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Abstract: Abstract
An effective protocol for cauliflower micropropagation and artificial seed production was optimised by (Rihan et al. 2012a; Rihan et al. 2012b; Rihan et al. 2011b; Rihan et al. 2012c). However, in order to be a viable alternative to traditional seeds, cauliflower artificial seeds need to show a high capacity to withstand abiotic stresses such as cold and desiccation. Therefore, in order to increase cauliflower abiotic stress tolerance, the effect of cold acclimation and drought on the cold tolerance of both cauliflower microshoots and mature plants were investigated. Moreover, the effect of cold and drought treatments on the induction of CBF/DREB1 gene regulation was tested. Both cold acclimation and drought improved the cold tolerance in both cauliflower microshoots and mature plants. However, whilst cold acclimation up-regulated CBF/DREB1 in cauliflower mature plants and microshoots, drought had the capacity only to up-regulate this gene in mature plants. Therefore, the high effect of cauliflower developmental stage on the CBF/DREB1 regulation was confirmed. Moreover, a small reduction in soil moisture had the capacity to upregulated this gene in mature cauliflower plants. The results presented in this study have an important role in the improvement of cauliflower micropropagation and the effectiveness of the artificial seed production protocol. Furthermore, the results contribute to an understanding of the cold tolerance mechanism in Brassica oleraceae var botrytis.

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Cover letter

- Research paper
- Title: “Upregulation of CBF/DREB1 and cold tolerance in artificial seeds of cauliflower (Brassica oleracea var. botrytis)“
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This paper is the first to confirm the effect of developmental stage on the induction of *CBF* gene in cauliflower under the effect of drought stimulation. It also has a high importance in improving the cold stress tolerance of cauliflower artificial seed, thus, the results reported in the current study help to improve the quality of the artificial seeds as a possible promising alternative of the traditional seeds. The partial sequence of *CBF* in cauliflower has been determined in the paper and this is a good step toward finding the full sequence of this gene in cauliflower.

We will be happy to provide any additional details you may require.

Your sincerely.

Dr Hail Rihan

Highlights:

1. The existence of the *CBF* pathway and its role in the cold tolerance of cauliflower artificial seeds.
2. The capacity of drought stimulation to upregulate *CBF* in mature cauliflower plants but not in the microshoots.
3. The capacity of a small reduction of soil moisture to up-regulate the *CBF* gene in cauliflower mature plants.
4. The partial sequence of *CBF* in cauliflower, which was found to be highly similar to that in DREB2-23 [*Brassica napus*]

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Upregulation of *CBF/DREB1* and cold tolerance in artificial seeds of cauliflower (*Brassica oleracea* var. *botrytis*)

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Abstract

An effective protocol for cauliflower micropropagation and artificial seed production was optimised by (Rihan et al. 2012a; Rihan et al. 2012b; Rihan et al. 2011b; Rihan et al. 2012c). However, in order to be a viable alternative to traditional seeds, cauliflower artificial seeds need to show a high capacity to withstand abiotic stresses such as cold and desiccation. Therefore, in order to increase cauliflower abiotic stress tolerance, the effect of cold acclimation and drought on the cold tolerance of both cauliflower microshoots and mature plants were investigated. Moreover, the effect of cold and drought treatments on the induction of *CBF/DREB1* gene regulation was tested. Both cold acclimation and drought improved the cold tolerance in both cauliflower microshoots and mature plants. However, whilst cold acclimation up-regulated *CBF/DREB1* in cauliflower mature plants and microshoots, drought had the capacity only to up-regulate this gene in mature plants. Therefore, the high effect of cauliflower developmental stage on the *CBF/DREB1* regulation was confirmed. Moreover, a small reduction in soil moisture had the capacity to unregulated this gene in mature cauliflower plants. The results presented in this study have an important role in the improvement of cauliflower micropropagation and the effectiveness of the artificial seed production protocol. Furthermore, the results contribute to an understanding of the cold tolerance mechanism in *Brassica oleraceae* var botrytis.

Key words: *CBF/DREB1,* cauliflower, cold tolerance, abiotic stress, artificial seed and micropropagation,

Introduction

It is widely known that the exposure of most temperate plants to non-freezing low temperature (0 to 5 ^{o}C) for a period of time (7-14 days) increases their freezing tolerance and this process is known as cold acclimation (Thomashow 1999). Because of its importance to agriculture, great efforts have been made and many experiments have been conducted to improve the understanding of this important phenomenon (Thomashow 2001). Multiple polygenic traits appear and various physiological and biochemical changes occur during the progress of acclimation and these changes often involve modifications in membrane lipid structure (Lynch and Steponkus 1987; Uemura and Steponkus 1994). Acclimation also causes an increase in the production of antioxidants, abscisic acid and compatible osmolytes such as soluble sugars and proline (Chen et al. 1993; Dörffling et al. 1997; Kishitani et al. 1994; Koster and Lynch 1992; Lynch and Steponkus 1987; Murelli et al. 1995; Nomura et al. 1995; Tao et al. 1998; Uemura and Steponkus 1994). The improvement of cold tolerance by acclimation involves abroad reprogramming of gene expression and metabolism. Recent studies describing full genome transcripts and mutational and transgenic plant analysis have provided a great deal of information about the complex transcriptional systems that function under cold acclimation (Jan et al. 2009).

It has been reported that there is a set of genes which are highly up-regulated during the process of acclimation and these genes encode a specific family of proteins called cold- regulated (COR) proteins (Gilmour et al. 2004). Several types of cold-regulated (*COR*) genes have been recognized in both monocotyledonous and dicotyledonous plants (Sharma et al. 2005; Sun et al. 2009). It has been demonstrated that abscisic acid (ABA) can have an important role in acclimation and it has been shown that ABA-dependent and ABA-independent pathways are the main two pathways intermediating the induction of *COR* genes expression. In the ABA-dependent pathway, the accumulation of endogenous ABA observed under the effect of cold

triggers the basic leucine zipper (bZIP) transcription factor, which then induces ABA-dependent *COR* genes through ABA-regulated elements (Uno et al. 2000; Xiong et al. 2002). Also it has been demonstrated that ABA accumulates under the effects of other environmental stresses such as drought (Leung and Giraudat 1998). The accumulation of ABA causes several physiological adaptations including stomatal closure and growth inhibition. Moreover, ABA induces the expression of several genes other than the *COR* genes(Kurkela and Franck 1990; Lång and Palva 1992).

In the ABA independent pathway, cold induces the expression of C-repeat binding factor (*CBF*) transcription factors. This family of genes has an essential role in activating downstream *COR* genes which in turn improve the freezing tolerance in plants (Sun et al. 2009). The *CBF* transcription factor has been identified and characterized in many plant species including rape (*Brassica napus*), broccoli (*Brassica oleracea*), alfalfa (*Medicago sativa*), tomato (*Lycopersicon esculentum*), corn (*Zea mays*), rice (*Oryza sativa*), strawberry (*Fragaria ananassa*), soybeans (Glycine max) wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) (Al-Issawi et al. 2015a; Al-Issawi et al. 2015b; Choi et al. 2002; Dubouzet et al. 2003; Francia et al. 2004; Gao et al. 2002; Owens et al. 2002; Rihan et al. 2014; Vágújfalvi et al. 2003).

Under drought stress a similar mechanism exists and dehydration-responsive element binding factor (DREB) are up-regulated. Both *CBF*s and *DREBs* are transcription factors which induce the expression of cold and dehydration stress regulated gene in plants (Gilmour et al. 1998; Liu et al. 1998a; Shinwari et al. 1998). These transcription factors bind to specific regulatory sequences in the promoters of cold and dehydration responsive genes. These sequences are C-repeat (CRT: TGGCCCGAC) and dehydration-responsive elements (DRE: TACCGACAT). Both of these sequences contain the highly conserved core 5-bp sequence of CCGAC, which has the capacity to regulate transcription under drought and low temperature and also under salinity (Baker et al. 1994; Gao et al. 2007; Yamaguchi-Shinozaki and Shinozaki 1994). Thus

CBF induces the expression of *COR* genes (the genes which contain the *COR* sequence) and these genes play an essential role in the improvement of plant abiotic stress resistance.

Cauliflower is species which demonstrates cold tolerance through a *CBF* mediated pathway (Hadi et al 2009, Rihan 2013) and also a remarkable capability for plant tissue culture. Cauliflower curd can be homogenised, sieved, grown into microshoots and converted to artificial seed (Kieffer et al 2006; Rihan et al 2012). Rihan et al., (2011a) reported high growth capacity of cauliflower artificial seeds in commercial substrates which is considered a promising step for their direct use *in vivo*. However, cauliflower artificial seeds should ideally show high cold and drought tolerance in order to survive the vagaries of establishment in the field and be a competitive alternative to traditional seeds. This study aimed to investigate the effect of cold acclimation and drought on the cold tolerance of cauliflower artificial seeds. Moreover, it aimed to investigate the effect of these parameters on the induction of *CBF/DREB1* gene expression at different developmental stages (microshoots and mature cauliflower plants) and to determine the partial sequence of *CBF/DREB1* gene in cauliflower.

