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DETERMINATION OF THE RELATIONSHIP BETWEEN STRESS (FROST AND SALT) RESISTANCE AND LEVEL OF PROLINE IN CAULIFLOWER (*Brassica oleracea* VAR. *BOTRYTIS*) AND BARLEY (*Hordeum vulgare*) PLANTS

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ABSTRACT

Proline continues to be the most studied molecule under abiotic stresses in plants. A study was conducted to determine the relationship between stress (frost & salt) resistance and proline level in cauliflower (*Brassica oleracea* var. *botrytis*) and barley (*Hordeum vulgare*) plants. Plants were stressed to low temperatures (-3 ºC to -9 °C) and in different salt solutions (0 to 300 mM). Damage increased significantly as temperature declined and salt concentration increased. During both stresses, damage was lower in plants that had been acclimated for increasing periods and 13-14 days of acclimation had the best positive effect. Proline accumulated in plants in accordance with increase in acclimation period. Significant negative correlation was observed between frost damage and proline content in both cauliflower and barley whilst there was hardly any relationship between salt acclimation and proline level. The role of proline accumulation during salinity needs therefore to be critically examined further.

***Key words**: Frost, salinity, proline, cauliflower, barley*

INTRODUCTION

Crop plants are frequently exposed to both biotic and abiotic stresses and must have mechanisms to tolerate these in order to survive. The tolerance of crop plants to salinity and drought is crucial in dryland and semi-dryland agriculture. In other regions tolerance to frost is important, especially during early spring growth. Resistance to stress is known to be multi-allelic and is often negatively correlated with yield factors (Fuller and Eed, 2003).

Water quantity and quality are important factors for agricultural production. Salinity in soil and in irrigation water is considered as a serious problem that restricts yield on 40 million hectares of irrigated land in the world. Increasing salinization of arable land will have devastating effects, resulting in 30% land loss within the next 25 years and up 50% by the year 2050 (Fuller, 2004 - unpublished). Increased salt tolerance in crops is widely recognized as an effective way to overcome the limitation of crop production in saline area.

Frost damage to crop and ornamental plants in the temperate regions is unpredictable yet can be devastating causing large economic losses (e.g., cauliflower worth £3.5 M was lost in SW England in 1987, Fuller, 2002). Unpredictability follows from the chaotic nature of the weather and the interaction of plants and the environment in terms of preparedness of plants for the onset of freezing conditions. The ability of plants to survive freezing is a desirable attribute in high latitudes and the occurrence and intensity of frosts is a common delimiter of plant distribution and cropping opportunity. The primary problem that plants face when exposed to freezing temperatures is ice formation. Ice crystals and the dehydration stress they impose can cause severe injuries to the living cells that can lead to the death of the plant. Reliable protection of crops from frost damage has always been an elusive goal.

An increase in the tolerance to stresses in crops could improve the profitability. For this, an understanding of the physiological, biochemical and genetic basis of tolerances in plants will be helpful for combining high yielding ability and good plant type under these stresses. Upon

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exposure to these prevalent stresses, many plants accumulate organic osmolyles, most commonly polyhydroxylic compounds (saccharides and polyhydric alcohols) and zwitterionic alkylamines (amino acids and quaternary ammonium compounds) (Hare *et al*., 1998). It is generally accepted that the increase in cellular osmolarity which results from the accumulation of non-toxic (thus 'compatible') osmotically active solutes is accompanied by the influx of water into, or at least a reduced efflux from, cells, thus providing the turgor necessary for cell expansion (Hare *et al*., 1998; Raymond, 1998).

Proline is one of the osmoprotecting molecules (osmolytes) which accumulates in many organisms and plants in response to environmental stresses (Strizhov *et al*., 1997). Rapid accumulation of free proline in tissues of many plant species as a response to salt, drought or temperature stress, has been associated with the ability of proline to act as an osmolyte, as protective agent for cytoplasmatic enzymes, as reservoir of nitrogen and carbon sources for post stress growth, or even as a stabilizer of the machinery for protein synthesis (Martinez *et al*., 1996). Proline accumulation could also serve hydroxyl radical scavenging, cryoprotection or regulation of redox potential (Aubert *et al*., 1999).

In plants, many reports indicate a positive correlation between proline accumulation and acclimation to osmotic stress (Eed, 2001). High proline content has been related to stress tolerance in a number of plant species (Deane *et al*., 1995). Results by various authorities suggested that proline accumulation could be used as a biochemical marker for increased stress tolerance in crops and vegetables (Fuller, 2004 – unpublished, Martinez *et al*., 1996).

Because environmental stresses represent severe agricultural constraints world-wide, studies on the regulation of proline biosynthesis in plants received attention and it continues to be the most studied molecule under abiotic stresses in plants.

