6.4

(g) Time Scale of DPPH Inhibition by Standard Antioxidant BHT

Standard antioxidants ascorbate (AsA), butylated hydroxytoluene (BHT) and Trolox had been tested for inhibition tests on standard Free Radical DPPH for a comparison with antioxidants extracted from seed associates.

Figure 3.50 (a) shows standard BHT reacts slowly on DPPH inhibition when recorded after 15, 30, 60, 120, 180 and 240 minutes of incubation at ambient condition (RT).

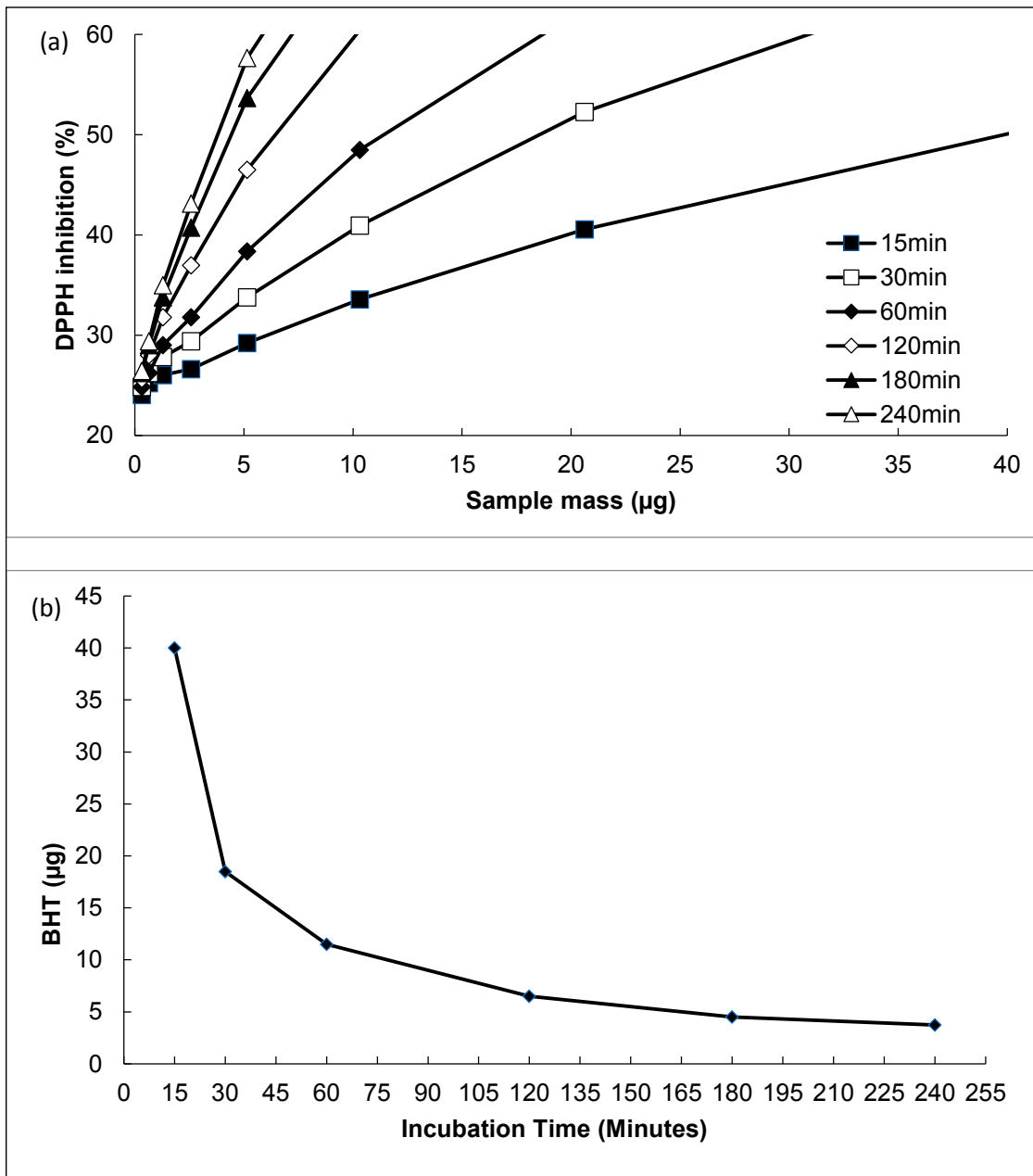


Figure 3.50: Effect of standard antioxidant BHT on DPPH inhibition, (a) incubation time (b) sample mass.

Figure 3.50 (b) shows the quantity of BHT required if DPPH inhibition was recorded at 15, 30, 60, 120, 180 or 240 minutes. The sample mass needed to inhibit 50% DPPH inhibition decreased with increase in sample reaction (assay recording) time.

Figure 3.51 shows the both standard AsA and BHT reacts very quickly to inactivate DPPH sample.

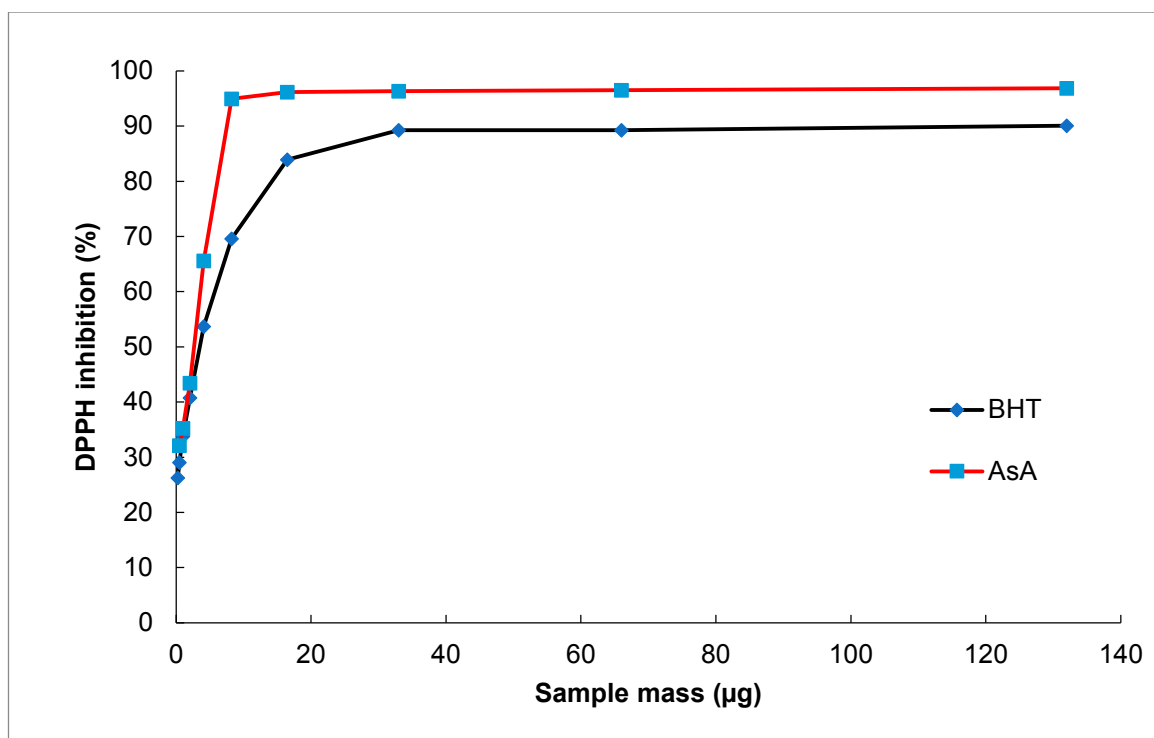


Figure 3.51: DPPH inhibition by standard antioxidants AsA and BHT dissolved in solvent [60/40 (v/v IMS/dH₂O)].

(h) Comparison of DPPH Inhibition Activity of Standard Antioxidant AsA and Trolox

In a further test, AsA and Trolox completed inhibition of DPPH in 15 minutes as seen in Figure 3.52 (a) and (b). Therefore either AsA or Trolox could be used as standard to evaluate tissue samples.

In this investigation, AsA was used as standard antioxidant equivalent.

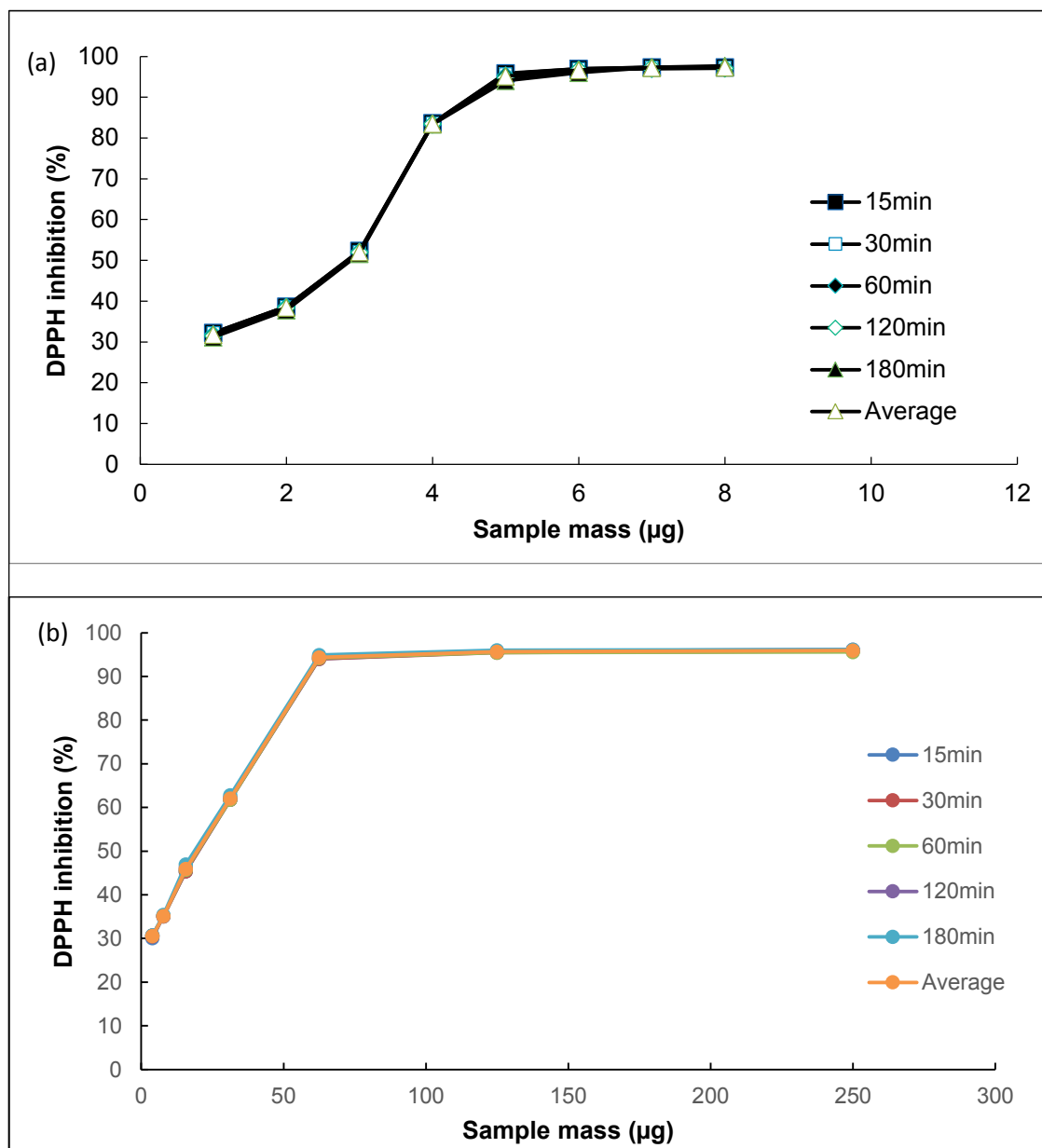


Figure 3.52: Scavenging of DPPH by standard antioxidants; (a) Ascorbate (AsA), and (b) Trolox.

(i) Qualitative DPPH Radical Scavenging Assay Using Thin Layer Chromatography

Figure 3.53 shows the DPPH free radical scavenging assay by thin layer chromatography (TLC) of extracts from endocarp, funiculus, testa and embryo along with three standard antioxidants (ascorbate (AsA), BHT and Trolox). Spots that produced a yellow colour on purple background were considered as antioxidants. Endoderm and funiculus have some activity consistent with polar ascorbate and some activity which is not consistent with either form of ascorbate, sometimes very

non-polar which might be vit-E (α -tocopherol). Presence of potent antioxidant activity in ten different *Combretum species* was evaluated using TLC analysis (Aderogba, et al., 2012), TLC profile using DPPH as a detection reagent indicted free radical scavenging of *Castilleja tenuiflora* (Alma, et al., 2012), Free radical scavenging activity of *Stachytarpheta angustifolia* leaf extract was also determined by DPPH scavenging activity detected by TLC (Awah & Verla, 2010).

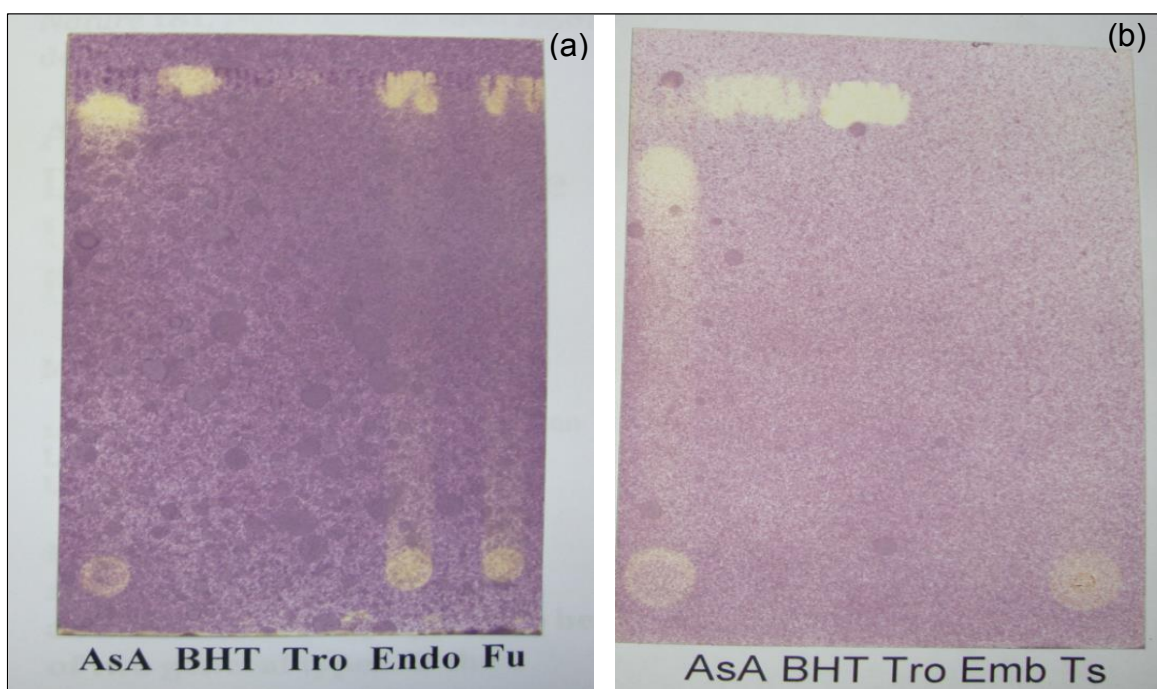


Figure 3.53: Both TLC plates are loaded with standard antioxidants AsA, BHT and Trolox. Plate (a) with extracts from Endoderm and Funiculus. Plate (b) loaded with extracts from Embryo and Testa.

AsA	Ascorbate
BHT	Butylated HydroxyToluene
Tro	Trolox
Endo	Endocarp
Fu	Funiculus
Emb	Embryo
Ts	Testa

(j) Stability of Antioxidant Extracts from Seed and Seed Associates Stored at 5°C and RT (Room Temperature)

During the entire experimental period, antioxidant extraction had been carried out on variously tested seeds and nuts. Sample stability of the extracted antioxidant samples held at 5°C and RT were tested for stability during storage.

Figure 3.54 (a & b) shows the DPPH inhibition activity of antioxidant samples extracted from seed, embryo, endocarp, funiculus and testa stored at 5°C and RT. Seed samples were tested up to 135 days, whereas the other samples were tested up to 142 days. It is interesting to note that all samples maintained antioxidant activity but embryo extract at 5°C showed increased activity as exhibited in Figure 3.54 (a). Whereas, Figure 3.54 (b) shows seed, embryo and endocarp extract needed more sample for 50% DPPH inhibition indicating loss of antioxidant stability.

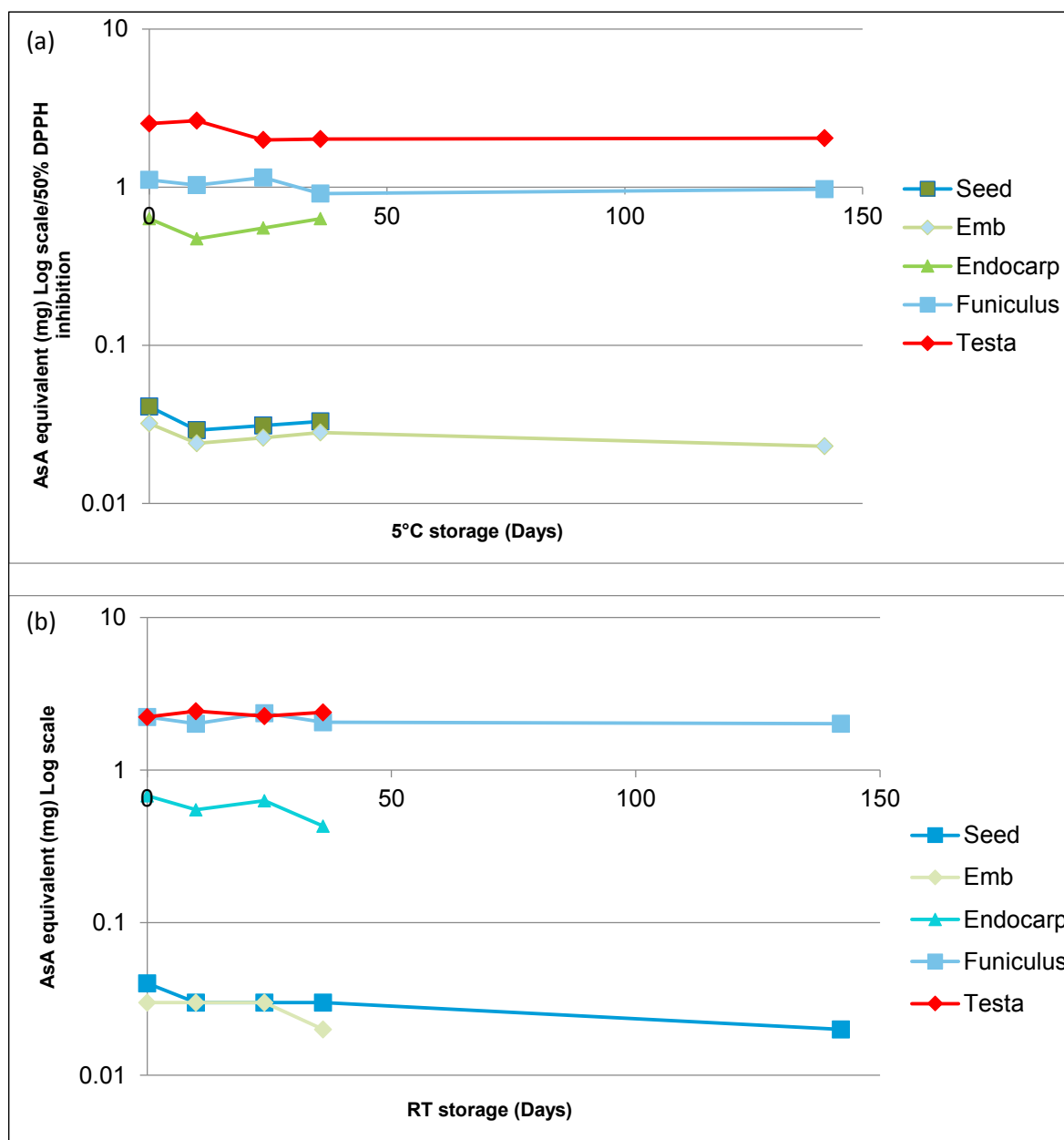


Figure 3.54: Antioxidant activity on DPPH inhibition of extracted samples from hazel seed and associates and stored at (a) 5°C and (b) RT in log scale.

3.7.3.2 Phase 2: Involvement of Antioxidants from Hazelnut Associates in Seed Physiology

Seeds and nuts after chilling or storage at 5°C or 15°C or room temperature would be tested for germination and therefore would be subjected to water stress as well. Hence, the impact of antioxidant activities on hazel seeds was tested.

Standard antioxidant ascorbate (AsA) had been tested for inhibition tests on standard Free Radical DPPH for a comparison with antioxidants extracted from hazelnut associates.

(a) Determination of Incubation Time for Antioxidant Activities

Reports indicate the incubation time prior to spectrophotometric assay varies from 15 minutes to 1 h (Sharma & Bhat, 2009), even up to 3 h (Contini, et al., 2008). Therefore, assay of the extracted samples from hazelnut associates to determine the suitable time for incubation to inhibit 50% DPPH needed to be established and follow throughout this investigation. Figure 3.55 (a, b, c and d) shows the increase in DPPH inhibition with increase in incubation time. In this test, DPPH inhibition was recorded at 30 minutes, 1 h, 2 h and 3 h. At the end of this test, it was decided to use 3 h incubation period at ambient room temperature for hazelnut embryo and associates.

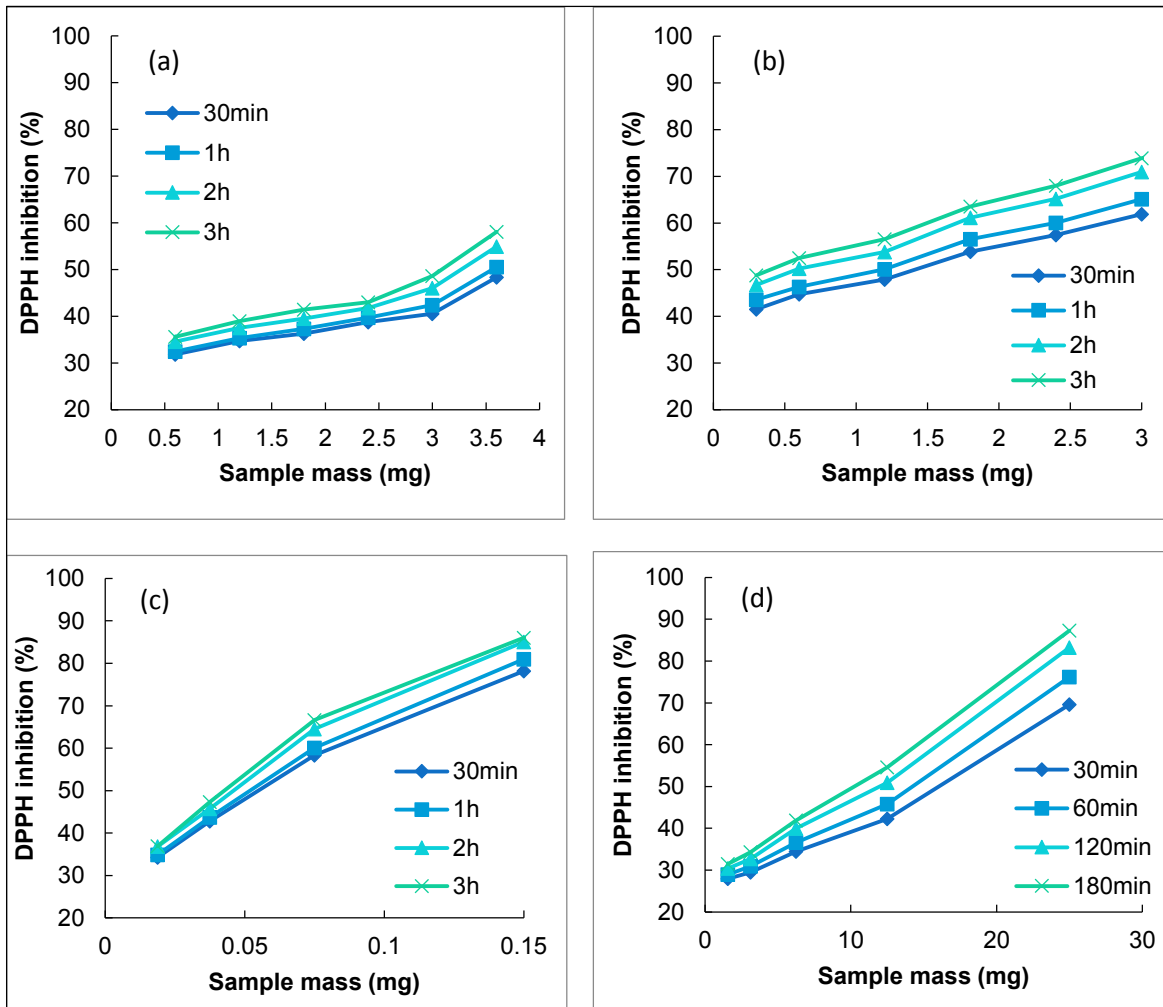


Figure 3.55: Incubation time of extracted antioxidants from hazelnut embryo and associate to inhibit standard DPPH. (a) Endocarp, (b) Funiculus, (c) Testa and (d) Embryo.

(b) Comparison of Antioxidant Activity of Seed and Embryo Extracts

Figure 3.56 shows seed had more antioxidant activities than the Embryo tissues. Seeds required 17 mg compared to 24 mg in embryo tissue for 50% DPPH inhibition. But on NFDM the quantity showed a much reduced value of 5.9 mg and 8.4 mg for seed and embryo respectively. As seed sample contained traces of testa pieces, seed extracts would give variable results because of unknown number and size of pieces of testa included during grinding. Because of the unavoidable mixing of testa pieces in seed extracts, embryo (devoid of testa) will give more dependable values. Hence, antioxidant activities from the embryo samples of the treated seeds were tested.

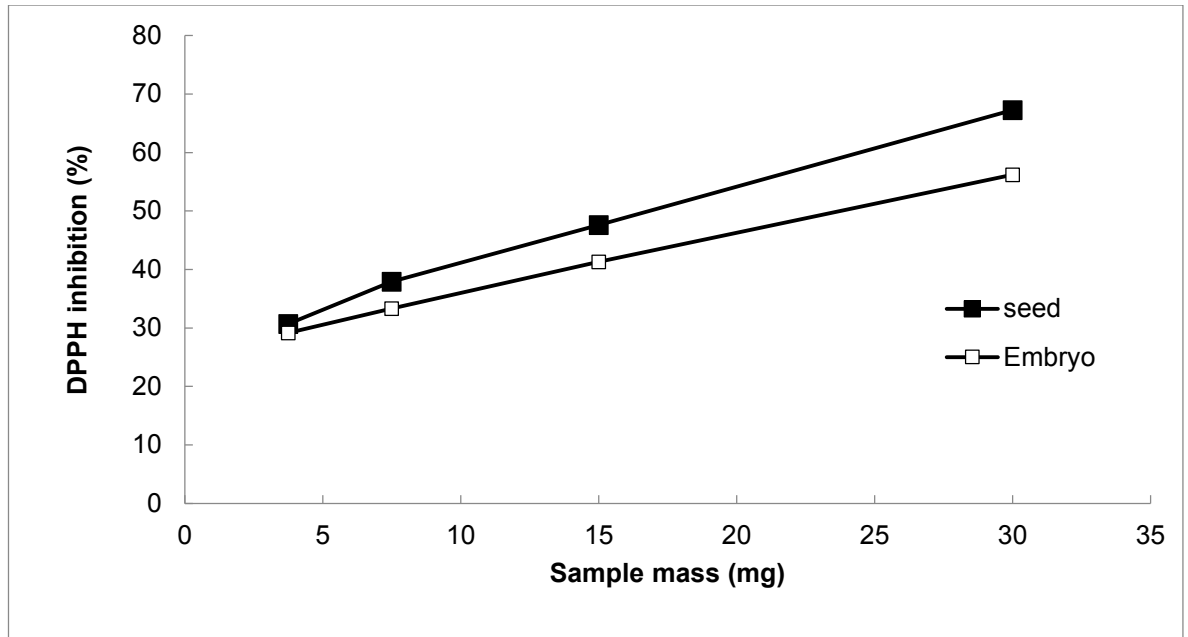


Figure 3.56: Comparison of standard DPPH inhibition by extracts from hazel seed and embryo tissues. Interpolating from the graph sample mass (mg) needed for 50% DPPH inhibition were estimated as below:

Test sample	Sample mass needed for 50% DPPH inhibition (mg)	Sample mass (mg) needed by NFDM
Seed	17	5.9
Embryo	24	8.4

(c) Role of Chilling on Antioxidant Extraction

Nuts had been chilled up to 6 weeks for dormancy breaking to achieve maximum seed germination. During chilling seeds remain enclosed within the pericarp or shell. Thus the endocarp and funiculus remain in close contact with the seed. The following results in Figure 3.57 show the response of chilling on antioxidant activities of hazelnut associates. It was very interesting to observe that testa required very small quantity of sample to achieve 50% DPPH inhibition compared to Funiculus and Endocarp as shown in Figure 3.57.

Therefore, it can be inferred that Testa which closely wrapped the embryo release most antioxidants. Funiculus connected to the tip of the seed where embryonic axis is located also released nearly 10 times more than pre-chilling state. These three

associates would therefore be providing antioxidants and would combat the free radicals as, when and where formed.

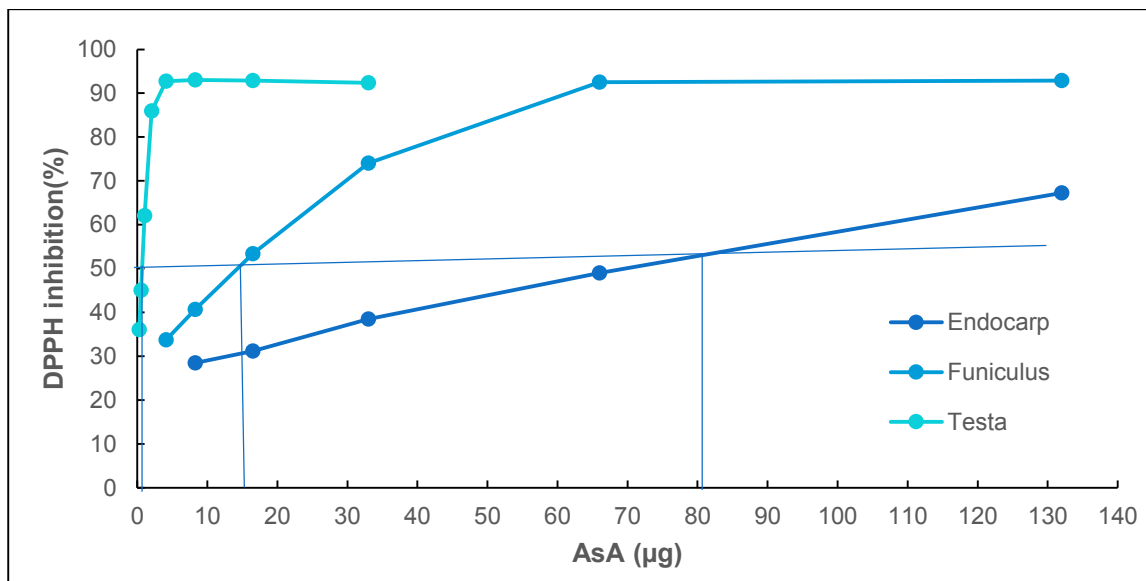


Figure 3.57: Effect of 6 weeks chilling at 5°C on antioxidant activity of hazel seed Endocarp, Funiculus and Testa on DPPH inhibition. Interpolating from the graph the amount of AsA required for 50% DPPH inhibition were estimated as below:

Sample	AsA (µg)
Endocarp	82
Funiculus	16
Testa	2

(d) Effect of Chilling on Sample Requirement for 50% Inhibition of DPPH

Seed associates are not available in bulk but need to be carefully collected from treated seeds or nuts for antioxidant extraction. Therefore, a test was done to assess the minimum amount of Endocarp, Funiculus and Testa needed for 50% inhibition of DPPH. Figure 3.58 shows the antioxidant activity of Endocarp, Funiculus and Testa from 6 weeks chilled hazelnuts. Testa, covering the embryo has the highest concentration of antioxidants followed by Funiculus and the lowest quantity is found in the Endocarp.

An interesting result is presented in Figure 3.58 which shows a gradual increase in sample mass needed for Endocarp to achieve 50% DPPH inhibition by antioxidants from un-chilled (OT) to 6 weeks chilled samples. This indicated that during chilling

antioxidants might have been used up from Endocarp. Whereas, Funiculus and Testa shows gradual reduction of sample mass needed for 50% DPPH inhibition. This indicated Funiculus and Testa released antioxidants from some bound form during chilling. Bound antioxidants may be scanty or lacking in Endocarp. Involvement of bound antioxidants has been reported in skin, pulp and seeds of miracle fruit (Inglett & Chen, 2011) and in whole grain rice (Min, et al., 2012).