Material and Methods

Cauliflower microshoot production

Large pieces of cauliflower curds (cv.Dionis) (1–5 cm) were sterilized by immersion in diluted un-thickened domestic bleach (10% v:v, 0.06% sodium hypochlorite) for 15 min., followed by a double wash with sterile distilled water. Explants were produced mechanically by eliminating the mass of non-responsive tissue (stem branches) and shaving off the upper meristematic layer using a sterilized scalpel whilst working in a laminar flow cabinet. The meristimatic clusters were then homogenized using a commercial blender (Waring model 800) at approximately

1,700 rev min^{-1} in liquid maintenance S23 medium (4.4 g L^{-1} MS salts (Murashige and Skoog 1962)) supplied by Sigma^{TM} and 3% w/v sucrose) for 30 sec to produce a homogenate of micro-explants. The micro-explants were size graded by passing the homogenate through a series of sieves with aperture sizes of 212, 300 and 600 µm (Endacotts Ltd). A small volume (74 µL) of the 212-300 µm homogenate fraction was cultured in 30 mL S23 medium, supplemented with 2 mg L^{-1} Kinetin and 1 mg L^{-1} IBA in 125 mL plastic pots. In order to preserve culture sterility, the culture media was supplemented with 1 mL L^{-1} PPM^{TM} (Plant Preservative Mixture) which was used with all treatments. The 26 day old cultures were divided into two groups:

The first group was transferred to the cold room at 4 °C for acclimation. Samples of microshoots, each consisting of 2 culture pots, were sampled at 0 (control), 1, 6, 12, 18, 24 hours after the transfer to the new temperature. These samples were stored at -80°C until the RNA was extracted. The samples (100 ± 10 mg) were then ground to a powder in liquid nitrogen with a mortar and pestle and the total RNA was isolated using the Spectrum plant total RNA kit (Sigma Aldrich: spectrum plant total RNA kit, Cat # STRN50) according to the manufacturer's instructions. The total extracted RNA was quantified using the Nano-drop 1000 spectophotometer method to estimate its concentration. The purity of the RNA was assessed spectrophotometrically by examining the absorbance ratio at 260 and 280nm. The reverse transcription was carried out using M-MLV Reverse Transcriptase (Sigma: M1302) in 20 µL volume. Sequence specific primers for *CBF/DREB1* (Forward primer 5-ACTTTCCTAACCGCCGAC, Reverse primer 5-TCTCAGCCTGAAAAGCCA-3) and for the Actin 1 mRNAs (endogenous control) (Forward primer 5-CCCAAAGGCCAACAGAGAGAAG-3-3) (Reverse primer 5-CACCAGAGTCCAGCACAATACC-3) were designed using Primer-BLAST (Ye et al. 2012) and synthesized by Eurofin MWG/ Operon (Germany).

151 The cDNA for the samples was used as a template for gel electrophoresis PCR (Applied
152 Biosystems, Veriti) (Sigma kit). A Master mix was prepared consisting of (for each sample) 1
153 µL Red tag polymerase + 2.5 µL Red tag polymerase buffer + 0.5 µL forward primer + 0.5 µL
154 reverse primer + 0.5 µL dNTPs + 18 µL sterile nuclease free water. The master mix was
155 prepared for all samples together and 23 µL from the mixture was added to 2 µL of each sample
156 in nuclease free 1.5 mL microcentrifuge tubes. The PCR thermal cycle was optimized to be as
157 follows, initial denaturation at 94 °C for 2 min once followed by 40 cycles of denaturation at
158 94 for 30 sec, annealing 57°C for 30 sec, extension at 72°C for 30 sec and then final extension
159 at 72 °C for 5 min and then 4°C ∞.

160 The PCR products were analysed using 1.4 % high melting agarose gel (Fisher, EP1356-100)
161 melted in TAE (Tris-acetate + EDTA) and with 0.005 % of SYBR^{TM} safe. The PCR products
162 were compared with a PCR 100bp low scale DNA ladder (Fisher BioReagents, BP2581-200)
163 consisting of 10 DNA fragments with sizes of 50, 100, 200, 300, 40, 500, 700, 1000, 1400,
164 1500, 2000 bp. Band intensities were semi-quantitatively measured using Image j software.
165 The same procedures were followed in all PCR experiments reported in this study.

166 The second group of microshoots was used for the production of artificial seeds. Microshoots
167 were mixed with sterilized (by tyndallisation) sodium alginate 2% (w/v) and dropped into a
168 sterilized (autoclaved) solution of calcium chloride 15 g L^{-1} using a sterilized pipette to form
169 gel beads. Microshoots were left in the calcium chloride for 30 min for full complexion of their
170 encapsulating beads. The artificial seeds were then transferred to S23 liquid media (without
171 plant growth regulators (PGRs)) for 30 min followed by a quick wash with sterile distilled
172 water.

173 The artificial seeds produced were divided into two groups. The first group was incubated at
174 4°C for 15 days for acclimation and the second group was used as a control (kept at room

temperature). Cultures were exposed to 16 h photoperiod. Frost tolerance analysis of both acclimated and non-acclimated artificial seeds was carried out to test the effect of acclimation process. Artificial seeds were exposed to different temperatures as follows, 20, 0, -2, -4, -6, -8, and -10°C. The artificial seeds were placed in sterile petri dishes together with a small piece of ice (prepared from sterilized water) to ensure ice nucleation. The petri dishes were placed in a Sanyo programmable chamber to the various freezing temperatures in sequence with a hold of two hours at each temperature. Samples were removed at the end of the 2 h hold of each temperature. Samples were then kept at 4°C overnight to thaw. The following day the artificial seeds were placed on S23 maintenance semi-solid media and cultures were incubated in a Snijder^{TM} growth cabinet at 22 °C with a 16 h photoperiod (PAR 177 µmol m^{-2} sec^{-1}). The conversion rates and the average fresh weights of plantlets produced were assessed after 27 days of culture. 10 replicate culture pots, each comprising four artificial seeds were used with each treatment.

The effect of mannitol on the development of cauliflower microshoots and artificial seed cold tolerance

Mannitol was added to media to simulate drought stress. Using an osmometer (Osmomat R), culture media with several osmotic potentials were prepared by the addition of mannitol as follows, mannitol free culture media osmotic potential of -0.47 Osmol kg^{-1} (this medium contained 3% sucrose), -0.7 Osmol kg^{-1} (12.22 g L^{-1} mannitol), -1.15 Osmol kg^{-1} (48.98 g L^{-1}). -1.60 Osmol kg^{-1} (79.79 g L^{-1}), -2.05 Osmol kg^{-1} (113.55 g L^{-1}), -2.50 Osmol kg^{-1} (147.33 g L^{-1}), -2.95 (181.121 g L^{-1}) and -3.40 Osmol kg^{-1} (259.99 g L^{-1}). The culture media prepared were used for the production of cauliflower microshoots (cv. Fremont). The cultures were left on a shaker at room temperature (20-22 ^{o}C) with16 hours day length provided by fluorescent lights, 80 µmol m^{-2} s^{-1} for 28 days after which the number and average weight of microshoots were evaluated. Four replicate culture pots were used for each treatment.

Samples of 15 day-old microshoots derived from the -0.47 (control), -2.05, -2.95 Osmol kg^{-1} treatments were used for the production of artificial seeds (other concentrations were not sampled as they adversely affected microshoot growth). Frost tolerance analysis of the artificial seeds was carried out to test the effect of the drought simulation on artificial seed cold tolerance. The artificial seeds were placed in sterile petri dishes with small piece of a sterile ice to ensure ice nucleation and placed in a chamber Sanyo programmed to fall to temperature of 0, -2, -4, -6, -8, -10 and -12°C with a hold of two hours at each temperature. Samples were moved at the end of the 2 h hold of each temperature and kept at 4°C overnight to thaw. Artificial seeds were then cultivated in maintenance semi-solid media S23. Artificial seed conversion rate was evaluated after 20 days of culture. Five lines (replicates) of six artificial seeds in each were cultivated in small plastic containers (10 x 10 x 8 cm) containing 75 ml of maintenance semi-solid S23 media and were used with each treatment. Three lines were used per container. The lines were distributed randomly between the containers. Each line was considered as a replicate.

The effect of mannitol on the induction of *CBF/DREB1* gene expression in cauliflower microshoots

Cauliflower microshoots (cv. Fremont) were produced and 25 day old microshoots were transferred to new culture medium containing several concentrations of mannitol -0.47 (control), -0.7, -1.15, -1.60, -2.05, -2.50, -2.95, -3.40 Osmol kg^{-1}. Samples of microshoots were derived from each mannitol concentration treatment after 0 (control), 1, 6, 12, 18, 24, 36 hours of the transfer to the new cultures. The samples were stored at -80°C until the RNA was extracted. Synthesis and amplification of *CBF/DREB1* cDNA was carried out and each PCR experiment was replicated three times.

The effect of low temperature treatment on the induction of *CBF/DREB1* gene expression in mature cauliflower plants

Eight cauliflower plants (cv. Aviso) were grown in pots placed in the greenhouse (Skarden Garden, Plymouth University) until they started forming curds. Four mature plants were transferred to Snijder cold cabinet at 4°C and 8 hours photoperiod while the others were left in the greenhouse as controls. Leaf samples, each consisting of 1 full leaf, were taken at 0 (control), 1, 6, 12, 18, 24 hours after transfer to the new temperature. The samples were kept at -80°C until the RNA was extracted. Synthesis and amplification of *CBF/DREB1* cDNA was carried out and PCR was replicated three times.