The purpose of this study was to determine the relationships between acclimation, stress resistance (frost and salt) and proline level in cauliflower (*Brassica oleracea* var. *botrytis*) and barley (*Hordeum vulgare*).

MATERIALS AND METHODS

Establishment of plants: Seeds of cauliflower (*Brassica oleracea* var. *botrytis* cv. Medailon) and barley (*Hordeum vulgare* cv. Pastoral) were sown in plastic module trays (10 ml plug volume) filled with sieved John Innes seed compost. Trays were placed in a glasshouse with a minimum temperature of 15 °C with automatic irrigation. Plants were fertilized weekly with 'Baby Bio' (NPK fertilizer, 23 ml per 18 litres of water). Plants were grown up to 4 weeks old when the cauliflower had 3 leaves and barley had 4 leaves.

Frost Resistance Test: A pilot test was carried out to determine the critical parameters for conducting the frost test. Three hundred sixty plants of each crop species were transferred from the glasshouse to a cold chamber (4 °C and 12h photoperiod) for acclimation (Fuller, 1993). On 0th day, 60 plants were placed into labelled boiling tubes. Fifteen plants (Controls) were left in the cold chamber and 8 plants were taken for proline analysis. The remaining 45 plants were placed in a Sanyo incubator and subjected to the following regime: 4 °C for 1 hour $\rightarrow$ -3 °C for 2 hours $\rightarrow$ -5 °C for 2 hours $\rightarrow$ -7 °C for 2 hours $\rightarrow$ -9 °C for 2 hours (for barley only). At -3 °C, a small amount of crushed ice was added to each tube to nucleate freezing of each plant and prevent supercooling (Fuller *et al.*, 1994). Ice was also added to the Control tubes. At the end of each test temperature [-3 °C, -5 °C and -7 °C (and -9 °C for barley)] 15 tubes were removed from the incubator and returned to the cold chamber to thaw overnight (Fuller, 1993). Both frost tested and control plants were then potted into compost in seed trays and placed in a net tunnel to recover for 14 days before scoring for survival. Frost tests were repeated with 2, 7, 10, 13 and 17 day acclimated plants.

Salt Resistance Test: A pilot test was undertaken to determine the critical parameters for conducting the salt acclimation level and 50 mM NaCl was chosen. Eight hundred plants of each crop species, with roots washed free of soil, were placed in a hydroponic system through polystyrene tiles into nutrient solution (Figure 1). Twenty two plastic tanks (87 litres volume) were filled with 40 litres of water followed by 140 ml each of A and B standard hydroponic solutions. Eight of the tanks were designated acclimation solutions and salt (NaCl) added to achieve 50 mM equivalent. Similarly, 0 mM, 100 mM, 200 mM and 300 mM of salt solutions (2 replicate tanks per treatment) were designated the test solutions and prepared. Initially, all plants were placed in the acclimation tanks in polystyrene tiles (16 cm^2, 16 plants per tile). On 0th day, 8 tiles from each of the two crop species were selected randomly from acclimation tanks and transferred to each treatment solution (1 tile per replicate). Three plants were taken for Proline analysis. This procedure was repeated on 2nd, 7th, 10th, 14th and 17th day of acclimation. After 2 weeks on the test solutions, plants were scored for survival. In all trays, an aerator to oxygenate the solution was connected and the solution surface completely covered by tiles to reduce evaporation loss. The electric conductivity of each solution was measured every week and necessary measures were taken to maintain the required nutrient concentrations.

Scoring System: The scoring system in both of the tests was as below:

Score	Cauliflower	Barley
0	Controls, no damage, vigorous regrowth	
1	no death, slight leaf damage / stunted regrowth	
2	substantial leaf damage and very limited or no regrowth	
3	almost or complete leaf damage but apex still alive	slight greenness
4	completely dead	

Figure 1: Hydroponic experimental design

Proline Analysis

The proline level in the plants was determined for both the pilot and actual tests. Selected plants were immediately frozen (-85 °C for 1 day) to stop all biochemical activities and then freeze dried (-35 °C, until constant weight) (Eed, 2001). Dried leaf (0.04 g), with 2 ml ultra clean water, was

finely ground in a pestle, taken into an eppendorf tube and centrifuged at 15000 rpm for 15 minutes. Three aliquots of the resultant supernatant (100 µl) were diluted to 1 ml in 3 crimptop sample vials.

The HPLC method was used to determine proline level in plants using a *Dionex AAA-Direct Amino Acid Analyser System*. A series of proline standards analysed by the Acid Ninhydrin Method (Bates et al., 1973) and the Dionex system, found a good linear fit between the two methods (Fuller and Paisey – unpublished) and concluded that the Dionex system is reliable, versatile, convenient and a suitable replacement for the acid ninhydrin method.

At first, 2 vials containing water were run by Dionex to clean the column and stabilize the detector's reading. Then, 5 standard samples were run to obtain a calibration curve for all analytes. Afterwards, vials containing prepared samples were placed in the autosampler and run overnight. The *Peaknet* software gave output curves for all analytes from which the area of proline was integrated.

