The Genetics and Physiology of Abiotic Stress Disorder in Swede (*Brassica napus var. napobrassica*)

By

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Swedes are extremely common as a root vegetable in Europe, USA, and Canada but are affected by the occasional presence of Brown Heart (BH) disorder affecting the marketable swede root. The incidence of BH has been reported worldwide however it is very difficult to breed resistance due to its sporadic occurrence with no external symptoms to select for. BH has been attributed to boron availability but attempts to link BH appearance definitively with boron deficiency have been difficult. Anecdotal evidence from breeders and growers highlighted the recent co-appearance of BH and frost injury in the field and it was postulated that if an association (physiological or genetic) can be determined between BH appearance and another more easily assessed trait such as frost susceptibility, then a frost tolerance screen may be developed as a useful surrogate method to screen for BH resistance.

Frost hardiness assessment of 12 swede genotypes including some F1 hybrids was carried out. Results showed that some genotypes (like Ag31, Me77c and Or13) were more susceptible to frost (EL_{50} circa -7 ˚C) whilst others (like Gr19 and Ly01) were classified as more tolerant. Breeder trials data from the UK and Germany over a 10 year period showed that 85% of the BH incidence was associated with genotypes that had the frost susceptible lines Ag31, Or13 or Me77c in their parentage. To investigate this association further, frost susceptible and tolerant genotypes, together with a number of their F1 hybrids, were evaluated in a field trial for their response to boron treatments (0.00, 1.35, 1.80 and 2.70 kg B ha^{-1}). At maturity, BH incidence and its severity was predominantly affected by genotype but could be ameliorated by boron application. Ag31 was confirmed to be the most susceptible to BH, and Or13 and Me77c were intermediate in their susceptibility. F1 hybrids between any two susceptible parents were also susceptible to BH. In contrast, genotypes Gr19 and Ly01 were confirmed to be highly resistant to BH and did not show any BH symptoms even at zero boron applied. F1 hybrids between resistant and susceptible lines demonstrated the BH resistant phenotype. Resistance to BH was therefore confirmed as a dominant trait with either a BHBH or BHbh genotype, whilst susceptibility was recessive bhbh. A degree of quantitative

variation existed in the severity of the BH suggesting that BH resistance was not a single gene effect. BH severity was significantly negatively correlated (r = - 0.632) with root boron content in susceptible genotypes. The genotypes which were BH resistant in this trial were also more tolerant to frost in screening tests and this association was investigated further at a molecular level.

Cold acclimation (CA) for 14 days at 4 ˚C positively affected the response of swede to frost, lowering the EL_{50} by -1.5˚C, and boron reduced the EL_{50} by -2.2˚C under non-acclimating conditions and by -1.2˚C under CA. Both boron and CA increased the catalase (CAT) and super oxidase dismutase (SOD) concentrations in swede leaves. Molecular analysis clearly demonstrated the presence of the *B.napus* cold response gene in swede, *BN115,* and was shown to be up-regulated due to both CA and boron application but differed between the two genotypes tested. The more frost resistant Gr19 showed a better response than the susceptible Ag31. Boron application reduced EL_{50} by -2.3˚C for Ag31 and -3.1˚C for Gr19.

Given the association between frost tolerance and BH resistance it is suggested that a frost test screen could be used as a useful surrogate method to screen for BH resistance in swede breeding programmes.

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Dedication:

This thesis is lovingly dedicated to….

My father who was always proud of me, he was always telling his friends, "Faiz will go to Europe to have his PhD", and that was even before I knew that I will have this scholarship in the UK. To you dad, I know you would be very happy holding this thesis in your hands but what shall I say,,,,, God bless you in your grave.

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Faiz

2014

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Author's Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance has been acknowledged.

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1- <u>Poster presentations:</u>

a. **F. Fadhel**, M.P. Fuller and A.J Jellings. The genetic and the physiology of abiotic stress disorder in swede. The Postgraduate Society Short Conference series 17 March 2011.

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   b. **F. Fadhel**, M.P. Fuller and A.J. Jellings. The genetic and the physiology of abiotic stress disorder in swede (*Brassica napus*). School of Biomedical Sciences Seminar for PhD students, 22 May 2013.

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   b. **F. T. Fadhel**, S. Burchett, S. Kennedy, A. J. Jellings and M. P. Fuller.The interrelationship between frost tolerance and brown heart disorder in swede (*Brassica napus* var. *napobrassica*). Under revision, submitted to the Journal of Agronomy and Crop Science.

List of abbreviations:

BH	Brown Heart
ms	Male sterility
LT50	Lethal temperature
EL50	50 % electrolyte leakage
REC %	Relative Electrical Conductivity
FE	Filed environment preconditioning
GH	Glasshouse preconditioning
CA	Cold acclimation preconditioning
NA	Non-acclimated
B	Boron
RG-II	Rhamnogalacturonan II
CAT	Catalase
SOD	Superoxide dismutase
ANOVA	Analysis of variances
CBF	C-repeat Binding Factor
cDNA	Complementary deoxyribonucleic acid
COR	Cold Regulated
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside Triphosphates
EC	Electrical Conductivity
IAA	Indole acetic acid
LSD	Least Significant Difference
QTL	Quantitative Trait Loci
H2O2	Hydrogen peroxide
UV	Ultraviolet
asl	Above sea level
KIN	Cold induced
LT	Low Temperature
N:P:K	nitrogen, phosphorus and potassium.
KNO3	Potassium nitrate
Ca (NO3)2•4H2O	Calcium nitrate
MgSO4•7H2O	Magnesium sulfate
NH4NO3	Ammonium nitrate
H3BO3	Boric acid
MnCl2•4H2O	Manganese (II) chloride
ZnSO4•7H2O	Zinc sulfate
CuSO4	Copper (II) sulfate
KH2PO4	Monopotassium phosphate

Chapter 1:

Introduction and Literature Review

1.1 Introduction:

Plants are subjected to a great variety of stresses which tend to restrict their growth and development and sometimes their survival. Stress, by most definitions, is considered as significant variation from the optimal conditions of life (Larcher, 2003) and stress induces responses and changes at all functional levels of the living organism. These changes and responses may initially be reversible but could become permanent. When a plant's ability to adjust to stress reaches its limit, then the latent damage becomes irreversible. In many fields of biology, the study of stress has gained great importance and a wide variety of approaches have been employed in order to gain more insight into the processes involved (Larcher, 2003).

Environmental stresses are broadly grouped as biotic and abiotic. Worldwide, abiotic stress is the most important adverse factor concerning the growth and production of crops and it is caused by non-living factors on which plant physiological systems are dependent. Abiotic stress is the negative influence of non-living factors on living organisms in a specific environment, and can be high light, low temperature, UV light, salinity, drought or atmospheric gases (SO_2, O_3, and NO). A large number of climatic factors are among the abiotic environmental stressors effective in the atmosphere in the soil and under water, including especially, radiation stress (high or low), and extremely high or low temperatures. Normally frost, frozen ground, covering of snow and ice are accompanied by low temperatures. In the soil, high salt and mineral concentrations, especially deficiency, may affect plant growth. Abiotic stresses are more injurious and harmful to the plant when they occur in combination (Tony et al., 2002). Surprisingly, molecular biologists have rarely addressed the co-occurrence of the impact on plants of different stresses. It has been revealed in recent studies that plant response to two different abiotic stresses combined is unique and cannot be extrapolated directly from the plants response to each of the stresses applied individually (Suzuki et al., 2005, Pnueli et al., 2002). Although plant tolerance to combined abiotic stresses is known as an aim of plant breeding (Heyne and Brunson, 1940, Jiang and Huang, 2001), not much

is investigated about the molecular mechanisms underlying plant acclimation to such combined abiotic stress (Rizhsky et al., 2004).

Abiotic stress symptoms vary between plant species and the factors causing the stress. Many of these abiotic factors have been studied deeply and specifically using the model species *Arabidopsis thaliana*, which belongs to the *Brassicaecae* family. Swede (*Brassica napus* var*. napobrassica*) is a horticultural root vegetable crop, common in Europe, and also a member of the *Brassicaceae*. One of the most important abiotic stress disorders affecting swede is Brown Heart (BH) disorder. BH makes swede roots unfit for human consumption on aesthetic grounds because the BH resembles a rot. In 1936 Hurst and Macleod were the first authors to suggest that BH is due to a lack of boron available to swede (Sanderson et al., 2002). However, BH could still occur even when sufficient amounts of boron were applied.