The remaining two plants were transferred from the greenhouse to the cold cabinet where they were kept for 15 days for acclimation and then the cold tolerance was tested by measuring electrical conductivity following freezing treatments. Four fully expanded upper cauliflower leaves were excised from each of acclimated and non-acclimated plants, fifteen leaf discs of 1 cm diameter each were cut and placed in labelled boiling tubes (75 mL volume) and exposed to freezing in the Sanyo^{TM} cabinet at 0 °C and programmed to -3, -6, -9, and -12 °C with a 2 h hold at each temperature. A small piece of ice was added to each tube at 0 °C to facilitate ice nucleation. Samples were taken at each temperature at the end of each 2 h hold and transferred to a refrigerator at 4°C to thaw overnight. Then 20 mL of distilled water was added to the tubes to fully cover the plant material and a lid was placed on each tube and incubated at 20 °C for 24 h and the Electrical Conductivity (EC1) of the solution measured. Tubes were then autoclaved at 121 °C for 15 min and again incubated for 24 h at 20 °C and then the EC was re-measured (EC2). The REC % was calculated as:

REC% = EC1/EC2 * 100 (Aronsson 1980; Levitt 1980). Three replicates (tubes) were used for each treatment at each temperature.

The effect of drought on the cold tolerance of cauliflower mature plants

Four cauliflower plants (cv. Aviso) were grown in pots in the green house (22 ± 2 °C) until they started forming curds. The plants were transferred to a Sanyo growth cabinet set at 23°C and 16 hours light (177 µmol m^{-2} sec^{-1}). The plants were irrigated to field capacity. Two plants were then irrigated regularly every three days and the other two were left without irrigation for 10 days. The frost resistance of irrigated and non-irrigated plants was analysed by measuring electrical conductivity of leaf discs following freezing treatment as described above. Both irrigated and non-irrigated were tested at different temperatures, control (0), -3, -6, -9, -12 °C. Three replicate tubes were used with each temperature for both irrigated and non-irrigated plants.

The effect of drought on the expression of *CBF/DREB1* in mature cauliflower plants

Four mature cauliflower plants (cv. Aviso) were cultivated in pots containing John Innes No. 1 compost and they were transferred from the greenhouse to a Snijder growth cabinet at 23°C and 8 hours light when they started forming curds. All of these plants were irrigated to field capacity. One of the pots was placed on a balance (Toledo, model 4714) and weights were recorded of regular intervals. The weights and the soil voltage measured using a Theta probe (Wavetek meterman, Delta T) were recorded every two days. Three measurements of soil voltage were carried out each time. The field capacity of the soil (FC) was considered to be 100% moisture and the moisture content was determined each two days using the following equation:

Moisture content %= Soil weight at FC – recorded weight/ Soil weight at FC ×100

A standard curve of soil moisture content and the Theta probe reading was plotted (Fig, 1). Samples (each consisting of one full expanded leaf) from the other three plants were taken for RNA extraction after 0, 1, 4, 8, 16, 24, 30 days of initial irrigation. Theta probe readings were

recorded when the samples were taken and using the standard curve, the Theta probe readings were used to calculate the moisture level at which the samples were obtained. At the same time stomatal conductance was measured using a Porometer (AP4 Delta-T devices Ltd). Three measurements of soil voltage and stomatal conductance were carried out each time. Corresponding leaf samples were taken and kept at -80°C until the RNA was extracted. Synthesis and amplification of *CBF/DREB1* cDNA was carried out. Each PCR experiment was repeated 3 times.

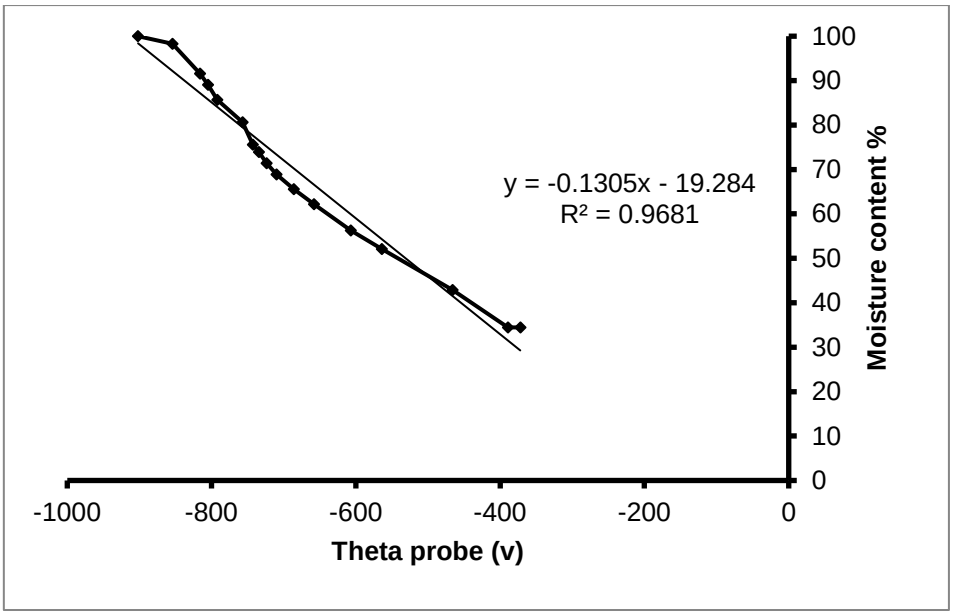


Fig 1. Standard curve of the relation between the Theta Probe reading and compost moisture content.

cDNA sequencing

Microshoot samples which had positive *CBF/DREB1* expression (previous experiments) were used to yield cDNA of this gene. cDNA sequence of *CBF/DREB1* detected was purified using a cleaning kit protocol (Qiagen, Cat. no. 28004) following the manufacture instructions. The purified DNA was subjected to sequencing by Eurofins MWG Operon (Germany). Multiple nucleotide sequence alignment and deduced amino acids sequences of *BoCBF/DREB1* comparison between the sequences obtained and other cold induced genes sequences were carried out using ClustalW 2. EMBL-EBI (Larkin et al., 2007) and BLAST (NCBI).

Statistical analysis

Each experiment was repeated 3-5 times. Results are presented as means ± standard error (SE). All data were subjected to analysis of variance (ANOVA) using Minitab software (version 15) and comparisons of means were made with least significant difference test (LSD) at 5% level of probability.

Results

The effect of acclimation on artificial seed cold tolerance

Acclimation improved the cold tolerance of artificial seeds. The conversion rate of non-acclimated artificial seeds significantly decreased at freezing temperature treatments lower than -4°C whilst the conversion rate of acclimated artificial seeds gave 100% conversion down to -8°C. Significant differences between acclimation and non-acclimation were evident at all temperatures lower than -4°C (P<0.001) (Fig, 2).

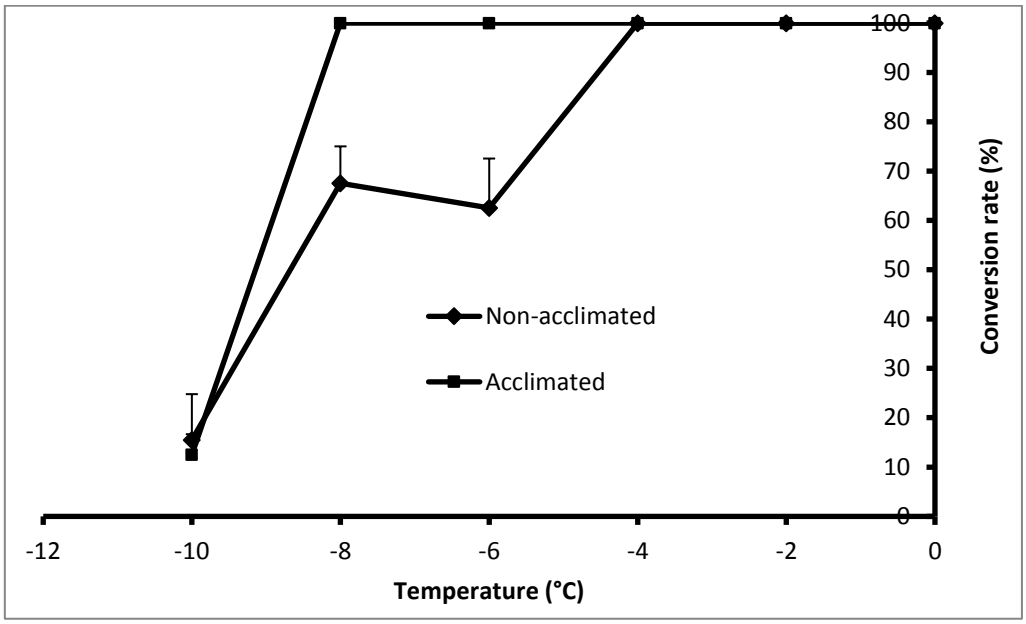
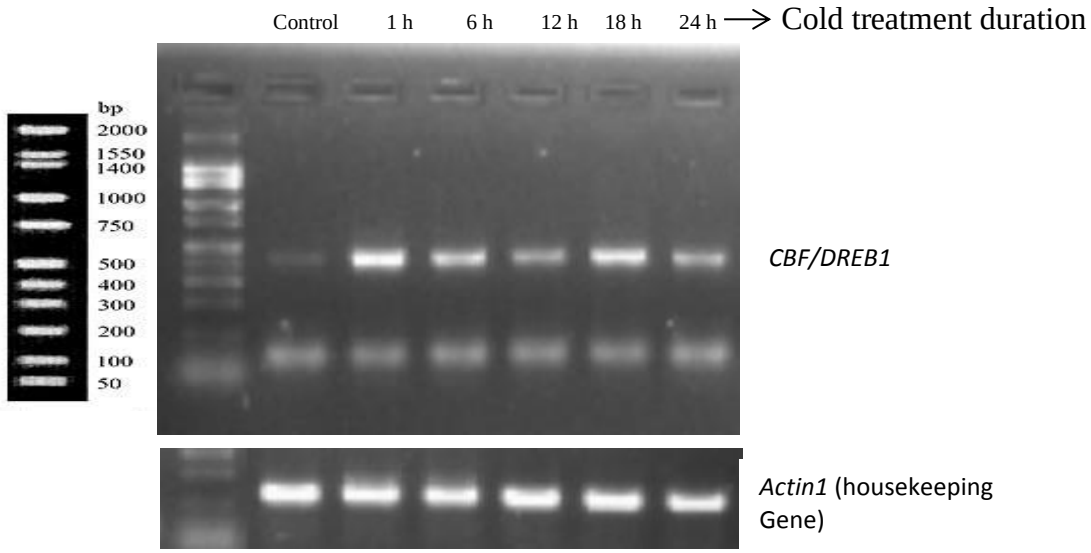