Statistical Analysis: Damage scores due to frost and salinity were analyzed with 'Anova' using Minitab v. 13.

RESULTS

Frost Test: In both crop species, frost damage was found to increase significantly ($P<0.001$) as the testing temperature declined. In comparison with controls, temperatures down to -3°C in cauliflower and -7°C in barley did not cause significant damage. As the acclimation period increased, both species demonstrated improved resistance to frost (Figure 2). For cauliflower acclimation for 13 days increased resistance by 2 points on the scoring scale at the testing temperature of -5°C and by 1 point on the scale at the testing temperature of -7°C. Similarly for barley over the same acclimation period resistance was increased by 1.5 points on the scoring scale at the testing temperature of -9°C.

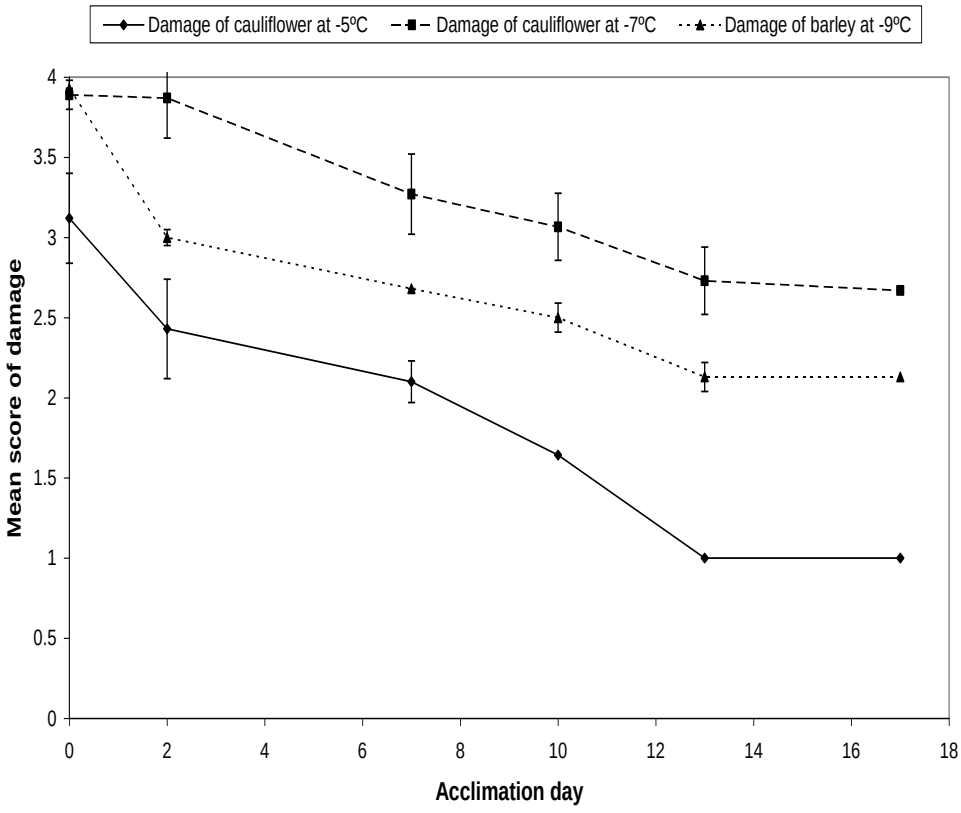

Figure 2: Frost damage in cauliflower and barley plants at different temperatures

With increase in acclimation period, the level of proline rose gradually in both crop species (Figure 3). After 17 days of acclimation, cauliflower plants raised proline level three times higher than that in barley plants. In barley, there was a significant negative linear correlation between proline content and mean damage score (r^2 = 0.982, P = 0.001, y = -0.4931x + 2.1247) (Figure 4) whilst significant negative curvilinear correlation was observed in case of cauliflower (r^2 = 0.76, P = 0.05, $y = 9.4794e^{-1.5623x}$).

Salt Test: Damage caused by the different salt test concentrations (0~300 mM) was found to be highly significant (P<0.001) at every acclimation period. No noticeable damage was observed in both crop species at 100 mM whilst leaf damage in barley and stunted growth in cauliflower were observed at 200 mM. Damage at 300 mM was prominent in both crop species. As the acclimation period increased, plants demonstrated improved resistance to salinity in different way. Acclimation did not play any role in cauliflower up to 10 days but after that the damage decreased significantly (Figure 5). In barley plants, damage decreased steadily after 2 days of acclimation period. Acclimation for 14 days increased resistance in cauliflower by 1.5 points and that in barley by 1 point on the scoring scale at the testing salt solution of 300 mM.