The incidence and severity of boron deficiency and the efficacy of boron fertilization alters from location to location and from year to year in ways that are not explained simply by differences in soil boron levels, cultivar, or agronomy.

The work presented here is part of an on-going project to examine the BH syndrome in UK bred varieties and presents important findings from a field trial on BH reporting both genetic and environmental influences on its incidence.

1.2 Abiotic stress:

Plants, as sessile and poikilothermic organisms, are constantly exposed to and need to survive variable and often unfavourable environmental conditions. Consequently, plant growth and productivity are greatly affected by various abiotic and biotic stresses factors. Abiotic stresses, including extremes in temperature, drought, high salinity, and nutrition deficiency, are in fact the principle causes of crop failure worldwide, and a threat to the agricultural industry. Any one of these factors can affect plant growth and development, or reduce plant production, and when of extreme impact, cause the death of plants.

Abiotic stress can be defined as the negative influence on living organisms by non-living factors within a specific environment. In order for non-living factors to

adversely affect the performance of the population or the physiology of individuals of the organism in a significant way, then these factors should influence the environment beyond its normal range of variation (Tony et al., 2002).

In order to escape or avoid stress, plants have evolved a myriad of strategies and mechanisms with behavioural, physiological, or morphological adjustments to adapt to a variety of stresses. These include stress avoidance, escape, and tolerance (this refers to traits that reduce the negative fitness impacts of damage) or resistance (traits that reduce damage) responses (Anurag et al., 2004). Experimental evidence has established that plants adapt to the surrounding environmental conditions on a daily, or even on an hourly basis using a variety of physiological mechanisms often mediated through plant hormones and through changes in gene regulation in anticipation of potential severe stress. The early events by which plants respond and adapt to environmental stresses include sensing of stress and subsequent signal transduction events that activate various physiological and metabolic responses. Accumulation of compatible solutes is one common mechanism that has been evolved by plants as a strategy to cope with various dehydrative abiotic stresses. Such compatible solutes include betaines and related compounds; polyols and sugars, such as mannitol, sorbitol, and trehalose; and amino acids, especially proline; all of which are low-molecular-weight, highly soluble compounds that are non-toxic at high concentrations and accumulate at the first signs of stress (Rhodes and Hanson, 1993a, MacNeil et al., 1999). Such responses are mediated via numerous physiological alterations involving numerous genes and regulatory circuits resulting in changes such as alteration of plasma membrane composition, changes in phytohormone levels, and changes in relative water content in the leaves, all of which eventually lead to gross phenotypic changes. These adaptive changes are correlated with a range of adaptive strategies in plants that demonstrate some tolerance to such stresses (Jenks and Wood, 2010).

The environmental factors causing stress and the time needed to present the effect as a stress vary, for example exposure to extremes of air temperature (at both a low level i.e. frost or a high level) can be stressful in just a few minutes

but soil water deficit may take days to weeks to manifest itself as stressful. Other factors like soil mineral deficiencies can take months to become stressful (Taiz and Zeiger, 2002).

The most harmful effect of abiotic stressors is when they take place in combination and require simultaneous different adaptation strategies and where adaptation to one stress negates or reduces the plants ability to develop tolerance to the second or further stresses. For example, plants which are nutrient stressed may have a lower ability to resist temperature extremes or drought.

Agriculture is highly affected by abiotic stresses and it has been estimated that more than 50% of a crops potential yield is missing because of the impact resulting from suboptimal climatic and soil conditions (abiotic stress) (Wang et al., 2007). In addition, abiotic stress has a defining role in determining the distribution of plant species. It is therefore of immense importance to both agriculture and the environment to understand the physiological processes that lead to stress injury and the adaptation and acclimation mechanisms of plants to environmental stress. The concept of stress is closely associated with that of stress tolerance, which is a plant's ability to carry on growing and developing under less than optimal environmental conditions. Abiotic stress covers many aspects as previously mentioned, however this review will concentrate on nutrient stress (especially the micronutrient boron) and low temperature stress as important factors involved in this study.

1.3 Swedes

The swede (*Brassica napus* var*. napobrassica*), also called Swedish turnip, turnip-rooted cabbage or Rutabaga (in the USA), is a crop believed to be originated from a hybrid between the turnip (*Brassica rapa*) and wild cabbage (*Brassica oleracea*) (Figure 1.1) as recently as the 17th century in Bohemia (a historical area and former kingdom in the Czech Republic). Like turnips, swedes are a member of the *Brassicaceae* family. The swede is similar to turnip in producing a large edible storage root; however, they differ in leaf characteristics and in minor details of root structure and shape. Swede leaves are like cabbage, bluish green and smooth; those of turnip are usually thin, hairy, and light green.

Turnip roots have little or no neck while swedes are slightly more elongated and have a thick leafy neck. Root flesh is yellowish and white in swedes but only white in turnip. Botanically speaking the marketable swede “root” is mostly a swollen hypocotyl with only its lower part being a true root, however for convenience, they will be referred to throughout this thesis by the common description as a “root” vegetable.

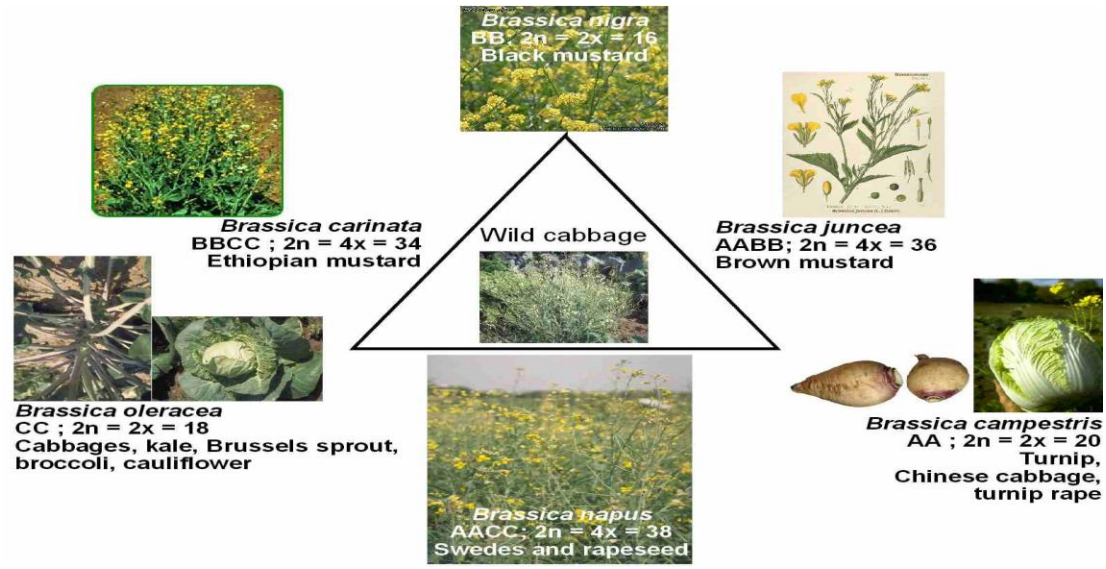


Figure 1.1: The triangle of U showing the relationships between Brassica genomes (Nagaharu, 1935).

Swedes are grown for both human and animal consumption. It was determined by researchers at the beginning of the 1900s that swede root flesh is a valuable energy source for young livestock. However, livestock farmers at that time were turning away from the brassica crops (which also included rape, kale, and turnips) because their production and utilization required much hand labour. Later, in the 1970s, researchers began to recognise the potential of using brassicas roots as forage crops and crop mechanisation had largely overcome the hand labour issues. The forage brassicas are generally considered to be high yielding forage crops with high nutritional quality available during the winter months when grass availability is low.