Fig 2, the effect of cold acclimation on artificial seeds cold tolerance assessed by their conversion rate at different low temperatures (LSD=10.11).

The effect of cold acclimation on the induction of *CBF/DREB1* expression in cauliflower microshoots

Acclimation treatments at 4°C induced the regulation of *CBF/DREB1* after just 1 hour of cold treatment where it showed maximum expression in comparison to other times (P<0.001) (Fig, 3).



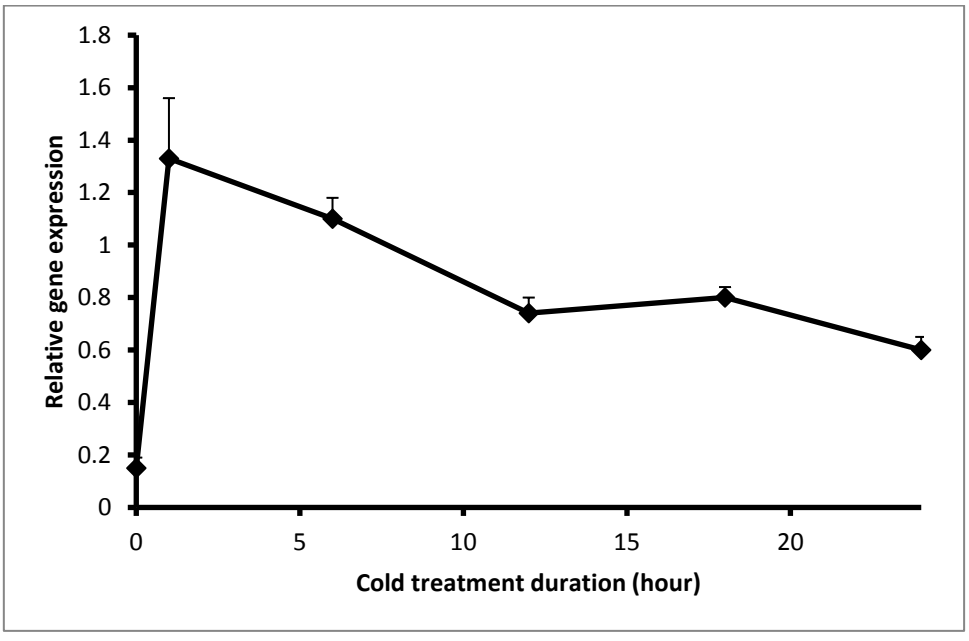


Fig 3. The effect of low temperature (4°C) for various exposure times on the relative induction of *CBF/DREB1* expression in cauliflower microshoots (LSD=0.285).

The effect of mannitol on the development of cauliflower microshoots

While various osmotic potential culture media had no significant effect on the number of growing microshoots (P=0.076), the effect on the average weights was highly significant (P<0.001). The higher the osmotic potential, the lower the average weight of microshoots (Figs, 4 and 5). The use of relatively high concentration of mannitol (-2.95 Osmol kg^{-1}) negatively affected the growth of cauliflower microshoots.

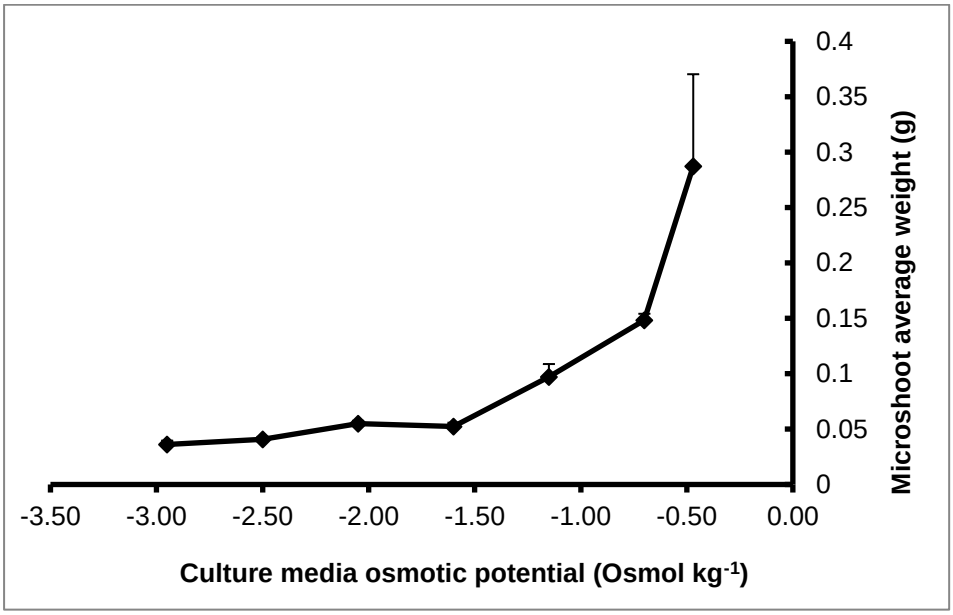


Fig 4. The effect of culture osmotic potential (manipulated by varying mannitol concentration) on the average weight of cauliflower microshoots (LSD=0.016)

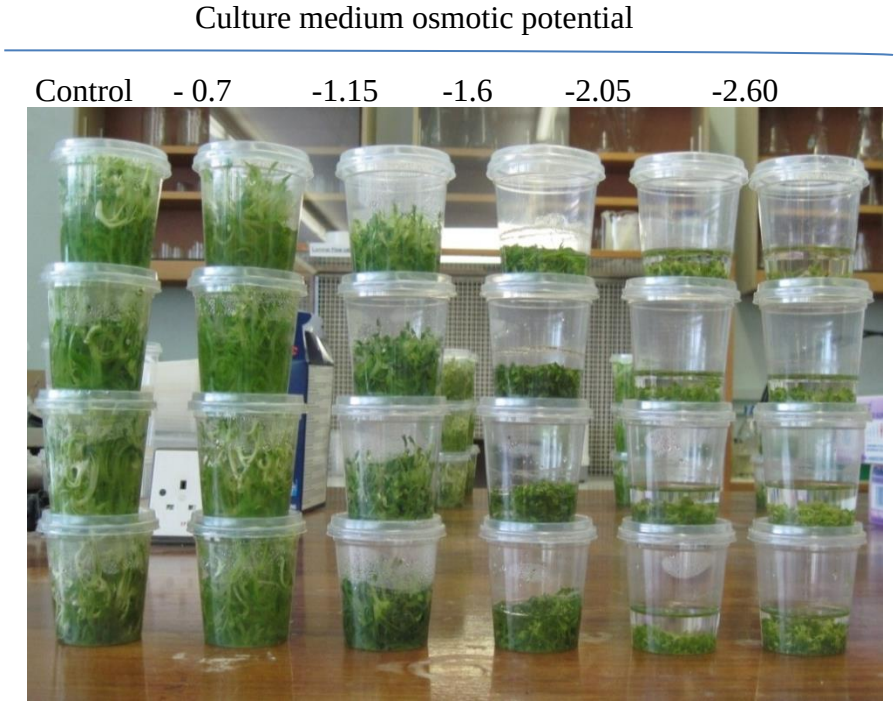


Fig 5. The effect of culture osmotic potential (manipulated by varying mannitol concentration) on the development of cauliflower microshoots

The effect of mannitol on artificial seed cold tolerance

Mannitol treatments had significantly (P<0001) positive effects on artificial seed cold tolerance when it was used at an osmotic potential of -2.05 Osmol kg^{-1} (147.33 g L^{-1}). While the artificial seeds produced using -2.05 Osmol kg^{-1} treated microshoots tolerated -10°C temperature, the conversion rate of the control sample decreased to less than 40% at this temperature. It was observed that the microshoots produced at -2.95 Osmol kg^{-1} were unsuitable to be encapsulated as artificial seeds since the conversion rate for them was very low even without low temperature treatment (control) (Figs, 6 and 7).