The level of proline rose rapidly in both crop species on 2nd day of acclimation (Figure 6), but appeared to decrease steadily after this and increase slightly again after 14 days. Initially, the level in barley was twice as much as that in cauliflower. There was hardly any significant relationship between salt damage and proline level in both crop species (Figure 7).

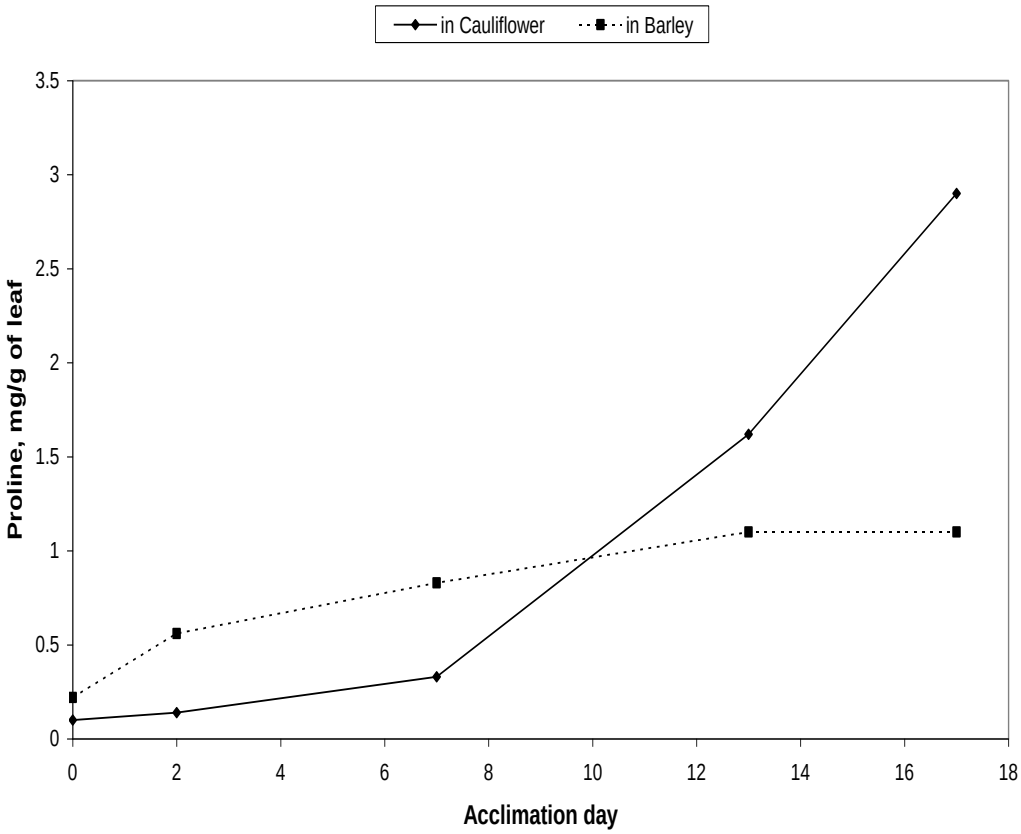

Figure 3: Level of proline rose in cauliflower and barley plants during acclimation at 4ºC

At the early stage of the acclimation period, proline level rose during salt stress was 10-12 times higher than that during low temperature stress in both crop species. But as acclimation period went on, the trend was not sustained.

DISCUSSION

Both cauliflower and barley clearly demonstrated frost resistance but to different degrees with cauliflower being killed at -7°C whilst barley survived to -9 ºC. Fuller (2004) showed that 10% of cauliflower seedlings were killed at -7°C. Plants die during freezing because of dehydration of cells due to an osmotic difference between cell water and ice formed in the apoplast space (Wisniewski and Fuller, 1999). About 80% of cell water is drawn out by the time temperature has fallen to -5°C and 90% by -10°C (Fuller, 2002).