Swede is a cool-weather crop and is extremely common as a root vegetable grown in the northern part of the USA and Europe, the UK and Canada. Swedes are a biennial and overwinter as a storage root. The swollen root in the

first season comprises the top region of the true root and the hypocotyl and together these form a big structure which is borne partially above ground. Swedes root to a depth of up to 1.50 m with lateral expansion of a fibrous root system of up to 0.60 - 0.75 m. Compared with other “root” crops swedes have relatively high dry matter content at harvest (10-12 %) (Langer et al., 1991).

Swede flesh colour is associated with flower colour whereby cultivars with white fleshed root have yellow flowers while the yellow fleshed forms have cream coloured flowers. The flesh colour is also associated with the colour of the top part of the root, which can be purple, green or bronze, due to the development of anthocyanin and/or chlorophyll pigments in the outer cells of the part of the root which is exposed to light.

Swede root shapes are usually described as globe or tankard but may be intermediate between these two. There appears to be no consistent correlation between colour and shape grouping and agricultural value, but green and dark-purple skinned forms are mainly considered to be frost-hardy, while the light-purple forms are more readily damaged by frost (Langer et al., 1991).

One general advantage of swedes compared to other root crops like turnip is frost hardiness, which enables them to be used late into the autumn and winter. In agro-climatic regions with mild winters such as the South west of the UK, swede may be grazed by sheep in the field throughout the winter period. However, of greatest commercial importance is their saleability for human consumption as a vegetable and it is in this regard that BH is of significance economically.

1.4 Brown Heart

Brown Heart (BH) is a physiological disorder appearing as an internal browning discolouration in marketable swede roots and continues to be a concern for vegetable swede growers with a reported rise in incidence (K. Coles Ltd. pers. com). As mentioned earlier, BH has been attributed to boron deficiency and the first connection between boron and BH was reported in 1936 by Hurst & MacLeod (Sanderson et al., 2002). Severe BH is often recorded in the absence of boron fertiliser application (Umesh and Cutcliffe, 1978). It was found that BH symptoms did not appear when the boron content of the root and young leaves were 27 and 56 µg g^{-1} DM respectively, whilst when it was 14 µg in roots and 17-20 µg in young leaves slight internal signs of brown discoloration were apparent. Boron deficiency, sometimes referred to as water core, appears at first as firm, water-soaked areas in the swede flesh. Severely affected roots may be off-shaped and can have a rough corky exterior appearance (Cutcliffe and Gupta, 1987).

Hurst and MacLeod (1936) noted that BH might not be corrected with boron application on alkaline soils or in conjunction with lime application. Likewise, Gupta and Cutcliffe (1972) found BH incidence to be greater upon liming of some Prince Edward Island soils. In Ontario, BH was reported in swedes grown on soils ranging in pH from 7 to 8 despite the fact that boron fertilizer was applied in sufficient amounts. Boron is found to be optimally available within a soil pH range of 4.5 to 6.5 (Figure 1.2) (Taiz and Zeiger, 2002). These reports clearly indicate that boron uptake by crops may be limited at normal alkaline soil pH. However, Smith and Anderson (1955) mentioned that during 20 years of investigations, soil content of boron had no relation to the incidence of boron deficiency symptoms in crops grown.

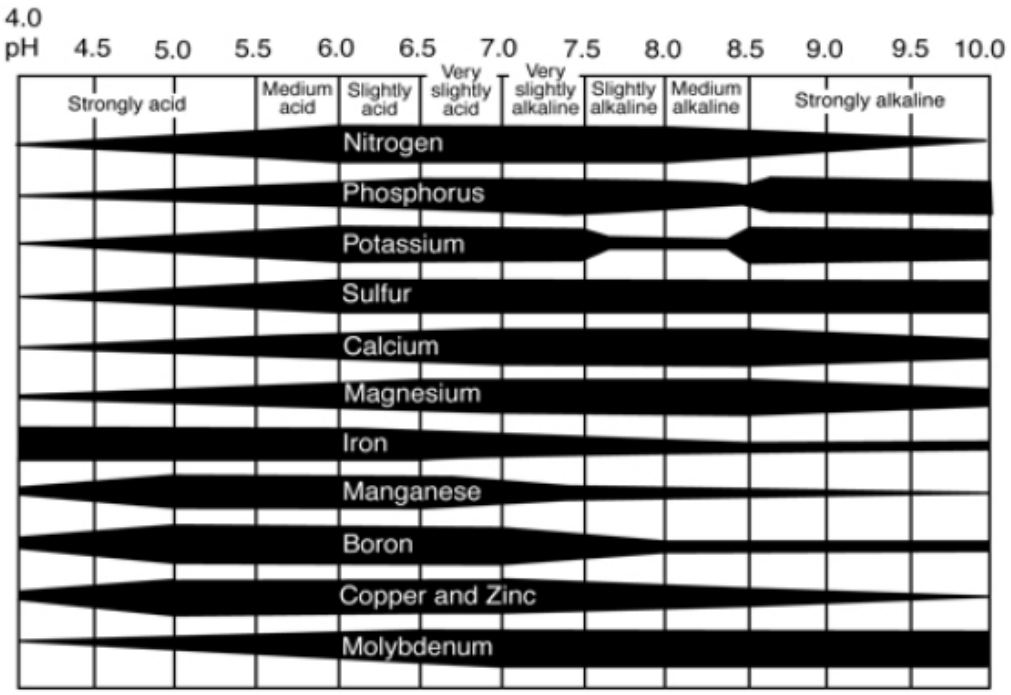


Figure 1.2: Influence of soil pH on the availability of nutrients elements in organic soils, the wider the bar, the greater the availability. Reviewed by Taiz and Zeiger 2002.

Only Sanderson et al. (2002) recommended growers to consider different factors other than initial level of soil boron, and also mentioned that several factors are likely to control BH appearance regardless of sufficient boron application. The application method of boron might also be a factor affecting BH appearance in swede roots. Umesh and Cutcliffe (1978) found that BH was controlled with broadcast applications of 2.24 kg B ha^{-1} but this could be reduced to 1.12 kg B ha^{-1} by band application and they also found that foliar sprays were an effective method of applying boron to control BH. It is also suggested that higher rates of boron are applied by growers to overcome factors such as improper mixing and uneven application. In Canada in the Atlantic provinces, boron application in bands at 2.24 kg ha^{-1} or broadcast at 3.36 kg ha^{-1} was recommended to assure commercial swede plantings free from BH. The timing of boron application was also found to be rather important in controlling BH appearance, Cutcliffe and Gupta (1987), in their experiments carried out at three locations, reported that two foliar applications of boron, each at 1 kg ha^{-1}, applied at 30 and 42-45 day after sowing provided a high level of

control of BH. However, boron applied at 40 days and repeated at 52-55 days did not provide acceptable control at all three locations. Generally BH incidence was increased as the time of foliar boron application was delayed at all locations of the study. Such variability in response to application rates and methods of application is indicative of the extreme solubility of mineral boron and its propensity to be leached away from the rooting zone of young developing plants. Soil boron on the other hand can be held within the organic fraction where it is mineralised slowly upon organic matter degradation but once mineralised is susceptible to the same risk of leaching. It is apparent from the literature that abiotic stress related to limited boron availability is a key factor leading to BH symptoms.

BH is a very difficult trait for which to breed resistance because it is very difficult to detect in the field as there are no exterior indications that are associated with the condition. It is therefore not possible to identify roots with BH by an external visual examination (Umesh and Cutcliffe, 1978, Shelp and Shattuck, 1987). The only way to assess if a plant is affected or not is to cut the root at the marketable stage and by this time no solution to remedy the condition is available to growers. Growers and managers of pack-houses therefore must take a sacrifice sample of roots and cut them transversely to detect the incidence of BH in a crop. Clearly if the incidence is above a critical threshold (20 – 30 % affected roots) then the whole crop could be condemned as not fit for sale (Personal communication).

Elsoms Seed Company is the only vegetable breeding company in the UK, and possibly in Europe, with an active breeding programme for swede. Utilising the new germplasm emerging from the Elsoms breeding programmes in association with novel agronomic methods, which include field covers to extend the growing season and reduce cabbage root fly infestations, a new range of stress symptoms are appearing in crops in the UK. In particular, there have been some observations of certain germplasms demonstrating symptoms of frost susceptibility combined with BH symptoms.Preliminary investigations by the author in commercial swede fields at the beginning of this project (2011) and meetings with a swede breeder and commercial growers led to the contention that BH appearance may possibly be associated with frost damage (Plate 1.1) or with low frost tolerance.