The effect of mannitol on the up-regulation of CBF/DREB1 gene in cauliflower microshoots

It was confirmed that none of the mannitol concentration used had the capacity to up-regulate *CBF/DREB1* gene whatever the exposure.

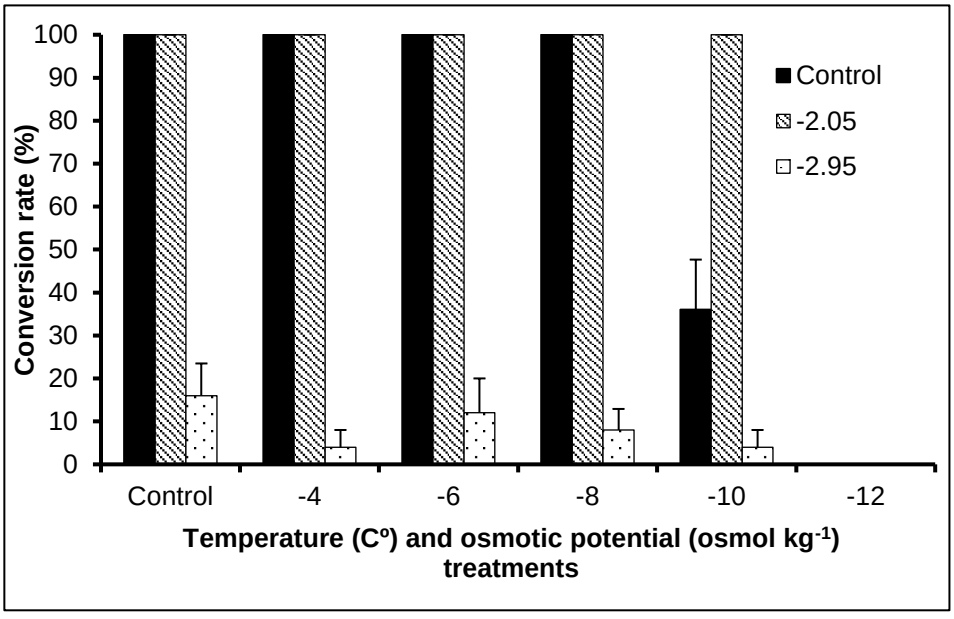


Fig 6, the effect of the culture osmotic potential (mannitol concentration) on the conversion rate of artificial seed treated following exposure to various freezing temperatures (LSD= 1.181).

Culture osmotic potential treatments → - 2.95 Control -2.05

Fig 7. The effect of culture osmotic potential (mannitol concentration) on the conversion rate of artificial seeds following exposure to -10 °C.

The effect of cold acclimation on cauliflower mature plant cold tolerance

It was observed that REC% increased following exposure to lower and lower sub-zero temperatures indicating increasing cell damage. The REC% was significantly lower in the leaf disc samples obtained from acclimated plants compared with those taken from non-acclimated plants (P=0.016). This clearly demonstrated that acclimation significantly improved the cold tolerance of mature cauliflower plants (Fig, 8).

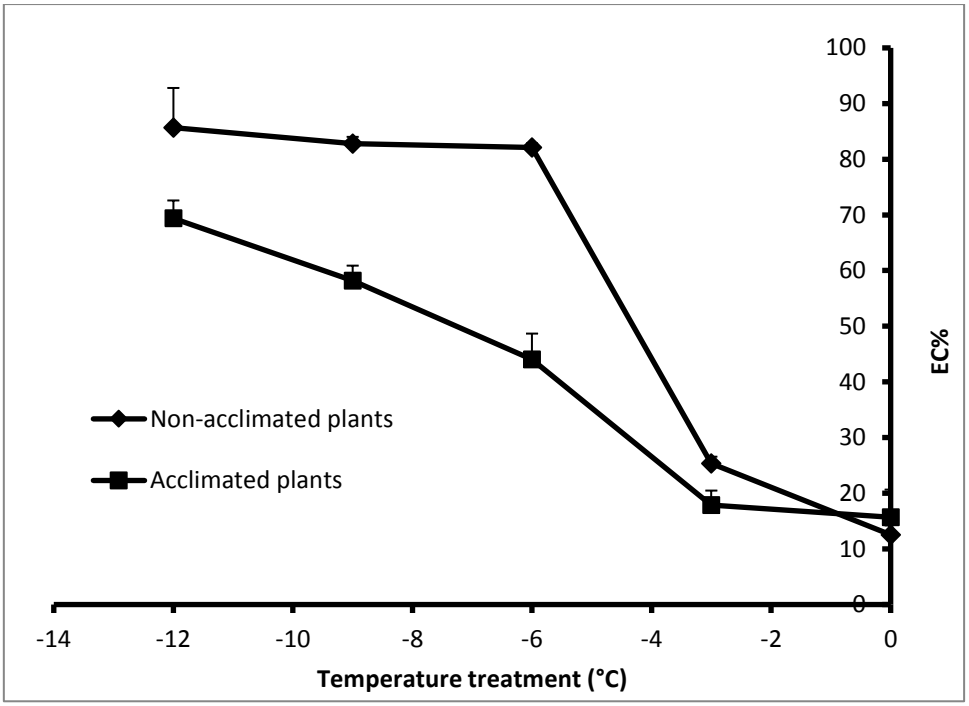
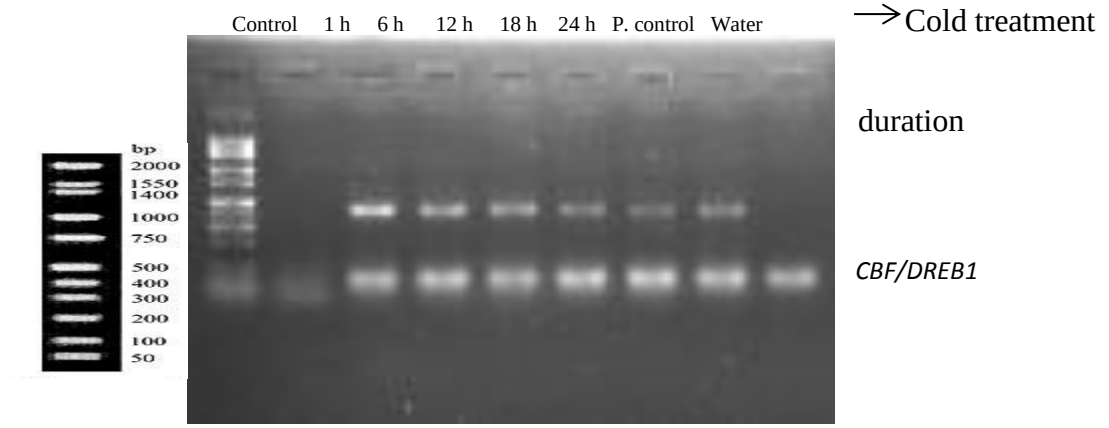


Fig 8. The effect of freezing temperature treatment on the relative electrical conductivity (REC %) of both acclimated and non-acclimated leaf discs taken from cauliflower mature plants (LSD=14.4).

The effect of cold acclimation on the induction of *CBF/DREB1* gene expression in mature cauliflower plants

Cold acclimation had the capacity to induce the expression of *CBF/DREB1* gene after one hour of cold treatment. However, the highest gene expression was observed after 12 h of cold treatment. (P = 0.012) (Fig, 9).



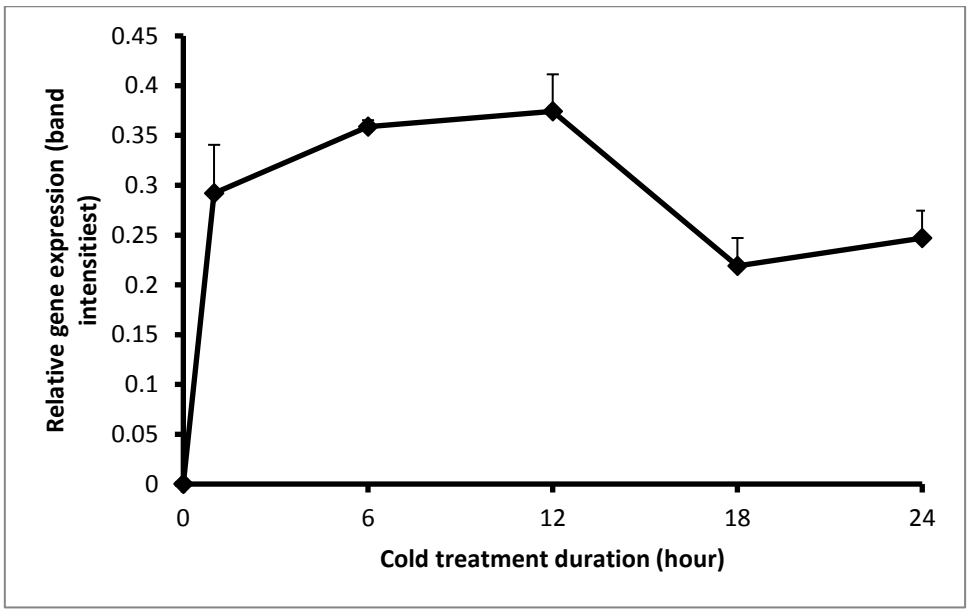


Fig 9. The effect of cold acclimation at (4°C) for different periods on the induction of *CBF/DREB1* in mature cauliflower plants (LSD = 0.098).