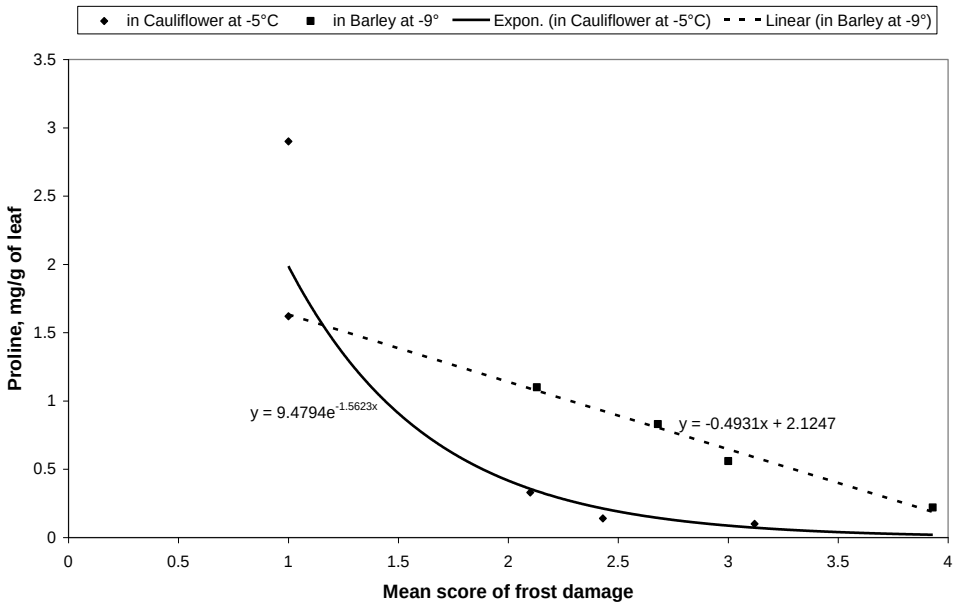

Figure 4: Relationship between frost damage and level of proline in cauliflower and barley plants

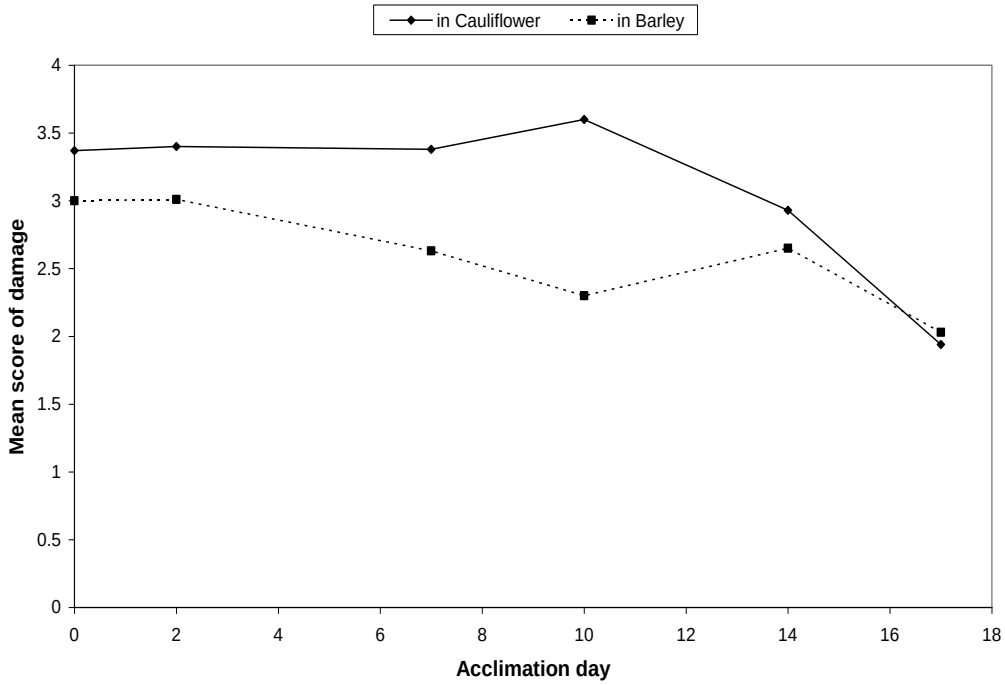

Figure 5. Salt damage of cauliflower and barley plants at 300mM concentration

Acclimation period has played an important role to lower frost damage in both crop species and 13-day-acclimation was the best in this regard. This was supported by Burchett (2000) who observed 50% kill of non-acclimated barley seedlings at -4.4°C and a significant increase in the frost resistance of plants acclimated for 14 days. Fuller (1993) observed that hardening is a relatively