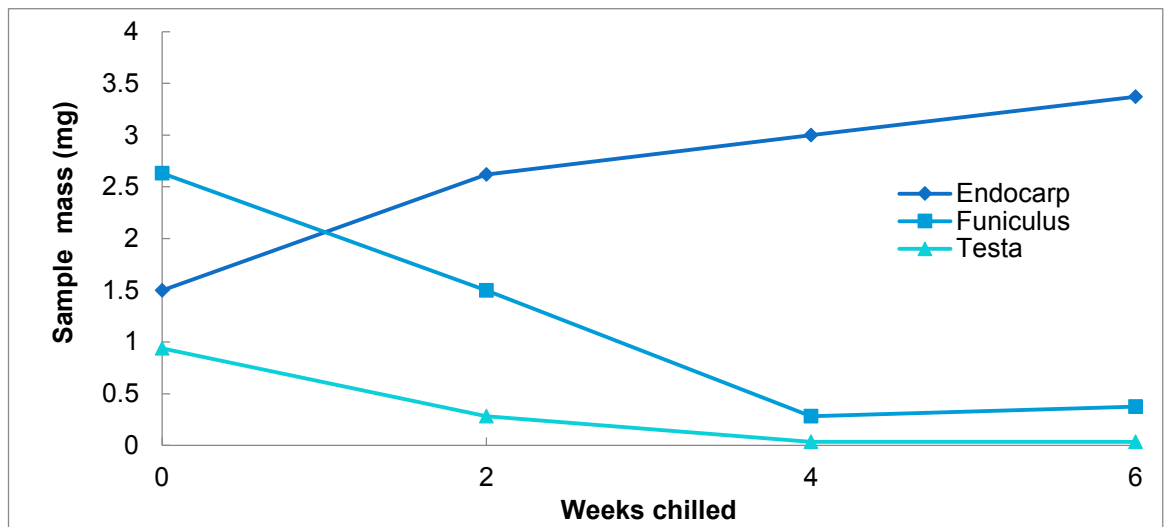


Figure 3.58: Comparison of Endocarp, Funiculus and Testa sample mass needed for DPPH inhibition during 6 weeks chilling. Interpolating from the graph sample mass (mg) needed for 50% DPPH inhibition were estimated as below:

Sample	Weeks chilled			
	0	2	4	6
Endocarp	1.5	2.62	3	3.37
Funiculus	2.63	1.5	0.28	0.38
Testa	0.94	0.28	0.035	0.035

(e) Effect of Storage of Dormant and Non-dormant Seeds at 5°C on Antioxidant Activities

Dormant to non-dormant (DND) seeds: Dormant hazelnuts were chilled for up to 6 weeks at 5°C to break seed dormancy. During the chilling process seeds were taken out at OT (control), 2, 4 and 6 weeks. Testa from each seed was carefully removed to get the embryo which was ground in a blender. Ground defatted, defatted tissue

samples from embryo were then extracted for antioxidants with 3 x 5 ml IMS/dH₂O (60/40) (v/v) solvent as described (section 2.7). Samples were then used for spectrophotometric assay to measure inhibition of DPPH by antioxidants.

Non-dormant to dormant (NDD) seeds: Non-dormant seeds resulted from the above treatment were used for this experiment. All non-dormant nuts were held in polyethylene bags without any added water for up to 6 weeks at 5°C. Required number of seeds was removed from the packet at OT (control), 2, 4 and 6 weeks. Embryo tissue samples from the above treatments were subjected to spectrophotometric assay to measure inhibition of DPPH. Results are presented in Figure 3.59 which shows a similar pattern in sample mass requirement for 50% DPPH inhibition from seeds of both DND and NDD treatments. Sample needed gradually decreased as chilling continued in DND seeds from 2 to 6 weeks. Similar pattern was shown in NDD samples. NDD seeds which were already non-dormant got more time at 5°C and therefore were able to produce more antioxidants. Hence, both treatments show reduction in seed mass needed for 50% DPPH inhibition during the 6 weeks test period.

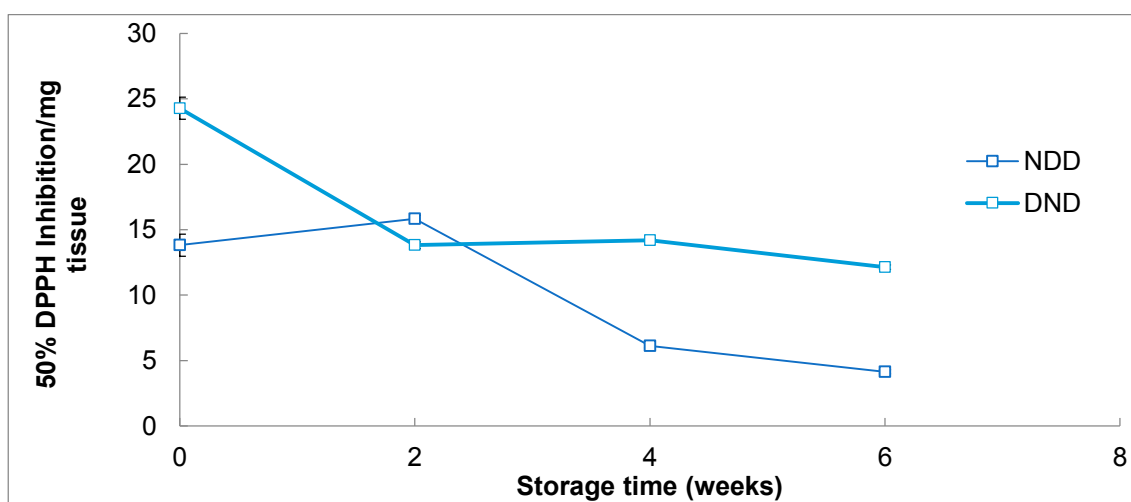


Figure 3.59: DPPH inhibition activities of hazel embryo extracts obtained from hazelnuts used in dormant to non-dormant and non-dormant to dormant treatments. Dormant seeds were chilled for 6 weeks at 5°C for non-dormant seeds and then these nuts were held at 5°C for 6 more weeks without any added water. Samples were taken out at 2, 4 and 6 weeks. Error bars indicate \pm 95%CI.

3.7.3.3 Recommendations

At the end of the various experiments conducted the following procedures were found to be useful for hazelnut associates extraction and assay protocol.

1. Care should be taken to exclude pathogens within the embryo/seed before grinding.
2. Embryo should be used instead of seed for antioxidant extraction.
3. All ground samples should be dried to remove seed moisture before extraction.
4. Sample should be defatted prior to extraction or antioxidant activities calculated on the basis of Non Fat Dry Matter evaluation.
5. Samples may be extracted after stirred up to 3 h or held for 24 h at ambient room temperature, followed by two more 1 h RT stirring. The pooled sample should be used for spectrophotometric assay.
6. Incubation of hazelnut sample extracts for inhibition measurement should be assayed up to 3 h.

3.7.4 Discussion

Hazel seeds become dormant shortly after shedding but can maintain viability up to year in storage. Seed water contents are decreased during storage hence the possibility of free radical attacks is expected (Priestley, et al., 1985). To counter stress related free radical attack, effective antioxidant systems present in the seed has been reported to protect living cells (Awah & Verla, 2010). Antioxidant phytochemicals have been extracted from hazelnut kernel, hazelnut skin, hard shell, green leafy cover and tree leaf (Shahidi, et al., 2007) and concluded that these hazelnut byproducts could be an excellent source of natural antioxidants. Literature

refers to differences in the assay protocol in various laboratories (Sharma & Bhat, 2009). Therefore, standardisation of the assay protocol were attempted to check the role of hazelnut associates (Table 3.6) in relation to the protection mechanisms of hazel seed. Previously, 22.5, 50, 80, 100, 250 μM free radical DPPH was used as standard in various laboratories, some of these are beyond spectrophotometric accuracy (Sharma & Bhat, 2009). Hence, in this investigation 60 μM of standard DPPH were be used to check antioxidant activity of extracted samples.

Extraction of antioxidants using hazel embryo (seed without testa) tissues was tested at 5°C, 15°C and at ambient room temperature (RT) which resulted in RT as the favourable temperature as 8.5 mg tissue sample was required for 50% inhibition of standard free radical DPPH (Fig. 3.44). Further tests to check the effect of stirring of the extraction medium did not show any significant difference between the stirred and non-stirred sets (Fig. 3.45). Since hazelnuts contain about 60% fat, sample used for DPPH inhibition were defatted to evaluate the difference between normal extraction (with fat) and non-fat dry matter (NFDM) (de-fatted) samples. The results clearly indicate that NFDM shows very little variations in DPPH inhibition among the treatments compared to normal extraction which show variations. Defatted samples also showed little variations hence, all extractions were performed after de-fatting and drying the samples overnight at 50°C prior to extraction (Fig. 3.46). Antioxidant extraction of the samples with dH_2O was compared with solvent extraction which resulted in solvent extraction was more efficient in terms of time taken to attain 50% inhibition of DPPH (Fig. 3.48). Similar test comparing solvent extract compared to buffer extract showed no significant difference (Fig. 3.49) as had been reported (Sharma & Bhat, 2009).

DPPH scavenging profile of standard antioxidants AsA, BHT and Trolox shows reaction time of AsA and Trolox (Figs. 3.52 a and b) is essentially instantaneous,

whereas BHT shows slower reaction and extends up to 240 minutes (Figs. 3.50 a and b). Literature records show 5, 20, 30 and 60 minutes incubation time in various laboratories (Sharma & Bhat, 2009). Assay of hazelnut associates to determine assay incubation time is shown in Figure 3.55 and the results gave identical radical scavenging profile. Since hazel seeds are oily with probability of fat soluble antioxidants (Vitamin-E), all DPPH inhibition tests will be incubated up to 180 minutes before assay (Contini, et al., 2008). TLC test shows the presence of both polar and non-polar antioxidants in all associates (Fig. 3.53) but embryo tissues show very little activity.

Stability of antioxidants in the extracted samples recorded up to 135 days at 5°C in testa, funiculus and embryo extracts and at RT, funiculus and seeds maintained up to the same period (Figs. 3.54 a and b).

Chilling of hazelnuts significantly increased antioxidant activity which shows testa has the highest activity followed by funiculus (Fig. 3.57). Endocarp on the other hand, show increase in sample mass needed to achieve 50% inhibition in radical scavenging activity (Fig. 3.58). It is not clear whether the decrease in antioxidant activity in endocarp was due to loss of potency or lack of antioxidants. Chilling at 5°C is the safest and convenient way to break hazel seed dormancy (Fig. 3.22). Figure 3.59 shows the decrease in embryo sample mass needed during chilling at 5°C for DND and also for NDD samples for radical scavenging indicating possible release of bound antioxidants. During the storage period, germination of dormant seeds increases with increase in chilling time (Fig. 3.22) and also record decrease in seed mass needed for free radical scavenging (Fig. 3.59) which indicates that more antioxidants are released with increasing chilling time.

All test results mentioned and discussed above points to the fact that hazelnuts are equipped with structures (associates) aimed to provide protection for the embryonic axes located in the seed inside the nut. Chilling which breaks hazel seed dormancy is aided by antioxidants from the associates (Fig. 3.59). The spongy endocarp which covers all around the seed recorded gradual decline in antioxidants (Fig. 3.58) but may be useful for providing a steady supply of moisture by slow imbibition thereby preventing the rush of water which might have resulted in membrane rupture and damage to the seed.

3.7.5 Summary

It has been observed that seeds may malfunction or perish due to infections. Cause of the infections and damages are not always due to pathogens, but attributed to free radical attacks as well. Free radicals increase with decreasing moisture content which results in water stress but the seeds have a natural gift of protection mechanism to counter the attack. Antioxidants present in the nut help to overcome the damaging impact of free radicals by quenching its action. Since antioxidants are also reported to be nutraceuticals, antitumor, anticancer, most research works on other fruits, seeds and even hazelnut antioxidants are focussed for its quality and quantity.

For assessment of antioxidant activities, inhibition of standard free radical DPPH has been used. But, some differences in the protocol for antioxidant assay in various laboratories points to the need to establish a more clarified methodology. At the end of a series of assays it was concluded to use the following extraction and assay protocol: i) Use solvent (IMS) for extraction, ii) allow 24 hour at room temperature for extraction, iii) embryo (seed without testa) tissues should be used instead seed tissues, v) incubation time of hazelnut samples for 50% inhibition of standard free radical assay should be three hours.

The extraction protocol thus achieved, were tested on hazel seed, embryo and hazelnut associates. Chilling of dormant hazelnuts which break hazel seed dormancy also showed reduction in sample mass of hazelnut associates viz., funiculus and testa to achieve 50% free radical inhibition with increase of chilling time. The seed associate endocarp showed exceptional result where more sample mass was required for 50% DPPH inhibition. It is likely that endocarp tissues either lost potency or lack antioxidants. The reduction in sample size in hazelnut associates could be due to release of bound antioxidants and also due to activation of seed's metabolic activities resulting in increased enzymatic antioxidant activities during chilling. Extracted samples whether stored at 5°C or ambient room temperature maintained stability of antioxidant activity when tested up to 135 days.

In this investigation, a substantial increase of antioxidants in hazelnut associates as a result of chilling was observed during the study of the viability of hazel seeds. A thorough literature review suggests that there is no previous specific report of hazelnut antioxidants for physiological protection of the seed itself.

The findings suggest the antioxidants present in hazelnuts are not only for other beneficial uses but first and foremost for its' own safety and propagation.

4 Recalcitrance in Hazelnuts

4.1 Test of Recalcitrance in Hazelnuts

4.1.1 Introduction

Experimental results in various research laboratories have placed hazelnuts in all three storage groups (Table 1.2). Hazel seeds show recalcitrance as shed with >40% water, but develops seed dormancy after a few days or weeks of ambient storage (Bradbeer, 1968). It is known that recalcitrant seeds cannot survive if seed water is reduced (Roberts, 1973). In case of hazelnuts, seed dormancy develops which prevents germination which might prevent damage as dormant seeds are not subjected to germination related metabolism. But to test for recalcitrance, hazel seeds must remain non-dormant during storage period. Therefore, in this experiment hazelnuts were subjected to 6 weeks chilling to break dormancy. These non-dormant seeds were used as control to another batch of non-dormant seeds imbibed in 10^{-4} M GA₃ to ensure non-dormant nature of the test seeds. Germination and storage behaviour of dormant recalcitrant *Myristica dactyloides* Gaertn. seeds were tested by exogenous application of GA₃ and IBA (indole butyric acid) and found to accelerate germination (Sivakumar, et al., 2006).

The objective of this experiment was to subject the non-dormant control and GA treated seeds to be stored at two contrasting temperatures, i) 5°C and ii) ambient room temperature (RT) and check their water activity, germinability, total viability and leachate conductivity during storage period. Prior to storage, both control and GA treated seeds were dehydrated to a lower moisture level and used for the 6 weeks experimental treatments.

4.1.2 Material and Methods

4.1.2.1 Nut Provenance

Hazelnuts of 2010 crop after usual air drying were stored in 5°C and then used for the following experimental treatments. Details of the provenance are given (section 2.1).

4.1.2.2 Seed Storage

Fully hydrated hazelnuts chilled for 6 weeks at 5°C with average seed moisture content 47.3% (FW) were used for this experiment. The chilling procedures are stated (section 2.10). Chilled nuts were cracked to get the non-dormant seeds. For germination test, all seeds were thoroughly washed in 1% (v/v) sodium hypochlorite solution for surface sterilization (section 2.11). The seeds were then dehydrated in ambient conditions for 5 days when seed moisture content was reduced to 23.1% (FW). At the end of which half of the seeds were imbibed in dH₂O and the other half of the seeds were soaked in 10⁻⁴M GA₃ (w/v) for 4 days in ambient laboratory conditions. On completion of four days rehydration, seeds were dehydrated again for 4 days prior to storage. Batch of 450 each of control and GA treated seeds were packed separately in re-sealable polyethylene bags and stored at 5°C and at room temperature (20 ± 2) until required for relevant tests. Required number of seeds was removed at 0, 2 and 6 weeks storage for physiological and analytical tests.

4.1.2.3 Analytical

The analyses included moisture content determination (section 2.4), water activity measurement (section 2.5) and leachate conductivity (section 2.6) were followed accordingly as stated therein.

4.1.2.4 Germination Assessment

Germination of control and variously tested seeds were carefully conducted and recorded as described (section 2.11). For germination, 50 seeds each was used as

control for i) dH₂O and ii) GA treated seeds. In the remaining tests 20 seeds were used per treatment, except 30 seeds were used in GA treated 5°C seeds (6W 5°C).

4.1.2.5 Viability Assessment

It had been observed that in some experiments all seeds may not germinate but still look healthy. Hence, the un-germinated seeds were subjected to TTC viability test as illustrated (section 2.12).

4.1.3 Results

4.1.3.1 Effects of 6 Weeks Storage of Non-dormant Control and GA Treated Seeds at 5°C

Effects of storage of non-dormant hazelnuts at 5°C on germination, viability, moisture content, water activity are detailed below.

Seed Germination and Viability

Figure 4.1 (a) shows control seeds which were chilled for 6 weeks at 5°C resulted in 96% seed germination and the remaining 4% un-germinated seeds proved to be viable by the TTC test. Therefore, this batch of seeds had 100% seed viability. This batch after 2 weeks storage at 5°C showed a fall in germination to 80%, with 10% showing viability by TTC test but 10% of the seeds became infected. On the other hand, after 6 weeks at 5°C seed germination dropped further and recorded 70% germination while the remaining 30% were viable by TTC test. So, at the end of 6 weeks at 5°C control seeds recorded 100% viability but also show a continuous reduction in seed germination.

Non-dormant seeds were imbibed in 10⁻⁴M GA₃ so that these seeds retain germinability by not becoming dormant during storage. These GA treated dehydrated seeds were also stored for up to 6 weeks at 5°C. Figure 4.1 (b) shows 100% control (pre-dehydration) seeds of this batch had seed viability as 96% of the seeds germinated and the remaining 4% were positive to TTC test. Although after

2 weeks storage, seeds from the same batch recorded 85% germination and 15% infection. Then after 6 weeks storage, this batch showed further reduction in germination to 63% and the remaining 37% of which did not germinate but were still viable as TTC test results indicated.

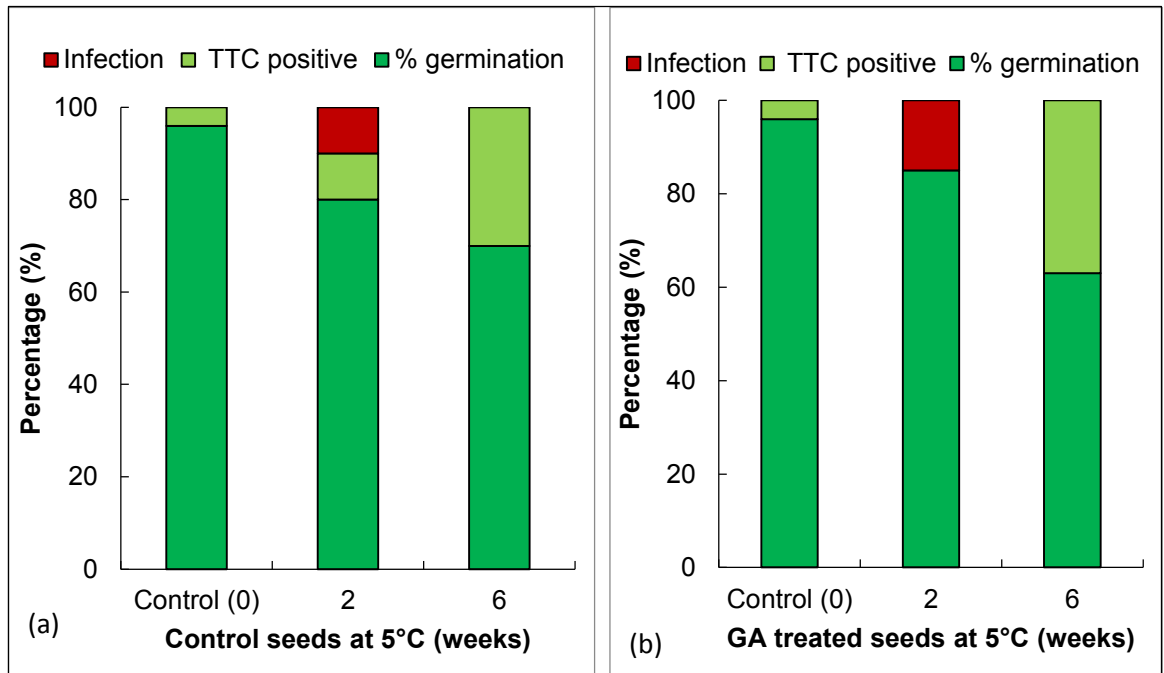


Figure 4.1: Effect of storage of non-dormant hazelnuts for 6 weeks at 5°C on seed germination and total viability. (a) Control seeds and (b) seeds imbibed in GA₃.

Seed Moisture Content

Figure 4.2 (a) exhibits control non-dormant nuts having seed MC of 42.3% but seeds stored at 5°C after appropriate dehydration and stored for 2 and 6 weeks contained 14.4 and 14.3% moisture content respectively. In GA treated seeds during storage at 5°C in Figure 4.2 (b) shows the control seeds in this test had 39.7% seed moisture and the dehydrated seeds were recorded as 20.3% after 2 weeks. Thereafter when tested after 6 weeks at 5°C remaining seeds contained 20.8% seed moisture.

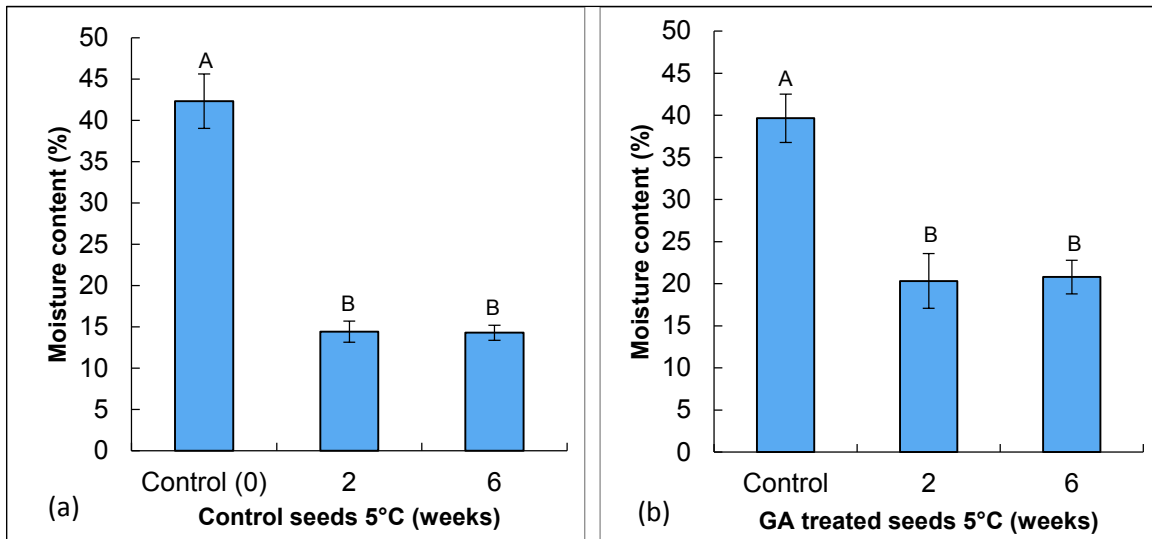


Figure 4.2: Effect of storage of non-dormant hazelnuts for 6 weeks at 5°C on moisture content. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey’s test, p <0.05). The values are mean of ten replicates ± SD.

(a) One-way ANOVA: MC versus Weeks

Source	DF	SS	MS	F	P
Weeks	2	5215.54	2607.77	302.19	0.000
Error	27	233.00	8.63		
Total	29	5448.54			

S = 2.938 R-Sq = 95.72% R-Sq (adj) = 95.41%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
C	10	42.330	A
2	10	14.420	B
6	10	14.300	B

Means that do not share a letter are significantly different

(b) One-way ANOVA: MC (5°C) + GA versus Weeks

Source	DF	SS	MS	F	P
Weeks	2	2430.7	1215.3	82.02	0.000
Error	27	400.1	14.8		
Total	29	2830.8			

S = 3.849 R-Sq = 85.87% R-Sq (adj) = 84.82%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
C	10	39.650	A
6	10	20.800	B
2	10	20.320	B

Means that do not share a letter are significantly different.

Seed Water Activity

During the 6 week storage period water activity of control seeds was 0.961 but reduced to 0.876 and 0.878 after 2 and 6 weeks storage respectively as shown in Figure 4.3 (a). Whereas, GA treated seeds in Figure 4.3 (b) shows water activity reduced from 0.964 in control to 0.917 both in seeds after 2 and 6 weeks storage at 5°C.

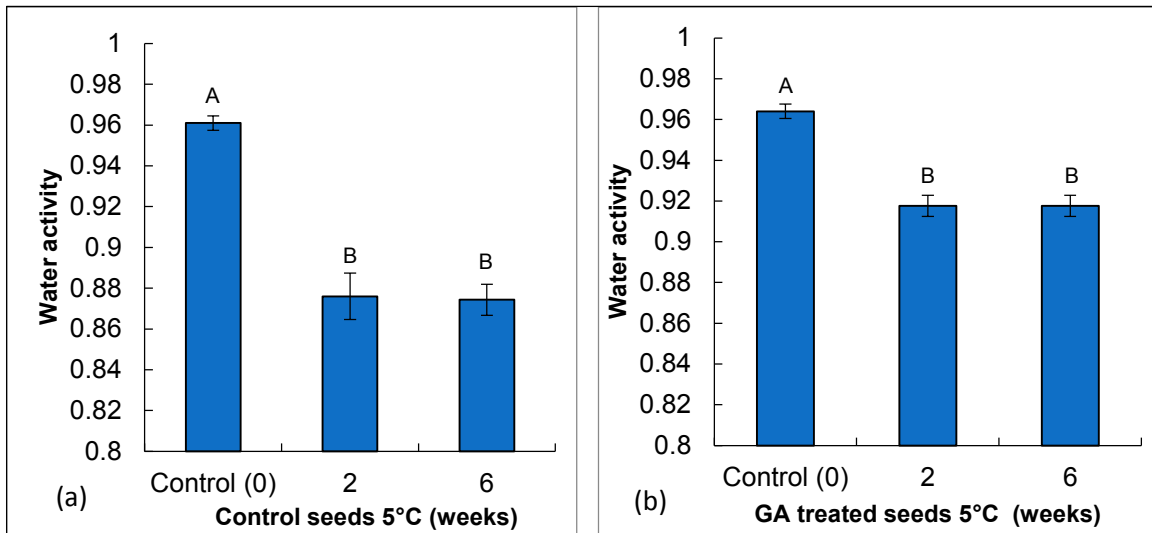


Figure 4.3: Effect of 6 weeks storage of non-dormant hazel seeds at 5°C on water activity. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey’s test, p <0.05). The values are mean of three replicates ± SD.

(a) One-way ANOVA: Water activity versus Weeks at 5°C

Source	DF	SS	MS	F	P
Weeks	2	0.0110552	0.0055276	441.03	0.000
Error	5	0.0000627	0.0000125		
Total	7	0.0111179			

S = 0.003540 R-Sq = 99.44% R-Sq (adj) = 99.21%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
C	2	0.96100	A
2	3	0.87600	B
6	3	0.87433	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Water activity versus Weeks at 5°C (+GA).

Source	DF	SS	MS	F	P
Weeks	2	0.0032202	0.0016101	416.40	0.000
Error	5	0.0000193	0.0000039		
Total	7	0.0032395			

S = 0.001966 R-Sq = 99.40% R-Sq (adj) = 99.16%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
C	2	0.964000	A
6	3	0.917667	B
2	3	0.917667	B

Means that do not share a letter are significantly different.

Conductivity of Seed Leachate

Leachate conductivity test results in Figure 4.4 (a) shows leachate activity of $6 \mu\text{S g}^{-1}$ in control and $6 \mu\text{S g}^{-1}$ after 2 weeks. Seeds after 6 weeks storage recorded a sharp increase to $10 \mu\text{S g}^{-1}$. In Figure 4.4 (b) seeds imbibed with growth hormone GA_3 and stored at 5°C shows leachate conductivity was reduced from $6 \mu\text{S g}^{-1}$ in control seeds to $5 \mu\text{S g}^{-1}$ in seeds stored for 2 weeks but after 6 weeks conductivity increased to $7 \mu\text{S g}^{-1}$.

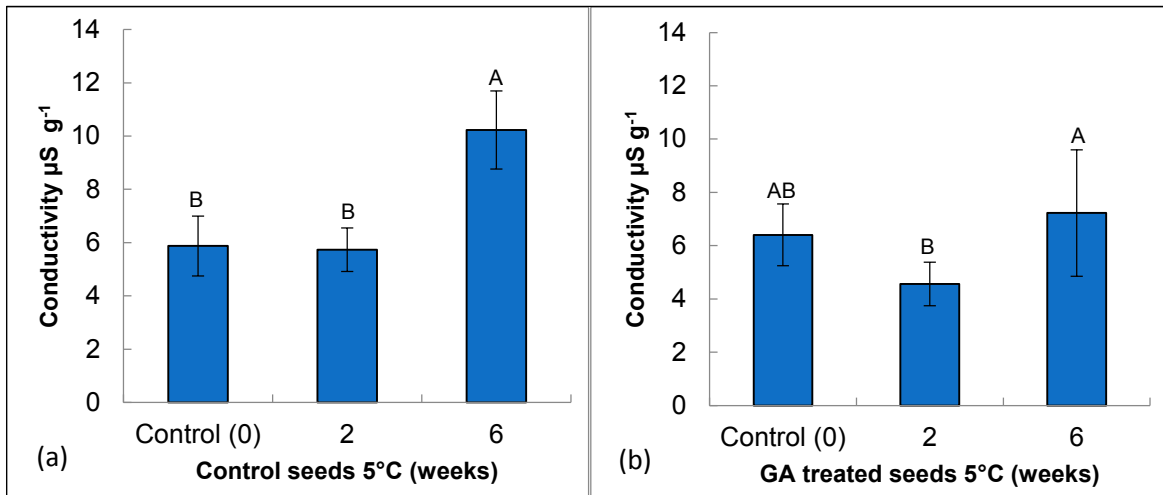


Figure 4.4: Effect of 6 weeks storage of non-dormant hazel seeds held at 5°C on leachate conductivity. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey’s test, p < 0.05). The values are mean of five replicates ± SD.

(a) One-way ANOVA: Leachate conductivity versus Weeks at 5°C.

Source	DF	SS	MS	F	P
Weeks	2	65.319	32.660	37.00	0.000
Error	12	10.592	0.883		
Total	14	75.911			

S = 0.9395 R-Sq = 86.05% R-Sq (adj) = 83.72%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
6	5	10.2280	A
C	5	5.8740	B
2	5	5.7320	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Leachate conductivity versus Weeks at 5°C (+GA).

Source	DF	SS	MS	F	P
Weeks	2	18.55	9.27	5.59	0.019
Error	12	19.90	1.66		
Total	14	38.44			

S = 1.288 R-Sq = 48.25% R-Sq (adj) = 39.62%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
6	5	7.224	A
C	5	6.402	A B
2	5	4.564	B

Means that do not share a letter are significantly different.

4.1.3.2 Effects of 6 Weeks Storage of Non-dormant Control and GA Treated Seeds at Room Temperature

Seed Germination and Total Viability

A set of non-dormant control seeds after appropriate dehydration were held at RT for up to 6 weeks. Figure 4.5 (a) shows germination drops from 96% to 80% after 2 weeks and drops further to 50% after 6 weeks storage. Germination tested after 6 weeks at room temperature show 30% of seeds which did not germinate were viable by TTC test. After 2 weeks, 20% seeds became infected. Similarly, 20% infection was also recorded after 6 weeks storage at room temperature.

GA₃ treated dehydrated seeds were also held at room temperature for up to 6 weeks. Figure 4.5 (b) shows a gradual reduction in seed germination and increase in infection. Control seeds showed 100% viability as recorded 96% germination and 4% was TTC positive. After 2 weeks at room temperature germination reduced to 80% and the remaining 20% were infected. Storage for 6 weeks was more detrimental as only 5% seeds germinated and another 5% showing viability being TTC positive but 90% of the seeds were infected.