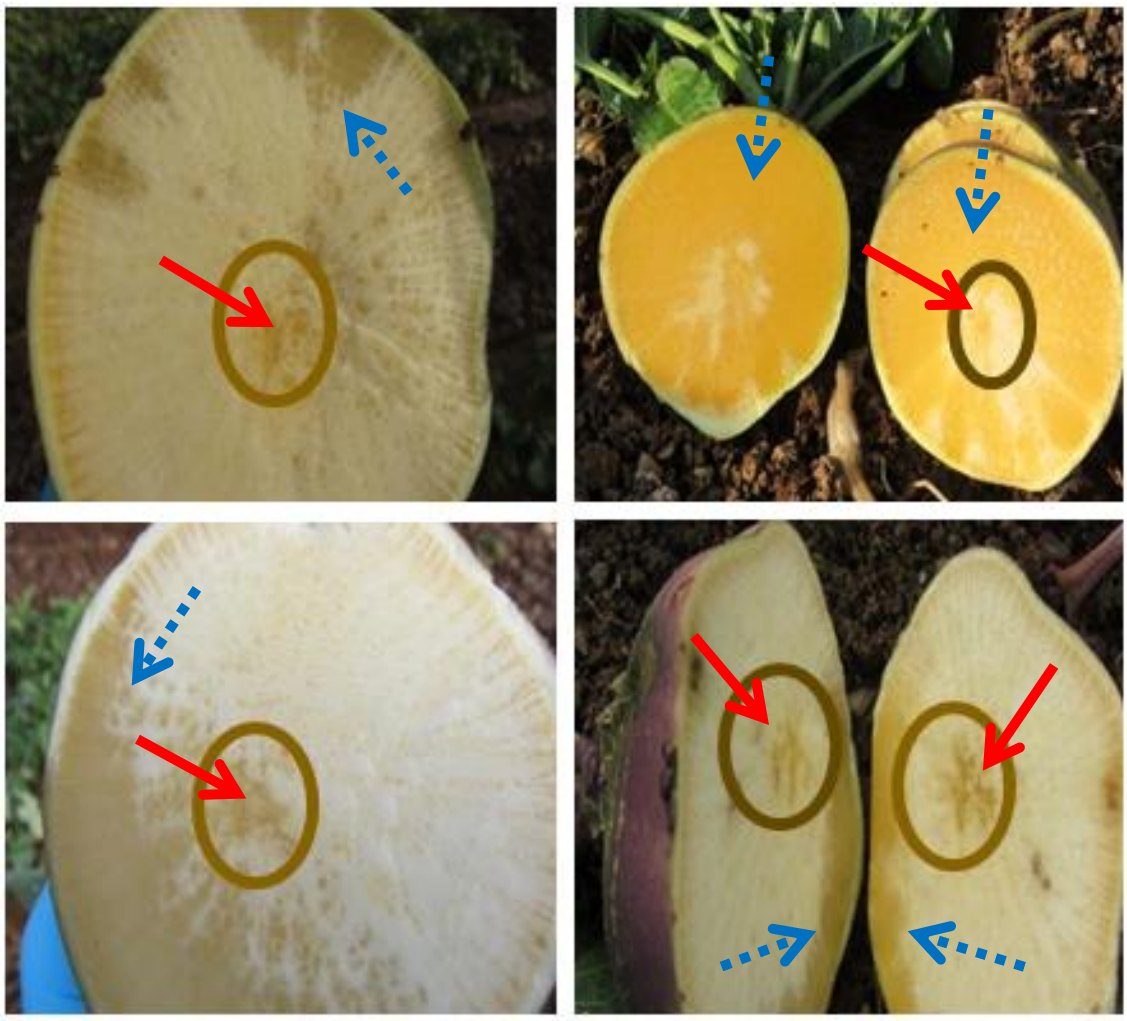


Plate 1.1: Co-appearance of brown heart appearance (Red sold arrow) and frost damage (Blue dotted arrow) in the field (photo taken by the researcher in the field).

The two factors, low temperature and boron, significantly affect plant growth and productivity in swede (Graham and Patterson, 1982) and were of interest in the present project. Although there are a few literature sources that mention boron and swede together there are none that mention swede response to low temperature. The literature studied here therefore presents the general impact of these two factors on plants. It was postulated early on in this investigation that if an association can be determined between frost and BH susceptibility then a frost-testing screen might be a useful surrogate method to screen for BH resistance.

1.4.1 Nutrient Abiotic Stress:

Nutrient deficiency can be caused by limitation of the essential nutrients that are important to agricultural production worldwide (Wang et al., 2002). According to their relative concentration in plant tissues; nutrients are often classified as macronutrients (N, K, Ca, Mg, P, S, and Si) or micronutrients (Cl, Fe, B, Mn, Na, Zn, Cu, Ni, and Mo). Inadequate supply of an essential element results in a nutritional disorder manifested by characteristic deficiency symptoms. These deficiency symptoms are the result of metabolic disorders which are related to the roles played by these essential elements in the normal plant (Taiz and Zeiger, 2002).

In swede, one of the most important nutrient deficiency disorders, as mentioned earlier, is BH, which is suggested to be related to the availability of boron. Boron is one of the micronutrients, along with phosphorus and silicon that are important in many plants physiological processes like maintaining structural integrity or in energy storage reactions and these elements appear in plant tissues as phosphate, borate, and silicate. The role of boron in plants is still not completely clear. Boron is a soluble and mobile element inside the plant, but this mobility appears to be related to the availability of boron in the soil. This suggests that the plant prefers to utilize newly taken up boron rather than to remobilize it. There are also limitations on boron availability from the soil to plants and this is particularly affected by soil acidity (Taiz and Zeiger, 2002). Acute boron deficiency can cause problems in many crops and can lead to rapid termination of root elongation, leaf shank growth and low sexual fertility (Fujiwara et al., 2010, Al-Amery et al., 2011); these can all be attributed to the reduction of cell expansion and reflect the importance of boron in cell wall development and function. In plant tissues boron is present in the cytoplasm in all subcellular compartments of the cytosol and vacuoles but the majority of boron is in the apoplasm bound in cell walls (Dannel et al., 2002). The segmentation of boron has been found to be considerably different within and between species even when under the same environmental conditions in highly uniform genotypes such as in *Arabidopsis thaliana*. The requirement of boron for normal growth in B-inefficient cultivars appears to be more than that in

B-efficient cultivars. In *Arabidopsis thaliana* the wild type shows greater boron partitioning than that in the mutant bor-1 (Takano et al., 2001).

Moreover, it has been reported by Noguchi et al. (2000a) and Takano *et al,.* (2001) that the transportation of boron from root to shoot in wild-type *A. thaliana* occurred only under low external concentration of boron, therefore Takano et al., (2001) suggested that another mechanism(s) is involved besides transpiration flow.

Each element has its function in the plant and the function of boron is to make complexes with cell wall constituents like mannitol, mannan and polymannuronic acid, and it is involved in cell elongation, nucleic acid metabolism, hormone responses and membrane function (Shelps, 1993). It has been found that the cell wall contains up to 90% of localized cellular boron in plants and abnormalities in the cell wall and organization of the middle lamella are the first symptoms of boron deficiency (Loomis and Durst, 1992). It has been proposed that the mechanism for crosslinking cell wall polymers is the formation of borate esters with the hydroxyl group of cell wall carbohydrates and/or glycoproteins. A relationship has been observed between plant boron content and pectin with boron protecting Ca^{+} in the cell wall by forming a crosslink in pectin. The first boron-containing pectic polysaccharide complex that has been isolated from plants was reported by Clarkson and Hanson (1980) and (Yamauchi et al., 1988), as RG-II-B (boron-rhamnogalacturonan-II). Kobayashi et al. (1996) purified the pectin fraction from radish root cell walls and demonstrated that the molecular weight of the RG-II-B complex reduced by half just by removing boron from it. The RG-II-B complex has been found in cell walls of more than 20 plant species including two from *Brassicaceae* (Matoh et al., 1996)

Boron deficient plants may show a wide variety of symptoms, depending on the species and the age of the plant and the severity of deficiency. A characteristic severe symptom is black necrosis of the young leaves and terminal buds with the necrosis of the young leaves occurring primarily at the base of the leaf blade. Stems may be unusually stiff and brittle and apical dominance may also be lost causing the plant to become highly branched. The terminal apices of the

affected branches soon become necrotic because of inhibition of cell division. Structures such as fruit, fleshy roots, and tubers may exhibit necrosis or abnormalities related to the breakdown of internal tissues.