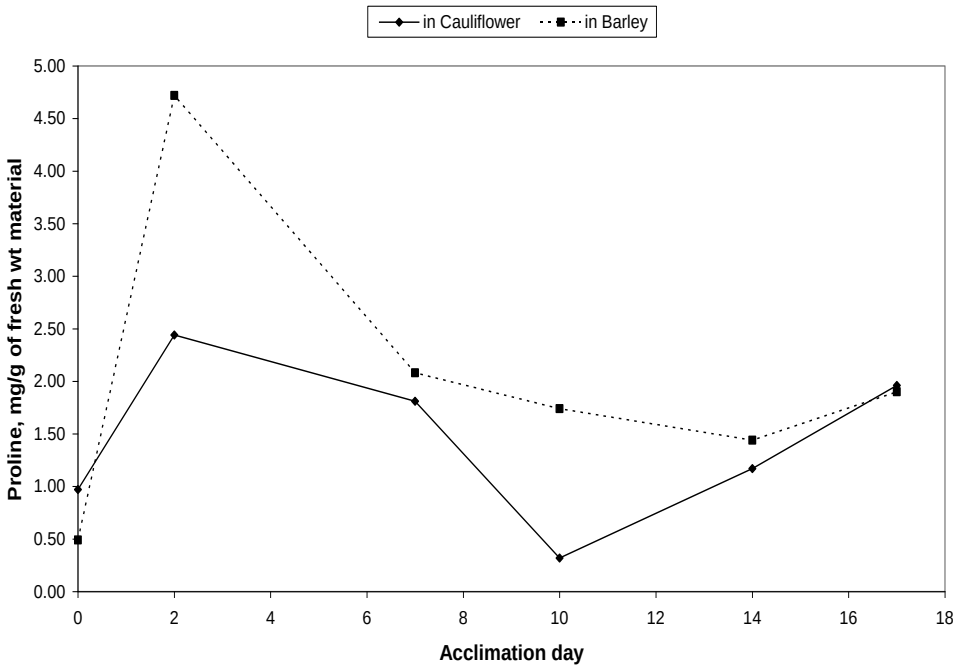

Figure 6. Proline level rose in cauliflower and barley plants during acclimation at 50 mM salt solution.

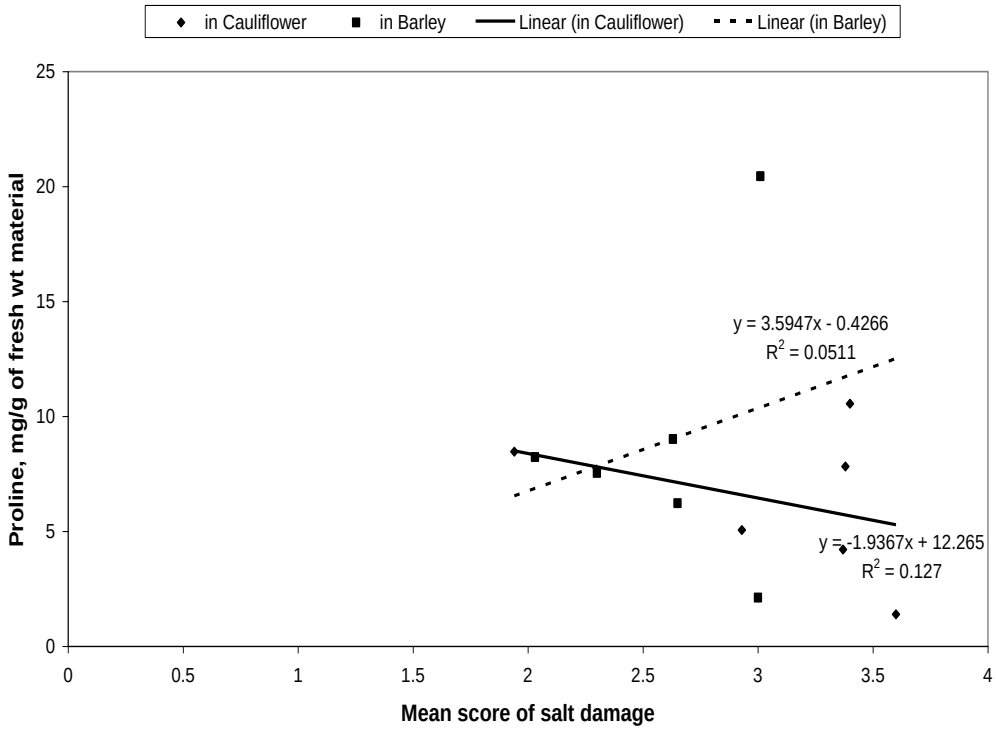

Figure 7. Relationship between salt damage and level of proline in cauliflower and barley plants

slow process taking some 5-10 days to complete. Fuller (1993) also tested cauliflower seedlings to -7°C when 2% unhardened plants survived and 80% survived after hardening. During acclimation a number of genes are up-regulated. These genes modify the cell membrane to become more fluid so as to able to withstand the collapsing of the cell during dehydration, accumulate of compatible solutes like proline, glycine betaine and sugars which raise the solute potential of the cell water to counterbalance the low water potential of extracellular ice, manufacture protection proteins (dehydrins) which help prevent important macromolecules from coming into contact with each other during dehydration, and manufacture antifreeze proteins which get exported from the cell to bind onto the extracellular ice to help modify and control its growth (Fuller, 2002). Both crop species in this study demonstrated salt resistance up to 200 mM concentration in different way with leaf damage in barley and stunted growth in cauliflower. They were hardly tolerant at 300 mM concentration. Eed (2001) found that 50-100 mM salt concentrations had no significant effect on growth of *B. oleracea* seedlings and they were completely killed above 450 mM. Choi *et al*., (2002) observed significant reduction of stem dry weight and thousand seed weight in winter barley at salt solution of 180 mM concentration. When salt concentration increases in growth medium, it increases Na concentration. Na has an antagonistic effect on K uptake i.e., it declines K concentration and thus reduces K/Na in the shoots. This nutritional imbalance has effect on enzymes and membranes. Eventually, physiological mechanisms such as stomatal movement, photosynthesis and transpiration are affected leading ultimately to the reductions in plant growth (Ashraf and McNeilly, 2004). Under salt stress, little energy is produced by photophosphorlation and phosphorylation in the respiratory chain, nitrogen assimilation is thus impaired and protein metabolism is disturbed causing impaired growth (Eed, 2001). Halperin *et al*., (1997) found that calcium transport to the shoot of barley is reduced in NaCl-stressed plants and proposed the ability to transport Ca to the shoot during salt stress as an index of salt tolerance.