The effect of drought on mature cauliflower plant cold tolerance and on the induction of *CBF/DREB1* gene expression in mature cauliflower plants

Drought significantly reduced the REC% when the leaf disks were treated at -6°C (P<0.003) which seemed to be the critical temperature where the effect of drought on the REC% values (frost damage) was clear. The REC% was about 60 % from drought plant at -6°C and it was about 90 % from irrigated plants (Fig, 10). At temperature lower than this, complete hill occurred.

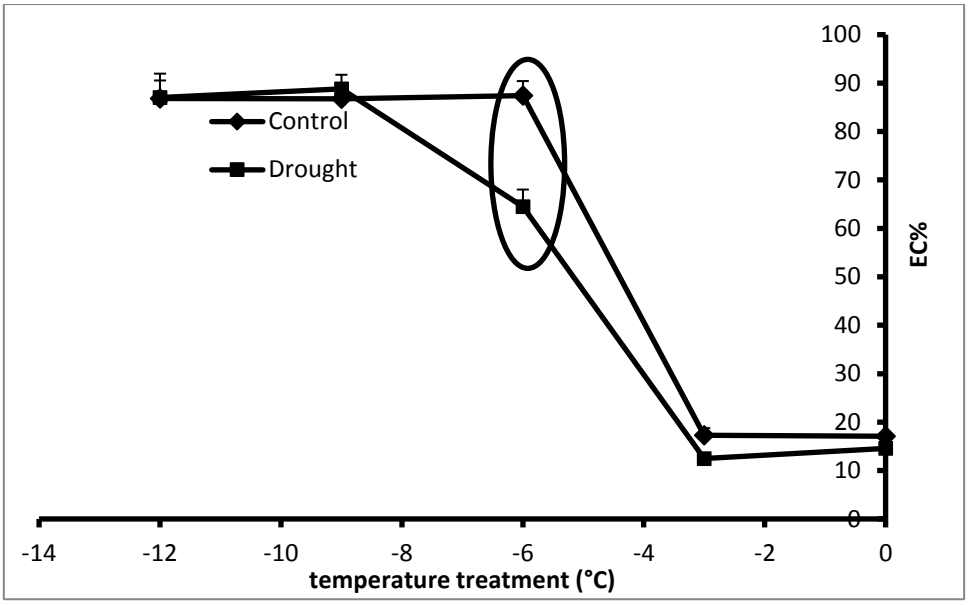
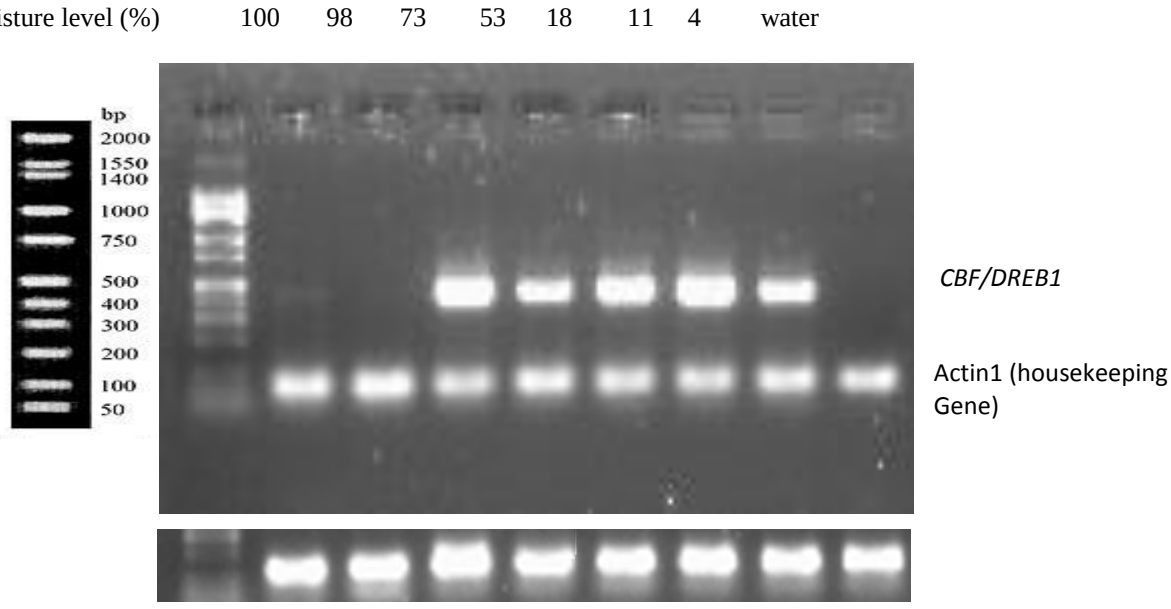
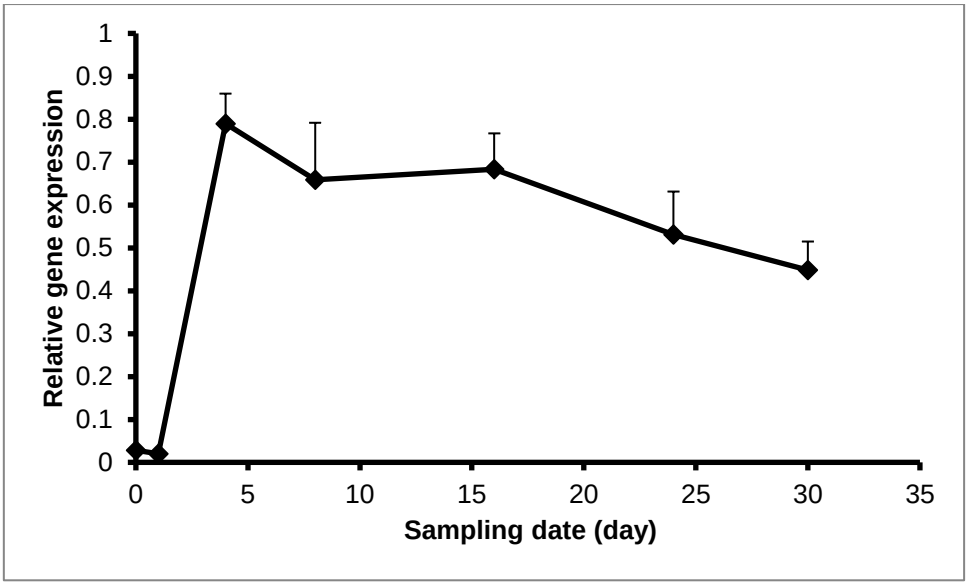


Fig 10, the effect of drought on the mature cauliflower frost damage under irrigation and drought (LSD=8.99 at -6°C).

Drought induced the expression of *CBF/DREB1* in cauliflower and it was observed that a reduction in compost moisture level to 73 % or less (4 days without irrigation) was needed to induce the expression of *CBF/DREB1* (Fig, 11).

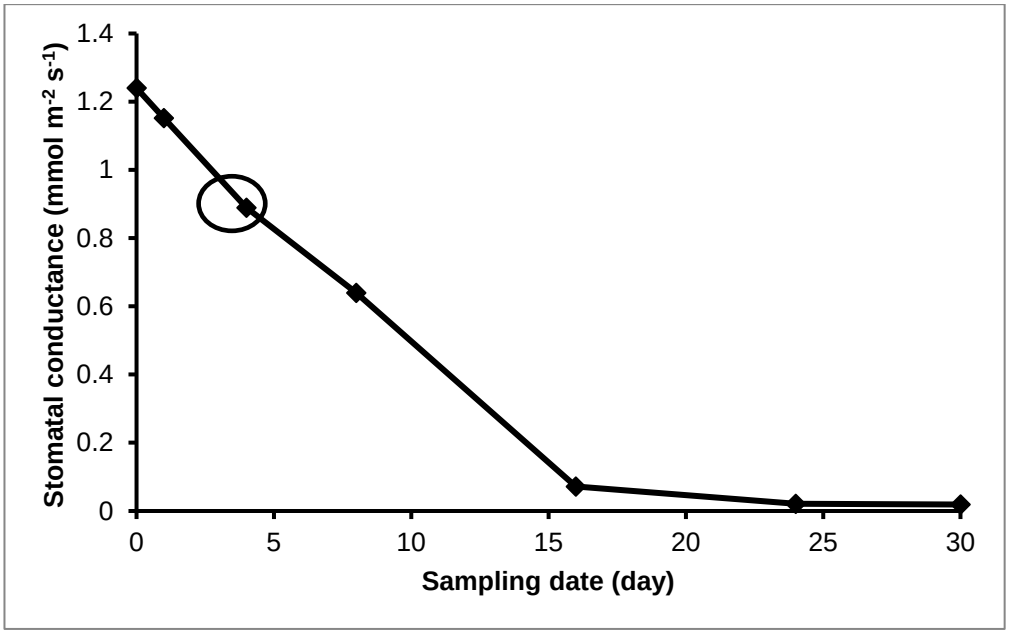




437

438 **Fig 11. The effect of sampling date on the induction of *CBF/DREB1* expression in the cauliflower mature plants**
439 **(LSD= 0.240)**