In swede, plants with severe deficiency demonstrate symptoms including mottling and tinting of foliage and death of the growing point. Rough skin on the root and longitudinal and transverse sections of the swelling root reveal water soaking and brown discoloration which is severe BH syndrome (Plate 1.2) (Thomas Wallace, 1943).

Plate 1.2: Severe boron deficiency symptoms in swede. A) Boron deficiency symptoms in leaves. B) Boron deficiency symptoms in root. C) Root longitudinal section of brown heart. http://www.hbci.com/~wenonah/min-def/swede.htm

Many studies have been carried out with boron and its effect on *Brassica napus* (oilseed rape and swede), which showed that the species is sensitive to boron deficiency. In addition, there appears to be variation among brassica genotypes in their response to boron deficiency (Du et al., 2002). Differences between genotypes in response to nutrition can be generally attributed to two mechanisms more than anything else: 1) plant ability to absorb nutrients, and 2) the translocation and utilization of nutrients in the plant (Taiz and Zeiger, 2002).

Boron is a mobile element both in the soil and inside the plant, and this mobility is related to the availability of boron in soil. Boron availability to plants is affected by soil pH which should be in range 4.5 to 6.5 for maximum availability (Figure 1.2) (Taiz and Zeiger 2002).

Analysis of swedes grown in soil with sufficient boron showed limited “in plant” remobilisation of boron while plants in soil with boron starvation demonstrated translocation of boron from older leaves to younger tissues. Swede genotypes that have a good capacity for translocation of boron to the swelling roots from the leaves are reported to always show less sensitivity to boron deficiency and BH disorders (Shlep and Shattuck, 1987). Research looking at the genetics of boron utilisation have shown that boron efficiency (defined as the capacity to utilise boron) is a dominant trait (Xu and Wang, 1998). A comparison between boron efficient and inefficient cultivars of swede revealed that the efficient cultivars showed light leaf colour, a short growing season and bolted early, and that this was closely related to their boron efficiency coefficient (defined as the ratio of yield from plants grown in the soil with 0.25 mg kg^{-1} to that from plants grown in the soil with 1.00 mg kg^{-1} of available boron) (Barrero et al., 2006).

1.4.2 Temperature abiotic stress:

Temperature is one of the most important environmental factors governing plant growth and development; it limits plant geographical distribution and frequently limits crop yield potential and actual yield. Low temperature has been widely studied for its important impact on plant growth and yield of crops because it shortens the growing season and affects the product quality of fruits or vegetables in storage (Tony et al., 2002). The effect of low temperature stress depends on the degree of severity and the duration of exposure. Injury may occur through chilling at temperatures between 15˚C and 0˚C for species that are extremely temperature sensitive and the seedling stage of plants is often the most susceptible to chilling injury. Both chilling sensitive and chilling resistant plants can be affected by temperatures below 0 ˚C especially when water changes to a solid state i.e. it freezes. Frost injury symptoms include: 1) surface lesions; 2) appearance of water-soaked tissues; 3) loss of water; 4) internal discoloration; 5) tissue damage; 6) ethylene production/quickened senescence; and 7) faster decay due to leakage of plant metabolites. Physiological changes due to frost can be either reversible (primary) or irreversible (secondary) injury. Reversible injury is characterised by a dysfunction in the plant caused by the first rapid response to the low temperature but the plant can continue normally if brought back into non-frost

temperatures. Irreversible (secondary) injury is the result of the primary injury when damage to the plasma membrane occurs and the metabolic machinery of the cell is impossible to repair leading to a permanently damaged state and cell death (Russell et al., 2006). A whole plant exposed to damaging frost can display regions of complete cell death e.g. to leaves, yet be able to recover through regrowth of undamaged meristems. Thus it is possible for a plant to demonstrate irreversible damage symptoms yet be able to survive the freezing event.

Plants can be classified, depending on their response to Low Temperature (LT), into three broad categories: LT-sensitive plants such as rice and maize; LT-insensitive plants like spinach; and tolerant plants such as temperate woody species. LT-sensitive plants are those that are chilling sensitive and if exposed to temperatures below 10 ˚C will undergo irreparable damage. For LT-insensitive plants primary injury occurs at temperatures below 0 ˚C whilst LT-tolerant plants can tolerate secondary injury due to an inherited ability to either avoid exposing its sensitive organs to freezing through a deciduous habit and/or developing transient hardiness. Plants insensitive to low temperature have either an inherited trait that confers the ability for its tissues to remain undamaged despite extra cellular freezing and dehydration or the ability of its essential fluids to supercool (without forming extra cellular ice). The induction of frost tolerance normally occurs in response to transient exposure to low non-freezing temperatures (between 10 ˚C and 0 ˚C) and this is known as cold acclimation. Such acclimation results in cellular and metabolic changes including increased accumulation of osmoprotectants such as sugars, amines, soluble proteins, proline and other compatible solutes via activation of low-temperature signal transduction pathways, and altered gene expression to provide improved protection to low-temperature stress at a cellular level (Jessop and Toelken, 1986, Gill and Vear, 1980).

The brassicas are classified as “cool season” plants meaning that they are relatively resistant to frost. Swede demonstrates a good adaptation to low temperature and it has the ability to germinate at 5 ˚C with the optimum germination temperature range of 15 ˚C to 29 ˚C. Swedes are reported to favour growing temperature in the range 15 ˚C to 20 ˚C (Taiz and Zeiger, 2002). In the

UK these characteristics mean that swedes can germinate at a range of soil temperatures and continue to make meaningful growth in the autumn and early winter.

There is a lot of evidence that shows that changes in gene expression occur due to low temperature and that these genes control many biochemical pathways leading to physiological changes from exposure to acclimating (low but not freezing) temperatures. These so called low-temperature responsive genes (LTR) or cold upregulated genes (COR) have been cloned and studied for a range of plant species (Hill, 1991). LTR/COR's typically belong to a regulon which is under the control of a low temperature induced transcription factor. Also, a number of related physiological adaptations including the up-regulation of LTR/COR's can occur due to the transiently increased amount of ABA, and are called ABA inducible. During acclimation, some of the up-regulated genes are expressed by both ABA-dependent and ABA-independent signalling pathways (Rhodes and Hanson, 1993).

Two 9-bp DNA elements have been identified in the promoter of *Arabidopsis* RD29A gene (Rhodes and Hanson, 1993) containing the low-temperature and drought-responsive element (CBF/DRE) with the core sequence CCGAC, which hereafter will be referred to as the low-temperature responsive element (LTRE). This was found to be necessary, and is the precursor, for the upregulation of low temperature responsive cold-induced LTR/COR genes. In most *Brassicas* acclimation is under the control of *CBF/DRE* transcription factors (Hadi et al., 2011, Blevins and Lukaszewski, 1998) and the gene *COR15* is a downstream LTR/COR gene that is commonly used by molecular biologists to determine if acclimation has been effective. For *Brassica napus* (oilseed rape) the downstream gene *BN115* can be used in place of *COR15* (Jaglo et al., 2001) but it was not known at the beginning of this study whether *BN115* exists in swede and whether it is up-regulated by cold acclimation.

Thomashow (1999) demonstrated that in *Arabidopsis* a change occurs in gene expression in response to low temperature. These genes were given various designations including COR (cold regulated), LTE (Low-temperature-induced), KIN (cold-induced) and RD (responsive to dehydration). The transcription levels

for these genes were found to increase dramatically within 2 to 4 hours of transferring plants to low temperature, and remain elevated for weeks if plants were kept at low temperature. But these genes quickly decreased in transcription levels to those in non-acclimated plant within hours if plants are returned to warm temperatures (de-acclimation). Studies with gene fusions of *COR15a, COR78/RD29a* and *COR6.6/KIN2* established that the promoter of these cold-induced genes is activated in response to both low temperature and drought. Other studies have indicated a common DNA regulatory element included within the promoter regions of these genes referred to as the CRT(C-repeat) / DRE (dehydration responsive element), that imparts responsiveness to both low temperature and dehydration stress (Wang et al., 2007, Rhodes and Hanson, 1993). *CBF1, CBF2* and *CBF3* were the first three of these DNA-binding proteins to have been designated. The genes for *CBF1-3* do not express if plants are grown at warm temperature (≈ 22˚C), but transferring plants to low temperature (e.g. 4˚C) showed rapid transcript accumulation for all three proteins within 15 min.