In this study, plants acclimated (in 50 mM) for longer period showed lower salt damage and 14-day-acclimation played the best role against damage.

There are some information it can be hypothesized that there should be major kill in unhardened plants and no kill in plants hardened for longer period (Fuller, 1993; Fuller *et al*., 1994; Burchett, 2000). This study could not show this result. Some possible reasons were: (1) the acclimation chamber could not be at 4°C all the time due to power disruption and mechanical faults, (2) the Sanyo incubator did not read the designed temperatures (-3°C to -9°C) properly, (3) Concentration and volume of the hydroponic solution could not be maintained as constant due to evaporation and transpiration losses.

When plants were acclimated at 4 ºC, both crop species raised proline level in accordance with increase in acclimation period. Significant correlations were found between frost damage and proline level in both crop species. Fuller *et al*., (unpublished), in *in- vitro* and *in-vivo* screened and selected cauliflower lines which were resistant to frost, found that leaf proline content increased markedly in all the lines. Fuller (2004) stated that the enhancement in proline levels under stress may be due to (1) prevention of feedback inhibition of the biosynthetic enzymes caused by sequestering of proline away from its site of synthesis or (2) by relaxed feed-back inhibition of the regulatory step enzyme by gene amplification or (3) decreased activity of enzymes involved in degradation of proline. Madan *et al*., (1995) described that proline biosynthesis can take place from glutamate as well as ornithine. Ornithine aminotransferase (OAT) catalyses the first step in the pathway of conversion of ornithine to proline. Pyrroline-5-carboxylate reductase is another enzyme involved in the biosynthesis of proline. Proline oxidase converts proline to glutamate thus this enzyme also influences the level of free proline.

Though plants could raise the level of proline during acclimation to low temperature but they failed to follow the same trend whilst acclimation at 50 mM salt solution during this study. Initially, proline level rose rapidly but it decreased steadily afterwards. There was hardly any relationship between salt damage and proline level.

In a number of studies (Choi *et al*., 2002; Pakniyat *et al*., 2003), proline accumulation was enhanced by several-fold, yet its role in imparting resistance to salt stress continues to be controversial. Some workers did not observe any appreciable increase in free proline content (Colmer *et al*., 1995; Jain *et al*., 1987) whilst others consider enhanced proline level merely a stress effect, rather than a cause of stress tolerance (Lutts *et al*., 1996). Aziz *et al*., (1998) and Ashraf (1989) reported a negative relationship between proline accumulation and salt tolerance in tomato and *Vigna mungo*, respectively. Yamaya *et al*., (1989) found that NaCl did not affect proline accumulation in barley seedlings. Madan *et al*., (1995), in different genotypes of *Brassica*, found that at 34-fold increase in proline level, the increase in the activities of proline biosynthesis was only 3-4 fold. Proline metabolism however, is known to be influenced by different abiotic stresses (Eed, 2001). The effect of salt stress may be at the level of gene expression, as a 6-fold increase in the relative abundance of P5C reductase mRNA was reported in soybean seedlings under salt stress (Delauney and Verma, 1990). The role of proline accumulation and its metabolism *vis-à-vis* tolerance to salinity therefore needs to be critically examined.

For frost test, plants were grown in a heated glasshouse where they suffered from drought stress. After frost test, the plants were placed in a cool net tunnel. So, growing period and recovery period were not identical. For salt test, plants were grown in the net tunnel. Growth was not vigorous like in the glasshouse possibly due to insufficient thermal days and day-degree. A further study is needed to undertake these experiments in controlled and ideal environment to observe the actual damage from frost and salinity as well as the level of proline.

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REFERENCES

Ashraf M and McNeilly T. 2004. Salinity tolerance in brassica oil seeds. Critical Reviews in Plant Sciences. 23(2):157-174.

Ashraf M. 1989. The effect of NaCl on water relations, chlorophyll, and protein and proline contents of two cultivars of blackgram (Vigna mungo L.). Plant Soil. 119:205-210.

Aubert S, Hennion F, Bouchereau A, Gout E, Bligny R and Dorne AJ. 1999. Subcellular compartmentation of praline in the leaves of the subantarctic Kerguelen cabbage Pringlea antiscorbutica R. Br. In vivo ^{13}C-NMR study. Plant, Cell and Environment. 22:255-259.