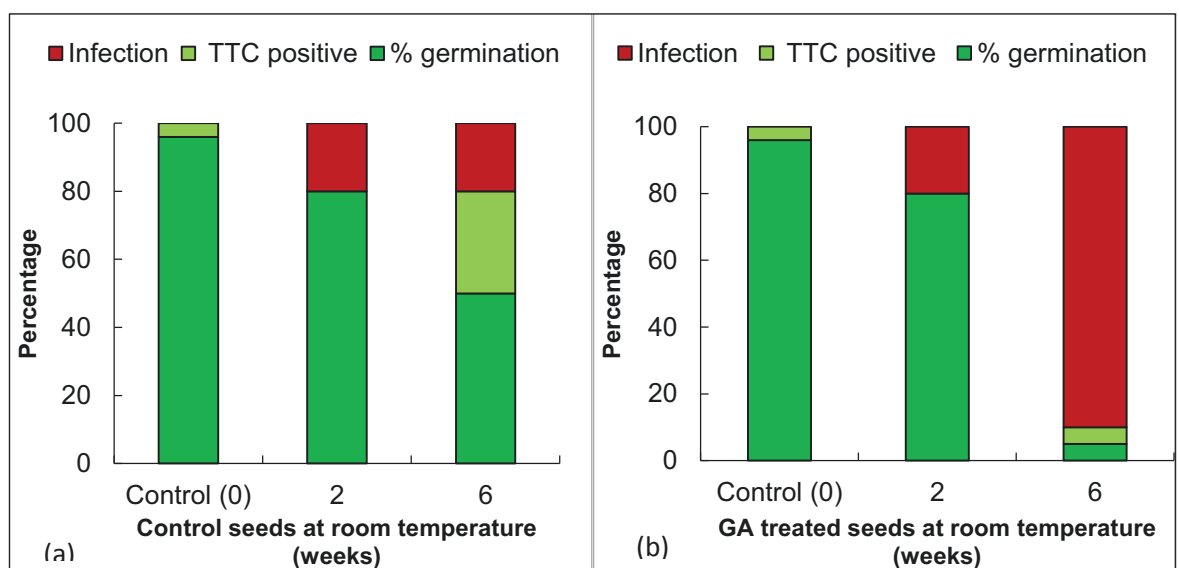


Figure 4.5: Effect of 6 weeks storage of non-dormant hazelnuts at room temperature on seed germination and total viability. (a) Control seeds and (b) seeds imbibed in GA₃.

Seed Moisture Content

Figure 4.6 (a) shows MC of control seeds having 42.3% but seeds dehydrated prior to storage maintained 15.1% after 2 weeks and 15.2% after 6 weeks storage at room temperature. Figure 4.6 (b) shows during 6 weeks storage, in GA treated seeds at room temperature seed moisture content was 39.7% in control seeds whereas 2 weeks stored seeds recorded 20.3% and 19.6% after 6 weeks storage.

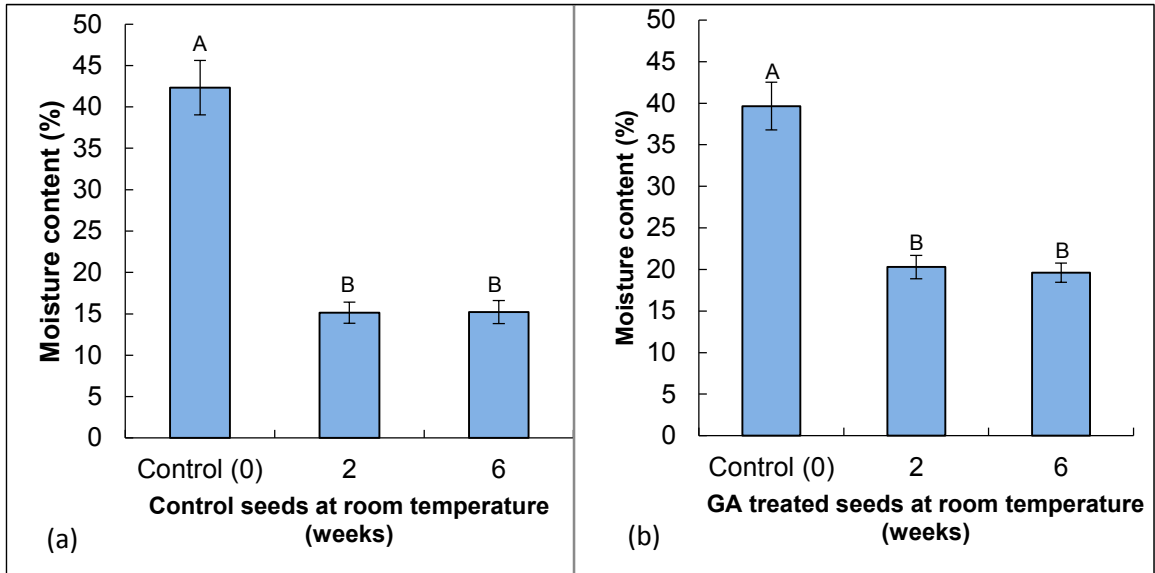


Figure 4.6: Effect of 6 weeks storage at room temperature on seed moisture content of non-dormant seeds. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey’s test, $p < 0.05$). The values are mean of ten replicates \pm SD.

(a) One-way ANOVA: Moisture Content versus Weeks at room temperature

Source	DF	SS	MS	F	P
Weeks	2	4914.17	2457.08	261.50	0.000
Error	27	253.70	9.40		
Total	29	5167.86			

S = 3.065 R-Sq = 95.09% R-Sq (adj) = 94.73%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
Control	10	42.330	A
6	10	15.210	B
2	10	15.150	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Moisture Content versus Weeks at room temperature (+GA).

Source	DF	SS	MS	F	P
Weeks	2	2589.58	1294.79	172.88	0.000
Error	27	202.22	7.49		
Total	29	2791.80			

S = 2.737 R-Sq = 92.76% R-Sq (adj) = 92.22%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
Control	10	39.650	A
2	10	20.290	B
6	10	19.610	B

Means that do not share a letter are significantly different.

Seed Water activity

Water activity of control seeds after room temperature storage as shown in Figure 4.7 (a) recorded a reduction from control value of 0.961 at control (O) to 0.878 each after 2 and 6 weeks storage. In GA treated seeds, water activity decreased from 0.964 in control to 0.917 recorded after 2 weeks storage and to 0.909 after 6 weeks as shown in Figure 4.7 (b).

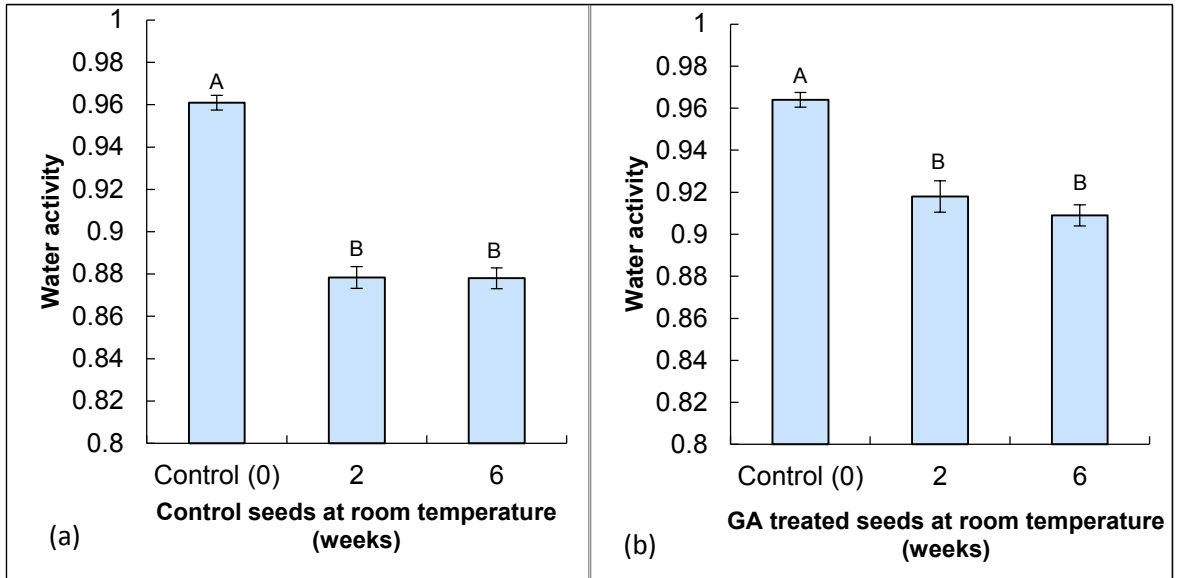


Figure 4.7: Effect of 6 weeks storage of non-dormant hazelnuts at room temperature on water activity of seeds. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey’s test, $p < 0.05$). The values are mean of three replicates \pm SD.

(a) One-way ANOVA: Water activity versus Weeks

Source	DF	SS	MS	F	P
Weeks	2	0.0102922	0.0051461	1378.42	0.000
Error	5	0.0000187	0.0000037		
Total	7	0.0103109			

S = 0.001932 R-Sq = 99.82% R-Sq (adj) = 99.75%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
Control	2	0.961000	A
2	3	0.878333	B
6	3	0.878000	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Water activity versus Weeks

Source	DF	SS	MS	F	P
Weeks	2	0.0039469	0.0019734	352.40	0.000
Error	5	0.0000280	0.0000056		
Total	7	0.0039749			

S = 0.002366 R-Sq = 99.30% R-Sq (adj) = 99.01%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
Control	2	0.964000	A
2	3	0.918000	B
6	3	0.909000	C

Means that do not share a letter are significantly different.

Conductivity of Seed Leachate

Figure 4.8 (a) shows leachate conductivity of seeds stored at room temperature increases from $6 \mu\text{S g}^{-1}$ in control seeds to $8 \mu\text{S g}^{-1}$ after 2 weeks but increased sharply to $11 \mu\text{S g}^{-1}$ after 6 weeks. Whereas, GA treated seeds in Figure 4.8 (b) shows conductivity in control seeds were recorded as $7 \mu\text{S g}^{-1}$ which after 2 weeks storage still recorded $6 \mu\text{S g}^{-1}$ but after 6 weeks at room temperature increased to $15 \mu\text{S g}^{-1}$.

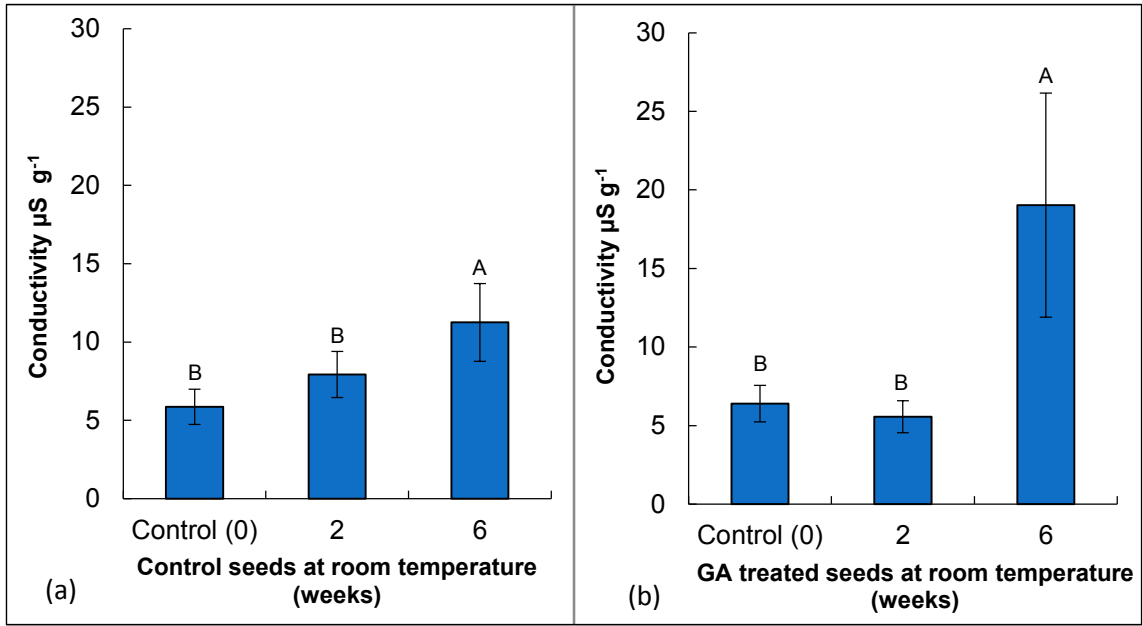


Figure 4.8: Effect of 6 weeks storage of non-dormant hazelnuts at room temperature on leachate conductivity of seeds. (a) Control seeds and (b) seeds imbibed in GA₃. Values with different letters in each chart are significantly different (Tukey’s test, p < 0.05). The values are mean of five replicates ± SD.

(a) One-way ANOVA: Leachate conductivity versus Weeks

Source	DF	SS	MS	F	P
Weeks	2	73.64	36.82	17.70	0.000
Error	12	24.97	2.08		
Total	14	98.61			

S = 1.443 R-Sq = 74.68% R-Sq (adj) = 70.46%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
6	5	11.252	A
2	5	7.930	B
Control	5	5.874	B

Means that do not share a letter are significantly different.

(b) One-way ANOVA: Leachate conductivity versus Weeks

Source	DF	SS	MS	F	P
Weeks	2	569.6	284.8	24.76	0.000
Error	12	138.0	11.5		
Total	14	707.6			

S = 3.391 R-Sq = 80.49% R-Sq (adj) = 77.24%

Grouping Information Using Tukey’s Method

Weeks	N	Mean	Grouping
6	5	19.036	A
Control	5	6.402	B
2	5	5.566	B

Means that do not share a letter are significantly different.

4.1.3.3 Overview of Seed Germination and Viability

Figure 4.9 shows a complete graphic representation of total viability test results. It is evident that seeds stored at 5°C or at room temperature gradually lose germinability and show viability by TTC test. During storage at either temperature domains, increase in seed loss due to infection is evident. These results therefore indicate the effects of stress on seed viability.

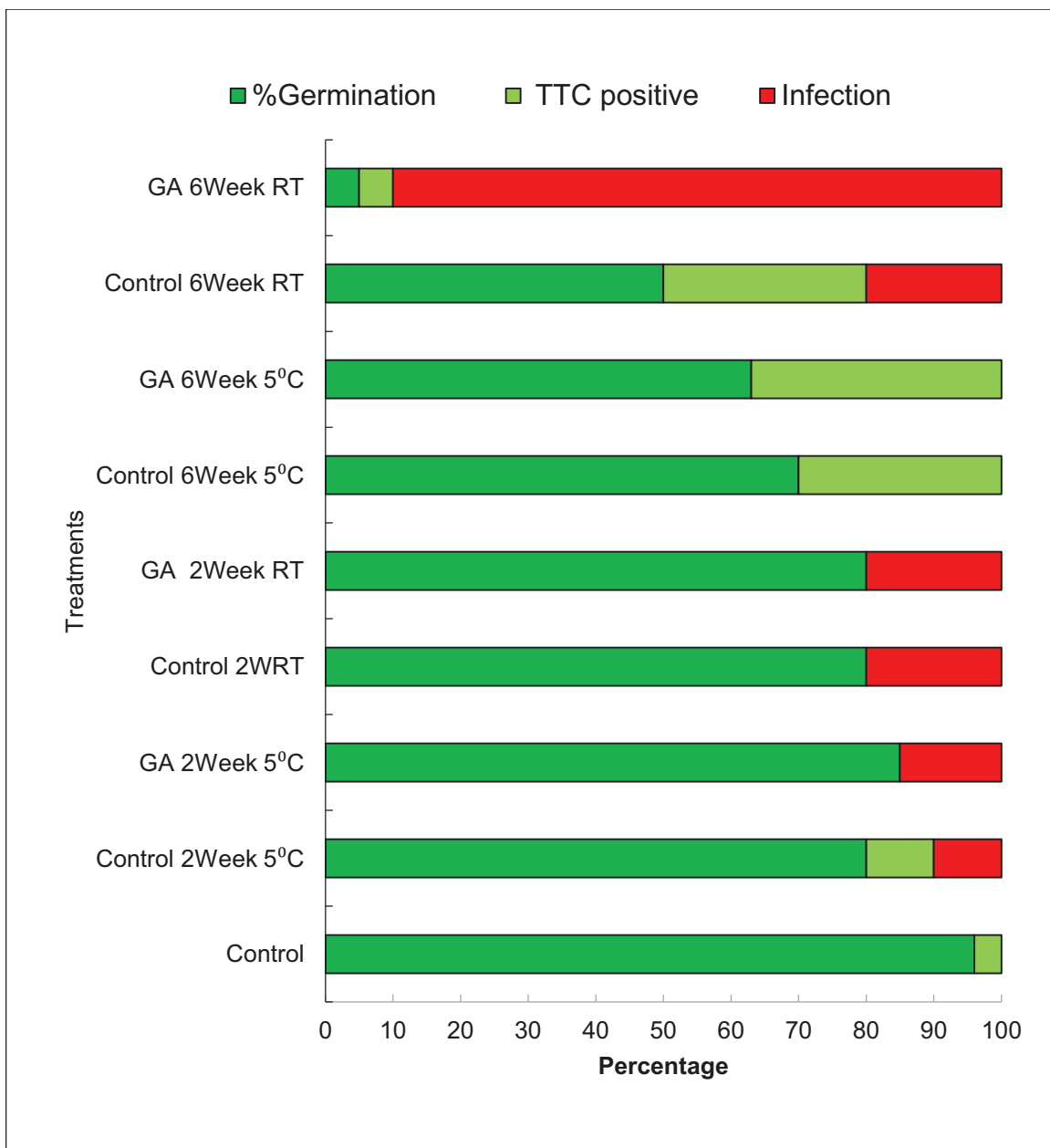


Figure 4.9: The pattern of seed viability loss and increase of infection during 6 weeks at 5°C and room temperature (RT) storage of non-dormant seeds.

4.1.4 Discussion

The placement of hazel seeds in all three storage groups by various research results (Table 1.2) has pressed for a re-visit to further or clarify the storage habit. The difficulty in assessing the storage habit of hazel seeds is complicated by seed dormancy imposed within a short period of storage after harvest (Bradbeer, 1968). Therefore, seeds were made non-dormant by chilling and adding dormancy breaking growth promoter GA₃ prior to the tests, dehydrated to lower moisture contents and held at 5°C and room temperature storage to check the impact on germination and viability tested for up to 6 weeks.

5°C Storage (Control)

Although ungerminated seeds retained viability as measured by TTC test, there was a gradual reduction in germination. Thus may be due to re-imposing of seed dormancy in reduced water content. Water activity of the control and dehydrated seeds were corresponding to the respective moisture content but increase in leachate conductivity indicated membrane damages and its impact for reduction in germination. It has been observed that seeds under stress show inactivity or reduced activity due to free radical actions resulting in dead embryonic axis or dead parts within the cotyledon as shown by TTC test (Hosomi, et al., 2011).

GA Treated Seeds at 5°C

Dehydrated seeds did not show any loss in seed moisture during storage, and water activity was reflective of the corresponding moisture content. Both control and GA treated seeds show a similar pattern of decrease in germination during the storage period at 5°C. The ungerminated seeds proved viable by TTC test. It may be related to the impact of added GA which exerts extra demand for germination related metabolic activities but were unable to continue due to reduced available water and

hence resulted in water stress and reduced germination. Test results also recorded increase in leachate conductivity with increase in storage time suggesting impact of membrane damages during storage.

Room Temperature Storage (Control)

At room temperature, storage of non-dormant hazelnuts did not show any noticeable reduction in moisture content up to 6 weeks but only half of the seeds germinated. Of the ungerminated seeds, TTC test proved 30% viable supporting dormancy imposition and remaining 20% showed infection indicates impact of water stress. Water activity was reflective of the moisture content at the respective states. Increase in leachate conductivity indicated a possible link to decreased germination. In *Acer saccharinum*, desiccated seeds gradually lost their germination capacity correlated with increase in electrolyte leakage from seeds (Pukacka & Ratajczak, 2006).

GA Treated Seeds at Room Temperature

Dehydrated GA treated seeds did not show noticeable reduction in moisture content during storage at room temperature and water activity was relative to the respective moisture content. Seed germination of control set was reduced drastically to only 5% and another 5% were TTC viable and the remaining 90% showed infection. The observations suggesting the storage of hazelnuts at higher temperature is severely affected by increase in infection and reduction in germination. Increase in leachate conductivity clarifies the impact of water stress as more severe at higher ambient temperature which accounts for the increased infection and drastic reduction in total viability. A sharp reduction of storability of *Theobroma cacao* (L.) seeds was associated with a number of physiological, biochemical and structural changes. There was a 3-fold increase in leachate conductivity, lower [¹⁴C] leucine

incorporation, and major ultra-structural changes of all cell membrane systems was detected (Hor, et al., 1984). Non-desiccated GA and IBA treated recalcitrant seeds of *Myristica dactyloides* Gaertn. maintained viability up to three months (Sivakumar, et al., 2006).

Decreasing germination and increasing leachate conductivity at both 5°C and room temperature shows that hazel seeds were subjected to water stress and therefore not favourable for storage with seed moisture content between 14-15% (FW). It is evident from the results that hydrated hazel seeds cannot be held at 5°C or room temperature without substantial loss of viability indicating recalcitrant storage habit (Pammenter et al., 1994). An extended storage time would add more insight on the chemical and biochemical implications on hazel seed storage and viability. Although germination and viability tests are indicators of recalcitrance but the unavoidable involvement of dormancy makes it more difficult to evaluate recalcitrance in hazelnuts. The observation that, hazel seeds maintain viability even at <6% (Fig. 3.30) seed moisture which is a characteristics of orthodox seeds needs to be biochemically tested.

Therefore other tests like role of LEA proteins and cryoprotectants will provide more information about hazel seed recalcitrance.

4.1.5 Summary

Hazelnuts were chilled for 6 weeks to break seed dormancy. The non-dormant seeds of control and GA treated seeds resulted in 96% germination and TTC test giving a total of 100% viability. These non-dormant seeds were dehydrated for 4 days prior to subsequent tests. Control seeds held at 5°C show reduction in germination to 70% whereas at room temperature germination reduced to 50% after 6 weeks storage. Seeds imbibed with GA and held at 5°C also recorded reduction

in germination to 63% whereas seeds held at the room temperature showed drastic reduction 5% germination after 6 weeks. The reduction in germination of seeds of both 5°C and room temperature is suggesting the impact of water stress as seen by increased leachate conductivity. The outcome of this experiment shows, non-dormant hazel seeds lose viability during storage whether at 5°C or at room temperature having seed water content between 14 to 15% further supporting its recalcitrant habit. Seeds imbibed with exogenous GA were not able to maintain viability even though the seed moisture content was about 20% (FW) showing effects of water stress. Increase in leachate conductivity during dry and hydrated storage in the embryonic axes of recalcitrant *Quercus robur* has been reported (Ntuli, et al., 2011). Other methods like role of Late Embryogenesis Abundant (LEA) proteins, cryoprotectants and storage sugars should be explored for testing seed recalcitrance in hazel seeds.

4.2 Storage of Hazelnuts at -20°C

4.2.1 Introduction

Various research groups have attempted to extend the storage life of hazelnuts (Tables 1.2 and 1.3). As a result of the inability for *ex situ* preservation of hazelnuts, intact hazelnut trees are reported to be preserved as *in situ* collections in 20 stations (seed banks) in 16 countries worldwide (Battencourt & Konopka, 1989).

For medium or long-term storage, hazelnuts need to be stored below freezing temperature at reduced seed moisture content (REF). Several experiments for cryopreservation of hazel embryonic axes had been tried. Cryopreservation of air-dried hazel embryonic axes in liquid nitrogen and their subsequent growth in culture medium has been reported (Pence, 1990). Cryopreservation and growth of hazel embryonic axes in culture medium has also been reported but had not been able to cryopreserve whole seeds, neither has there been any information on the ability to regenerate plants (Gonzalez-Benito & Perez, 1994; Normah, et al., 1994; Reed, et al., 1994). Recently, cryopreservation of the whole hazel seed had been reported (Michalak, et al., 2013). In that experiment, hazel seeds survived liquid nitrogen (LN, -196°C) and germinated if the seed moisture content was between 0.05 - 0.10 gg⁻¹ but seedling emergence was possible at the seed moisture content between 0.08 - 0.10 gg⁻¹. The responses of the emerged seedlings have not been described. Liquid nitrogen storage has the dual advantage of an extremely low temperature and a non-oxidising gas phase although the extreme temperature may cause physical damage to whole seeds. Micropropagation of hybrid hazelnut cultivars has also been tested which is claimed to be difficult due to recalcitrant nature (Garrison, et al., 2013).

'Ultra-dry' ambient seed storage has been suggested at less than 5% seed moisture content (Zheng & Jing, 1998). This observation has been countered (Ellis & Roberts, 1998) referring to reports that storage of some species at ambient temperatures

results in more rapid loss of seed viability than at about 5% moisture content at -20°C (Ellis & Roberts, 1980). Storage of 'ultra-dry' seeds, based on germination records, has shown that refrigerated storage is needed to achieve the seed longevities required for *ex situ* germplasm conservation (Walters, et al., 1998).

Ex situ germplasm conservation of hazelnut is safer than *in situ* conservation. It is interesting to note that cryopreservation is a high technology method. To avoid these seeds may be most conveniently stored at either 0 - 5°C or -20°C. The most striking observation is *Coffea arabica* L. pioneer species for the term 'intermediate' storage habit by (Ellis, et al., 1991a) and later stated the species to possess an 'exceptional storage habit', after surviving 10 yr at -20°C (Hong & Ellis, 2002).

There are few experimental results on the establishment and performance of seedlings grown from the diverse storage methods mentioned above. Even though hazelnuts has been placed in all three storage groups (Table 1.2), taking the advantage of its seed dormancy, the present investigation was set to test whether 'ultra-dry' hazel seeds can be held at -20°C and follow up with seedling performance.

4.2.2 Materials and Methods

4.2.2.1 Nut Provenance

Hazelnuts of 2008, 2010 and 2011 harvest were used in this experiment. Details of the seed provenance are outlined (section 2.1).

4.2.2.2 Nut Storage

Procedures for storage of hazelnuts are described (section 2.3). For this experiment hazelnuts of the respective years were air dried for 7 days on laboratory table in ambient temperature before placing them in polyethylene bags. During this drying period seed moisture were below <6% (fresh weight basis). The sealed bags were kept in -20°C refrigerator till the time needed for viability experiments.

Table 4.1: Seed moisture content and storage period of desiccated hazelnuts at -20°C

Crop year	MC % (FW)	Weeks at -20°C
2008	5.8 ± 0.7	189
2010	3.5 ± 0.5	80
2011	3.9 ± 0.5	36

4.2.2.3 Chilling

Hazelnuts of 2008, 2010 and 2011 crop were held in storage at -20°C for 189, 80 and 36 weeks respectively (Table 4.1). These batches of nuts were then chilled for 6 weeks at 5°C according to the procedure stated (section 2.10).

4.2.2.4 Moisture Content Determination

Moisture content of the seeds from nuts after storage time in -20°C refrigerator followed by 6 weeks chilling at 5°C was determined as described (section 2.4).

4.2.2.5 Seed Germination

Seed germination was carried out after chilling as detailed (section 2.11).

4.2.2.6 Seedling Growth

Germinated seeds from each crop year were used for seedling growth to observe the physical features. A batch of 30 healthy germinated seeds from each crop year were planted in compost trays as detailed (section 2.13). During the growth period, time for seedling emergence was recorded. At the end of 2 weeks, seedling height and internode numbers were also recorded.

4.2.3 Results

4.2.3.1 Seed Germination and Total Viability

Seed moisture content and number of weeks of -20°C storage of 2008, 2010 and 2011 crop after storage has been mentioned in Table 4.1. Hazelnuts at the end of the usual 6 weeks chilling at 5°C were cracked and seeds were surface sterilized

as described (section 2.11). For germination test 100 seeds were used from each crop. Germination was recorded daily. Another batch of hazelnuts from 2008 crop was kept at 5°C. Figure 4.10 shows that seeds from -20°C storage exhibit similar germination pattern. Seeds of 2008, 2010 and 2011 crop recorded 87, 83 and 70% final germination respectively. Whereas, 2008 hazelnuts held at 5°C for 189 weeks resulted in 30% germination.

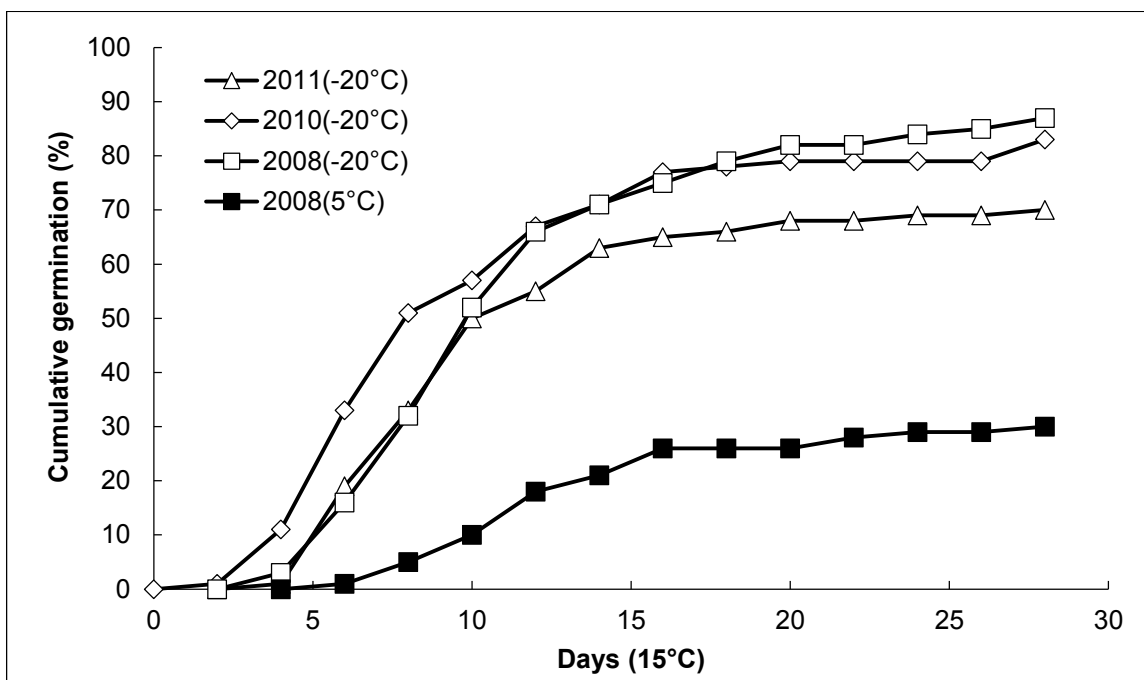


Figure 4.10: Newly harvested hazelnuts of 2008, 2010 and 2011 crop after desiccation were held in -20°C for 189, 80 and 36 weeks respectively. A batch of 2008 nuts was held at 5°C for 189 weeks. Nuts after storage period were chilled for 6 weeks at 5°C at the end of which the seeds were subjected to germination test in a 15°C incubator.

Germination lag phase in Figure 4.11 shows that seeds from -20°C storage of 2008, 2010 and 2011 began germination after 4, 2 and 4 days respectively, whereas seeds held at 5°C germinated on the 6th day.

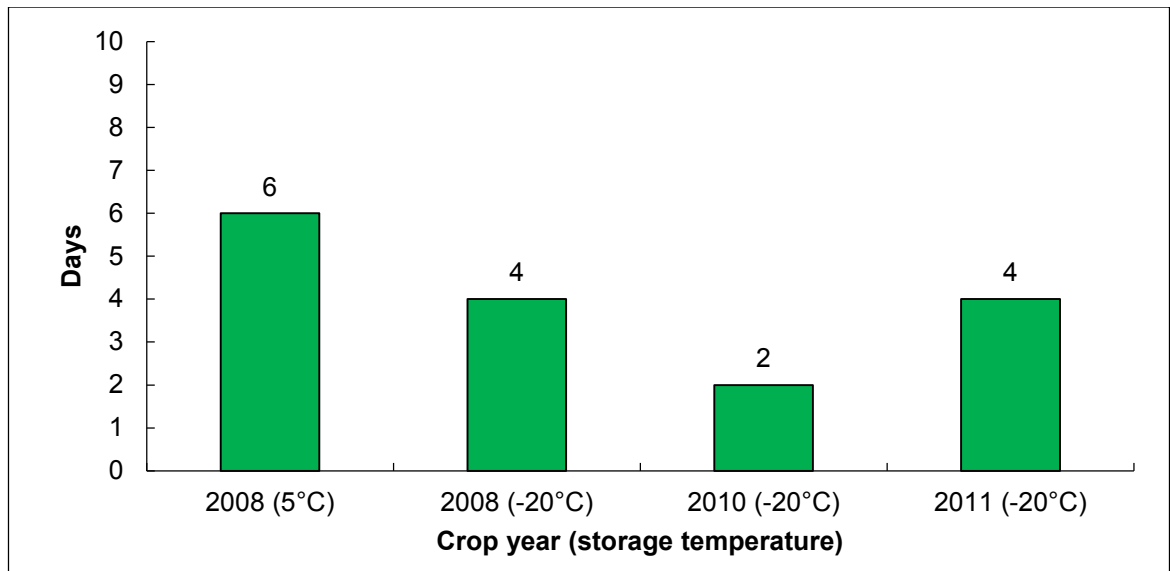


Figure 4.11: Average lag phase of first germination of seeds subjected to storage at 5°C and -20°C followed by 6 weeks chilling at 5°C. Storage treatments of hazelnuts are as detailed in Fig. 4.10.

Figure 4.10 shows that not all of the seeds germinated at the end of the 28 days germination time. Thus, ungerminated seeds were evaluated for viability by TTC test. Figure 4.12 showed 87% seeds germinated and 13% were infected from 2008 crop. Seeds of 2010 and 2011 showed 8 and 11% seeds respectively did not germinate but were viable. The seeds also show 9% and 19% infection in 2010 and 2011 seeds respectively. As a control, 2008 hazelnuts stored at 5°C shows only 30% germination, 11% TTC viable and 59% were infected.