440 It was confirmed that the lower the moisture level in the soil, the lower the stomatal
441 conductance (Figure 12. The stomatal conductance, when the *CBF/DREB1* was up-regulated,
442 was determined and found to be 0.889 mmol m^{-2} s^{-1} (Fig, 12)



443

444 **Fig 12.The effect of sampling date on the stomatal conductance of cauliflower full extended leaves.**

445

The sequences of *CBF/DREB1* alignment

Forward

GAGGTGAGGGAGCCAAACAAGAAATCTAGGATTTGGCTCGGTACTTTCCTAACAGCCGA

GATCGCAGCCCGTGCTCACGACGTCGCCGCCATAGCCCTCCGCGGCAAATCAGCTTGTCT

CAATTTTGCCGACTCCGCTTGGCGGCTCCGTATCCCGGAGACAACATGCCCCAAGGAGAT

TCAGAAGGCGGCTGCTGAAGCCGCGGTGGCTTTTCAGGCTGAGATAAATAATACGACGG

CGGATCATGGCATTGACGTGGAGGAGACGATCGTGGAGGCTATTTTCACGGAGGAAAAC

AACGATGGTTTTTATATGGACGAGGAGGAGTCCATGTTCGGGATGCCGGCCTTGTTGGCT

AGTATGGCGGAAGGTAGCTTTTGCC

Reverse

ATATGGACTCCTCCTCGTCCATATAAAAACCATCGTTGTTTTCCTCCGTGAAAATAGCCTC

AACGATCGTCTCCTCCACGTCAATGCCATGATCCGCCGTCGTATTATTTATCTCAGCCTGA

AAAGCCACCGCGGCTTCAGCAGCCGCCTTCTGAATCTCCTTGGGGCATGTTGTCTCCGGG

ATACGGAGCCGCCAAGCGGAGTCGGCAAAATTGAGACAAGCTGATTTGCCGCGGAGGGC

TATGGCGGCGACGTCGTGAGCACGGGCTGCGATCTCGGCTGTTAGGAAAGTACCGAGCC

AAATCCTGGATTTCTTGTTTGGCTCCCTCACTTCACACACCCACTTACCTGAGTGTCTCAG

Fig 13. *CBF/DREB* sequence (BLAST (NCBI)) Fasta sequences :(F premix 52..433 of sequence) (R premix 25..385 of sequence).

The nucleotide sequence of cDNA isolated from *Brassica oleracea* var. *botrytis* was determined (Figures, 13). This sequence was compared with *CBF/DREB1* DNA sequences reported for other plant species and the results showed significant similarities with several plant species (Table 1).

Table 1 Alignment of DNA sequences of *CBF* gene isolated from cauliflower microshoots in nucleotide database using nucleotide query (BLAST-NCBI)

Accession	Description	Max score	Total score	Query cover	E value	Iden t
AF499033.1	Brassica napus CBF-like protein CBF16 (CBF16) mRNA, complete cds	669	669	99%	0	98%
AY444875.1	Brassica napus DREB2-3 mRNA, complete cds	662	662	99%	0	98%
EU727155.1	Nicotiana tabacum DREB1 mRNA, complete cds	617	617	99%	2.00E-173	96%
AY437878.1	Brassica napus DREB2-1 mRNA, complete cds	617	617	99%	2.00E-173	96%

AY444876.1	Brassica napus DREB2-23 mRNA, complete cds	612	612	100%	8.00E-172	96%
EU136731.1	Brassica juncea DREB1B mRNA, complete cds	593	593	99%	3.00E-166	95%
GQ866977.1	Raphanus sativus CBF1 mRNA, complete cds	584	584	100%	2.00E-163	94%
EF219470.1	Brassica rapa subsp. Pekinensis dehydration responsive element binding protein 2-19 gene, partial cds	582	582	100%	7.00E-163	94%
AF499032.1	Brassica napus CBF-like protein CBF7 (CBF7) mRNA, complete cds	582	582	99%	7.00E-163	94%
AF084185.	Brassica napus dehydration responsive element binding protein mRNA, complete cds	582	582	99%	7.00E-163	94%

It was observed from the results of sequencing that the nucleotide sequences were similar to different *CBF/DREB1* genes in the *Brassicaceae*.

Amino Acid sequence

The cDNA sequence was translated to amino acid sequence. The amino acid sequence was blasted using NCBI software and compared with the amino acid sequence in other plants. The results indicated a high similarity with different plant species (Table, 2) (Fig, 14).

Table 2, Alignment of amino acid sequences of CBF gene isolated from cauliflower microshoots using P1 primers in protein database using nucleotide query (BLAST-NCBI)

Description	Similarity (%)	Accession	Reference
DREB2-23 [Brassica napus]	98	AAR20499.1, 214 aa	(Zhao et al., 2006)
CBF-like protein CBF16	98	AAM18960.1, 215 aa	(Gao et al., 2002)
CBF-like protein CBF5 [Brassica napus]	96	AAM18958.1, 214 aa	(Gao et al., 2002)
CBF [*Brassica napus*]	95	ADN28047.1, 146 aa	(Zhao and Song, unpublished)
DREB2-3 [*Brassica napus*]	98	AAR20498.1, 215 aa	(Zhao et al., 2006)
DREB2-1 [*Brassica napus*]	95	AAR11858.1, 215 aa	(Zhao et al., 2006)
DREB2-2 [*Brassica napus*]	95	AAR20497.1, 214 aa	(Zhao et al., 2006)
DREB-like protein 1 [*Brassica rapa* subsp. *pekinensis*]	95	ACL12046.1, 214 aa	(Wang et al, unpublished)
CBF1 [*Raphanus sativus*]	95	ACX48435.1, 215 aa	(Li and Gao, unpublished)
CBF-like protein [*Brassica rapa* subsp. *pekinensis*]	94	AAY43345.1, 214 aa	(Zhang et al., 2006b)
DREB1 [*Nicotiana tabacum*]	94	ACE73693.1, 215 aa	(Liu and Feng, unpublished)

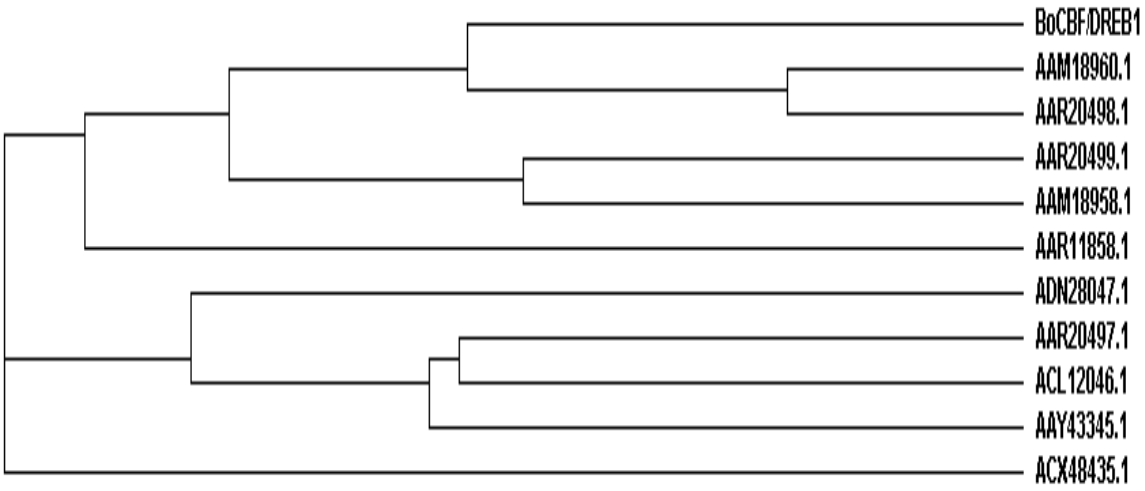


Fig 14. Phylogenic relation of the *BoCBF/DREB1* deduced amino acid sequence.

The phylogram is based on the alignment of amino acids sequence of *Brassica oleracea* v. botrytis *BoCBF/DREB1* and the following proteins from the members of *Brassicacea* and other families. DREB2-23 [*Brassica napus*], CBF-like protein CBF16 [*Brassica napus*], CBF-like protein CBF5 [*Brassica napus*], CBF [*Brassica napus*], DREB2-3 [*Brassica napus*], DREB2-1 [*Brassica napus*], DREB2-2 [*Brassica napus*], *DREB*-like protein 1 [*Brassica rapa subsp. pekinensis*], CBF1 [*Raphanus sativus*], DREB1 [*Nicotiana tabacum*], CBF-like protein [*Brassica rapa subsp. pekinensis*]. The values show tree graph distances. The tree was constructed with ClustalW2 EMBL-EBI (Larkin et al., 2007).