Like *Arabidopsis, Brassica napus* is a member of the *Brassicaceae* family and it can acclimate to cold, and so *Brassica napus* var. *napobrassica* is expected to have a *CBF* cold-response pathway. Shleps, (1993) and Dale and Krystyna (1998) demonstrated at least four *CBF*-like proteins that have the *CBF* signature sequences are encoded in *Brassica napus* (oilseed rape), and about 90% of these proteins are identical to each other and about 75% identical in sequence to *CBF1* in *Arabidopsis*. *Brassica napus CBF*-like gene transcript levels accumulate rapidly upon exposing plants to low temperature (within 30 min) in just the same way that occurs with the *CBF1-3* genes in *Arabidopsis*. Transcript levels reach their maximum during a few hours of plants being exposed to low temperature, and after that, they decrease but stay at higher levels compared to non-acclimated plants (Shleps, 1993, Dale and Krystyna 1998).

In transgenic *Brassica napus* the constitutive expression of *Arabidopsis CBF1-3*, activates expression of downstream genes namely *BN115* and *BN*28, (homologous to the *CBF*-targeted *Arabidopsis COR* genes *COR15a* and *COR6.6* respectively). Furthermore it has been shown that transformation of *B.*

napus with the CBF genes of *Arabidopsis* results in an increase in freezing tolerance (Shelps, 1993). Physiological frost hardiness experiments using electrolyte leakage (EL) showed that leaf tissue from non-acclimated control plants of *Brassica napus* showed frost hardiness values measured as EL_{50} (50% Electrical Leakage) of -2.1 ˚C, compared to about -4.7 ˚C for leaf tissue from cold-acclimated CBF over expressing plants. In cold acclimated plants, CBF-expression results in an increase in the plants ability to tolerate freezing. Leaf tissue from cold acclimated *Brassica napus* plants had an EL_{50} value of -8.1˚C, but the EL_{50} of tissues from cold-acclimated CBF expressing plants was about -12.7˚C. This indicates clearly that the CBF cold-response pathway of *Brassica napus* is closely related to the pathway of the CBF cold-response of *Arabidopsis* (Shelps, 1993). This upholds recent work which shows that the CBF response pathway is a highly conserved pathway in both brassicas and cereals and is probably present in all temperate plant species (Hadi et al., 2011, Al-Issawi et al., 2013).

1.5 Boron and Low temperature:

Temperature not only has a great impact on plant growth but also on plant responses to low boron. The extent of the low temperature and boron interaction varies between plant species and between varieties due to their different ability to tolerate both low temperature and variations in boron efficiency (Moraghan and Mascagni, 1991). The mechanism that links low temperature with the boron response however remains unclear. A better understanding of this mechanism (temperature x boron interaction) may eventually assist breeders to develop better varieties and enable farmers to grow better crops under less than optimal temperature conditions as frequently occur in temperate climates.

In the literature, reports about studies conducted under controlled conditions concerning the interaction between low temperature and boron nutrition in plant are very limited. To date, studies on plant boron deficiency affected by low temperature have been reported only on wheat, cassava, sunflower and oilseed rape.

Plant growth requirement for boron is influenced by environmental components including temperature, light, humidity and soil water conditions (Moraghan and Maseagni; 1991; Gupta, 1993; Shorrocks, 1997). Temperature is one of the key environmental factors which affect plant boron nutrition and a plant's ability to tolerate frost could be affected by boron status. Stoker and Tolman, (1941), Combrink et al. (1995) and Ye C et al. (1997) reported that plants with an adequate boron concentration will be more able to tolerate low temperature injuries. The interactions between boron and low temperature on plant growth may alter depending on the range of the temperature experienced as well as the plant species.

Low temperature influence on plant responses to boron could be due to increased susceptibility of plants to low temperature injury as a result of a poor boron status *in planta*. It has been postulated that plants or plant parts with low boron are less able to resist frost due to structural deterioration of cell walls and altered membrane integrity (Zhengqianye 2004).

Low temperatures reduce the uptake and/or translocation of boron leading to deficiency in plants. Boron uptake and boron translocation rate from root to shoot were dramatically decreased by low root zone temperature especially at low soil boron level (Forno et al., 1979). It was noted that greater boron application will be necessary for plant growth when soil temperature is below a critical threshold. Zhengqian (2004) demonstrated that plant boron deficiency was induced by deterioration of boron translocation into growing shoot parts in addition to the decrease in boron uptake rate and boron translocation rate, and greater shoot to root ratio

It appears that plant tolerance to low temperature might be affected by boron status and plants with an adequate boron application were reported to be less injured by low temperature (Wang et al., 1999, Ye et al., 1997). In field experiments of oilseed rape, plants supplied with boron were injury free or had reduced frost injury in comparison to plants that had no boron applied (Zhengqian, 2004). In muskmelon, boron application improved the growth and fruit quality and chilling injuries decreased in harvested fruit during cold storage at 5 ˚C (Combrink et al., 1995 quoted in Zhengqian, (2004).

These limited results indicate that boron nutritional status may play a crucial role in plant cellular response to low temperature, but how these two factors are linked physiologically and genetically and what mechanisms are involved remains unresolved. In particular, to the author's knowledge, there has been no research conducted on how boron affects plant molecular response to low temperature and how the low temperature affects either boron transporter or remobilisation genes. Indeed, there has been virtually no molecular based research published in the literature on swedes since this is a minority vegetable crop species and not a target plant.

The aim of this study was to explore the genetics and physiological links between BH and frost tolerance in swede leading to a potential reduction in BH in swede crops grown for human consumption:

This led to the following objectives being formulated:

a) Develop a frost hardiness screen for genotypes of swede.

b) Investigate the BH syndrome in swede from a physiological and genetic perspective.

c) Determine the association between BH syndrome, frost tolerance, and boron.

d) Investigate the molecular basis of plant response to nutrient abiotic stress (boron deficiency) and low temperature.

Chapter 2:

General Materials and Methods

2.1 Plant materials:

A total of 18 genotypes of swede were used in this project in three experiments (Chapter 3, 4, and 5). Seeds were kindly supplied by Elsoms Seed Ltd., (Spalding, Lincolnshire, UK) who are the only vegetable breeding company in the UK with an active swede breeding programme. The genotypes included parent lines produced by single seed decent for at least 6 generations which had undergone some directed "cleaning" to eliminate off-types. Some of these improved parent lines had male sterile (ms) variants produced using the cytoplasmic Ogura male sterility system. A number of selected F_1 hybrids (test hybrids) created using these ms lines were also included. Not all genotypes were used in each of the three experiments; the start was with 11 genotypes which were selected randomly from the Elsoms gene bank and used in the 1st Experiment (Chapter 3) – part one (Table 2.1). In part two of the 1st Experiment, 6 genotypes were eliminated where no seed stocks were available and the swede breeders were no longer including them in their breeding programme. Then following on from the results obtained in the 1st Experiment, 12 genotypes (Table 2.2) were used in the 2nd Experiment (Chapter 4) and according to the results obtained from this experiment, only 2 genotypes (Ag31 and Gr13) were chosen for the 3rd Experiment (Chapter 5). Prior to the 3rd Experiment, a pilot experiment was carried out using only one genotype (Gr13). For commercially sensitive reasons any variety or parent lines names have been suppressed and code names substituted for all genotypes.

Table 2.1: The codes of the 11 genotypes of swede used for frost screening (1st Experiment) in part one (seedling hardiness) and part two (REC %).