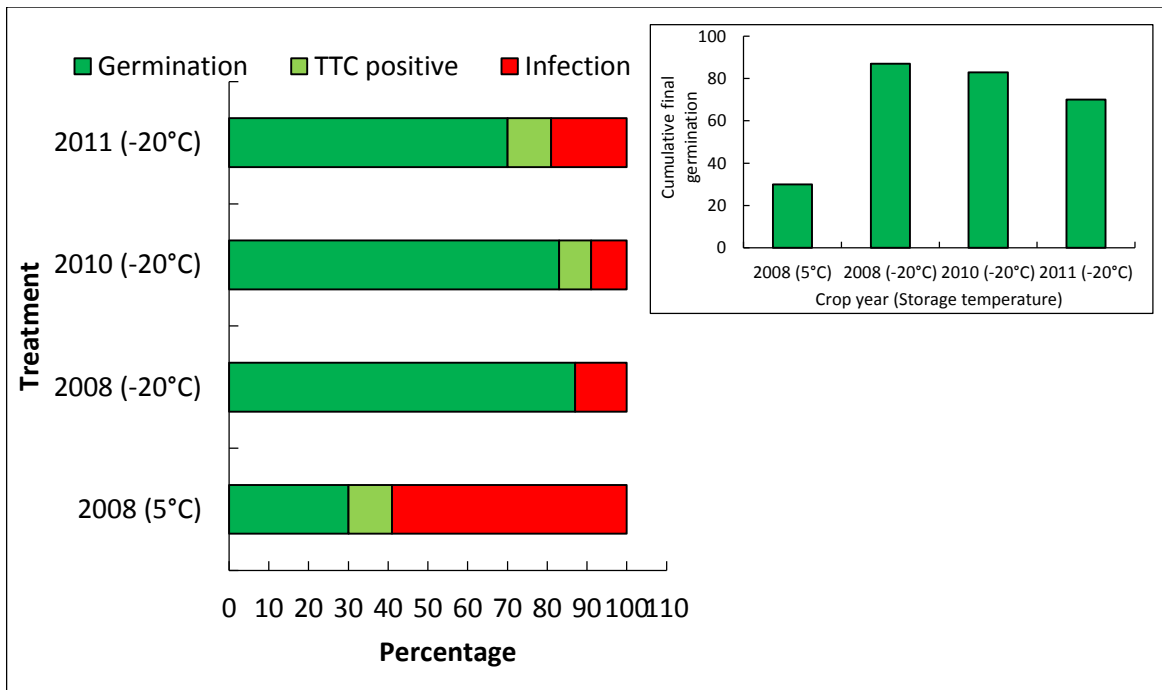


Figure 4.12: Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seed viability. Nuts held 5°C and -20°C were given 6 weeks chilling at 5°C prior to germination test. Inset showing the final germination of 5°C and -20°C stored seeds. Storage treatments of hazelnuts are as detailed in Fig. 4.10.

Seeds from both 5°C and -20°C after chilling were subjected to germination test. Figure 4.13 (a) shows mean daily germination of seeds held at -20°C were higher than from 2008 crop held at 5°C. Similar response was also observed in seeds from 2008 crop at 5°C in peak value (Fig. 4.13 b) and also in germination index (Fig. 4.13 c).

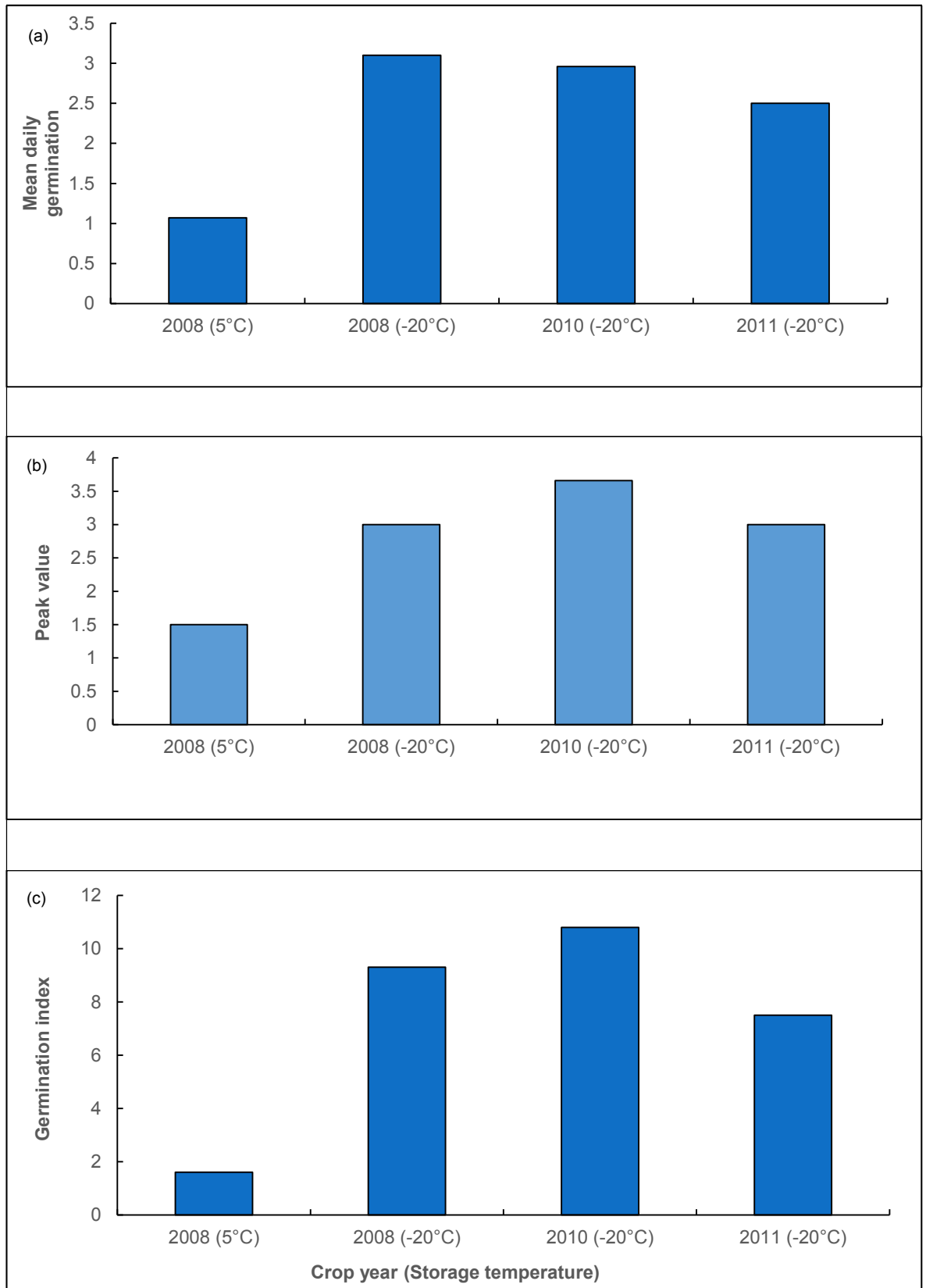


Figure 4.13: Effect of 6 weeks chilling of hazelnuts held at 5°C and -20°C on; (a) mean daily germination, (b) peak value and (c) germination index. Storage treatments of hazelnuts are as detailed in Fig. 4.10.

4.2.3.2 Seed Moisture Content and Water Activity

Figure 4.14 (a) shows seed moisture content after 6 weeks chilling records between 40 - 47% and the water activity of the seeds show between 0.969 - 0.982 (Fig. 4.14 b).

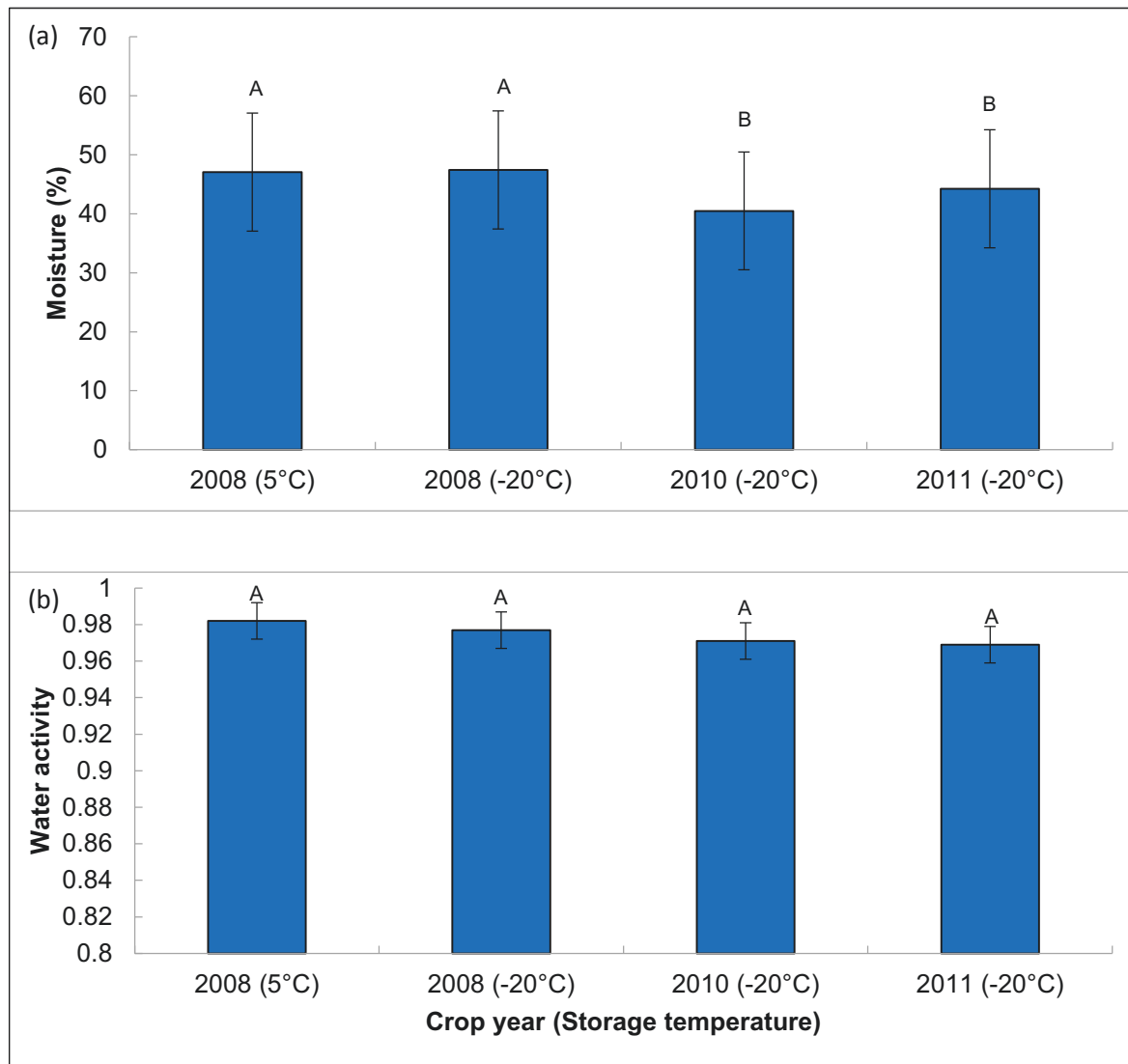


Figure 4.14: Effects of chilling of hazelnuts post storage at 5°C and -20°C on seeds: (a) moisture content; (b) water activity. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). Moisture content values are mean of ten replicates \pm SD; water activity values are mean of three replicates \pm SD. Storage treatments of hazelnuts are as detailed in Fig. 4.10.

Fig. 4.14 (a)

One-way ANOVA: %MC versus Sample year

Source	DF	SS	MS	F	P
Sample year	3	38.130	12.710	32.77	0.000
Error	33	12.799	0.388		
Total	36	50.929			

S = 0.6228 R-Sq = 74.87% R-Sq (adj) = 72.58%

Grouping Information Using Tukey's Method

Sample year	N	Mean	Grouping
2008 (5°C)	10	5.7700	A
2008 (-20°C)	10	5.7700	A
2011 (-20°C)	7	3.9571	B
2010 (-20°C)	10	3.6000	B

Means that do not share a letter are significantly different

Fig. 4.14 (b)

One-way ANOVA: Water activity versus Sample year

Source	DF	SS	MS	F	P
Sample year	3	0.2133	0.0711	1.07	0.416
Error	8	0.5329	0.0666		
Total	11	0.7462			

S = 0.2581 R-Sq = 28.59% R-Sq (adj) = 1.81%

Grouping Information Using Tukey's Method

Sample year	N	Mean	Grouping
2008 (5°C)	3	0.9823	A
2008 (-20°C)	3	0.9767	A
2010 (-20°C)	3	0.9713	A
2011 (-20°C)	3	0.6690	A

Means that do not share a letter are significantly different.

4.2.3.3 Conductivity of Seed Leachate

Seeds after storage at 5°C and -20°C and subsequent chilling were tested for leachate conductivity. Figure 4.15 shows the 2008 nuts held at 5°C leached similar conductivity to seeds from -20°C batches except the seeds from 2011 batch which showed lesser leachate conductivity.

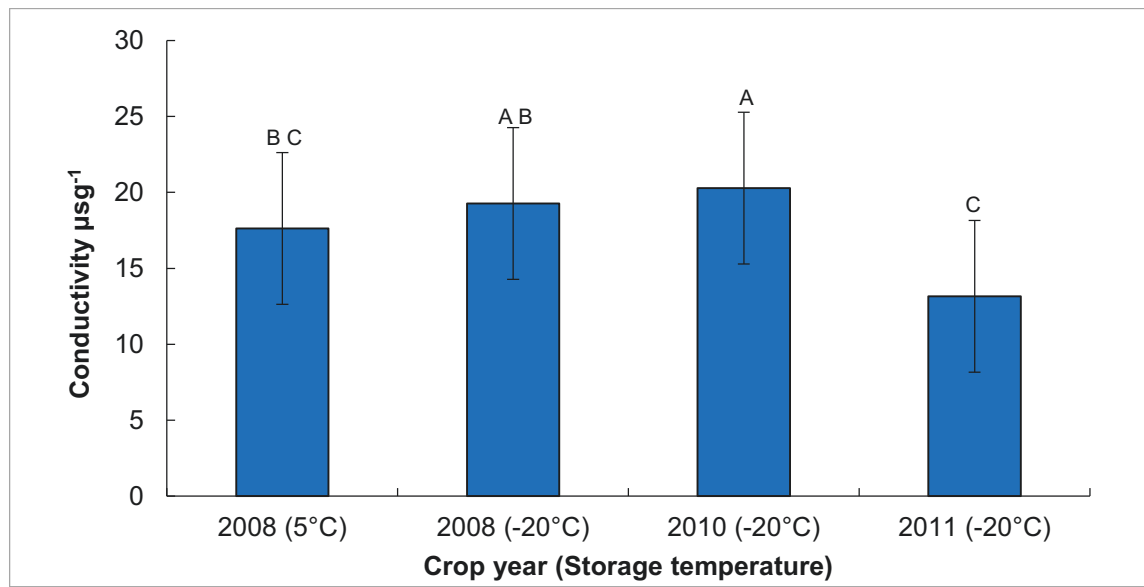


Figure 4.15: Effect of 6 weeks chilling of hazelnuts held at 5°C and -20°C on leachate conductivity. Values with different letters in the chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of five replicates \pm SD. Storage treatments of hazelnuts are as detailed in Fig. 4.10.

One-way ANOVA: Leachate conductivity versus Sample year

Source	DF	SS	MS	F	P
Sample year	3	98.14	32.71	9.17	0.000
Error	36	128.38	3.57		
Total	39	226.52			

S = 1.888 R-Sq = 43.32% R-Sq (adj) = 38.60%

Grouping Information Using Tukey's Method

Sample year	N	Mean	Grouping
2010 (-20°C)	10	11.720	A
2008 (-20°C)	10	9.890	A B
2008 (5°C)	10	9.277	B C
2011 (-20°C)	10	7.333	C

Means that do not share a letter are significantly different.

4.2.3.4 Seedling Performance

Seed germination was followed up with seedling performance test. After 2 weeks of growth, normal and abnormal seedling numbers were counted. Figure 4.16 shows germinated seeds from 2008 (5°C) crop showed only 17% normal seedlings but 83% exhibited various types of abnormalities. Seeds of each crop held at -20°C showed more than double in number of normal seedlings compared to 2008, 5°C crop thereby decreasing the abnormal seedlings from these seeds.

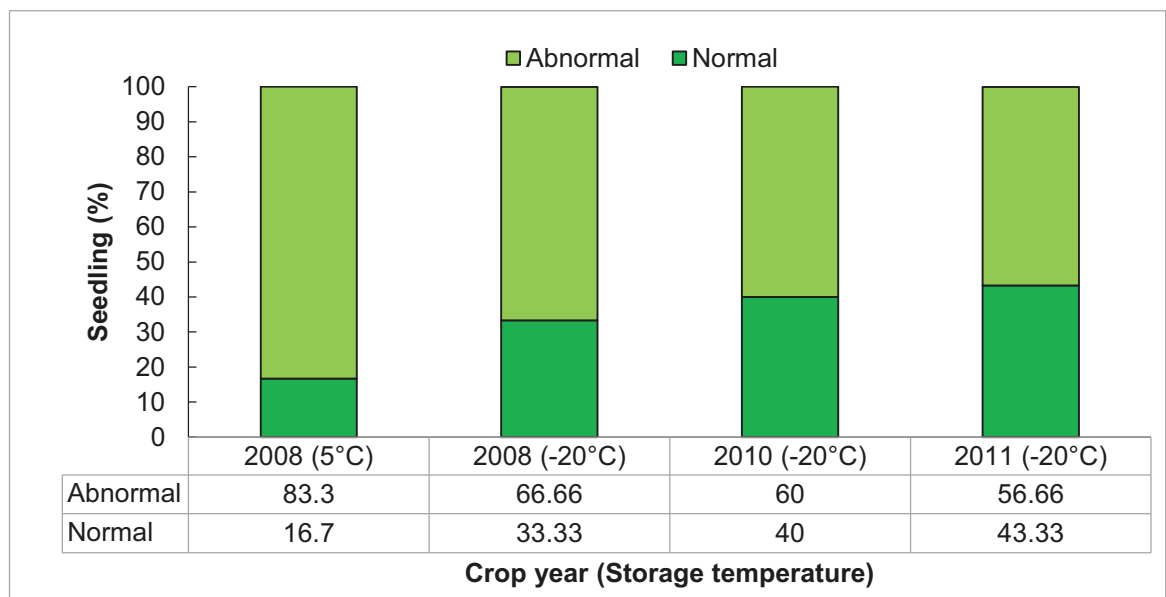


Figure 4.16: Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seedling performance. Hazelnuts held 5°C and -20°C were given 6 weeks chilling at 5°C prior to germination test. Germinated seeds were sown in ambient conditions and performance recorded after 2 weeks as detailed in Fig. 4.10. Storage treatments of hazelnuts are detailed in Table 4.1.

The effects of storage of hazelnuts at 5°C and -20°C on seedling establishment are shown in Figure 4.17. Seedling emergence time from seeds stored at 5°C needed more than 9 days whereas the germinated seeds from -20°C storage required less than 8 days (Fig. 4.17 a). Average height of the seedlings grown for 2 weeks show seedlings from 5°C storage seeds recorded 118 mm but the seedlings from each of -20°C crop were above 140 mm (Fig. 4.17 b). Internode numbers in all treatments however, show quite close to each other as seedlings from 5°C or -20°C contained between 7.4 - 8 internodes (Fig. 4.17 c).

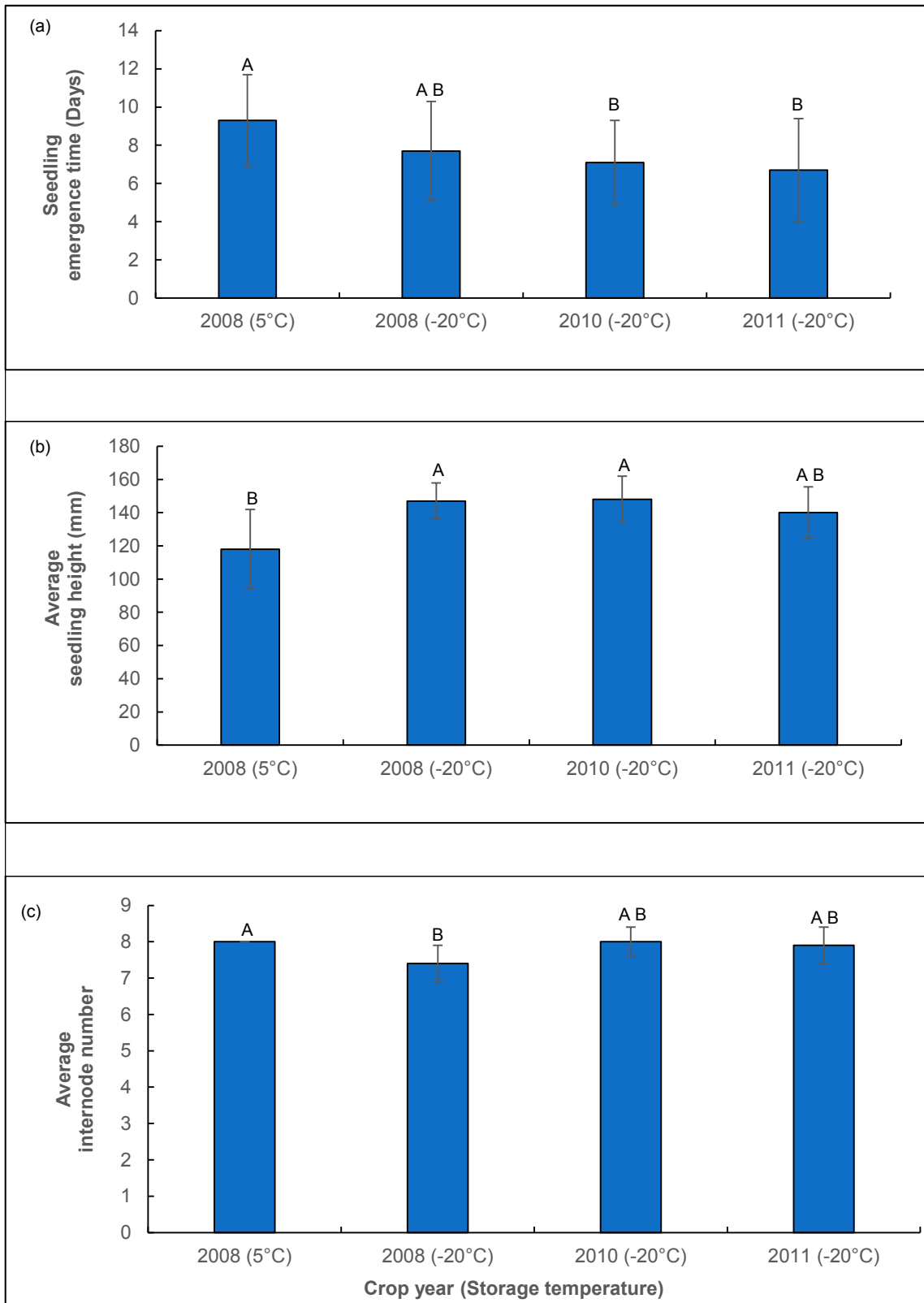


Figure 4.17: Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seedling performance: (a) Seedling emergence time (days), (b) average seedling height (mm) and (c) average internode numbers recorded after 2 weeks growth period. Values with different letters in each chart are significantly different (Tukey's test, $p < 0.05$). The values are mean of 30 replicates \pm SD. Seed germination of hazelnuts are as detailed in Fig. 4.10.

Fig 4.17(a) One-way ANOVA: Emergence day versus Sample year

Source	DF	SS	MS	F	P
Sample year	3	71.92	23.97	3.93	0.011
Error	86	524.40	6.10		
Total	89	596.32			

S = 2.469 R-Sq = 12.06% R-Sq (adj) = 8.99%

Grouping Information Using Tukey's Method

Sample year	N	Mean	Grouping
2008 (5°C)	14	9.357	A
2008 (-20°C)	18	7.722	A B
2010 (-20°C)	30	7.133	B
2011 (-20°C)	28	6.679	B

Means that do not share a letter are significantly different.

Fig 4.17(b) One-way ANOVA: Seedling height (mm) versus Sample year

Source	DF	SS	MS	F	P
Sample year	3	2934	978	4.34	0.011
Error	34	7665	225		
Total	37	10600			

S = 15.02 R-Sq = 27.68% R-Sq (adj) = 21.30%

Grouping Information Using Tukey's Method

Sample year	N	Mean	Grouping
2010 (-20°C)	11	147.91	A
2008 (-20°C)	10	147.00	A
2011 (-20°C)	13	140.15	A B
2008 (5°C)	4	118.25	B

Means that do not share a letter are significantly different.

Fig 4.17(c) One-way ANOVA: Internode numbers versus Sample year

Source	DF	SS	MS	F	P
Sample year	3	2.394	0.798	3.71	0.021
Error	34	7.317	0.215		
Total	37	9.711			

S = 0.4639 R-Sq = 24.65% R-Sq (adj) = 18.00%

Grouping Information Using Tukey's Method

Sample year	N	Mean	Grouping
2010 (-20°C)	11	8.0000	A
2008 (5°C)	5	8.0000	A B
2011 (-20°C)	12	7.9167	A B
2008 (-20°C)	10	7.4000	B

Means that do not share a letter are significantly different.

At the end of the 2 weeks' recording of seedling features viz., seedling emergence, height and internode numbers; seedlings were not discarded but grown up to 13 weeks. At the end of 13 weeks, seedling height and internode numbers were recorded. A comparative recording of 2 week and 13 week growth of the height and internode numbers are shown in Figure 4.18. Growth rate of seedling height among the 5°C and -20°C (2010 and 2011) treated seeds follows the same pattern after 13 weeks as seen at the end of 2 weeks in Figure 4.18 (a). Seedlings of 2008 (-20°C) batch showed decreased height when recorded after 13 weeks. Increase in internode numbers in all sets show a similar pattern as observed in Figure 4.18 (b).

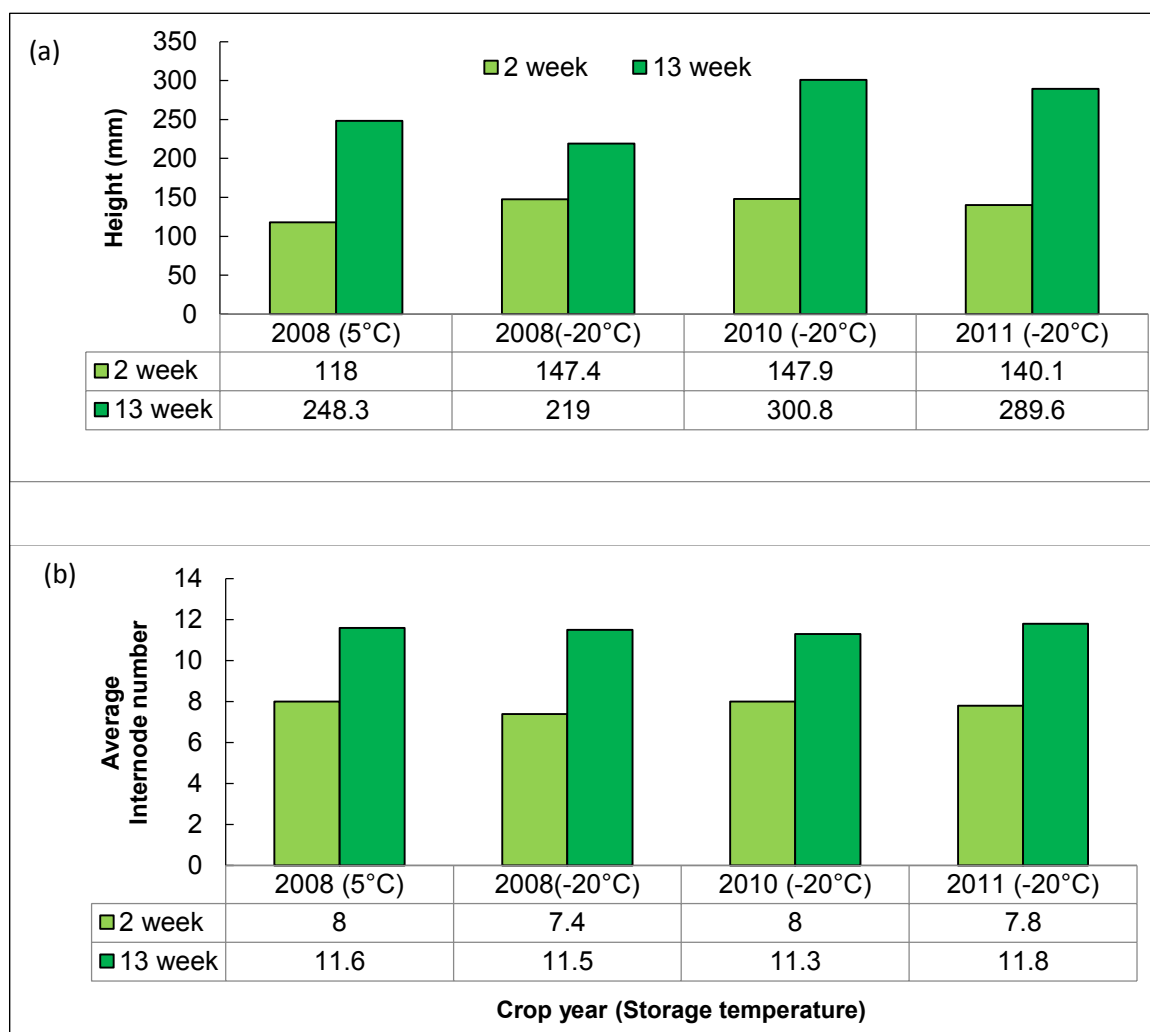


Figure 4.18: Effect of storage of newly harvested hazelnuts at 5°C and -20°C on seedling performance: (a) seedling height (mm) and (b) internode numbers recorded after 2 and 13 weeks growth period. Seed germination of hazelnuts are as detailed in Fig. 4.10.

Photograph in Figure 4.19 shows the physical appearances of the seedlings after 13 weeks growth. The various features of a seedling, viz., roots, stem, leaf, petiole, internode and apical bud look normal in these seedlings.

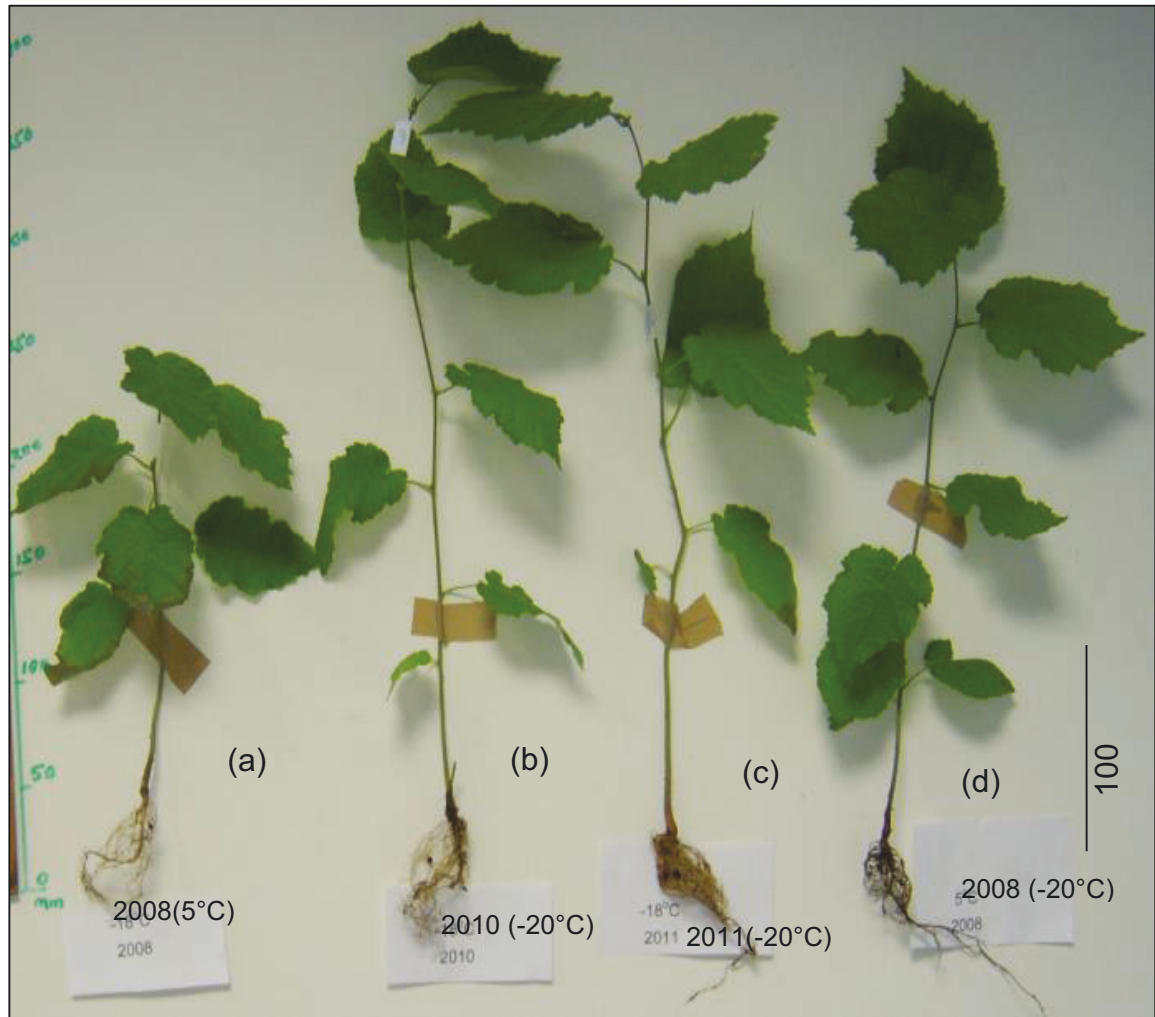


Figure 4.19: Photograph showing typical 13 week old seedlings grown from hazelnuts held in -20°C , and 5°C . After appropriate storage nuts were chilled for 6 weeks before germination test. Germinated seeds were sown for seedling performance. Seed germination of hazelnuts are as detailed in Fig. 4.10.