Discussion

Cold acclimation which is defined as the expose of plant to low, non-freezing temperature, has been reported to increase the cold tolerance in many plant species (Gilmour et al. 2000; Jan et al. 2009; Shinozaki and Yamaguchi-Shinozaki 1996; Thomashow 1999; Thomashow 2001). In terms of *Brassica olearacea* var botrytis, the experiments presented here demonstrated the capacity of cauliflower tissue cultures (microshoots and artificial seeds) to be cold acclimated. At the molecular level, cold acclimation requires recognition of low temperature by cell signalling processes and as a consequence large modifications of gene expressions takes place in order to eventually enable the plants to survive the low temperature (Lee et al. 2005; Seki et al. 2002). Several studies have demonstrated that the *CBF/DREB1* (CRT/DER binding factor) is the central pathway participating in the up-regulation of cold acclimation (Choi et al. 2002; Dubouzet et al. 2003; Francia et al. 2004; Gao et al. 2002; Owens et al. 2002; Shinozaki et al.

2003; Smallwood and Bowles 2002; Stitt and Hurry 2002; Sung et al. 2003; Vágújfalvi et al. 2003; Xiong et al. 2002). The current results confirmed that the *CBF/DREB1* gene was upregulated under the effect of low temperature in cauliflower and that the peak of gene expression was observed one hour after transfer to acclimating temperatures. These results confirm the important role of the "*CBF* regulon" in the improvement of cold tolerance in cauliflower microshoots.

Dehydration is one of the main mechanisms which imposes stress on cells during freezing temperatures. When the temperature drops below the freezing point, ice formation begins in the extracellular spaces of the plant tissue and as a consequence, the water moves from inside the cell to the extracellular spaces since the chemical potential of ice is less than that in liquid water and the cell begins to dehydrate. Freezing injury could therefore be caused by the effect of plant cell dehydration (Thomashow 2001). It is clear that tolerance to freezing and to drought could include the action of shared genes. Many studies have reported that the induction of *CBF* expression has positive impact not just on cold tolerance but also on drought and salinity tolerance (Kasuga et al. 1999; Liu et al. 1998a). In view of this finding, the capacity of increasing the osmotic potential (drought simulation) on both cold tolerance and up-regulation of *CBF/DREB1* gene was investigated using different concentrations of mannitol. Mannitol had negative effects on the growth rate of microshoots and it is assumed that this was mainly through the increase of culture osmotic potential since mannitol is not absorbed by plant cells. It was observed that the increase of culture osmotic potential improved the cold tolerance of the cauliflower artificial seeds when used to obtain an osmotic potential of -2 Osmol kg^{-1} in the culture medium. This simulation of drought however did not have the capacity to induce the expression of *CBF/DREB1* regardless of the concentration of mannitol used. However, it has been reported that a multifaceted network of genes is involved in cold tolerance and that the *CBF* regulon only cannot clarify all differences in phenotype cold tolerance (McKhann et al.

2008). The cold stress cause changes in expression of hundreds of genes resulting in the increase of hundreds of metabolites some of which are known to have an important effect in the improvement of plant cold tolerance (Jan et al. 2009).

The failure of mannitol to induce the expression of *CBF/DREB1* gene in cauliflower microshoots raised an important question as to whether the failure of *CBF/DREB1* gene up-regulation under simulated drought was due to the developmental stage of cauliflower (microshoots) or whether is it related to the plant species. To date there are no records in the literature of investigations of DREB induction using drought. It was therefore necessary to investigate the effect of cold acclimation and drought on the up-regulation of *CBF/DREB1* gene in mature cauliflower plants. Both cold acclimation and drought had the capacity to increase the cold tolerance and to upregulate *CBF/DREB* gene in cauliflower mature plants.

The technique of EC (electrical conductivity) was used to analyse the frost resistance in acclimated and non-acclimated mature cauliflower plants since the cellular membrane systems are the main place of freeze-induced injury caused by severe cellular dehydration which occurs upon ice formation in the extracellular spaces (Fuller et al. 2006; Hadi et al. 2011; Thomashow 2001). The injury of cell membranes is the principle on which the electrical conductivity test is based. It is supposed that individual cells become progressively leakier under the increase of frost stress, therefore the electrical conductivity is used to measure the collective average of cell damage caused by freezing. The electrolyte leakage evaluation contains the measurement of electrical conductivity of pure water in which detached samples have been located after a freezing thaw cycle (Lindén 2002). The use of REC% using leaf discs derived from mature cauliflower leaves was found to be an effective methodology for evaluating frost damage in cauliflower mature plants and the positive effect of acclimation on the frost tolerance of mature cauliflower was confirmed. The effect of low temperature was demonstrated to induce the expression of the *CBF/DREB1* gene which resulted in the improvement of cold tolerance.

553 The effect of drought on the induction of cold tolerance of cauliflower was investigated using
554 the REC% technique. It was confirmed that drought can have a significantly positive influence
555 on cauliflower cold tolerance. Moreover, the current results showed that the drought had the
556 capacity to induce the expression of *CBF/DREB1* gene in mature cauliflower plants. The soil
557 moisture level and the stomatal conductance points, in which the *CBF/DREB1* was up-
558 regulated, were determined. Leaf stomatal conductance has been considered a good selection
559 criterion for drought resistance (Ashraf and Oleary 1996) and it has been reported that a fast
560 stomatal response could be a drought resistance mechanism to save soil water for later use and
561 to maintain a high leaf water potential (Jones 1974). What was found to be interesting in the
562 current study was that a small reduction on the soil moisture (to about 70% of the field capacity)
563 had the capacity to induce the up-regulation of the *CBF/DREB1* gene in mature cauliflower
564 plants. Furthermore, the stomatal conductance was relatively high at the point of which
565 *CBF/DREB1* was up-regulated. Such a relatively high stomatal conductance allows a high level
566 of gas exchange and as a consequence a high level of photosynthesis (Mediavilla and Escudero
567 2004). The up-regulation of *CBF/DREB1* has also been reported to lead to increases in sugars,
568 proline and many other solutes in plant tissue resulting in high potential osmotic required to
569 keep the stomata open and maintain gas exchange and growth under relatively drier conditions
570 (Pérez-Pérez et al. 2009).

571 The current study showed that cold acclimation had the capacity to induce the expression of
572 *CBF/BREB1* gene in both microshoots and mature plants, and the capability of drought to up-
573 regulate this gene in mature plant but not in cauliflower microshoots. It seems that the
574 cauliflower developmental stage and the culture environment has an effect on the capacity for
575 *CBF/DREB1* up-regulation. This result agrees with Beck et al (2004) who reported that plant
576 injury caused by low temperature depends on the plant developmental stage. Prasil et al (2004)
577 indicated that cold tolerance of wheat depends on the growth stage and demonstrated that the

cold tolerance of wheat decreases significantly after vernalisation and that is due to the failure in the up-regulation of *CBF* genes after this stage of growth.

The comparison of the isolated partial sequences isolated from cauliflower (*Brassica olearceae* var botrytis) with the *CBF/DREB1* sequences in other *Brassica* species showed high similarity (more than 90%). The similarity between the *BoCBF/DREB1* partial sequence and the *CBF/DREB1* sequences in other *Brassica* species confirms that this gene is in the genome of *Brassica oleracea*. However, further investigation is required to identify the remaining *BoCBF/DREB1* sequence.

Deduced amino acid sequence of the *BoCBF/DREB1* partial sequence in comparison with other Brassica species showed 90% homology and showed an identical conserved AP2 domain. The AP2 domain may play a crucial role in recognition of DNA binding sequence in the promoter of cold responsive genes (Liu et al. 1998b; Sakuma et al. 2002). Among the six member *Brassica* species in the triangle of U (U, 1935), the sequence from *B. oleracea* showed high resemblance with the species *B. napus*, *B. juncea*, and *B. rapa*. For the remaining two species, *B. nigra* and *B. carinata*, no *CBF* genes have been reported in the literature. However, this homology was found to be more than 90% when compared with plants other than *Brassicas* such as *Nicotiana tabacum*.

Conclusion

It was confirmed in current study that cauliflower plants could be cold acclimated regardless the developmental stage (microshoots and mature plants) and that cold acclimation increased the cold tolerance of cauliflower mature plants and microshoots. The capacity of low temperature to up-regulate *CBF/DREB1* in both microshoots and mature plants was also confirmed.

Whilst drought and simulated drought improved the cold tolerance in both cauliflower microshoots and mature plants, it only had the capacity to up-regulate *CBF/DREB1* in mature plants but not in microshoots. The level of soil moisture and the stomatal conductivity at which *CBF/DREB1* was up-regulated in mature cauliflower plants, was determined and it was demonstrated that a small reduction in soil moisture (70%) had the capacity to up-regulate *CBF/DREB1* in mature plants of cauliflower. This is considered to be an important finding that could have significant practical applications in the field.

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