Aziz A, Tanguy JM and Larther F. 1998. Stress-induced changes in polyamine and tyramine levels can regulate proline accumulation in tomato leaf discs treated with sodium chloride. Physiol. Plant. 104:195-202.

Bates L, Waldren R and Teare I. 1973. Rapid determination of free proline for water-stress studies. Plant and

Burchett S. 2000. The mechanism of action of liquid seaweed extracts in the manipulation of frost resistance in winter barley (Hordeum vulgare L). PhD Thesis, The University of Plymouth, Devon, UK.

Choi WY, Kim YM and Park JH. 2002. Effects of salt treatment on yield and physiological characteristics of flag leaf at heading stage in winter barley. Korean Journal of Crop Science. 47:409-412.

Colmer TD, Epstein E and Dvorak J. 1995. Differential solute regulation in leaf blades of various ages in salt sensitive wheat and a salt-tolerant wheat x Lophopyrum elongatum (Host.) A. Love amphiploid. Plant Physiol. Biochem. 108:1715-1724.

Deane CR, Fuller MP and Dix PJ. 1995. Selection of hydroxyproline-resistant proline-accumulating mutants of cauliflower (Brassica oleracea var. botrytis). Euphytica. 85:329-334.

Delauney AJ, Verma DPS. 1990. A soybean Delta[1]-pyrroline-5-carboxylate reductase gene was isolated by functional complementation in Escherichia coli and is found to be osmoregulated. Molecular and General Genetics. 221:299-305.

Eed MH. 2001. The resistance to salt of Brassica sps. and improved resistance by direct selection and mutagenesis. PhD Thesis, The University of Plymouth, Devon, UK.

Fuller M. 2002. Frost sensitivity in temperate crop plants. Journal of the Royal Agricultural Society of England. 163:22-29.

Fuller M. 2004. Cauliflower – the educated cabbage. Inaugural speech. The University of Plymouth, Devon, UK. 29th January, 2004.

Fuller MP and Eed MHI. 2003. The development of multiple stress-resistant cauliflower using mutagenesis in conjunction with a microshoot tissue culture technique. Acta Horticulturae. 618:71-76.

Fuller MP, White GG and Charman A. 1994. The freezing characteristics of cauliflower curds. Annals of Applied Biology. 125:179-188.

Fuller MP. 1993. Varietal differences in frost hardiness of cauliflower. Aspects of Applied Biology. 34:179-182.

Halperin SJ, Kochian LV, Lynch JP. 1997. Salinity stress inhibits calcium loading into the xylem of excised barley (Hordeum vulgare) roots. New Phytol. 135: 419-427.

Hare PD, Cress WA and Staden JV. 1998. Dissecting the roles of osmolytes accumulation during stress. Plant, Cell and Environment. 21:535-553.

Jain RK, Dhawan RS, Sharma DR and Chowdhury JB. 1987. Salt tolerance and proline accumulation: a comparative study in salt tolerant and wild type cultured cells of egg plant. Plant Cell Reports. 6:382-384.

Lutts S, Kinet JM and Bouharmont J. 1996. Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (Oryza sativa L.) cultivars differing in salinity tolerance. Plant Growth Regul. 19:207-218.

Madan S, Nainawatee HS, Jain RK and Chowdhury JB. 1995. Proline and Proline Metabolising Enzymes in in-vitro selected NaCl-tolerant Brassica juncea L. under salt stress. Annals of Botany. 76:51-57.

Martinez CA, Maestri M and Lani EG. 1996. In vitro salt tolerance and proline accumulation in Andean potato (Solanum spp.) differing in frost resistance. Plant Science. 116:177-184.

Pakniyat H, Kazemipour A and Mohammadi GA. 2003. Variation in salt tolerance of cultivated (Hordeum vulgare L.) and wild (H. spontaneum C. Koch) barley genotypes from Iran. Iran Agricultural Research. 22:45-62.

Raymond MJ. 1998. Aspects of drought-induced proline accumulation in Arabidopsis and barley. MPhil Thesis, The University of Exeter, UK.

Strizhov N, Abraham E, Okresz L, Blickling S, Zilberstein A, Schell J, Koncz C and Szabados L. 1997. Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 Arabidopsis. The Plant Journal. 12(3):557-569.

Wisniewski M and Fuller MP. 1999. Ice nucleation and deep supercooling: new insights using infrared thermography. In: Cold Adapted Organisms- Fundamentals and Applications. R Margesin and F Schinner (Eds). Springer Verlag. pp. 105-108.

Yamaya T and Matsumoto H. 1989. Accumulation of asparagines in NaCl-stressed barley seedlings. Berichte des Ohara Institut fur Land-wirtschaftliche Biologie Okayama Universitat. 19:181-188.