4.2.4 Discussion

4.2.4.1 Storage of Nuts at -20°C

Seeds held at -20°C were not able to carry out any metabolic activities. Therefore, to reactivate seed metabolism, the nuts were thawed and chilled for 6 weeks which allowed seeds to imbibe sufficient water and begin metabolic activities resulting in germination when transferred to higher temperature (Bradbeer, 1968).

Being equally imbibed, seed moisture content or water activity among the batches showed similar responses. Storage of hazelnuts of 2010 and 2011 for 80 and 36 weeks respectively also resulted in 83 and 70% seed germination. These results showed that hazel seeds with seed moisture content <4% can survive after the standard 6 weeks chilling.

The adverse impact of storage at 5°C was indicated in germination lag phase compared to storage at -20°C. Further signs of stress resulting from 5°C storage were expressed in MDG, PV and GI, which were significantly lower than the -20°C stored seeds. Increase in infection and reduction in germination indicated that 5°C is not suitable for storage of hazelnut and suggested non-orthodox mechanisms. Storage at either temperature did not show any noticeable difference in leachate conductivity indicating that membrane disruption was not a limiting parameter. This suggested that some stress processes were mitigated by seed storage at both storage temperature and post chilling treatments.

The TTC test results (sections 3.3, 3.5 and 3.7) gave an indication of the impact of stress on seed tissues. Thus free radical attack resulted with various levels of cellular damages, which led to several types of seedling abnormalities. Therefore, seed health should be assessed properly before subjecting hazelnuts to different tests. Also, in case of expected adversities, appropriate remedial methods should be applied before seeds are passed through stress related tests. For example, increases in chilling times may ensure better seed recovery rates.

4.2.4.2 Seedling Performance

The deleterious effects of 5°C storage on seeds are shown as 17% normal seedlings resulted, whereas the -20°C stored seeds produced more than double normal seedlings. Specifically, storage at 5°C resulted in delayed seedling emergence and

reduced height indicating impact on metabolism. Nevertheless, there was no significant difference in internode number (Fig. 4.17), which may be an inherent genetic property or genetically inherent to the species. Also, 2-week and 13-week seedlings showed similar growth patterns and suggested that seed damage was most likely done through pre-storage preparation method. Hence, as discussed in 4.2.4.1, more care is needed to improve performance in the post storage recovery.

Results from seed germination and seedling performance showed that although seed germination and development of some normal seedlings resulted after 189 weeks at 5°C, storage at 5°C is not necessarily more beneficial compared to -20°C storage. On the other hand, storage at -20°C seemed essential for high rates of seed germination and normal seedling establishment. The main indication is that hazelnut has been classified in all three storage groups and thus, if we accept hazelnut as recalcitrant (Hong & Ellis, 1996) or intermediate (Ellis, et al., 1990) then this germination and seedling establishment is appreciable. In case hazelnut is orthodox as claimed (Gosling, 2007; Michalak, et al., 2013), then it is evident from this investigation that it was made to behave as an orthodox. Therefore, improvements in experimental techniques to verify the status may be required.

Seed germination (<10%) and 50% seedling production of germinated hazel seeds held in LN have been reported (Michalak, et al., 2013). However, <10% of seed germination is not very encouraging hence alternative storage methods and/or conditions that result in higher germination rates must be explored. Cryopreservation of hazel embryonic axes provides opportunities for storage of a large number of samples in a small space but requires a high-tech methodology for restoration of viability and seedling production (Reed, et al., 1994). Therefore, germination of hazel seeds after 189 weeks of storage at -20°C, and establishment

of seedlings, identified the possibility of medium-term storage. Seed storage at 5°C and -20°C has been most effective for orthodox seeds (Ellis, et al., 1996). For hazelnut, the seeds were made to behave as orthodox. This was possible due to dormancy in hazel seeds where chemicals such as ABA block germination metabolism and, as a result, protect the seeds from metabolic stress.

Preservation of whole seed provides appropriate natural and balanced nutrition for the emerging seedlings which are not available when seed size is reduced (half seed) or only the embryonic axes are used to accommodate growth of increased numbers on exogenous nutrients. It has been reported that removal of one whole cotyledon or two half cotyledons results in smaller seedlings while embryonic axes on their own failed to produce any seedlings (Rendon, 1983). Furthermore, reduction in seed moisture content before germination and seedling development also resulted in reduced seedling performance (section 3.1). Therefore, whole seeds should be preserved and used for seedling production to give the developing embryonic axes the natural and normal quota of nutrition.

4.2.5 Summary

Investigations of hazelnut storage conditions are needed to clarify the storage habit as three opposing conditions are recorded in literature (Table 1.2).

In this study, it is observed that hazelnuts with seed moisture content <6% (FW) survived 189 weeks at 5°C and -20°C. Seed germination and seedling growth with normal features was greater at -20°C than at 5°C. Storage at 5°C showed more detrimental features in germination process, viz., reduced MDG, PV and GI and similar weak performance in seedling emergence time and height compared to -20°C stored seeds. Since seeds from both conditions resulted in germination and seedlings, more experiments are needed to improve both results. The ability of hazel

seeds to tolerate dehydration to <6% (MC), retain seed viability for 189 weeks resulting in 30 and 87% germination at 5°C and -20°C respectively indicate this recalcitrant seed may possess some inherent desiccation tolerant mechanism more commonly associated with an orthodox habit. This is the first report of successful storage of hazel seeds for such long periods.

The need to improve hazelnut propagation and safe storage is getting more interesting with emerging reports of the production of anticancer drug Taxol in hazelnut plants. Hazelnuts are reported to have high Taxol content which is reported to be more active than commercial drugs (Bemani, et al., 2012). The potential relevance of hazelnut in this aspect will increase its market demand which will not only maintain but increase in global orchard sizes.

5 General Discussion

Studies have placed hazelnuts in all three storage groups, viz., recalcitrant, intermediate and orthodox. Besides the organoleptic, nutritional and nutraceutical properties, the importance of hazelnuts has increased tremendously with the discovery of the anticancer drug Taxol. Therefore, critical investigations on the various physiological and biochemical aspects of the germination, viability and storage became inevitable.

Since hazelnuts are non-dormant at harvest, but develop seed dormancy after a few days of harvest, the physiological studies for this programme/thesis focused on several chemical and biochemical aspects of seed dormancy and recalcitrance.

5.1 Tests of Seed Dormancy

This investigation was based on testing seed dormancy by several techniques. Thus, some chemical and biochemical aspects like water activity in relation to germination necessitated seed viability test by tetrazolium test, leachate conductivity and role of antioxidants. Also, all tests related to seed dormancy need a supporting seed germination result.

5.1.1 Effects of Stabilisation Treatment

The first test was the stabilisation of moisture content in the seeds and nuts to check the impact on germination and seedling development. Variability in germination related to seed moisture content has been reported in *Avicennia* (Berjak, et al., 1984), *Hevea brasiliensis* (Normah, 1987), *Nephelium lappaceum* & *Artocarpus heterophyllus* (Normah, et al., 1994). However, in this study, hazel seed germination did not indicate any effect of moisture content stabilisation, remaining constant of around 80% in all treatments (section 3.1). Recalcitrant *Telfaria occidentalis* (Nkang, et al., 2000) showed similar germination enhancement by limited desiccation. In a further investigation with the same species (Nkang, et al., 2003) reported that seeds

desiccated for 6 days at either 5°C or 25°C showed increased germination but a decline thereafter when moisture content dropped below 30% on the day 9. Increases in germination of recalcitrant *Aesculus hippocastanum* L. and *Quercus robur* L. by desiccation were also reported (Tompsett & Pritchard, 1998; Finch-Savage & Blake, 1994).

For hazelnuts, seedling growth from the germinated seeds exposed to stabilisation treatments as described in section 3.1 resulted in some abnormal seedlings, possibly attributed to volatile gas accumulation in the stabilisation container which affected the plumular part of the embryonic axis. Reports suggested that desiccation could be harmful particularly in recalcitrant seeds due to the involvement of acetaldehyde, methanol and ethanol in the deterioration of *Ligustrum japonicum*, *Quercus serrata*, *Quercus myrsinaefolia* and *Camilla japonica* (Akomito, et al., 2004). The differences in germination rate and health of seedlings between seeds and intact nuts (Figure 3.3 c) may be due to additional stress on seeds within nuts as they are enclosed in a smaller environment and exposed to concentrated volatiles than seeds removed from the shell and stored in the container where they have more space and diluted volatiles. But overall health of individual seeds is the main deciding parameter for germination and seedling establishment.

Stabilisation treatment resulted in hazel seeds maintaining between 21-24% MC which is within the range classified as 'Type 2 water'. This parameter predicts damage of intermediate seeds (Vertucci & Farrant, 1995) where this water level is not protective of the organelles since protein structure destabilises and free radical production degrades enzymes. In the current study, survival of hazel seed indicated the involvement of some inherent protective mechanisms which countered these negative impacts. Based on per 100 g seed sample, hazel seeds are reported to

contain 2.2 mg ascorbic acid as well as 2-5% sucrose along with raffinose and stachyose which are linked to membrane stabilisation (Duke, 1989). These could be involved in the protection mechanism in hazel seeds.

Results from this experiment emphasized three aspects of seed physiology:

- i) dormancy is not imposed if germination is tested immediately after dehydration
- ii) seed germination does not necessarily reflect normal/healthy seedling establishment, and
- iii) a variety of abnormalities in seedling performance may result from (stress related) desiccation of seeds which should be addressed prior to germination test.

5.1.2 Effects of Dormancy Reversal Treatments

Two dormancy reversal tests were designed using non-dormant hazelnuts with successive alternation of storage temperature (Treatment 1: 5°C-15°C-5°C; Treatment 2: 15°C-5°C-15°C; 6 weeks at each temperature). Cumulative results of Treatment 1 showed that seed moisture was maintained between 31 and 28% compared to 36% in the control during the three alternations. Final seed germination was recorded between 72 and 83% compared to 80% in control. This result is in line with the findings of Bradbeer (1988), who reported that hazel seeds can survive from 6 months to a year in storage. In Treatment 1, seeds underwent two terms at 5°C and once at 15°C. It is likely that dual storage at 5°C helped in GA synthesis hence no change in final germination (Ross & Bradbeer, 1971a). Delayed germination, reduced MDG, PV and GI at 15°C indicated an impact of water stress while the final germination showed no significant difference between the treatments.

Significance in the tests was supported by seedling performance. The impact of water stress can be seen in increased time for seedling emergence, increase of abnormal seedlings and decreased seedling height and internode numbers at different stages in the Treatment 1 (Table 3.2). These changes may be due to the detrimental effect of water stress on the embryonic axes and cotyledons. Water stress might have led to free radical production, damaging seed tissues. In Treatment 2 was more detrimental (Table 3.3) and two terms at 15°C led to a larger decline in seed germination (to 28%), emergence time, increase in abnormal seedlings, seedling height decreases and reduction in internode number at different stages. These were also attributed water stress.

The test results showed seed dormancy was not imposed in hydrated storage but the reduction in germination was probably due to stress which is visible in seedlings. This experiment revealed that seed moisture and temperature needed to be monitored and managed carefully to protect the seed against the detrimental actions of water stress. Further tests targetting the impact of water stress on the role of water activity in relation to free radicals and leachate conductivity, at 5°C and ambient temperature (RT) storage, need to be assessed for the source of damage and possible mechanism of prevention.

5.1.3 Dormancy Breaking in Hazel Seeds

Several chemical treatments have been used to break hazel seed dormancy (Table 1.5). Other work has used chilling, which substitutes the natural method of stratification, to break dormancy. The most common method involves 6 weeks chilling in hazelnuts (Bradbeer, 1968; Ross & Bradbeer, 1971a; Williams, et al., 1973; Arias, 1976). This practice was followed in this thesis. A gradual increase in dormancy breaking (Fig. 3.22), increased germination and reduced infection (section 3.4) was observed with increase in chilling time. Seeds achieved full

imbibition after two weeks chilling and water activity values were corresponded to seed MC. Chilling increased antioxidant activities (section 3.4.3.3). Total elimination of infection after 6 weeks chilling suggested a pathogen repressing mechanism and the role of endophytes was a possible suggestion as indicated in Figure 3.35 and 3.40.

5.2 Recalcitrance in Hazelnuts

5.2.1 Test of Recalcitrance in Hazelnuts

Seed germination was used as a test of recalcitrance in hazel seeds. Desiccated non-dormant seeds and seeds imbibed in gibberellic acid were stored for up to 6 weeks at 5°C or at ambient room temperature. Viability tests showed a gradual reduction in germination but ungerminated seeds showed viability by TTC test (Fig. 4.1). Water activity during the storage periods were corresponding to moisture content. Sharp increases in leachate conductivity indicated damage. Similar increases in leachate conductivity with decreases in seed performance were reported for recalcitrant of *Kielmeyera coriacea* Mart. seeds (Ramos, et al., 2012). Also, other studies reported increased leachate conductivity with poor performance in radish seeds (Mavi, et al., 2014). Storage in ambient conditions showed a reduction in germination and an increase in infection and increase in leachate conductivity (sections 4.4 and 4.8). In particular, the decline in germination of non-dormant hazel seeds stored at 5°C or at ambient room temperature indicated its closeness to seed recalcitrance. Nevertheless, impacts of desiccation on protective sugars and LEA proteins are essential to assess the desiccation tolerance as this species has been placed in the orthodox group by Gosling (2007) and Michalak, et al. (2013). Non-desiccated GA and IBA treated recalcitrant seeds of *Myristica dactyloides* Gaertn. maintained viability up to three months (seed MC 34% FW) stored at 20°C (Sivakumar, et al., 2006). Exogenous application of growth regulators

have released dormancy and increased germination in seeds showing physiological dormancy (Baskin and Baskin, 1998). Although Pritchard and Tompsett (1995) stated that a combination of recalcitrance and dormancy are rare phenomenon but the observations in this investigation contributes to the knowledge of such a rare case.

5.2.2 Effects of Storage of Non-dormant Hazelnuts at 5°C and at Ambient Temperature

Non-dormant hazelnuts (section 3.3) were held for one treatment of 6 weeks storage each at 5°C and at room temperature to investigate the imposition of dormancy. Storage of non-dormant hazelnuts at 5°C resulted in increased germination (Fig. 3.16 a), unchanged MDG/PV/GI values (Fig. 3.17 a), and good viability of non-germinated seeds (Fig. 3.16 a). On the other hand, there was an increase in lag phase and reduced seed moisture, indicating some physiological stress and the first sign of infection (Table 3.4). The deleterious impact of stress on membranes was evident from the increase in leachate conductivity (Fig. 3.20 a). Interestingly, increase in antioxidant activity was observed (Fig. 3.21 a) suggesting an antioxidant system activated to counter the free radical actions (Bailly, 2004; Pukacka & Ratajczak, 2006; Zhang, et al., 2011; Luo, et al., 2012).

The relatively small impact of 5°C compares with dramatic impact of storage of hydrated non-dormant hazelnuts at RT. This resulted in a considerable reduction of seed germination (Fig. 3.16 b) and sharp decrease in MC and water activity (sections 3.3.3.7 and 3.3.3.8). While TTC viability test showed that un-germinated seeds were viable, there was significant infection (Table 3.5). The impact of RT storage on hydrated seeds is expressed in the delay of first germination, sharp reductions of MDG, PV and GI values (Fig. 3.17) indicated the damaging effects of hydrated storage at RT.

The benefit of TTC test is that it exposes the damaged areas and hence gives the clue for improving seed health to overcome the defects. Figure 3.15 represents a cross section of some distinct staining patterns in seeds which showed the biologically active tissues and less active areas as a result of various treatments and possible impact of free radicals. Thus, the TTC tests (Fig. 3.15 a) revealed some faintly stained cotyledons indicating weak dehydrogenase reaction with white areas revealing the total dead tissue. Therefore, it is assumed that free radical actions may have caused the seedling damages which resulted as abnormal plants developed from faintly stained cotyledons and embryonic axis, reduced growth developed from faintly stained cotyledons and dead shoot resulted from dead plumule. Observations from various experiments indicated that TTC test results must be checked carefully to assess the level or extent of damages. It has been observed that TTC staining pattern of seeds under stress show inactivity or dead parts within the cotyledon (Schafer, et al., 2002) or even the dead *Acer* and *Ulnas* embryonic axis (Phartyal, et al., 2003).

Leachate conductivity more than doubled compared to control after 6 weeks, correlates to increase in infection and delay in germination lag phase (Table 3.5). Although only one DPPH assay was possible as the samples were lost during experiment, the result obtained indicated that the stressed seed had intensified the synthesis or release of antioxidants to counter free radical threats. Similarly, leachate conductivity has been reported to increase with decrease in seed performance in recalcitrant seeds of *Quercus nigra* L. (Bonner, 1996) and in *Kielmeyera coriacea* Mart. seeds (Ramos, et al., 2012).

5.2.3 Acclimatisation of Hazel Seeds

Seeds need to be held in storage for propagation and continuation. A fundamental observation is that seed storage depends on seed moisture content (Roberts, 1973).

The acclimatisation test of lowering hazel seed moisture to <6% (FW) and 45 weeks storage at 5°C resulted in two observations:

i) seeds showed 40% viability by TTC test directly following the storage at reduced moisture. Examination of the TTC treated seeds revealed areas where damage was most prevalent. (Fig. 3.31).

ii) seeds subjected to a further 6 weeks chilling with imbibing of exogenous water (dormancy breaking treatment) resulted in profuse infection and thus the test was discarded (result not shown).

Therefore, hazel seeds can survive desiccation to <6% MC but the failure of germination and evidence of damage revealed by the TTC test, which vividly identified the areas and severity of stress, revealed that seed on its own is not suitable for survival but needs to be within the shell and stored as nuts. Consequently, naked seed became vulnerable to pathogen during the 6 week chilling. It is thus suggested that all moisture reduction and chilling tests should be done with whole hazelnuts. Although Rendon (1983) reported germination of whole hazelnuts after prolonged chilling, germination of seeds after the appropriate treatments are preferable as this method reduces germination time.

5.2.4 Infections in Hazel Seeds

Seed loss due to pathogen is detrimental for seed preservation and seedling propagation. The types of pathogens reported for hazel are listed (Duke, 1989; Abdel-Hafez & Saber, 1993). During the present investigation, seed infections were observed and hence some aspects of hazel seed pathogen were tested. The presence of pathogens is evident in Figures 3.34, 3.35 and 3.37, while Figure 3.36 showed a healthy seedling despite the parent seed contained pathogens. Subsequent pathogen testing showed the presence of pathogens in all parts of

hazelnut viz., pericarp, testa, cotyledon and axis (Fig. 3.39). Infection was recorded during chilling of dormant seeds (hazelnuts) but eliminated at the end of chilling treatment (6 weeks) (Fig. 3.23). The unstained parts in Figure 3.40 may be the result of free radical attacks on the pathogens during stress period or the impact of phytoalexins produced by the tissues in response to pathogens (Ahuja, et al., 2012). It is encouraging to note that appropriate chilling inactivated pathogens by an as yet undetermined mechanism. Another interest in hazelnut is the discovery that they produce the anticancer drug Taxol (Hoffman, et al., 1998; Boone, et al., 2000) linked to endophytes, which has increased the importance of hazelnuts from food to medicinal uses.

5.2.5 Free Radicals and Antioxidants in Hazelnuts

Exploration of antioxidants from hazelnut byproducts has been linked to commercial advantage (Shahidi, et al., 2007) but the importance of antioxidants for the physiological and protection benefits to hazel seed has not been examined. A series of experiments in the current study revealed that hazel seed associates contain antioxidants which showed increased activity with increase in chilling time (Fig. 3.58). Figure 3.21 shows that the tissues of non-dormant seeds held at 5°C or at ambient temperature released antioxidants even when under water stress. At 5°C, even though no external water was added, a steady increase in activity was noted. A similar trend was recorded in the seeds held at ambient temperature indicating that, once hydrated, antioxidant enzymes are possibly activated and release antioxidants (Tommasi, et al., 2006). The increase in antioxidant contents in seed associates during chilling are, therefore, vital for the safety and longevity of hazel seed as was also shown in dormancy breaking treatment where 6 weeks chilling broke seed dormancy and eliminated infection. Therefore, the role of antioxidant in relation to pathogen action during chilling needs further tests.

5.3 Storage of Hazelnuts at -20°C

Experiments linked with seed dormancy and recalcitrance have shown that viability increases with an increasing chilling period but has adverse effects on storage of partially or fully hydrated seeds. Hence, desiccation of seeds to low moisture content to make them behave as orthodox was tested. Desiccated seeds (hazelnuts) held at 5°C up to 189 weeks retained 30% germinability although 60% were infected. The same batch held in -20°C for 189 weeks resulted in 87% germination and 13% were infected as shown in Figure 4.10 and 4.12. Seedlings grown from the 5°C batch showed 17% normal, while those from the -20°C samples recorded 33%. Development of healthy (normal) seedlings is a criterion that would confirm the success of the test. Hazelnuts of 2010 harvest stored for 80 weeks at -20°C having 3.5% MC resulted in 83% germination whereas the 2011 harvest recorded 70% germination after 36 weeks but also recorded 9 and 19% infection respectively. Normal seedlings from the germinated seeds showed 40 and 43% in 2010 and 2011 samples, respectively. Although seeds survived desiccation to low moisture content and subjected to 5°C and -20°C storage, it cannot be considered orthodox or intermediate unless confirmatory investigation of protective sugars and LEA proteins is conducted. Infection in all seeds from -20°C and 5°C requires an identification of the endophytes. Thus, is the infection due to pathogens, seed batch health or free radicals?

Cryopreservation of whole hazel seed, leading to germination and seedling production (Michalak, et al., 2013), indicated an orthodox habit. However, the investigation of protective sugars and LEA proteins is required to confirm its storage nature. To facilitate cryopreservation in recalcitrant *Acer saccharinum* seeds, embryonic axes were separated and desiccated to low moisture content (Pukacka & Ratajczak, 2006). Although this practice allows embryonic axes to be preserved,

a drop in antioxidants has been reported during desiccation (Hendry, et al., 1993). It is assumed, therefore, that embryonic axis on its own is more vulnerable to free radical attacks during regeneration as it is devoid of nutrient supply from cotyledons which is also a vast source of antioxidant enzymes (Hendry, et al., 1993). In this study, the vulnerability of embryonic axes to FR attack was evident in acclimatised hazel embryonic axes (Fig. 3.31) where, although 60% axes were damaged, the remaining 40% showed viability.

5.4 Summary

A brief account of the results of various tests on seed dormancy and recalcitrance on hazelnuts is as follows: The first objective for this investigation was to determine the dormancy breaking mechanism. Figure 5.1 summarises the role of hazelnut associates in releasing antioxidants for combating the impact of free radicals to prevent any stress during dormancy breaking. As indicated in Column (A), desiccation and ageing of seeds has been suggested by other authors (Kranner, et al., 2008) as a mechanism leading to fatality of hazel seeds. This was overcome by appropriate chilling of hazelnuts in this experiment as shown in Column (B). The role of hazel seed associates in providing antioxidants during chilling had been the best indicator of seed survival post stress period (dormant, dehydrated seeds). The involvement of seed associates has been elaborated in Figure 3.58 and 3.59.

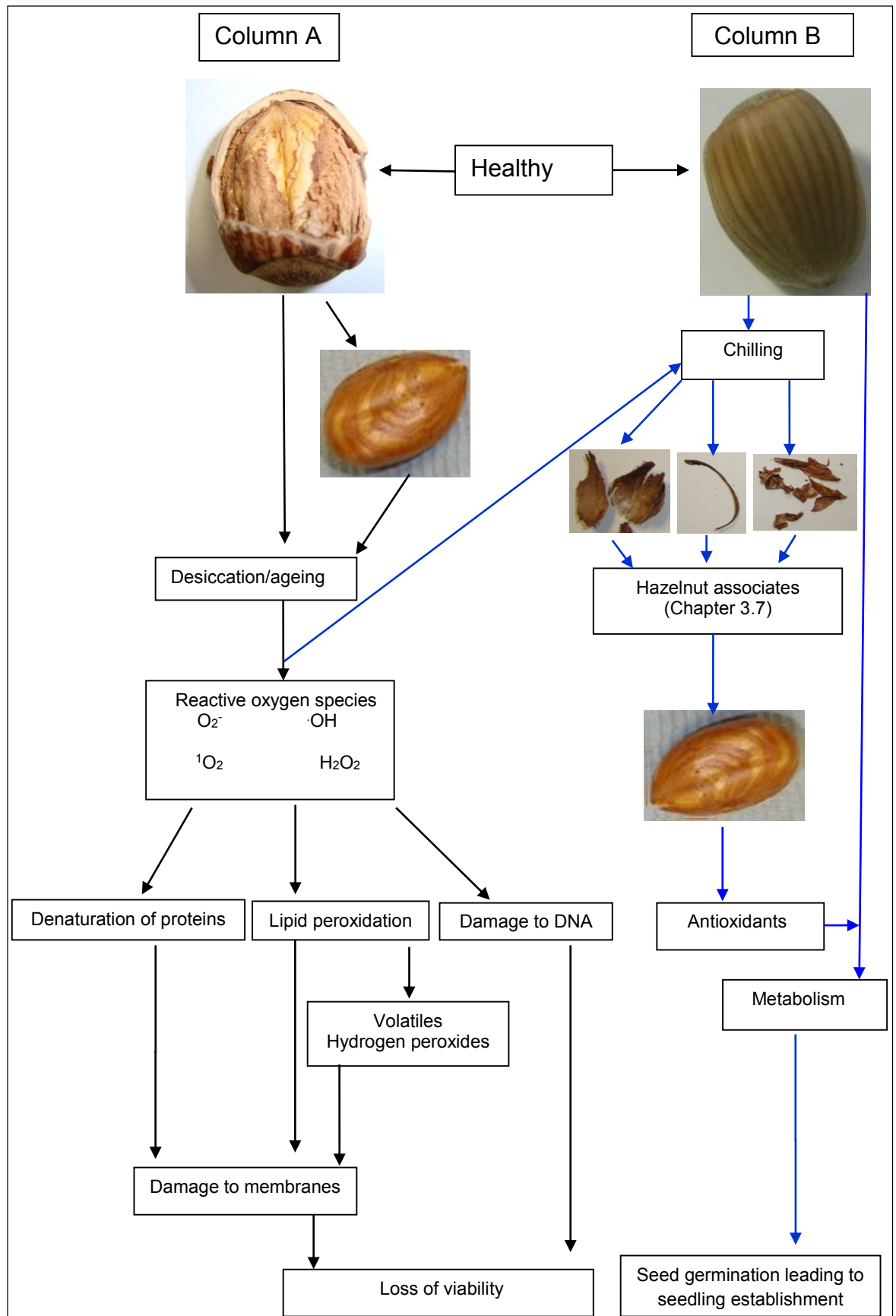


Figure 5.1: Role of chilling on restoration of hazel seed viability. A) Simplified model of free radical initiated viability loss proposed (incorporated from (Kranner, et al., 2008) and B) counter action of antioxidants by chilling hazelnuts from this experiments. Blue lines indicate the modifications tested in this experiment.

Figure 5.2 compares the effects of dehydrated and hydrated storage at 5°C and at room temperature for 6 and 189 weeks. Although moist storage at 5°C was better than room temperature storage in terms of seed germination, and 30% seeds survived 189 weeks at 5°C, a variety of staining indicated that ungerminated seeds were subject to stress as shown in Column A. The seedlings developed from the germinated seeds may show the abnormalities presented in Column B.

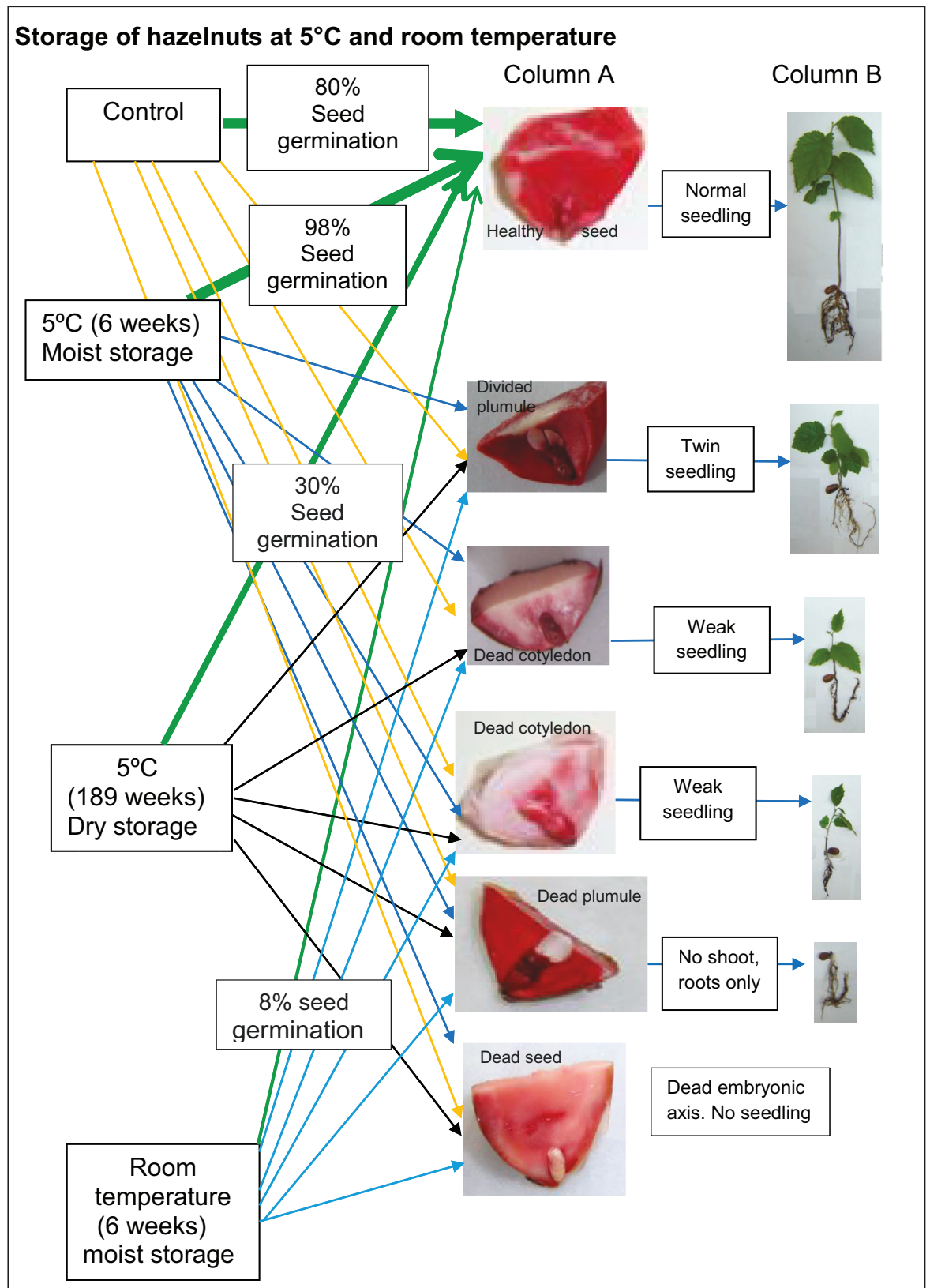


Figure 5.2: Viability of hazelnut seeds after storage for 189 weeks at 5°C and 6 weeks each at 5°C and room temperature. A) TTC test done on un-germinated seeds reveal the following staining patterns which likely correspond to the seedling indicated. B) Germinated seeds after physiological tests result in the varieties of normal and abnormal seedlings shown vertically. Included images are illustrative of different defects and are not necessarily taken from the specific treatments included in this figure. See also Figure 5.3.