No.	Genotypes used in part one	Genotypes used in part two
1	Li713	Gr19
2	Gr19	Or13
3	Or13	Ly01
4	E20y	Me77c
5	We19	E20y
6	Di06	___
7	Ly01	___
8	Me77c	___
9	Ro107	___
10	Yn139	___
11	Sh107	___

Table 2.2: The codes of the 12 genotype of swedes used in the field trial (2nd Experiment) (ms = male sterile).

Genotypes		
Parent lines	**ms Parent lines**	**F_1 Hybrids**
Gr19	ms. Gr19	ms. Gr19 X Ly01
		ms. Gr19 X Or13
Ag31	ms. Ag31	ms. Ag31 X Gr19
		ms. Ag31 X Or13
Or13	__	ms. Or13 X Me77c
Ly01	__	__
Me77c	__	__

2.2 Growing conditions:

Genotypes were sown and grown in different growth conditions depending on the aim of each experiment. The 1st and 3rd experiments were carried out in semi-controlled conditions in a glasshouse with an air temperature of 20 ˚C max and 10 ˚C min using plastic seedling trays. In the 1st Experiment, John Innes No.1 seed compost was used whilst for the pilot experiment and the 3rd Experiment standard grade horticultural perlite (William Sinclair Horticulture Ltd) + Hoaglands hydroponic solution was used. The 2nd Experiment was conducted in the field in a silty-clay loam of the Denbigh series with a pH of 6.7 surrounded by a commercial crop.

2.3 Hydroponic solution:

A standard Hoaglands solution was used to irrigate plants in the 3rd Experiment and the pilot experiment (see appendix 6 for formulation) and Stock solutions with three levels of boron. 0 µM B L^{-1}, 30 µM B L^{-1} and 60 µM B L^{-1} (Appendix 6) were prepared. Plants received their respective treatment from sowing to sampling stage, so that plants irrigated with 0 µM B L^{-1} Hoaglands received no boron for the whole period of growth. For the pilot experiment, standard Hoagland was used first before starting with the boron treatments which were only two, 0 µM B L^{-1}, 30 µM B L^{-1}. Hoaglands solutions were stored in the dark at room temperature.

2.4. Frost tolerance evaluation:

2.4.1 Cold acclimation before frost test.

Plants at the seedling stage or at an advanced growth stage were acclimated in a cold room at 4 ˚C (Appendix 1) with a light intensity 7.6-µmol m^{-2} sec^{-1} and an 8-hour photoperiod for 14 days before being exposed to freezing temperatures. Plants were irrigated as required during the acclimation period. Non-acclimated plants were maintained in the glasshouse. Samples were taken for the physiological test for frost hardiness at the end of cold acclimation period (14 days). For molecular analysis samples were taken at the beginning (0 hour) and the end of acclimation (14 days).

2.4.2 Frost hardiness at seedling stage.

Swede genotypes were screened for their ability to tolerate frost by exposing acclimated seedlings to freezing temperatures in a controlled environment frost chamber (SANYO M533) at 0, -2, -4, -6, -8 and -10 ˚C with a 2 hours hold at each temperature to ensure the equilibration of freezing (Appendix 2). Samples were taken by removing plants from the frost chamber at the end of each hold period and placing them immediately at 4˚C where they were left overnight to defrost before being returned to the glasshouse to recover for two weeks before measuring the recovery percent. The numbers of surviving plants were counted and the percentage plant survival calculated. The temperature at which 50% of plants were killed (LT_{50}) was assessed using a logistic differential equation for curve fitting by SPSS statistical software V.18:

$$S\% = 100/ (1+e^{a-rt}).$$

Where (S) is the percentage of survival, (a) and (r) are constants, (t) the temperature (Robert, 1974).

2.4.3 Relative Electrical Conductivity (REC %).

Where plants were too big to fit into the frost chamber or where the plants were required for other purposes the relative electrical conductivity (REC %) was calculated as an indicator of cell damage due to frost. Leaf disc samples (5 disc of about 1 cm diameter) for each treatment were collected and placed into 75 mL boiling tubes. All samples were placed in the frost chamber together and chilled to 0˚C for 2 hours and the first sample taken and placed immediately at 4˚C. The frost chamber was then programmed to reduce the temperature by 2˚C every 2 hours, small pieces of ice added to each tube to facilitate ice nucleation and the freezing programme started. After 2 hours at -2˚C the second sample (-2) was removed and placed immediately at 4˚C to thaw. This procedure was continued so as to produce 6 samples (0, -2, -4, -6, -8 and -10 ˚C). All samples were kept overnight at 4˚C to facilitate slow thawing.

The REC % was calculated as follows:

1. After 24 hours in the cold room at 4˚C; 30 mL of distilled water was added to all tubes and a lid was placed on each tube to prevent evaporation and incubated at 20 ˚C for a further 24 hours.
2. The electrical conductivity of the bathing solution was then measured for each tube (EC1).
3. Samples were then autoclaved for 15 minutes at 121 ˚C.
4. After the autoclave all tubes were again left 24 hours at 2˚C to equilibrate.
5. The electrical conductivity was measured again (EC2).
6. REC% was calculated as:

$$REC\% = EC_1/EC_2 \times 100 \text{ (Aronsson and Eliasson, 1970)}$$

The freezing temperature that damaged 50% of the plant cells (EL_{50}) was estimated by the logistic differential equation for curve fitting using SPSS statistical software v.18:

$$D\% = a\,(e^{-bt}).$$

Where (D) is the percentage of damaged tissues, (a) and (b) are constants, (t) the temperature (Robert, 1974).

2.5 Boron concentration determination of plant tissue:

Plant roots were sampled from the field, cleaned and chopped into small pieces (3 - 5 cm) and stored at -20 ˚C prior to freeze drying them for 3 – 5 days to constant weight using an Edwards Super Modulyo freeze drier (Plate 2.1). Dry weight samples of 0.25 g were then hot digested in 5 mL of concentrated (70%) nitric acid (16 Molar) for two hours on a hot plate (80 ˚C) using special Teflon bombs for acid digestion made of a material that is safe for boron concentration determination (not borosilicate glass) (Plate 2.2). Digested samples were cooled down then diluted to 25 mL using 2% nitric acid. After each digestion the Teflon bombs were cleaned using concentrated nitric acid (5 mL) for 2 hours on the hot plate at 80˚C. The Teflon bombs then washed with 2% nitric acid and dried. This was in order to avoid any boron contamination that may remain from previous samples that might affect the results of the next digestion. Boron

content was determined using a Plasma Spectrometer (X-Series ICP-MS) (Plate 2.3).

Plate 2.1 Edwards Super Modulyo freeze drier

Plate 2.2: Teflon bombs for acid digestion

Plate 2.3: plasma spectrometer (X-Series ICP-MS)

2.6 Molecular analysis:

2.6.1 mRNA extraction:

The mRNA was isolated from plant tissues using Plant Total RNA Kit (STRNA50) (Sigma: Plant Biotechnology) according to the method of Ausubel (2001) and Farrell (1998) and the protocol was as follow:

Preparation instructions

1. **Wash Solution 2 Preparation**

The plant total RNA kit (STRNA50) supplies wash solution as a concentrate, therefore prior to first time use, 60 mL of absolute alcohol (100% ethanol) was added to the supplied concentrated wash solution 2, briefly mixed and tightly capped to prevent evaporation. The diluted solution was stored at room temperature and used accordingly with the extraction procedure.

2. **Plant tissue preparation:**

2.1 Grind plant tissue.

Plant tissues were harvested and submerged in liquid nitrogen as soon as possible to prevent RNA degradation. Sampled plant tissues were then kept at - 80˚C. Before the start of grinding of the tissues; RNase ZAP (Sigma kit, Cat# R2020) spray was used and sprayed on to all surfaces and equipment used in the extraction process in order to avoid any trace of RNA contamination. Using a mortar and pestle, plant tissue was ground in liquid nitrogen to a fine powder. RNA yield is often dependent upon how fine the plant tissue has been ground. The mortar was kept cold (placed in dry ice) and the plant tissues were kept frozen at all times.

2.2 Weigh tissue sample:

When liquid nitrogen had evaporated from the frozen tissue powder samples of approximately 100 mg (90–110 mg and should not be exceeded 110 mg per tube) tubes were quickly weighed and placed in a 2 mL microcentrifuge tube which had been pre-chilled on dry ice or in liquid nitrogen. The weighed samples were kept on dry ice or at -80°C before lysis solution was added.