The impact of desiccation and storage of hazelnuts at 5°C and -20°C involving germination, TTC staining and seedling production is illustrated in Figure 5.3. Hazelnuts held at -20°C for up to 189 weeks compared to the same batch was held at 5°C and subjected to 6 weeks chilling at 5°C resulted in higher germination. The observation of the staining pattern of the ungerminated seeds shown in Figure 5.3 (column A) and the possible resulting seedling pattern in Figure 5.3 (column B) confirms stress inflicted on the seeds during the treatments. In general, this experiment showed that hazelnuts can be held at -20°C for medium term storage with a subsequent success of 87% germination.

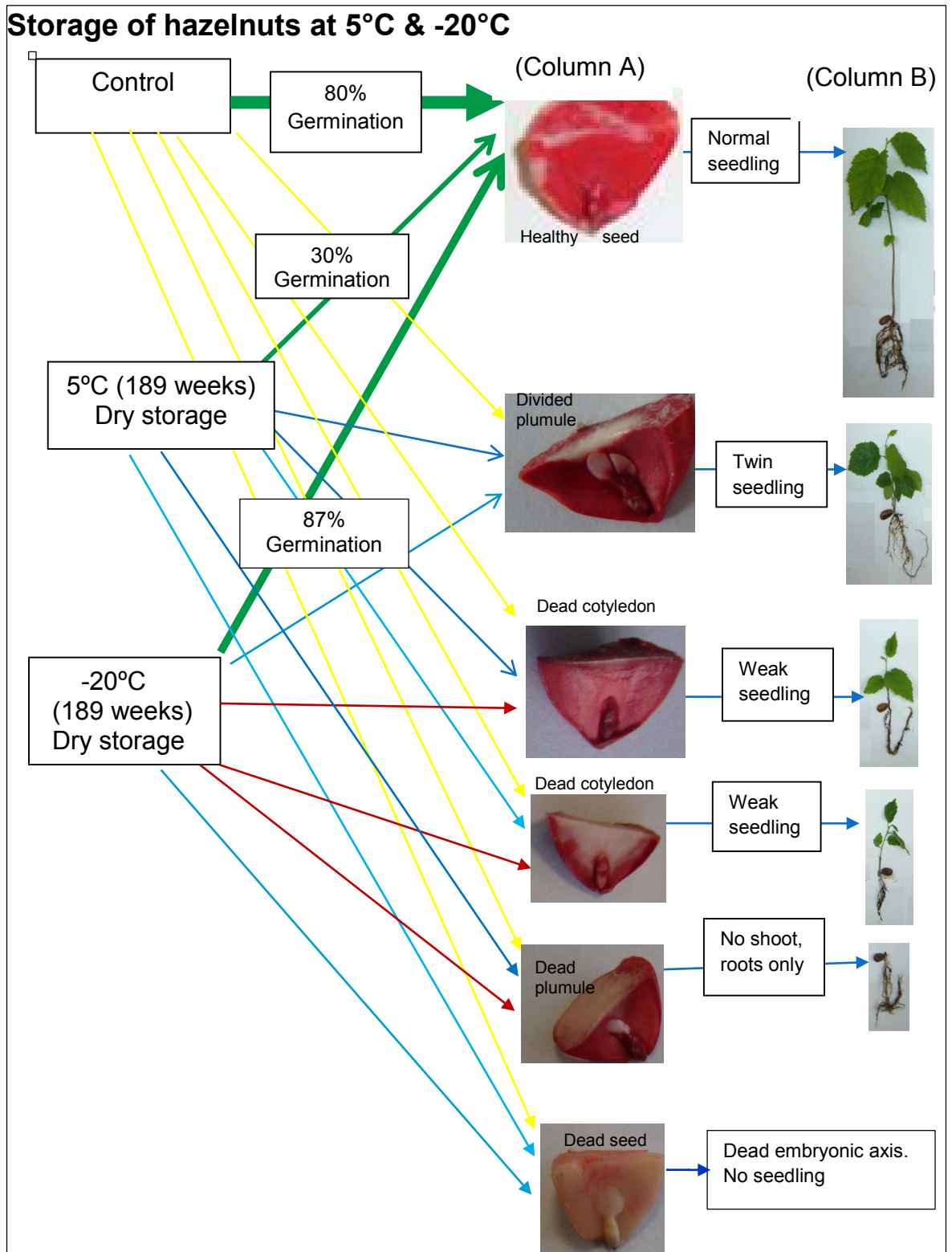


Figure 5.3: Viability of hazelnut seeds after storage for 189 weeks at 5°C and -20°C. Column A) TTC test done on un-germinated seeds reveal the following staining patterns which likely correspond to the seedling indicated. Column B) Germinated seeds after physiological tests result in the varieties of normal and abnormal seedlings shown vertically. See also Figure 5.2.

A model in Figure 5.4 follows the pathways that lead to loss of viability in hazel seeds as well as the by-pass that enable seeds to undergo chilling and regain full viability via protective antioxidants from seed associates.

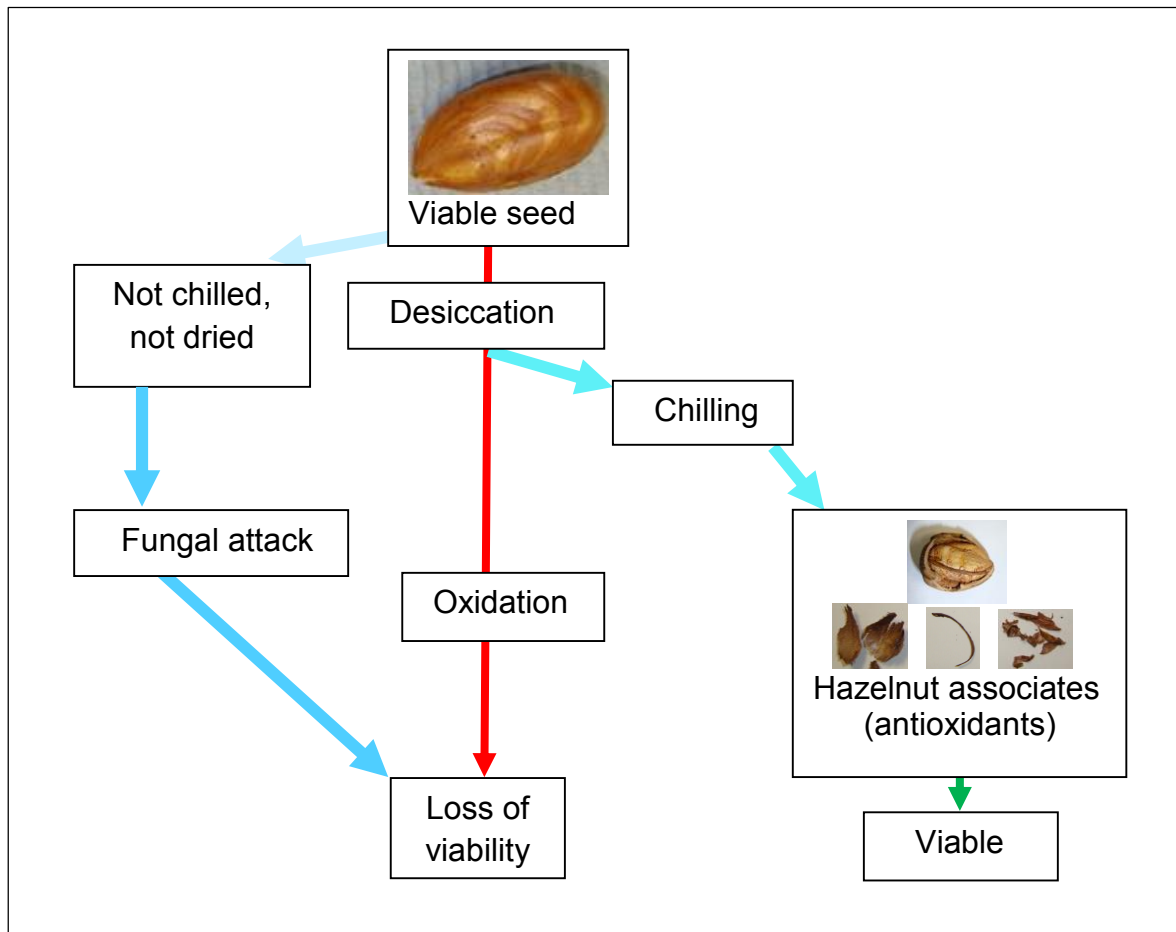


Figure 5.4: A model to illustrate the role of antioxidants in the viability of desiccated hazel seeds. Chilling activates antioxidants which counters the free radicals to enable seeds' normal metabolic activities leading to germination.

6 Conclusions

Due to commercial importance ranging from food values to medicinal importance, continuation of hazelnuts become important. Hazelnuts are considered as recalcitrant as seeds are shed with >40% seed moisture and develop dormancy after a few days of storage and lose viability after a year following harvest. It is not easy to test for recalcitrance in hazelnuts as the seeds also show dormancy. As discussed in section 1.8.2, results from various laboratories has placed hazelnuts in all three storage groups (recalcitrant, orthodox or intermediate) thereby drawing more interest in further investigations. Results in this thesis support the suggestion that hazel has some properties associated with recalcitrance, but also can be made to show orthodox properties. This conclusion, that hazel can be either orthodox or recalcitrant depending on the circumstances, is similar to conclusions drawn by Ellis, et al., (1990) when studying *Coffea arabica* L. but has not previously been considered for hazel.

Thus, tests for viability following 5°C and room temperature storage show recalcitrance nature as the seeds show reduced germination and water stress damages caused by free radicals as revealed by TTC staining and membrane damages revealed by increased leachates (see results in section 4.1). Damages to seeds were more prominent at room temperature than at 5°C. While results in section 3.5 show that when seed moisture content was reduced by dehydration-rehydration-dehydration and acclimatisation test, storage at 5°C resulted in partly oily and oily seeds, but also some apparently normal healthy seeds. The TTC test suggested that about half of the normal looking seeds were viable (Fig. 3.31) but were affected by profuse infection and failed to germinate. These observations fit well with the suggestions made by Pammenter et al (1994) explaining why stored hydrated recalcitrant seeds die.

On the other hand, results discussed in section 4.2 show that hazelnuts were made to behave as orthodox seed by dehydrating to <6% (FW) MC and could be stored for 189 weeks either at 5°C or at -20°C. Chilling of these seeds restored in 30% and 87% germination respectively. The seedlings produced show normal features indicating normal metabolic activities. Results (Figs. 4.10, 4.16 and 4.19) show that storage at -20°C were preferable to 5°C for medium term storage. This ability to survive at low moisture and temperature is a characteristic of orthodox seeds (Roberts, 1973). Recently it has also been demonstrated that hazel seeds can survive storage in Liquid N₂ (Michalak, et al., 2013).

These findings clearly point towards the possibility that hazel seed contains some protective mechanism and support reports that desiccation sensitivity and storage behaviour of recalcitrant seeds differ greatly among species (Pammenter, et al., 1994) and suggestions that there is a continuum of recalcitrant seed behaviour, with species showing highly, moderately or minimally recalcitrance, depending on their degree of desiccation sensitivity, hydrated life span, and also chilling sensitivity (Farrant, et al., 1988; Berjak, et al., 1989). A look at the desiccation protective sugars and LEA proteins in hazelnuts would provide more evidence for its position in this proposed seed storage habit continuum.

Presence of antioxidants in hazel byproducts (Shahidi, et al., 2007) as a natural source of antioxidants raises the need to check whether their involvement in physiological application is related to germination.

The first task was to optimise the assay for antioxidant activity in hazel as differences have been observed in the most commonly used DPPH assay protocol in various laboratories (Sharma & Bhat, 2009). A series of tests were conducted on hazelnuts

to optimise the extraction method which explores incubation time, extraction conditions and storage of extracted samples (section 3.7.3 Phase 1).

The next step was to look at the distribution of activity. While many authors have reported the importance of antioxidants in seed viability (Abele, 2002; Alpert, 2005), there are few reports on the distribution of antioxidant activities within a seed such as hazel or on the impact of storage conditions on the distribution of antioxidant activity. In this thesis, the order of local levels of antioxidant activity was found to be:

Embryo tissue < Endocarp < Funiculus < Testa.

This is first time this differential distribution of activity has been observed in hazelnut and hazel seed associates in this detail.

Chilling increased activity as revealed by the reduced sample mass of hazel seed associates needed to achieve 50% inhibition (Fig. 3.58) and embryo (seed without testa) (Fig. 3.59) indicating chilling possibly activates the enzymes and also release antioxidants from bound forms. Results (section 3.7) suggest antioxidants present in the seed and seed associates are closely linked to counter free radical attacks and protect the embryonic axes to proliferate into a healthy seedling. The damages seen (Figs. 3.15, 3.31, 3.40 and 3.41) in the seed parts possibly results in abnormal seedlings (Fig. 3.5). Hence, restoring normal health of seed by adequate recovery techniques (chilling) after any stress related exposure is proposed as a pre-requisite to a high yield of healthy seedlings.

Various chemical and environmental (chilling) methods have been used in breaking dormancy in hazelnuts (Bewley and Black, 1994). In this investigation chilling had been used as the method of dormancy release. Several chemical and biochemical

tests were applied to monitor the impact of various environmental regimes on seed viability and function.

Initial tests to stabilise seed moisture content showed that reduction in seed moisture did not impose dormancy as most seeds germinated but seedlings showed various abnormalities. To further investigate seed dormancy, reversibility of dormancy was tested and the germination results showed one term at 15°C was less harmful than two terms at 15°C (Figs. 3.9 a, b) but still resulted in many abnormal seedlings. In a further test, results show hydrated storage was also detrimental for seed viability as germination was reduced and the seedlings grown showed abnormalities including reduction in seedling height, internode numbers as in previous tests, supporting previous suggestions (Pammenter, et al., 1994). Tetrazolium test for viability revealed the impact of free radicals due to water stress and also showed the areas of damages. Impact of water stress is higher at room temperature, as revealed by increase in non-germinated but viable seeds by TTC test (Figs. 3.16 a, b), membrane damage shown by increased leachate conductivity and infection.

Taken together, these results suggest that while, once imbibed, seed tissues activate inherent antioxidant systems, these changes could not always cope with the overall stress impact like membrane damages.

Dormancy breaking test by 6 weeks chilling showed that seed germination gradually increased with increase in chilling time, shown by increase in seed vigour as germination index increased and increase in antioxidant activities with a reduction of seed mass needed for inhibiting free radical DPPH (Figs. 3.58 & 3.59). The gradual elimination of seed infection at the end of 6 weeks chilling (Fig. 3.23) opens an interesting question whether chilling activates phytoalexins, the antimicrobial and

often antioxidative substances (Ahuja, et al., 2012) synthesized de novo by plants that accumulate rapidly at areas of pathogen infection to counter the endophytes. The above chemical and biochemical responses of the seeds during imposition and breaking of dormancy possibly clarifies the observation of various abnormalities in seedlings (TTC test) recorded in stabilisation tests (Figs. 3.5, 5.2 and 5.3). Therefore, it can be assumed that seed germination does not necessarily reflect seeds ability to result into a healthy seedling but proper health of the seed need to be restored prior to germination tests.

7 Future Work

Results in this thesis and elsewhere leave the question 'is hazel seed orthodox or in a particular spot at continuum of recalcitrance'. To firmly understand seed germination in dormancy and recalcitrance, research on chemical/bio-regulators or signalling chemicals/proteins as well as the genetic expression pattern in hazel seeds are needed to be explored. To consolidate the position of hazelnut's storage habit, the role of other bio-markers: cryoprotectants and LEA proteins in hazel seeds might provide some insight into the survival of desiccated hazel seeds and probably determine its storage habit or link the seed in a continuum of recalcitrance (Berjak & Pammenter, 2000).

The encouraging observation of increase in antioxidant activities by hazel seed associates and reduction of infection during chilling, and presence of pathogens in seeds as well as some localised infected areas within the cotyledon indicates the presence of endophytes. It has been suggested that hazel endophytes are linked with production of anticancer drug Taxol, used as the best known drugs for the treatment of breast, ovarian and some other cancers (Ojima, et al., 2002). The discovery of Taxol in hazelnuts (Hoffman, et al., 1998) was a turning point for the hazelnuts. The report that hazelnuts are the master producer of Taxol and more active than commercial taxol is more encouraging (Bemani, et al., 2012). Therefore, it is difficult to accept the findings that fungi has no role in taxol production (Heinig, et al., 2013). The observations above in Taxol production clearly emphasises the need to further the involvement of hazelnut endophytes.

8 References

- Abdel-Hafez, A. & Saber, S. M., 1993. Mycoflora and mycotoxin of hazelnut (*Corylus avellana* L.) and walnut (*Juglans regia* L.) seeds in Egypt. *Zentralbl Mikrobiol*, 148(2), pp. 137-147.
- Abele, D., 2002. The radical life-giver. *Nature*, Volume 420, p. 27.
- Aderogba, M., Kgatle, D., McGaw, L. & Eloff, J., 2012. Isolation of antioxidant constituents from *Combretum apiculatum* sbsp. *Apiculatum*. *S Afr J Bot*, Volume 79, pp. 125-131.
- Ahuja, I., Kissen, R. & Bones, A., 2012. Phytoalexins in defense against pathogens. *Trends Plant Sci*, 17(2), pp. 73-90.
- Akomito, T. et al., 2004. Involvement of acetaldehyde in seed deterioration of some recalcitrant woody species through the acceleration of aerobic respiration. *Plant Cell Physiol*, 45(2), pp. 201-210.
- Alasalvar, C., Amaral, J. S., Satir, G. & Shahidi, F., 2009a. Lipid characteristics and essential minerals of native Turkish hazelnut varieties (*Corylus avellana* L.). *Food Chem*, Volume 113, pp. 919-925.
- Alasalvar, C., Amaral, J. S. & Shahidi, F., 2006b. Functional lipid characteristics of Turkish Tombul hazelnut (*Corylus avellana* L.). *J Agricult Food Chem*, pp. 10177-10183.
- Alasalvar, C., Karamac, M., Amarowicz, R. & Shahidi, F., 2006. Antioxidant and antiradical activity in extracts of hazelnut kernel (*Corylus avellana* L.) and in hazelnut green leafy cover. *J Agricult Food Chem*, Volume 54, pp. 4826-4832.
- Alasalvar, C. et al., 2009b. Antioxidant activity of hazelnut skin phenolics. *J Agricult Food Chem*, Volume 57, pp. 4645-4650.
- Alasalvar, C., Pelvan, E. & Amarowicz, R., 2010. Effects of roasting on taste-active compounds of turkish hazelnut varieties (*Corylus avellana* L.). *J Agricult Food Chem*, Volume 58, pp. 8674-8679.
- Alasalvar, C., Shahidi, F. & Cadwallader, K., 2003b. Comparison of Natural and Roasted Turkish Tombul Hazelnut (*Corylus avellana* L.) Volatiles and Flavor by DHA/GC/MS and Descriptive Sensory Analysis. *J Agricult Food Chem*, 51(17), pp. 5067-5072.
- Alasalvar, C., Shahidi, F., Liyana-Pathirana, C. M. & Ohshima, T., 2003a. Turkish Tombul Hazelnut (*Corylus avellana* L.): 1. Compositional Characteristics. *J Agricult Food Chem*, 51(13), pp. 3790-3796.
- Aliniabee, M. T., 1983. Monitoring the Filbertworm, *Melissopus latiferreanus* (Lepidoptera: Olethreutidae), with Sex Attractant Traps: Effect of Trap Design and Placement on Moth Catches. *Environ Entomol*, 12(1), pp. 141-146.
- Allens Farm, 2014. *Allen Farm Kent Cobnuts*. [Online] Available at: <http://www.cobnuts.co.uk/order.htm> [Accessed 10 07 2014].

Alma, M. et al., 2003. Screening chemical composition and in vitro antioxidant and antimicrobial activities of the essential oils from *Origanum syriacum* L. Growing in Turkey. *Biol Pharm Bull*, Volume 26, pp. 1725-1729.

Alma, R.-L. et al., 2012. Variation in antioxidant properties and phenolics concentration in different organs of wild growing and greenhouse cultivated *Castilleja tenuiflora* Benth. *Acta Physiol Plantarum*, 34(6), pp. 2435-2442.

Alpert, P., 2005. The limits and frontiers of desiccation-tolerant life. *Integr Comp Biol*, Volume 45, pp. 685-695.

Amaral, J. S. et al., 2006a. Tocopherol and tocotrienol content of hazelnut cultivars grown in Portugal. *J Agricult Food Chem*, Volume 54, pp. 1329-1336.

Ames, S., Shigenaga, M. & Hagen, T., 1993. Oxidants, antioxidants and degenerative diseases of aging. *Proc Nat Acad Sci USA*, Volume 90, pp. 7915-7922.

Andriotis, V. M. & Ross, J. D., 2003. Isolation and characterisation of phytase from dormant *Corylus avellana* seeds. *Phytochemistry*, 64(3), pp. 689-699.

Andriotis, V. M. & Ross, J. D., 2004. Isolation and partial characterisation of acid phosphatase isozymes from dormant oilseed of *Corylus avellana* L. *Planta*, 219(2), pp. 346-358.

Arc, E. et al., 2012. Cold stratification and exogenous nitrates entail similar functional proteome adjustments during *Arabidopsis* seed dormancy release. *J Proteome*, Volume 11, pp. 5418-5432.

Arias, I. E., 1976. *Studies on the dormancy and germination of seeds of Corylus avellana* L., *PhD Thesis*. London: University of London.

Arias, I. E., Williams, P. M. & Bradbeer, J. W., 1976. Studies in seed dormancy. IX. The role of gibberellin biosynthesis and the release of bound gibberellin in the postchilling accumulation of gibberellin in seeds of *Corylus avellana* L. *Planta*, Volume 131, pp. 135-139.

Ashraf, M. & Harris, P. J. C., 2004. Potential biochemical indicators of salinity tolerance in plants. *Plant Sci*, Volume 166, pp. 3-16.

Asomaning, J. M., Olympio, N. S. & Sacande, M., 2011. Desiccation Sensitivity and Germination of Recalcitrant *Garcinia kola* Heckel Seeds. *Res J Seed Sci*, Volume 4, pp. 15-27.

Awah, F. M. et al., 2010. Free radical scavenging activity and immunomodulatory effect of *Stachytarpheta angustifolia* leaf extract. *Food Chem*, Volume 119, pp. 1409-1416.

Awah, F. M. & Verla, A. W., 2010. Antioxidant activity, nitric oxide scavenging activity and phenolic contents of *Ocimum gratissimum* leaf Extract. *J Med Plants Res*, 4(24), pp. 2479-2487.

Bailly, C., 2004. Active oxygen species and antioxidants in seed biology. *Seed Sci Res*, 14(2), pp. 93-107.

- Bailly, C., El-Maarouf-Bouteau, H. & Corbineau, F., 2008. From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. *C. R. Biol*, Volume 331, pp. 806-814.
- Bai, X. et al., 2011. *Nitric oxide enhances desiccation tolerance of recalcitrant *Antiaris toxicaria* seeds via protein S-nitrosylation and carbonylation*, Cambridge: PLOS ONE.
- Bao, J. P. & Zhanj, S. L., 2011. Changes in germination, storage materials and antioxidant enzyme activities in pear (*Pyrus betulaefolia* Bge. and *Pyrus calleryana* Dcne.) stock seeds during cold stratification. *Seed Sci Technol*, 39(3), pp. 655-659.
- Barbedo, C. J. & Bilia, D. A. C., 1998. Evolution of research on recalcitrant seeds. *Sci Agric*, Volume 55(Especial), pp. 121-125.
- Barbedo, C. J., Centeno, D. C. & Figueiredo-Rieiro, R. C. L., 2013. Do recalcitrant seeds really exist?. *Hoehnea*, 40(4), pp. 583-593.
- Barbour & Brinkman, 2004. The genus *Corylus*. In: *Woody Plant Seed Manual (on-line version)*. Georgia: USDA Forest Service's National Seed Laboratory, Dry Branch, pp. 434-437.
- Barton, L. V., 1965. *Dormancy in seeds imposed by the seed coat*. Berlin: Springer Berlin Heidelberg.
- Baskin, C. C. & Baskin, J. M., 1998. *Seeds - ecology, biogeography, and evolution of dormancy and germination*. San Diego, CA, USA: Academic Press.
- Baskin, C. C., Thompson, K. & Baskin, J. M., 2006. Mistakes in germination ecology and how to avoid them. *Seed Sci Res*, Volume 16, pp. 165-168.
- Baskin, J. M. & Baskin, C. C., 2004. A classification system for seed dormancy. *Seed Sci Res*, Volume 14, pp. 1-16.
- Bass, L. N., 1975. Seed storage of *Carica papaya* L. *Hortscience*, 10(3), p. 232.
- Batlla, D. & Benech-Arnold, R. L., 2006. The role of fluctuations in soil water content on the regulation of dormancy. *Seed Sci Res*, 16(1), pp. 47-59.
- Battencourt, E. & Konopka, J., 1989. *Directory of Germplasm Collections. 6.11 Temperate Fruits and Tree Nuts*. Rome: International Board for Plant Genetic Resources.
- Bazin, J. et al., 2011. Role of relative humidity, temperature, and water status in dormancy alleviation of sunflower seeds during dry after-ripening. *J Exp Bot*, Volume 62, pp. 627-640.
- Becwar, M. R., Stanwood, P. C. & Lehonardt, K. W., 1983. Dehydration effects on freeze characteristics and survival in liquid nitrogen for desiccation-tolerant and desiccation-sensitive seeds. *J Am Soc Hort Sci*, Volume 108, pp. 613-618.
- Bemani, E., Ghanati, F., Rezaei, A. & Jamshidi, M., 2013. Effect of phenylalanine on Taxol production and antioxidant activity of extracts of suspension-cultured hazel (*Corylus avellana* L.) cells. *J Nat Med*, 67(3), pp. 446-451.

Bemani, E., Ghanati, F., Yousefzadeh, L. & Boroujeni, F. K., 2012. Antioxidant activity, Total Phenolics and Taxol Contents Response of Hazel (*Corylus avellana* L.) cells to Benzoic acid and cinnamic acid. *Not Bot Horti Agrobo*, 40(1), pp. 69-73.

Berjak, P. & D. Dumet, D., 1996. Cryopreservation of seeds and isolated embryonic axes of neem (*Azadirachta indica* A. Juss.). *Cryo-letters*, Volume 17, pp. 99-104.

Berjak, P., Dini, M. & Pammenter, N. W., 1984. Possible mechanisms underlying differing dehydration responses in recalcitrant and orthodox seeds: desiccation associated sub-cellular changes in propagules of *Avicennia marina*. *Seed Sci Technol*, Volume 12, pp. 365-384.

Berjak, P., Farrant, J. M. & Pammenter, N. W., 1989. The basis of recalcitrant seed behaviour. In: R. B. Taylorson, ed. *Recent advances in the development and germination of seeds*. New York: Plenum Press, pp. 89-108.

Berjak, P. & Pammenter, N. W., 1994. Recalcitrance is not an all-or-nothing situation. *Seed Sci Res*, Volume 4, pp. 263-264.

Berjak, P. & Pammenter, N. W., 2000. What ultrastructure has told us about recalcitrant seeds. *Rev Bras Fisiol Veg*, Volume 12, pp. 22-55.

Berjak, P. & Pammenter, N. W., 2008. From *Avicennia* to *Zizania*: seed recalcitrance in perspective. *Ann Bot*, Volume 101, pp. 213-228.

Berjak, P., Pammenter, N. W. & Vertucci, C. W., 1992. Homoiohydrous (recalcitrant) seeds: developmental status, desiccation sensitivity and the state of water in axes of *Landolphia kirkii* Dyer. *Planta*, Volume 186, pp. 249-261.

Berjak, P., Vertucci, C. W. & Pammenter, N. W., 1993. Effects of developmental status and dehydration rate on characteristics of water and desiccation-sensitivity in recalcitrant seeds of *Camellia sinensis*. *Seed Sci Res*, Volume 3, pp. 155-166.

Beuchat, L. R. & Scouten, A. J., 2002. Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157:H7 on alfalfa seeds. *J Appl Microbiol*, 92(3), pp. 382-95.

Bewley, J. D., 1979. Physiological aspects of desiccation tolerance. *Ann Rev Plant Physiol*, Volume 30, pp. 195-238.

Bewley, J. D., 1997a. Seed germination and dormancy. *Plant Cell*, Volume 9, pp. 1055-1066.

Bewley, J. D., 1997b. Breaking down the wall -- a role for endo- β -mannanase in release from seed dormancy?. *Trends Plant Sci*, 4(12), pp. 464-469.

Bewley, J. D. & Black, M., 1982. *Physiology and Biochemistry of Seeds*. Berlin: Springer-Verlag.

Bewley, J. D. & Black, M., 1994. *Seeds: Physiology of development and germination*. Second edition ed. New York and London: Plenum Press.

Bewley, J. D. & Oliver, M. J., 1992. Desiccation-tolerance in vegetative plant tissues and seeds: protein synthesis in relation to desiccation and a potential role for protection and repair mechanisms. In: C. B. Osmond & G. Somero, eds. *Water*

and Life: a comparative analysis of water relationships at the organismic, cellular and molecular levels. Berlin: Springer-Verlag, pp. 141-160.

Black, M., 1996. Liberating the radicle. A case for softening up. *Seed Sci Res*, Volume 6, pp. 39-42.

Blois, M. S., 1958. Antioxidant determination by the use of a stable free radical. *Nature*, Volume 181, pp. 1199-1200.

Bonner, F., 1986. *Technologies to Maintain Biological Diversity*. Washington D.C.: Office of Technology Assessment.

Bonner, F. T., 1990. Storage of seeds: potential and limitations for germplasm conservation. *Forest Ecol Management*, Volume 35, pp. 35-43.

Bonner, F. T., 1996. Response to Drying of Recalcitrant Seeds of *Quercus nigra* L. *Ann Bot*, Volume 78, pp. 181-187.

Boone, R. B., Hoffman, A., Daley, L. & Arbogast, B., 2000. How Taxol was found in hazelnut trees. *Australian Nutgrower*, Volume 14, p. 36.

Bovi, M. L. A., Martins, C. C. & Spiering, S. H., 2004. Desidratação de sementes de quatro lotes de pupunheira: efeitos sobre a germinação e o vigor. *Hort Bras*, Volume 22, pp. 109-112.

Bradbeer, J. W., 1968. Studies in seed dormancy. IV. The role of inhibitors and gibberellin in the dormancy and germination of *Corylus avellana* L. seeds. *Planta (Berlin)*, Volume 78, pp. 266-276.

Bradbeer, J. W., 1988. *Seed dormancy and germination*. Glasgow and London: Blackie.

Bradbeer, J. W., Arias, I. E. & Nirmala, I. S., 1978. The role of chilling in the breaking of seed dormancy in *Corylus avellana* L. *Pestic Sci*, Volume 9, pp. 184-186.

Bradbeer, J. W. & Colman, B., 1967. Studies in seed dormancy 1. The metabolism of [214C] acetate by chilled seeds of *Corylus avellana* L. *New Phytol*, Volume 66, pp. 5-15.

Bradbeer, J. W. & Floyd, V. M., 1964. Nucleotide synthesis in hazel seeds during after-ripening. *Nature*, Volume 201, pp. 99-100.

Bradbeer, J. W. & Pinfield, N. J., 1967. Studies in seed dormancy. III. The effects of gibberellin on dormant seeds of *Corylus avellana* L. *New Phytol*, Volume 66, pp. 515-523.

Bradford, K. J. & Chandler, P. M., 1992. Expression of dehydrin-like proteins in embryos and seedlings of *Zizania palustris* and *Oryza sativa* during dehydration. *Plant Physiol*, Volume 99, pp. 488-494.

Brand-Williams, W., Cuvelier, M. E. & Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. *Lebensmittel-Wissenschaft und-Technologie/Food Sci Technol*, Volume 28, pp. 25-30.

Briggs, D. E., Sole, S. M. & Latham, P., 2009. Tetrazolium Staining, Mitochondria, and Barley Quality. *J Inst Brew*, 115(1), pp. 41-48.

Brinkman, K. A., 1974. *Corylus L. In Seeds of Woody Plants in the United States. Agriculture Handbook No 450*. Washington DC: Forest Service, USDA.

Bruce, W. & Horn, B. W., 2005. Colonization of wounded peanut seeds by soil fungi: selectivity for species from *Aspergillus* section *Flavi*. *Mycologia*, 97(1), pp. 202-217.

Bryant, G., Koster, K. L. & Wolfe, J., 2001. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Sci Res*, Volume 11, pp. 17-25.

Caffrey, M., Fonseca, V. & Leopold, A. C., 1988. Lipid-sugar interactions. *Plant Physiol*, Volume 86, pp. 754-758.

Castro, Y. G. P. & Krug, H. P., 1951. Experiências sobre germinação e conservação de sementes de *Inga edulis*, espécie usada em sombreamento em cafeeiros. *Ciência e Cultura*, Volume 3, pp. 263-264.

Chien, C.-T. & Lin, T.-P., 1999. Effects of moisture content and temperature on the storage and germination of *Cinnamomum camphora* L. Sieb.). *Seed Sci Technol*, Volume 27, pp. 315-320.

Chin, H. F., 1988. *Recalcitrant Seeds – A status report*. Rome, Italy: IBPGR.

Chiruvella, K. K., Mohammed, A. & Ghanta, R. G., 2014. Factors influencing the seed germination of *Soymida febrifuga* (Roxb.) A. Juss. (Meliaceae). *Tra J Sci*, Volume 2, pp. 121-131.

Christensen, C. M., 1972. Microflora and seed deterioration. In: E. H. Roberts, ed. *Viability of Seeds*. London: Chapman and Hall.

Cimino, G., Passerini, A. & Toscano, G., 2000. Removal of toxic cations and Cr(VI) from aqueous solution by hazelnut shell. *Water Res*, 34(11), pp. 2955-2962.

Close, T. J., Fenton, R. D. & Moonan, F., 1993a. A view of plant dehydrins using antibodies specific to the carboxy terminal peptide. *Plant Mol Biol*, Volume 23, pp. 279-286.

Close, T. J. et al., 1993b. Dehydrin: the protein. In: T. J. Close & E. A. Bray, eds. *Plant responses to cellular dehydration during environmental stress. Current topics in plant physiology*. Rockville: American Society of Plant Physiologists, pp. 104-118.

Close, T. J., Kortt, A. A. & Chandler, P. M., 1989. A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Mol Biol*, Volume 13, pp. 95-108.

CNSF, 1993. *Fiches de production de plants en pépinière*. Ouagadougou: Centre National de Semences Forestières.

Conklin, J. R. & Sellmer, J. C., 2009. Germination and Seed Viability of Norway Maple Cultivars, Hybrids, and Species. *HortTechnology*, 19(1), pp. 120-126.

- Contini, M., Baccelloni, S., Massantini, R. & Anelli, G., 2008. Extraction of natural antioxidants from hazelnut (*Corylus avellana* L.) shell and skin wastes by long maceration at room temperature. *Food Chem*, 110(3), pp. 659-669.
- Crane, J., Miller, A. J., Van Roekel, J. W. & Walters, C., 2003. Triaglycerols determine the unusual physiology of *Cuphea* seed. *Planta*, Volume 217, pp. 699-708.
- Czabator, F. J., 1962. Germination value: an index combining speed and completeness of pine seed germination. *For Sci*, Volume 8, pp. 386-396.
- Daws, M. I. et al., 2006. Variable desiccation tolerance in *Acer pseudoplatanus* seeds in relation to developmental conditions: a case of phenotypic recalcitrance?. *Funct Plant Biol*, Volume 33, pp. 59-66.
- Daws, M. I. et al., 2004. Developmental heat sum influences recalcitrant seed traits in *Aesculus hippocastanum* across Europe. *New Phytol*, Volume 162, pp. 157-166.
- De Knop, K. J. et al., 2011. Age-related sensitization profiles for hazelnut (*Corylus avellana*) in a birch-endemic region. *Pediatr Allergy Immunol*, 22(1pt2), pp. e139-e149.
- Dehgan, B. & Yuen, C. K. K. H., 1983. Seed morphology in relation to dispersal, evolution and propagation of *Cycas*, L. *Bot Gaz*, Volume 144, pp. 412-418.
- Del'Aquila, A. & Spada, P., 1992. Regulation of protein synthesis in germinating wheat embryos under polyethylene glycol and salt stress. *Seed Sci Res*, Volume 2, pp. 75-80.
- Delahaie, J. et al., 2013. LEA polypeptide profiling of recalcitrant and orthodox legume seeds reveals ABI3-regulated LEA protein abundance linked to desiccation tolerance. *J Exp Bot*, 64(14), pp. 4559-4573.
- Delgado, L. F. & Barbedo, C. J., 2012. Water potential and viability of seeds of *Eugenia* (Myrtaceae), a tropical tree species, based upon different levels of drying. *Braz Arch Biol Technol*, Volume 55, pp. 583-590.
- Demirkaya, M., Dietz, K. J. & Sivritepe, H. O., 2010. Changes in Antioxidant Enzymes during Ageing of Onion Seeds. *Not Bot Hort Agrobot Cluj*, 38(1), pp. 49-52.
- Dhindsa, R. S. & Bewley, J. D., 1976. Water stress and protein synthesis. IV responses of a drought-tolerant plant. *J Exp Bot*, Volume 27, pp. 513-523.
- Di Matteo, V. & Esposito, E., 2003. Biochemical and therapeutic effects of antioxidants in the treatment of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. *Curr Drug Targets*, Volume 2, pp. 95-107.
- Dickie, J. B., May, K., Morris, S. V. A. & Titley, S. E., 1991. The effects of desiccation on seed survival in *Acer platanoides* L. *Seed Sci Res*, Volume 1, pp. 149-162.
- Dickie, J. B. & Pritchard, H. W., 2002. Systematic and evolutionary aspects of desiccation tolerance in seeds. In: M. Black & H. Pritchard, eds. *Desiccation and*

survival in plants: drying without dying. Wallingford: CAB International, pp. 239-259.

Dickie, J. B. & Smith, R. D., 1992. *Limits to the survival of essentially orthodox seeds at low moisture contents in some woody species. Poster in Fourth international workshop on seeds: basic and applied aspects of seed biology*. Paris, ASFIS.

Diplock, A. T. et al., 1998. Functional food science and defence against reactive oxidative species. *Br J Nutr*, 80(Suppl. 1), pp. S77-S112.

Downs, M. P. & Cavers, P. B., 2000. Effects of wetting and drying on seed germination and seedling emergence of bull thistle, *Cirsium vulgare* (savi) Ten. *Can J Bot*, 78(12), pp. 1545-1551.

Droge, W., 2002. Free radicals in the physiological control of cell function. *Physiol Rev*, Volume 82, pp. 47-95.

Duda-Chodak, A. & Tarko, T., 2007. Antioxidants Properties of Different Fruit. *Acta Sci Pol Technol Aliment*, 6(3), pp. 29-36.

Duke, J. A., 1989. *Handbook of Nuts*. Boca Raton, Florida: CRC Press.

Duke, J. A., 2000. *Handbook of Nuts: Herbal Reference Library*. Boca Raton : CRC Press.

Dure, L., 1993. A repeating 11-mer amino acid motif and plant desiccation. *Plant J*, Volume 3, pp. 363-369.

Dure, L. et al., 1989. Common amino acid sequence domains among the LEA proteins of higher plants. *Plant Mol Biol*, Volume 12, pp. 475-486.

Dure, L., Greenway, S. C. & Galau, G. A., 1981. Developmental biochemistry of cottonseed embryogenesis and germination: changing messenger ribonucleic acid populations as shown by in vitro and in vivo protein synthesis. *Biochemistry*, 20(14), pp. 4162-4164.

Ebo, D. G. et al., 2012. Hazelnut allergy: A multi-faced condition with demographic and geographic characteristics. *Acta Clin Belg*, 67(5), pp. 317-321.

Ellis, E. R., Hong, T. D. & Roberts, E. H., 1990. An Intermediate category of seed behaviour? I. COFFEE. *J Exp Bot*, 41(9), pp. 1167-1174.

Ellis, R. H., 2011. Rice seed quality development and temperature during late development and maturation. *Seed Sci Res*, Volume 21, pp. 95-101.

Ellis, R. H. et al., 1996. Survival of dry and ultra-dry seeds of carrot, groundnut, lettuce, oilseed rape and onion during five years hermetic storage at two temperatures. *Seed Sci Technol*, Volume 24, pp. 347-358.

Ellis, R. H., Hong, T. D. & Roberts, E. H., 1991a. An Intermediate category of seed behaviour? II. EFFECTS OF PROVENANCE, IMMATURITY, AND IMBIBITION OF DESICCATION - TOLERANCE IN COFFEE. *J Exp Bot*, 42(5), pp. 653-657.

Ellis, R. H., Hong, T. D. & Roberts, E. H., 1991b. Effect of temperature and moisture on the germination of papaya seeds. *Seed Sci Res*, 1(1), pp. 69-72.

- Ellis, R. H. & Roberts, E. H., 1980. Improved equations for the prediction of seed longevity. *Ann Bot*, Volume 45, pp. 13-30.
- Ellis, R. H. & Roberts, E. H., 1998. How to store seeds to conserve biodiversity. *Nature*, Volume 395, p. 758.
- Engles, J. & Engelmann, F., 1998. Introductory statement. *Seed Sci Res*, Volume 2, pp. 1-2.
- Ezumah, B. S., 1986. Germination and storage of neem (*Azadirachta indica* A. Juss.) seeds. *Seed Sci Technol*, Volume 14, pp. 593-600.
- FAOSTAT, 2014. *FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS*. [Online] Available at: <http://faostat3.fao.org/browse/Q/QC/E> [Accessed 23 October 2014].
- Farrant, J. M., Berjak, P. & Pammenter, N. W., 1985. The effect of drying rate on viability retention of recalcitrant propagules of *Avicennia marina*. *S Afr J Bot*, 51(6), pp. 432-438.
- Farrant, J. M., Pammenter, N. W. & Berjak, P., 1986. The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. *Physiol Plant*, Volume 67, pp. 291-298.
- Farrant, J. M., Pammenter, N. W. & Berjak, P., 1988. Recalcitrance - a current assessment. *Seed Sci Technol*, Volume 16, pp. 155-166.
- Farrant, J. M., Pammenter, N. W. & Berjak, P., 1989. Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a study on three unrelated species. *Planta*, Volume 178, pp. 189-198.
- Fenner, M. & Thompson, K., 2005. *The Ecology of Seeds*. Cambridge: Cambridge University Press.
- Finch-Savage, W. E., 1992. Seed development in the recalcitrant species *Quercus robur* L.: germinability and desiccation tolerance. *Seed Sci Res*, Volume 2, pp. 17-22.
- Finch-Savage, W. E., 1995. *The role of developmental studies in research on recalcitrant and intermediate seeds*. Humlebaek, Denmark, IPGRI.
- Finch-Savage, W. E., 1998. *Farm Woodland Tree Seed*. Wellesbourne, Warwick: Horticulture Research International.
- Finch-Savage, W. E. & Blake, P. S., 1994. Intermediate development in desiccation-sensitive seeds of *Quercus robur* L. *Seed Sci Res*, Volume 4, pp. 127-133.
- Finch-Savage, W. E., Blake, P. S. & Clay, H. A., 1996. Desiccation stress in recalcitrant *Quercus robur* L. seeds results in lipid peroxidation and increased synthesis of jasmonates and abscisic acid. *J Exp Bot*, Volume 47, pp. 661-667.
- Finch-Savage, W. E., Pramanik, S. K. & Bewley, J. D., 1994. The expression of dehydrin proteins in desiccation-sensitive (recalcitrant) seeds of temperate trees. *Planta*, Volume 193, pp. 478-485.

- Finkel, T. & Holbrook, N. J., 2000. Oxidants, oxidative stress and the biology of ageing. *Nature*, Volume 408, pp. 239-247.
- Flores, P. C., Poggi, D., Garcia, S. M. & Gariglio, N. F., 2011. Topographic tetrazolium testing of Black Walnut (*Juglans nigra* L.) seeds. *Seed Sci Technol*, Volume 39, pp. 230-235.
- Francini, A. et al., 2006. Enzymatic and non-enzymatic protective mechanisms in recalcitrant seeds of *Araucaria bidwillii* subjected to desiccation. *P Physiol Biochem*, 44(10), pp. 556-563.
- Frankland, B., 1961. Effect of gibberellin and kinetin and other substances on seed dormancy. *Nature*, Volume 192, p. 678.
- Frankland, B. & Wareing, P. F., 1962. Changes in Endogenous Gibberellins in Relation to Chilling of Dormant Seeds. *Nature*, Volume 194, pp. 313-314.
- Frankland, B. & Wareing, P. F., 1966. Hormonal regulation of seed dormancy in hazel (*Corylus avellana* L.) and beech (*Fagus sylvatica* L.). *J Exp Bot*, Volume 17, pp. 596-611.
- Franks, P. J. & Drake, P. L., 2003. Desiccation-induced loss of seed viability is associated with a 10-fold increase in CO₂ evolution in seeds of the rare tropical rainforest tree *Idiospermum australiense*. *New Phytol*, 159(1), pp. 253-261.
- Fu, X. X., Liu, H. N., Zhou, X. D. & Shang, X. L., 2013. Seed dormancy mechanism and dormancy breaking techniques for *Cornus kousa* var. *Chinensis*. *Seed Sci Technol*, 41(3), pp. 458-463.
- Galau, G. A., Hughes, D. W. & Dure, L., 1986. Abscisic acid induction of cloned cotton late embryogenesis-abundant (lea) mRNAs. *Plant Mol Biol*, Volume 7, pp. 157-170.
- Gamene, C. S., Kraak, H. L., Van Pijlen, J. G. & De Vos, C. H. R., 1996. Storage behaviour of neem (*Azadirachta indica*) seeds from Burkina Faso. *Seed Sci Technol*, Volume 24, pp. 441-448.
- Garrison, W., Dale, A. & Saxena, P. K., 2013. Improved shoot multiplication and development in hybrid hazelnut nodal cultures by ethylenediamine di-2-hydroxyphenylacetic acid (Fe-EDDHA). *Can J Plant Sci*, Volume 93, pp. 511-521.
- Gest, N., Gautier, H. & Stevens, R., 2013. Ascorbate as seen through plant evolution: the rise of a successful molecule?. *J Exp Bot*, 64(1), pp. 33-53.
- Gonzalez-Benito, M. E. & Perez, C., 1994. Cryopreservation of embryonic axes of two cultivars of hazelnut (*Corylus avellana* L.). *Cryo-letters*, Volume 15, pp. 41-46.
- Gordon, A. G. & Rowe, D. C. F., 1982. *Seed Manual for Ornamental Trees and Shrubs. Forestry Commission Bulletin 59*, London: Her Majesty's Stationery Office.
- Gosling, P. G., 1991. Beechnut storage: a review and practical interpretation of the scientific literature. *Forestry*, Volume 64, pp. 51-59.
- Gosling, P. G., 2007. *Raising trees and shrubs from seeds*. Leicester: Forestry Commission Publications.

- Gosling, P. G., McCartan, S. A. & Peace, A. J., 2009. Seed dormancy and germination characteristics of common alder (*Alnus glutinosa* L.) indicate some potential to adapt to climate change in Britain. *Forestry*, 82(5), pp. 573-575.
- Gosling, P. G. & Ross, J. D., 1980a. A comparison of petiolar growth of isolated hazel cotyledons in response to stratification and exogenous GA3. *J Exp Bot*, 31(122), pp. 737-742.
- Gosling, P. G. & Ross, J. D., 1980b. Pentose phosphate metabolism during dormancy-breakage in *Corylus avellana* L. *Planta (Berl.)*, Volume 148, pp. 362-366.
- Gosling, P. G. & Ross, J. D., 1981. Peroxidase levels in the cotyledon of hazel seed (*Corylus avellana* L.). *Phytochemistry*, Volume 20, pp. 31-33.
- Groot, S. P. C. & Karssen, C. M., 1987. Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. *Planta*, Volume 171, pp. 525-531.
- Groot, S. P. C., Kieliszewska-Rokicka, B., Vermeer, E. & Karssen, C. M., 1988. Gibberellin-induced hydrolysis of endosperm cell walls in gibberellin-deficient tomato seeds prior to radicle protrusion. *Planta*, Volume 174, pp. 500-504.
- Grzybowski, C. R., Ohlson, O. C., Carvalho da Silva, R. & Panobianco, M., 2012. Viability of barley seeds by the tetrazolium test. *Rev bras sementes*, 34(1).
- Gunaseena, H. P. M. & Marambe, B., 1995. Storage technology for neem seeds: Sri Lanka experienc. *Multipurpose Tree Species Network* , 4(1), pp. 6-7.
- Guney, M. S., 2013. Utilization of hazelnut husk as biomass. *Sustain Energy Technol Assessments*, Volume 4, pp. 72-77.
- Hand, S. C. et al., 2011. LEA Proteins During Water Stress: Not Just for Plants Anymore. *Ann Rev Physiol*, Volume 73, pp. 115-134.
- Hanna, M. A. & Xu, Y. X., 2010. Evaluation of Nebraska hybrid hazelnuts: nut/kernel characteristics, kernel proximate compositions, and oil and protein properties. *Indust crop Prod*, 31(1), pp. 84-91.
- Hansen, K. S. et al., 2009. Component-resolved in vitro diagnosis of hazelnut allergy in Europe. *J Allerg Clin Immunol*, 123(5), pp. 1134-1141.
- Harrington, J. F., 1972. Seed storage and longevity. In: T. Kozlowski, ed. *Seed Biol*. New York and London: Academic Press, pp. 145-240.
- Hartman, W., 1989. Influence of light and GA3 on germination of *Corylus avellana* after dry storage. *Gartenbanwissenschaft* , Volume 54, pp. 256-260.
- Hay, F. R., Smith, R. D., Ellis, R. H. & Butler, L. H., 2010. Developmental changes in the germinability, desiccation tolerance, hardseedness, and longevity of individual seeds of *Trifolium ambiguum*. *Ann Bot*, Volume 105, pp. 1035-1052.
- Hedstrom, C. S., Shearer, P. W., Miller, J. C. & Walton, V. M., 2014 . The Effects of Kernel Feeding by *Halyomorpha halys* (Hemiptera: Pentatomidae) on Commercial Hazelnuts. *J Econ Entomol*, 107(October 1), pp. 1858-1865.

- Heinig, U., Scholz, S. & Jennewein, S., 2013. Getting to the bottom of Taxol biosynthesis by fungi. *Fungal Diversity*, Volume 60, pp. 161-170.
- Heit, C. E., 1967. Propagation from seed. XI. Storage of deciduous tree and shrub seeds. *Am Nurseryman*, 126(12), pp. 86-94.
- Hendry, G. A. F., 1993. Oxygen, free radical processes and seed longevity. *Seed Sci Res*, Volume 3, pp. 141-153.
- Hendry, G. A. F. et al., 1993. Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. *New Phytol*, Volume 122, pp. 273-278.
- Hilhorst, H. W. M., 1995. A critical update on seed dormancy. 1. Primary dormancy. *Seed Sci Res*, Volume 5, pp. 61-73.
- Hincha, D. K. & Thalhammer, A., 2012. LEA proteins: IDPs with versatile functions in cellular dehydration tolerance. *Biochem Soc Trans*, Volume 40, pp. 1000-1003.
- Hoffman, A. et al., 1998. Bioprospecting for Taxol in angiosperm plant extracts. *Spectroscopy*, Volume 13, pp. 22-32.
- Holmes, G. D. & Buszewicz, G., 1958. The storage of temperate forest tree species. *For Abstr*, Volume 19, pp. 313-322.
- Hong, T. D. & Ellis, E. H., 1996. *A protocol to determine seed storage behaviour*. Rome, Italy: IPGRI.
- Hong, T. D. & Ellis, R. H., 1992. Development of desiccation tolerance in Norway maple (*Acer platanoides* L.) seeds during maturation drying. *Seed Sci Res*, Volume 2, pp. 169-172.
- Hong, T. D. & Ellis, R. H., 1995. Interspecific variation in seed storage behaviour within two genera - *Coffea* and *Citrus*. *Seed Sci Technol*, Volume 23, pp. 165-181.
- Hong, T. D. & Ellis, R. H., 1998. Contrasting seed storage behaviour among different species of *Meliaceae*. *Seed Sci Technol*, Volume 26, pp. 77-95.
- Hong, T. D. & Ellis, R. H., 2002. Optimum moisture status for the exceptional survival of seeds of *Arabica* coffee (*Coffea arabica* L.) in medium term storage at -20°C. *Seed Sci Technol*, 30(1), pp. 131-136.
- Hor, Y. L., Chin, H. F. & Mohammed, Z. K., 1984. The effect of seed moisture and storage temperature on the storability of cocoa (*Theobroma cacao*) seeds. *Seed Sci Technol*, Volume 12, pp. 415-420.
- Hor, Y. L. et al., 2005. Optimal Hydration Status for Cryopreservation of Intermediate Oily Seeds: *Citrus* as a Case Study. *Ann Bot*, Volume 95, pp. 1153-1161.
- Hosomi, S. T. et al., 2011. Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. *Seed Sci Technol*, Volume 39, pp. 178-189.
- Huang, R. Z., Guo, X. Q., Fang, X. T. & Li, Y. L., 2006. Effect of cold-warm-cold, stratification treatment on physiological and biological characteristics of *Taxus*

chinensis var. mairei seeds. *Journal of Fijian Normal University (Natural Science Edition)*, Volume 22, pp. 95-98.

IBPGR, 1976. *Report of IBPGR Working Group on Engineering, Design and Cost Aspects of Long-term Storage Facilities*, Rome: International Board for Plant Genetic Resources.

Igawa, T. et al., 2007. Reduced Contamination by the Fusarium Mycotoxin Zearalenone in Maize Kernels through Genetic Modification with a Detoxification Gene. *Appl Environ Microbiol*, 73(5), pp. 1622-1629.

Ijtin, W. S., 1957. Drought resistance in plants and physiological processes. *Ann Rev Plant Physiol*, Volume 3, pp. 341-363.

Inglett, G. E. & Chen, D., 2011. Contents of phenolics and flavonoids and antioxidant activities in skin, pulp and seeds of miracle fruit. *J Food Sci*, 76(3), pp. 479-482.

IPGRI, 1976. *Report of IBPGR Working Group on Engineering, Design and Cost Aspects of Long-term Storage Facilities*, Rome: International Board for Plant Genetic Resources.

ISTA, 1999. International Rules for seed testing. *Seed Sci Technol*, Volume 27, pp. 1-333.

ISTA, 2003. *ISTA Working Sheets on Tetrazolium Testing*. Bassersdorf: ISTA.

Jain, P., Bharadwaj, M. & Bakshi, J., 2013. Comparative study of enzyme dehydrogenase activity in *Cicer arietinum* and *Arachis hypogea*. *Recent Res Sci Technol*, 5(2), pp. 16-17.

Jakopic, J. et al., 2011. HPLC–MS identification of phenols in hazelnut (*Corylus avellana* L.) kernels. *Food Chem*, Volume 124, pp. 1100-1106.

Jamalomidi, M. & Gholami, M., 2013. Effect of desiccation on antioxidant enzymes activity of recalcitrant tea (*Camellia sinensis* L.) seeds. *Intl Res J Appl Basic Sci*, 5(4), pp. 417-421.

Jarvis, B. C., 1966. *Nucleotide metabolism in dormant and non-dormant seeds*, PhD Thesis. Wales: University of Wales.

Jarvis, B. C., 1975. The role of seed parts in the induction of dormancy of hazel (*Corylus avellana* L.). *New Phytol*, Volume 75, pp. 491-494.

Jarvis, B. C., 1979. The influence of cotyledons on embryonic axes during Induction of Dormancy in *Corylus avellana* L. *Physiol Plant*, 45(4), pp. 363-366.

Jarvis, B. C., Frankland, B. & Cherry, J. H., 1968. Increased nucleic-acid synthesis in relation to the breaking dormancy of hazel seed by gibberellic acid. *Planta (Berl.)*, Volume 83, pp. 257-266.

Jarvis, B. C. & Wilson, D., 1978. Factors Influencing Growth of Embryonic Axes from Dormant Seeds of Hazel (*Corylus avellana* L.). *Planta*, Volume 138, pp. 189-191.

- Jarvis, B. C., Wilson, D. & Fowler, M. W., 1978. Growth of isolated axes from dormant seeds of hazel (*Coryllus avellana* L.). *New Phytol*, Volume 80, pp. 117-123.
- Jones, H. A., 1920. Physiological study of maple seeds. *Bot Gaz*, Volume 69, pp. 127-152.
- Kagaya, M., Tani, T. & Kachi, N., 2005. Effect of hydration and dehydration cycles on seed germination of *Aster kantoensis* (Compositae). *Can J Bot*, Volume 83, pp. 329-334.
- Kang, H.-M., Chen, K., Bai, J. & Wang, G., 2012. Antioxidative system's responses in the leaves of six *Caragana* species during drought stress and recovery. *Acta Physiol Plant*, 34(6), pp. 2145-2154.
- Kano, M. et al., 2005. Antioxidative activity of anthocyanins from purple sweet potato *Ipomea batatas* cultivar *Ayamurasaki*. *Biosci Biotechnol Biochem*, Volume 69, pp. 979-988.
- Kentish Cobnut Association, 2014. *Kentish Cobnuts Association*. [Online] Available at: <http://kentishcobnutsassociation.org.uk/the-cobnut/technical-information/> [Accessed 10 07 2014].
- Kermode, A. R., 1990. Regulatory mechanisms involved in the transition from seed development to germination. *Crit Rev Plant Sci*, Volume 9, pp. 155-195.
- Kermode, A. R. & Bewley, J. D., 1988. The role of maturation drying in the transition from seed development to germination. V. Responses of the immature castor bean embryo to isolation from the whole seed: a comparison with premature desiccation. *J Exp Bot*, Volume 38, pp. 487-497.
- Khanna, P. K., Kumar, A., Chandra, R. & Verma, V., 2013. Germination behaviour of seeds of *Withania somnifera* (L.) Dunal: a high value medicinal plant. *Physiol Mol Biol Plants*, 19(3), pp. 449-454.
- Kibinza, S. et al., 2006. Sunflower seed deterioration as related to moisture content during ageing, energy metabolism and active oxygen species scavenging. *Physiol Plant*, 128(3), pp. 496-506.
- Kidd, F., 1914. The controlling influence of carbon dioxide in the maturation, dormancy and germination of seeds. II. *Proc Royal Soc London*, Volume 87, pp. 609-625.
- Kim, H. J. et al., 2004. Evaluation of antioxidant activity of Australian tea tree (*Melaleuca alternifolia*) oil and its components. *J Agric Food Chem*, Volume 52, pp. 2849-2854.
- Kim, J.-K. et al., 2002. The first total synthesis of 2,3,6-tribromo-4,5-dihydroxybenzyl methyl ether (TDB) and its antioxidant activity. *Bull Korean Chem Soc*, 23(5), pp. 661-662.
- King, M. W. & Roberts, E. H., 1979. *The storage of recalcitrant seeds. Achievements and possible approaches*. Rome: IBPGR.
- King, M. W. & Roberts, E. H., 1980 (b). The desiccation response of seeds of *Citrus limon* L. *Ann Bot*, Volume 45, pp. 489-492.

- King, M. W. & Roberts, E. H., 1980(a). Maintenance of recalcitrant seeds in storage. In: H. F. Chin & E. H. Roberts, eds. *Recalcitrant Crop Seed*. Kuala Lumpur: Tropical Press, pp. 53-89.
- Kingston, D. G. I., 1994. Taxol: the chemistry and structure-activity relationship of a novel anticancer agent. *Trends Biotechnol*, Volume 12, pp. 222-227.
- Koopman, M. J. F., 1963. Results of a number of storage experiments conducted under controlled conditions (other than agricultural seeds). *Proc Int Seed Test Ass*, 28(4), pp. 853-860.
- Koster, K. L., Webb, M. S., Bryant, G. & Lynch, D. U., 1994. Interactions between soluble sugars and POPC(1-palmitoyl-2-oleolphosphatidylcholine) during dehydration: vitrification of sugars alters the phase behaviour of the phospholipid. *Biochimic Biophys Acta*, Volume 1193, pp. 143-150.
- Kovach, D. A. & Bradford, K. J., 1992. Imbibitional damage and desiccation tolerance of wild rice (*Zizanium palustris*) seeds. *J Exp Bot*, Volume 43, pp. 747-757.
- Kowalski, R. M. & Kawecki, Z., 1982. Physiology of breaking hazel seed dormancy. 1. Germination of stratified seeds in the year of harvest and after storage for one year. *Roczniki Nauk Rolniczych, Seria A*, Volume 105, pp. 179-190.
- Koyuncu, F., 2005. Breaking seed dormancy in black mulberry (*Morus nigra* L.) by cold stratification and exogenous application of gibberellic acid. *Acta Biol Cracoviencia Series Botanica*, Volume 47, pp. 23-26.
- Kranner, I. & Birtic, S., 2005. A Modulating Role for Antioxidants in Desiccation Tolerance. *Integr Comp Biol*, Volume 45, pp. 734-740.
- Kranner, I., Birtic, S. & Pritchard, H. W., 2008. *Kew Plants People Possibilities*. [Online] Available at: http://www.kew.org/science/directory/projects/SA_OxidativeStressDe.html [Accessed 16 02 2009].
- Kris-Etherton, P. M. et al., 2002. Bioactive compounds in foods: Their role in the prevention of cardiovascular disease and cancer. *Am J Med*, 113(9B), pp. 71S-88S.
- Krishnan, P. N., Kumar, A. C. & Babu, K. P., 2002. Seed storage and viability of *Myristica malabarica* Lam. An endemic species of Southern Western Ghats (India). *Seed Sci Technol*, Volume 30, pp. 651-657.
- Kusari, S., Singh, S. & Jayabaskaran, C., 2014. Rethinking production of Taxol (paclitaxel) using endophyte biotechnology. *Trends Biotechnol*, 32(6), pp. 304-311.
- Lakon, G., 1949. The topographical tetrazolium method for determining the germination capacity of seeds. *Plant Physiol*, Volume 24, pp. 389-394.
- Lamarca, E. V. et al., 2013. Maturation of *Eugenia pyriformis* seeds under different hydric and thermal conditions. *An Acad Bras Ciênc*, Volume 85, pp. 223-233.

- Lamarca, E. V., Silva, C. V. & Barbedo, C. J., 2011. Limites térmicos para a germinação em função da origem de sementes de espécies de *Eugenia* (Myrtaceae) nativas do Brasil. *Acta Bot Bras*, Volume 25, pp. 293-300.
- Lane, B. G., 1991. Cellular desiccation and hydration: developmentally regulated proteins, and their maturation and germination of seed embryos. *FASEB J*, Volume 5, pp. 2893-2901.
- Lee, J., Koo, N. & Min, D. B., 2004. Reactive oxygen species, aging, and antioxidative nutraceuticals. *Compr Rev Food Sci Food Safety*, Volume 3, pp. 21-33.
- Leopold, A. C., 1990. Stress in plants: adaptation and acclimation mechanisms. In: R. G. Alscher & J. R. Cumming, eds. *Coping with desiccation*. New York: Wiley-Liss, pp. 37-56.
- Leviator, S., Shoseyov, O. & Wolf, S., 1995. Involvement of endomannanase in the control of tomato seed germination under low temperature conditions. *Ann Bot*, Volume 76, pp. 1-6.
- Leymarie, J. et al., 2012. Role of Reactive Oxygen Species in the Regulation of Arabidopsis Seed Dormancy. *Plant Cell Physiol*, 53(1), pp. 96-106.
- Li, B. L. & Foley, M. E., 1997. Genetic and molecular control of seed dormancy. *Trends Plant Sci*, Volume 2, pp. 384-389.
- Li, C. & Sun, Q. W., 1999. Desiccation sensitivity and activities of free radical-scavenging enzymes in recalcitrant *Theobroma cacao* seeds. *Seed Sci Res*, 9(3), pp. 209-217.
- Li, L. & Ross, J. D., 1990. Lipid mobilisation during dormancy-breakage in oilseed of *Corylus avellana* L. *Ann Bot*, 66(5), pp. 501-505.
- Linnington, S. H. & Pritchard, H. W., 2001. *Genebanks*. In *Encyclopedia of Biodiversity*. San Diego, CA, USA: Academic Press.
- Lin, T. P. & Wu, J. C., 1995. Seed storage behaviour of *Michelia compressa* (Max.) Sargent. *Seed Sci Technol*, Volume 23, pp. 309-319.
- Li, S. et al., 2013. Methods for breaking the dormancy of eastern redbud (*Cercis canadensis*). *Seed Sci Technol*, 41(1), pp. 27-35.
- Liu, D., Yu, H. L., Li, F. L. & Guo, H. H., 2011. An analysis of dormancy and dormancy release in *Taxus chinensis* var. *mairei* seeds. *Seed Sci Technol*, 39(1), pp. 29-43.
- Liu, Q. et al., 2014. Germination of recalcitrant *Baccaurea ramiflora* seeds. *ScienceAsia*, Volume 40, pp. 101-105.
- Liu, Y., Kermodé, A. R. & El-Kassaby, Y. A., 2013. The role of moist-chilling and thermo-priming on the germination characteristics of white spruce (*Picea glauca*) seed. *Seed Sci Technol*, 41(3), pp. 321-335.
- Locatelli, M. et al., 2010. Total antioxidant activity of hazelnut skin (Nocciola Piemonte PGI): Impact of different roasting conditions. *Food Chem*, 119(4), pp. 1647-1655.

- Luo, Y. L. et al., 2012. Storage behaviour and antioxidant activities of *Mimusops elengi* seeds subjected to different drying rates. *Seed Sci Technol*, Volume 40, pp. 354-364.
- Lüttkopf, D. et al., 2002. Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol Immunol*, 38(7), pp. 515-525.
- Magan, N., Cayley, G. & Lacey, J., 1984. Effect of Water Activity and Temperature on Mycotoxin Production by *Alternaria alternata* in Culture and on Wheat Grain. *Appl Environ Biol*, 47(5), pp. 1113-1117.
- Magini, E., 1962. Forest seed handling, equipment and procedures: II Seed treatments, storage, testing and transport. *Unasylva*, 16(1), pp. 20-35.
- Manfre, A. J., LaHatte, G. A., Climer, C. R. & Marcotte Jr, W. R., 2009. Seed dehydration and the establishment of desiccation tolerance during seed maturation is altered in the *Arabidopsis thaliana* mutant *atem6-1*. *P Cell Physiol*, Volume 50, pp. 243-253.
- Marrero, P., Padilla, D. P., Valdes, F. & Nogales, M., 2007. Comparison of three chemical tests to assess seed viability: the seed dispersal system of the Macaronesian endemic plant *Rubia fruticosa* (Rubiaceae) as an example. *Chemoecology*, 17(1), pp. 47-50.
- Mavi, K., Mavi, F., Demir, I. & Matthews, S., 2014. Electrical conductivity of seed soak water predicts seedling emergence and seed storage potential in commercial seed lots of radish. *Seed Sci Technol*, 42(1), pp. 76-86.
- McIntyre, G. I., 1987. The role of water in the regulation of plant development. *Can J Bot*, Volume 65, pp. 1287-1298.
- McPherson, J. E. & McPherson, R. M., 2000. *Stink bugs of economic importance in America north of Mexico*. Boca Raton(Florida): CRC.
- Mehlenbacher, S. A., 1991. Genetic resources in temperate fruit and nut crops. *Acta Hort*, Volume 290, pp. 789-836.
- Michalak, M., Plitta, B. P. & Chmielarz, P., 2013. Desiccation sensitivity and successful cryopreservation of oil seeds of European hazelnut (*Corylus avellana*). *Ann Appl Biol*, Volume 163, pp. 351-358.
- Miller, J. C. & Miller, J. N., 1988. *Statistics for analytical chemistry*. 2nd ed. Chichester: Ellis Horwood.
- Miller, W. A. & Roy, K. W., 1982. Mycoflora of soybean leaves, pods and seeds in Mississippi. *Can J Bot*, Volume 60, pp. 2716-2723.
- Mimica-Dukic, N., Bozin, B., Sokovic, M. & Simin, N., 2004. Antimicrobial and Antioxidant Activities of *Melissa officinalis* L. (Lamiaceae) Essential Oil. *J Agric Food Chem*, 52(9), pp. 2485-2489.
- Min, B. et al., 2012. Free and phenolic concentrations, antioxidant properties, and profiles of proanthocyanidins and anthocyanins in white grain rice (*Oryza sativa* L.) of different bran colours. *Food Chemistry*, Volume 133, pp. 715-722.

- Molnar, T. J., 2014. *Extension: American Research-based Learning Network*. [Online] Available at: <http://www.extension.org/pages/26616/growing-hazelnuts-for-biofuel-production> [Accessed 02 08 2014].
- Molnar, T. J., Goffreda, J. C. & Funk, R., 2010. Survey of Resistance to *Anisogramma anomala* from Different Geographic Locations. *HortScience*, 45(5), pp. 832-836.
- Morohashi, Y., 2002. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. *J Exp Bot*, Volume 53, pp. 1643-1650.
- Msanga, H. P., 1996. *Effect of fruit ripeness stages and seed moisture content on storability and germination of neem (Azadirachta indica) seed*. Arusha, Tanzania, Danida Forest Seed Centre (DFSC), pp. 201-209.
- Naithani, S. C. & Chaitanya, K. S., 1994. Role of superoxide, lipid peroxidation and superoxide dismutase in membrane perturbation during loss of viability in seeds of *Shorea robusta* Gaertn.f. *New Phytol*, Volume 126, pp. 623-627.
- Nayal, J. S., Thapliyal, R. C., Rawat, M. M. C. & Phartyal, S. S., 2000. Desiccation tolerance and storage behaviour of neem (*Azadirachta indica* A. Juss.) seeds. *Seed Sci Technol*, Volume 28, pp. 761-767.
- Newton, R. J., Hay, F. R. & Ellis, R. H., 2013. Seed development and maturation in early spring-flowering *Galanthus nivalis* and *Narcissus pseudonarcissus* continues post-shedding with little evidence of maturation in planta. *Ann Bot*, Volume 111, pp. 945-955.
- Nguyen, H. D. T., Nickerson, N. L. & Seifert, K. A., 2013. Basidioascus and Geminibasidium a new lineage of heat-resistant and xerotolerant basidiomycetes. *Mycologia*, Volume 105, pp. 1231-1250.
- Nikolaeva, M. G., 1967. *Physiology of deep dormancy in seeds*. Washington, DC, USA: National Science Foundation.
- Nikolaeva, M. G., 2004. On criteria to use in studies of seed evolution. *Seed Sci Res*, Volume 14, pp. 315-320.
- Nkang, A., Omokaro, D. & Egbe, A., 2000. Effects of desiccation on the lipid peroxidation and activities of peroxidase and polyphenoloxidase in seeds of *Telfaria occidentalis*. *Seed Sci Technol*, Volume 28, pp. 1-9.
- Nkang, A., Omokaro, D., Egbe, A. & Amanke, G., 2003. Variations in fatty acid proportions during desiccation of *Telfaria occidentalis* seeds harvested at physiological and agronomic maturity. *Afr J Biotechnol*, 2(2), pp. 33-39.
- Nkomo, M. & Kambizi, L., 2009. Effects of pre-chilling and temperature on seed germination of *Corchorus olitorius* L. (Tiliaceae) (Jew's Mallow), a wild leafy vegetable. *Afr J Biotechnol*, 8(6), pp. 1078-1081.
- Nomaguchi, M., Nonogaki, H. & Mororhashi, Y., 1995. Development of galactomannan-hydrolyzing activity in the micropylar endosperm tip of tomato seed prior to germination. *Physiol Planta*, Volume 94, pp. 105-109.

- Normah, M. N., 1987. *Effects of Temperature on Rubber (Hevea brasiliensis Muell.-Agr.) Seed Storage, PhD Thesis*. Malaysia: Universiti Pertanian Malaysia.
- Normah, M. N., Reed, B. M. & Yu, X., 1994. Seed storage and cryoexposure behaviour in hazelnut (*Corylus avellana* L. cv. Barcelona). *Cryo-letters*, Volume 15, pp. 315-322.
- Ntuli, T. M., Finch-Savage, W. E., Berjak, P. & Pammenter, N. W., 2011. Increased Drying Rate Lowers the Critical Water Content for Survival in Embryonic Axes of English Oak (*Quercus robur* L.) Seeds. *J Integr P Biol*, 53(4), pp. 270-280.
- Obroucheva, N. V. & Antipova, O. V., 2004. The Role of Water Uptake in the Transition of Recalcitrant Seeds from Dormancy to Germination. *Russ J P Physiol*, 51(6), pp. 848-856.
- Obroucheva, N. V., Lityagina, S. V., Novikova, G. V. & Sin'kevich, I. A., 2012. Vacuolar status and water relations in embryonic axes of recalcitrant *Aesculus hippocastanum* seeds during stratification and early germination. *Ann Bot*, Volume 12, pp. 1-14.
- Ojima, I., Geney, R., Ungureanu, I. M. & Li, D., 2002. Medicinal chemistry and chemical biology of new generation taxane antitumor agents. *IUBMB Life*, Volume 53, pp. 269-274.
- Oliveira, I. et al., 2008. Chemical composition, and antioxidant and antimicrobial activities of three hazelnut (*Corylus avellana* L.) cultivars. *Food Chem Toxicol*, Volume 46, pp. 1801-1807.
- Oliveira, I. et al., 2007. Hazel (*Corylus avellana* L.) leaves as source of antimicrobial and antioxidative compounds. *Food Chem*, Volume 105, pp. 1018-1025.
- Oracz, K. et al., 2007. ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J*, 50(3), pp. 452-65.
- Oracz, K. et al., 2009. The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. *Plant Physiol*, 150(1), pp. 494-505.
- Orthen, B., Popp, M. & Smirnoff, N., 1994. Hydroxyl radical scavenging properties of cyclitols. *Proceedings of the Royal Society of Edinburgh. Section B (Biological Sciences)*, Volume 102, pp. 269-272.
- Ozdemir, F. & Akinci, I., 2004. Physical and nutritional properties of four major commercial Turkish hazelnut varieties. *J Food Eng*, Volume 63, pp. 341-347.
- Packa, D., Kwiatkowski, J., Graban, Ł. & Lajsner, W., 2014. Germination and dormancy of *Sida hermaphrodita* seeds. *Seed Sci Technol*, 42(1), pp. 1-15.
- Pammenter, N. W. et al., 1994. Recalcitrance is not an all-or-nothing situation. *Seed Sci Res*, Volume 4, pp. 263-264.
- Pandey, D. K., 1988. Electrolyte efflux into hot water as a test for predicting the germination and emergence of seeds. *J Hort Sci*, Volume 63, pp. 601-604.

- Pandey, D. K., 1989. Ageing of French bean seeds at ambient temperature in relation to vigour and viability. *Seed Sci Technol*, Volume 17, pp. 41-47.
- Pandey, S. et al., 2013. Breaking seed dormancy in sweet gourd (*Momordica cochinchinensis*). *Seed Sci Technol*, 41(1), pp. 133-136.
- Pasquini, S., Braidot, E., Petrusa, E. & Vianello, A., 2011. Effect of different conditions in recalcitrant seeds of holm oak (*Quercus ilex* L.) during germination. *Seed Sci Technol*, 39(1), pp. 165-177.
- Pawlowski, T. A., 2009. Proteome analysis of Norway maple (*Acer platanoides* L.) seeds dormancy breaking and germination: influence of abscisic and gibberellic acids. *BMC P Biol*, Volume 9, p. 48.
- Pawlowski, T. A., 2010. Proteomic approach to analyze dormancy breaking of tree seeds. *Plant Mol Biol*, Volume 73, pp. 15-25.
- Pedro, L.-L. & Ellis, R. H., 2002. Seed storage behaviour of *Fagus sylvatica* and *Fagus crenata*. *Seed Sci Res*, 12(1), pp. 31-37.
- Pence, V. C., 1990. Cryostorage of embryo axes of several large-seeded temperate tree species. *Cryobiology*, Volume 27, pp. 212-218.
- Pereira, W. V. S., Faria, J. M. R., Tonetti, O. A. O. & Silva, E. A. A., 2012. Tolerância à dessecação em sementes de *Tapirira obtusa* procedentes de diferentes ambientes. *Rev Bras Sementes*, Volume 34, pp. 388-396.
- Perez, A., Reyes, M. N. & Cuevas, J., 1980. Germination of two papaya varieties: Effect of seed aeration, K-treatment, removing of the sarcotesta, high temperature, soaking in distilled water, and age of seeds. *J Agric Uni Puerto Rico*, Volume 64, pp. 173-180.
- Phartyal, S. S., Thapliyal, R. C., Nayal, J. S. & Joshi, G., 2003. Assessment of viability of *Acer caesium* and *Ulmus wallichiana* seeds through the tetrazolium test. *Seed Sci Technol*, Volume 31, pp. 691-700.
- Pinfield, N. J., 1965. *Gibberellic Acid and Seed Dormancy*, PhD Thesis. Cardiff: University of Wales.
- Pinfield, N. J. & Stobert, A. K., 1969. Gibberellin-stimulated nucleic acid metabolism in cotyledons and embryonic axes of *Corylus avellana* (L.) seeds. *New Phytol*, Volume 68, pp. 993-999.
- Poulsen, K. M., 1993. Predicting the storage life of beech nuts. *Seed Sci Technol*, Volume 21, pp. 327-337.
- Poulsen, K. M. & Kundsén, H., 1999. Viability constants based on eight years storage of beech nuts (*Fagus sylvatica* L.). *Seed Sci Technol*, Volume 27, pp. 1037-1039.
- Powell, A. A., Oliveira, M. A. & Mathews, S., 1986. The role of imbibitional damage in determining the vigour of white and coloured seed lots of dwarf French beans (*Phaseolus vulgaris*). *J Exp Bot*, Volume 37, pp. 716-722.

- Priestley, D. A., Warner, B. G., Leopold, A. C. & McBride, M. B., 1985. Organic free radical levels in seeds and pollen. The effect of hydration and ageing. *Physiol Planta*, Volume 70, pp. 88-94.
- Pritchard, H. W., 1991. Water Potential and Embryonic Axis Viability in Recalcitrant Seeds of *Quercus rubra*. *Ann Bot*, 67(1), pp. 43-49.
- Pritchard, H. W. & Pendergast, F. G., 1986. Effects of desiccation and cryopreservation on the in vitro viability of embryos of the recalcitrant seed species *Araucaria hunsteinii* K. Schum. *J Exp Bot*, Volume 37, pp. 1388-1397.
- Pritchard, H. W., Tompsett, P. B., Manger, K. & Smidt, W. J., 1995. The effect of moisture content on the low temperature responses of *Araucaria hunsteinii* seed and embryo. *Ann Bot*, 76(1), pp. 79-88.
- Pukacka, S., 1989. The effect of desiccation on viability and phospholipid composition of *Acer saccharinum* L. seeds. *Trees*, Volume 3, pp. 144-148.
- Pukacka, S., 1993. Phospholipase D activity during long-term storage of *Acer platanoides* seeds in the imbibed state and desiccation of *Acer saccharinum* seeds. *Acta Physiol Plant*, Volume 15, pp. 147-153.
- Pukacka, S., 1998. Changes in membrane fatty acid composition during desiccation of seeds of silver maple. *Seed Sci Technol*, Volume 26, pp. 535-40.
- Pukacka, S. & Ratajczak, E., 2006. Antioxidative response of ascorbate-glutathione pathway enzymes and metabolites to desiccation of recalcitrant *Acer saccharinum* seeds. *J Plant Physiol*, Volume 163, pp. 1259-1266.
- Pyrzyska, K. & Anna Pękal, A., 2013. Application of free radical diphenylpicrylhydrazyl (DPPH) to estimate the antioxidant capacity of food samples. *Anal Methods*, Volume 5, pp. 4288-4295.
- Qaderi, A. et al., 2012. Hazel (*Corylus avellana* L.) as a new source of taxol and taxanes. *J Med Plants*, 11(41), pp. 66-77.
- Ramos, K. M. O., Matos, J. M. M., Martins, R. C. C. & Martins, I. S., 2012. Electrical Conductivity Testing as Applied to the Assessment of Freshly Collected *Kielmeyera coriacea* Mart. Seeds. *ISRN Agronomy*, Article ID 378139(2012), p. 5.
- Reed, B. M., 1999. *National Clonal Germplasm Repository, Appendix 2b*. Corvallis(Oregon): USDA.
- Reed, B. M., Normah, M. & Xiaoling, Y., 1994. Stratification is necessary for successful cryopreservation of axes from stored hazelnut seed. *Cryo-letters*, Volume 15, pp. 377-384.
- Rees, A. R., 1963. A large-scale test of storage methods for oil palm seed. *J West Afr Inst Oil Palm Res*, 4(13), pp. 46-51.
- Reiner, L. & Loch, U., 1975. Temperature and barley dormancy. *Cereal Res Commu*, Volume 4, pp. 107-110.
- Rendon, G. A., 1983. *Seed dormancy and germination in Corylus avellana L.*, PhD Thesis. London: University of London.

- Ren, J. & Tao, L., 2003. Effect of hydration-dehydration cycles on germination of seven Calligonum species. *J Arid Environ*, 55(1), pp. 111-122.
- Ricci, D. et al., 2005. Chemical composition, antimicrobial and antioxidant activity of the essential oil of *Teucrium marum* (Lamiaceae). *J Ethnopharmacol*, 98(1-2), pp. 195-200.
- Rigby, N. M. et al., 2008. The purification and characterisation of allergenic hazelnut seed proteins. *Mol Nut Food Res*, 52(1), pp. S251-S261.
- Roberts, E. H., 1963. The effects of inorganic ions on dormancy in rice seed. *Physiol Plant*, Volume 16, pp. 732-744.
- Roberts, E. H., 1973. Predicting the storage life of seeds. *Seed Sci Technol*, Volume 1, pp. 499-514.
- Rodríguez-Gacio, M. C., Iglesias-Fernández, R., Carbonero, P. & Matilla, A. J., 2012. Softening-up mannan-rich cell walls. *J Exp Bot*, 63(11), pp. 3976-3988.
- Roederer, Y. & Bellefontaine, R., 1989. Can neem seed be expected to keep their germinative capacity for several years after collection? *Forest Genetic Resources Information*, Volume 17, pp. 30-33.
- Ross, J. D., 1970. *Hazel seed gibberellins and inhibitors in relation to dormancy and germination*, PhD Thesis. London: University of London.
- Ross, J. D. & Bradbeer, J. W., 1968. Concentrations of gibberellins in chilled hazel seeds. *Nature (London)*, Volume 220, pp. 85-86.
- Ross, J. D. & Bradbeer, J. W., 1971a. Studies in seed dormancy.V. The content of endogenous gibberellins in seeds of *Corylus avellana* L. *Planta (Berlin)*, Volume 100, pp. 288-302.
- Ross, J. D. & Bradbeer, J. W., 1971b. Studies in seed dormancy. VI. The effects of growth retardants on the gibberellin content and germination of chilled seeds of *Corylus avellana* L. *Planta (Berlin)*, Volume 100, pp. 303-308.
- Sacande, M. et al., 1996. Intermediate storage behaviour of neem (*Azadirachta indica*) seeds from Burkina Faso. In: A. S. Oue'draogo, K. Poulsen & F. Stubsgaard, eds. *Improved method for the handling and storage of intermediate/recalcitrant tropical forest tree seeds*. Rome: International Plant Genetic Resources Institute (IPGRI), pp. 101-104.
- Sattler, S. E. et al., 2004. Vitamin E Is Essential for Seed Longevity and for Preventing Lipid Peroxidation during Germination. *The Plant Cell*, 16(6), pp. 1419-1432.
- Schafer, H., Schulte, E. & Thier, H. -P., 2002. A novel and simple approach for assessing the freshness of hazelnuts. *Eur Food Res Technol*, Volume 215, pp. 249-254.
- Schopmeyer, C. S., 1974. *Seeds of woody plants in the United States*. Washington DC: USDA Forest Service.

- Shahidi, F., Alasalvar, C. & Liyana-Pathirana, C. M., 2007. Antioxidant phytochemicals in hazelnut kernel (*Corylus avellana* L.) and hazelnut byproducts. *J Agric Food Chem*, Volume 55, pp. 1212-1220.
- Shannon, P. R. M., Sharrard, R. M. & Jarvis, B. C., 1981. Messenger RNA in embryonic axes of dormant and non-dormant seeds of hazel (*Corylus avellana* L.). *Plant Cell Physiol*, Volume 22, pp. 1293-1304.
- Sharma, O. & Bhat, T., 2009. DPPH antioxidant assay revisited. *Food Chemistry*, Volume 113, pp. 1202-1205.
- Shelar, V. R., 2008. Role of mechanical damage in deterioration of soybean seed quality during storage- a review. *Agric. Rev*, 29(3), pp. 177-184.
- Shereena, J. & Salim, N., 2006. Influence of Seed Moisture Content and Leakage on Germination and Viability in *Pisum sativum* L. Seeds. *Int J Bot*, Volume 2, pp. 427-430.
- Shewry, P., Pinfield, N. & Stobart, A., 1972. The glycerides and acyl fatty acids of germinating hazel seeds. *Phytochemistry*, Volume 11, pp. 2149-2154.
- Simon, E. W., 1984. Early events in germination. In: D. R. Murray, ed. *Seed Physiology, Germination and reserve mobilization*. Australia: Acad Press, pp. 77-115.
- Simsek, O., Arici, M. & Demir, C., 2002. Mycoflora of hazelnut (*Corylus avellana* L.) and aflatoxin content in hazelnut kernels artificially infected with *Aspergillus parasiticus*. *Food / Nahrung*, 46(3), pp. 194-196.
- Sivakumar, V. et al., 2006. Germination requirements and storage behavior of *Myristica dactyloides* Gaertn. seeds. *Seed Sci Technol*, 34(3), pp. 729-733.
- Sivasubramaniam, K. & Selvarani, K., 2012. Viability and vigor of jamun *Syzygium cumini* seeds. *Brazilian Journal of Botany*, 35(4), pp. 397-400.
- Slate, G. L., 1969. Filberts - including varieties grown in the east. In: R. A. Jaynes, ed. *Handbook of North American Nut Trees*. Tennessee: Northern Nut Growers Association.
- Sökmen, M. et al., 2004. In Vitro Antioxidant, Antimicrobial, and Antiviral Activities of the Essential Oil and Various Extracts from Herbal Parts and Callus Cultures of *Origanum acutidens*. *J Agric Food Chem*, 52(11), pp. 3309-3312.
- Souza, C. R., Ohlson, O. C., Gavazza, M. I. A. & Panobianco, M., 2010. Tetrazolium test for evaluating triticale seed viability. *Rev Bras Sementes*, 32(3), pp. 47-54.
- Staniek, A., Woerdenbag, H. J. & Kayser, O., 2009. *Taxomyces andreanae*: a presumed Paclitaxel producer demystified. *Planta Med*, Volume 75, pp. 1561-1566.
- Stanwood, P. C., 1985. In Cryopreservation of plant cells and organs . In: K. Kartha, ed. Boca Raton: CRC Press, pp. 200-226.
- Stanwood, P. C. & Bass, L. N., 1981. Seed germplasm preservation using liquid nitrogen. *Seed Sci Technol*, Volume 9, pp. 423-437.

- Stierle, A., Strobel, G. & Stierle, D., 1993. Taxol and taxane production in *Taxomyces andreanae*, an endophytic fungus of Pacific yew. *Science*, Volume 260, pp. 214-216.
- Still, D., Kovach, D. & Bradford, K., 1994. Development of desiccation tolerance during embryogenesis in rice (*Oryza sativa*) and wild rice (*Zizania palustris*). *Plant Physiol*, Volume 104, pp. 431-438.
- Stobart, A. K. & Pinfield, N. J., 1970. Glycerol utilization in seeds of *Corylus avellana* (L). *New Phytol*, Volume 69, pp. 939-949.
- Strobel, G. et al., 1996. Taxol from *Pestalotiopsis microspora*, an endophytic fungi of *Taxus wallachiana*. *Microbiology*, Volume 142, pp. 435-440.
- Sukesh & Chandrashekar, K. R., 2013. Effect of Temperature on Viability and Biochemical Changes During Storage of Recalcitrant Seeds of *Vatica chinensis*. *Int J Bot*, 19(2), pp. 73-79.
- Sung-Sook, C., Dhiraj, A., Yuan-Tong, L. & Kalidas, S., 2005. Phenolic antioxidants from clonal oregano (*Origanum vulgare*) with antimicrobial activity against *Helicobacter pylori*. *Process Biochemistry*, 40(2), pp. 809-816.
- Suszka, B. & Tylkowski, T., 1980. Storage of the acorns of the English oak (*Quercus robur* L.) over 1-5 winters. *Arboretum Kornickie*, Volume 25, pp. 199-229.
- Swarbrick, J. T., 1965. Storage of cocoa seeds. *Expl Agric*, Issue 1, pp. 201-207.
- Szilagyi-Zecchin, V. et al., 2014.. Identification and characterization of endophytic bacteria from corn (*Zea mays* L.) roots with biotechnological potential in agriculture. *AMB Express*, Volume 4, p. 26.
- Tang, A., 2012. Desiccation-induced changes in viability, lipid peroxidation and antioxidant enzyme activity in *Mimusops elengi* seeds. *Afr J Biotechnol*, 11(44), pp. 10255-10261.
- Tang, A., Zhao, Z. & Luo, A., 2013. Deep physiological dormancy in desiccation-sensitive seeds of *Osmanthus fragrans* (Oleaceae). *Seed Sci Technol*, 41(3), pp. 336-344.
- Tilden, R. L. & West, S. H., 1985. Reversal of the effects of ageing in soybean seeds. *Plant Physiol*, Volume 77, pp. 584-586.
- Tillman, P. G., Aldrich, J. R., Khrimian, A. & Cottrell, T. E., 2010. Pheromone attraction and cross-attraction of *Nezara*, *Acrosternum*, and *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae) in the field. *Environ Entomol*, 39(2), pp. 610-617.
- Tommasi, F., Paciolla, C. & Arrigoni, O., 1999. The ascorbate system in recalcitrant and orthodox seeds. *Physiol Planta*, Volume 105, pp. 193-198.
- Tommasi, F., Paciolla, C., Pinto, M. C. & Laura De Gara, L. D., 2006. Effects of storage temperature on viability, germination and antioxidant metabolism in *Ginkgo biloba* L. seeds. *Plant Physiol Biochem*, 44(5-6), pp. 359-368.

- Tompsett, P. B., 1984. Desiccation studies in relation to the storage of Araucaria seed. *Ann Appl Biol*, Volume 105, pp. 581-586.
- Tompsett, P. B., 1987. *A review of the literature on storage of dipterocarp seeds*. Umea, Swedish University of Agricultural Sciences.
- Tompsett, P. B., 1987. Desiccation and storage studies on Dipterocarpus seeds. *Ann Appl Biol*, 110(2), pp. 371-379.
- Tompsett, P. B. & Pritchard, H. W., 1993. Water status changes during development in relation to the germination and desiccation tolerance of Aesculus hippocastanum L. seeds. *Ann Bot*, Volume 71, pp. 107-116.
- Tompsett, P. B. & Pritchard, H. W., 1998. The effect of chilling and moisture status on germination, desiccation tolerance and longevity of Aesculus hippocastanum L. *Ann Bot*, 82(2), pp. 249-261.
- Tunnacliffe, A., Hinch, D. K., Leprince, O. & Macherel, D., 2010. LEA Proteins: Versatility of Form and Function. Dormancy and Resistance in Harsh Environments. *Top Curr Gen*, Volume 21, pp. 91-108.
- Tunnacliffe, A. & Wise, M. J., 2007. The continuing conundrum of the LEA proteins. *Naturwissenschaften*, Volume 94, pp. 791-812.
- Tylkowski, T., 1999. Generatywne rozmrażanie leszczyny posppolitej (*Corylus avellana* L.). *Arboretum Kornickie*, Volume 44, pp. 87-96. In Polish with English Abstract.
- Uzun, A., Sozen, E. & Acikgoz, E., 2013. Seed dormancy and germination of *Vicia sativa* subsp. *nigra* and *Vicia sativa* subsp. *macrocarpa*. *Seed Sci Technol*, 41(1), pp. 137-142.
- Valerie, G., Finch-Savage, W. E., Quick, W. P. & Atherton, N. M., 2000. Putative desiccation tolerance mechanism in orthodox and recalcitrant seeds of the genus *Acer*. *Seed Sci Res*, Volume 10, pp. 317-327.
- Valio, I. F. M. & Ferreira, Z. L., 1992. Germination of seeds of *Myrciaria cauliflora* (MART.) BERG (Myrtaceae). *R Bras Fisiol Veg*, 4(2), pp. 95-98.
- Valko, M. et al., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Intl J Biochem Cell Biol*, Volume 39, pp. 44-84.
- Vandelook, F., Bolle, B. & Van Assche, J. A., 2007. Seed Dormancy and Germination of the European *Chaerophyllum temulum* (Apiaceae), a Member of a Trans-Atlantic Genus. *Ann Bot*, 100(2), pp. 233-239.
- Varghese, B. et al., 2011. Differential drying rates of recalcitrant *Trichilia dregeana* embryonic axes: a study of survival and oxidative stress metabolism. *Physiol Plantarum*, Volume 142, pp. 326-338.
- Vashisth, A. & Nagarajan, S., 2009. Germination Characteristics of Seeds of Maize (*Zea mays* L.) Exposed to Magnetic Fields under Accelerated Ageing Condition. *J Agricult Physiol*, Volume 9, pp. 50-58.
- Vaughan, J. G., 1970. *The structure and utilization of oil seeds*. London: Chapman and Hall.

- Vertucci, C. W. & Farrant, J. M., 1995. Acquisition and loss of desiccation tolerance. In: M. Negbi & J. Kigel, eds. *Seed development and germination*. New York: Marcel Dekker, pp. 237-271.
- Vertucci, C. W. & Leopold, A. C., 1987. The Relationship between Water Binding and Desiccation Tolerance in Tissues. *Plant Physiol*, Volume 85, pp. 232 - 238.
- Vidensek, N., Lim, P., Campbell, A. & Carlson, C., 1990. Taxol Content in Bark, Wood, Root, Leaf, Twig and Seedling from Several Taxus Species. *J Nat Prod*, 53(6), pp. 1609-1610.
- Villalobos, V. M. & Engelman, F., 1995. Ex situ conservation of plant germplasm using biotechnology. *World J Micro Biotech*, Volume 11, pp. 375-382.
- Vleeshouwers, L. M., Bouwmeester, H. J. & Karssen, C. M., 1995. Redefining seed dormancy: An attempt to integrate physiology and ecology. *J Ecol*, Volume 83, pp. 1031-1037.
- Walters, C., 2000. Levels of recalcitrance in seeds. *Rev Bras Fisiol Veg*, Volume 12(especial), pp. 7-21.
- Walters, C., Farrant, J. M., Pammenter, N. W. & Berjak, P., 2002. Desiccation Stress and Damage. In: M. Black & H. W. Pritchard, eds. *Desiccation and Survival in Plants Drying Without Dying*. London: CABI Publishing, p. 275.
- Walters, C. et al., 1998. Refrigeration can save seeds economically. *Nature*, Volume 395, p. 758.
- Wang, S. et al., 2000. Taxol from Tubercularia sp strain TF5, an endophytic fungus of Taxus mairei. *FEMS Microb Lett*, Volume 193, pp. 249-253.
- Wang, Y., 2013. *A Novel System to Study Seed Recalcitrance and Dormancy-Comparative Proteomics Between Two Spartina Species*, PhD Thesis. Louisiana: Louisiana State University.
- Wani, M. C. et al., 1971. Plant antitumor agents. VI. The isolation and structure of Taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J Am Chem Soc*, Volume 93, pp. 2325-2327.
- Wareing, P. F., 1965. Endogenous inhibitors in seed germination and dormancy. In: W. Ruhland, ed. *Encyclopedia of Plant Physiology*. Heidelberg, Berlin: Springer, pp. 909-924.
- Williams, P. M., Bradbeer, J. W., Gaskin, P. & MacMillan, J., 1974. Studies in seed dormancy. VIII. The identification and determination of gibberellins A1 and A9 in Seeds of Corylus avellana L. *Planta (Berlin)*, Volume 117, pp. 101-108.
- Williams, P. M., Ross, J. D. & Bradbeer, J. W., 1973. Studies in seed dormancy. VII. The abscisic acid content of the seeds and fruits of Corylus avallana L. *Planta (Berlin)*, Volume 110, pp. 303-310.
- Wood, A. & Bradbeer, J. W., 1967. Studies in seed dormancy. II. The nucleic acid metabolism of the cotyledons of Corylus avellana L. *New Phytol*, Volume 66, pp. 17-26.

- Wood, C. B., Pritchard, H. W. & Amritphale, D., 2000. Desiccation-induced dormancy in papaya (*Carica papaya* L.) seeds is alleviated by heat shock. *Seed Sci Res*, 10(2), pp. 135-145.
- Xia, K., Hill, L. M., Li, D. & Walters, C., 2014. *Factors affecting stress tolerance in recalcitrant embryonic axes from seeds of four Quercus (Fagaceae) species native to the USA or China*, Oxford: Oxford University Press.
- Xu, Y. X. & Hanna, M. A., 2009. Synthesis and characterization of hazelnut oil-based biodiesel. *J Ind Crop Prod*, Volume 29, pp. 473-479.
- Xu, Y. X. & Hanna, M. A., 2010. Evaluation of Nebraska hybrid hazelnuts: Nut/kernel characteristics, kernel proximate composition, and oil and protein properties. *J Ind Crop Prod*, 31(1), pp. 84-91.
- Xu, Y. X., Hanna, M. A. & Josiah, S. J., 2007. Hybrid hazelnut oil characteristics and its potential oleochemical application. *J Ind Crop Prod*, Volume 26, pp. 69-76.
- Yang, Y., Zhao, H., Barrero, R. A. & Zhang, B., 2014. Genome sequencing and analysis of the paclitaxe-producing endophytic fungus *Penicillium aurantiogriseum* NRRL 62431. *BMC Genomics*, Volume 15, p. 69.
- Yap, S. K., 1981. Collection, germination and storage of dipterocarp seeds. *Malay Forestry*, Volume 44, pp. 281-300.
- Ye, N. et al., 2012. Ascorbic acid and reactive oxygen species are involved in the inhibition of seed germination by abscisic acid in rice seeds. *J Exp Bot*, 63(5), pp. 1809-1822.
- Zaidman, B.-Z., Ghanim, M. & Vaknin, Y., 2010. Effect of seed weight on seed vigour and early seedling growth of *Jatropha curcas*, a biodiesel plant. *Seed Sci Technol*, Volume 38, pp. 757-766.
- Zhang, L. et al., 2011. Testing methods for seed quality of *Ammomum villosum*. *Zhongguo Zhong Yao Za Zhi*, 36(22), pp. 3086-90.
- Zhang, W. & Gusta, L. V., 2010. Germination response of black and yellow seed coated canola (*Brassica napus*) lines to chemical treatments under cold temperature conditions. *Plant Growth Regul*, 60(2), pp. 105-114.
- Zheng, G.-H. & Jing, X.-M., 1998. Ultradry seed storage cuts cost of gene bank. *Nature*, Volume 393, pp. 223-224.
- Zhu, Q. Y. et al., 2002. Antioxidative activities of oolong tea. *J Agric Food Chem*, Volume 50, pp. 6929-6934.