3. Preparation of Lysis Solution/ 2-ME Mixture:

Lysis solution was mixed with 2-mercaptoethanol (2-ME) before use. A pre-determined amount (depends upon sample number) of lysis solution was transferred to a clean conical tube. 10 µL of 2-ME was added for every 1 mL of Lysis solution and mixed briefly. Each RNA preparation delivered 500 µL of the mixture. For pipetting allowance some extra solution was made when working with multiple samples. The preparation of lysis solution/2-ME mixture was made as close to the time of use as possible because old prepared mixture (more than one day) results in RNA yield reduction.

4. Assemble Column and Collection Tube:

A Filtration column (blue retainer ring) was inserted into a 2 mL collection tube. The collection tube was closed with the lid to be used in step two under the procedure. Likewise, the red retainer ring binding columns were inserted into 2 mL collection tubes and lids were closed. Binding columns were left for use in step three of the procedure.

Procedure

All centrifugation steps were performed at room temperature at maximum speed (14,000 – 16,000 x *g*) in a standard microcentrifuge.

1- Lyse tissue sample

In a 2 mL collection tube, 500 µL of the mixture (Lysis solution / 2 ME) was pipetted to a weight of 100 mg of frozen plant tissue powder. This was vortexed immediately and vigorously for at least 30 seconds. Samples were then incubated at 56˚C for 3 – 5 minutes. Vortexing or shaking of the samples during or after the heat incubation was strongly avoided.

2- Pellet Cellular Debris

The samples were centrifuged for three minutes at maximum speed in order to pellet cellular debris.

3- Filter Lysate

The supernatant lysate was pipette into a filtration column (blue retainer ring) seated in a 2 mL collection tube by positioning the pipette tip at the bottom of the tube but away from the pellet. Sometimes there was a layer of floating particulates and in such cases the pipette tip was positioned below the floating layer and away from the pellet before pipetting the supernatant. It was of no consequence if some of the floating particulates were carried over to the filtration column as long as pellets can be avoided. Caps were then closed and centrifuged at maximum speed for 1 minute in order to remove residual debris. The flow-through lysate was collected and saved.

4- Bind RNA to Column

- Add Binding solution

500 µL of binding solution was pipetted into the clarified lysate and mixed immediately and thoroughly by pipetting for at least 5 times and sometimes the vortex was briefly used.

- Bind RNA

A volume of 700 µL of the binding solution and lysate mixture was pipetted into a binding column seated in a 2 mL collection tube. The Lid was closed and centrifuged for 1 minute at maximum speed to bind the RNA. The flow-through liquid was decanted and the collection tube was tapped (upside down) briefly on clean absorbent paper in order to drain the residual liquid. Then columns were returned to the collection tube, the remaining mixture was pipetted to the column, and the process was repeated.

5- First Column Wash

Each column was pipetted with 500 µL of wash solution 1. Columns caps were tightly closed and centrifuged for one minute at 13,000 rpm. The flow-through liquid was decanted and the collection tubes were tapped (upside down) briefly on a clean absorbent paper in order to drain the residual liquid. Then columns were returned to the collection tube.

6- Second Column Wash

Diluted wash solution 2 volume of 500 µL was pipetted into the column. Columns caps were tightly closed and centrifuged for one minute. The flow-through liquid was decanted and the collection tubes were tapped (upside down) briefly on a clean absorbent paper in order to drain the residual liquid. Then columns were returned to the collection tube.

7- Third Column wash: Step 6 was repeated.

8- Dry Column

Without any additions, the columns were centrifuged for 1 minute to dry. The column tube assembly was carefully removed from the centrifuge in order to avoid splashing the residue flow-through liquid on the dried columns.

9- First Elution

Dried columns from step 8 were transferred into new 2 mL collection tubes. A 50 µL of elution solution was pipetted directly onto the centre of the binding matrix inside the column and tubes closed tightly. After one minute columns-tubes were centrifuged for one minute only. The purified RNA was now in the flow-through eluate and ready for immediate use or storage at –20°C (short term) or –80°C (long term).

10- Second Elution:

The Second Elution was optional. Sometimes the expected RNA yield was < 20 mg and an additional 10–30% of RNA yield could be recovered from the column with another elution. In such situations, columns were transferred into new, clean 2 mL collection tubes and 30-50 µL of elution solution was pipetted directly onto the centre of the binding matrix inside the column. They were then centrifuged at maximum speed for 1 minute to elute. The purified RNA was in the flow-through eluate and ready for immediate use or storage at -20 °C (short term) or -80 °C (long term). Extracted RNA was quantified with a Nanodrop (NanoVuePluce) to estimate its concentration. In addition, the purity of RNA was measured as the absorbance using A260/A280 ratio procedure (Warburg and Christian, 1942). Nucleic acids have a higher absorbance at 260nm than at 280 nm and therefore, the A260/A280 ratio was expected to be ≥ 2 for pure samples.

2.6.2 cDNA preparation:

Before starting *cDNA* preparation, extracted RNA for all samples was purified of any trace contamination of DNA using DNase I (Sigma: AMP-D I). DNase and its buffer 1µL each were added to 10 µL volume of each sample and mixed gently. Samples were then incubated at room temperature for 15 minute then 1 µL stop solution (50 mM EDTA) was added to stop the reaction of DNA digestion and to bind the calcium and magnesium ions and to inactive the DNase I which was then denatured by incubating samples for 10 minutes at 70 ˚C. Up to 2 µg of total RNA from each sample was reverse transcribed to cDNA using High capacity RNA-to-cDNA kit (Applied Biosystems, PN 4352604):

Procedure

The following reagents were used:

1. High capacity RNA-to-cDNA kit (PN 4352604).
    - RT buffer Mix 2X.
    - RT Enzyme Mix 20X.
2. Molecular grade water (product code W 1754).

To synthesize single-strand cDNA from total RNA using the RNA-to-cDNA kit; up to 2 µg total RNA was mixed with Nuclease-free H_2O to 9 µL final volume. A mixture of 1 µL Enzyme mix and 10 µL RT buffer was added to the sample (9 µL) to make total reaction volume of 20 µL. The reaction was then incubated in a Thermocycler for 37 ˚C for 60 minutes then stopped by heating to 95 ˚C for 5 minutes and then held at 4 ˚C. The product (cDNA) was stored at -20 ˚C for later use in real-time quantitative PCR or ordinary PCR.

Ordinary PCR technique was used to investigate the expression of genes of interest in sampled leaves and roots stored at -80 ˚C. The expression pattern of the gene of interest, *BN115,* was normalised with the constitutively expressed housekeeping gene *Actin 1*. Primers for genes were designed with gene sequences obtained from Blast software and gave the following templates.

Actin 1: Forward 5´- CCCAAAGGCCAACAGAGAGAAG-3´ and Reverse 5´- CACCAGAGTCCAGCACAATACC-3´,

BN115: Forward 5´- AAAACTGAGCTCGTCGTCGT-3´ and Reverse 5´- CGCGTAATCCGAAGCTCTCT-3´ and

Primers were all ordered from Eurofins MWG.

2.6.3 Polymerase Chain Reaction (PCR)

- **Ordinary PCR**

The following master mix was prepared:

1. 1µL Red-Taq (sigma: product code D 4309).
2. 2.5 µL Red-Taq buffer (product code B5926).
3. 0.5 µL forward and reverses primers.
4. 0.5 µL dNTPs.
5. 18 µL Molecular water.

A total volume of 23 µL of the above mixture was combined with 2 µL cDNA to give a final volume 25 µL of reaction mixture which was run under the following cycle in a Thermocycler: initial denaturation at 94˚C for 2 minutes once followed by 40 cycles of (denaturation at 94˚C for 30 s, annealing at 60°C for 30 s, extension at 72˚C for 30 s) and a final extension at 72˚C for 5 minutes and then held at 4°C. An agarose gel (2 %) was prepared by dissolving 1.4 g of agarose (DNase and RNase free, Invitrogen cat# BPE 1356.100) in 70 mL of 10X TAE buffer (Tris-acetate + EDTA, Invitrogen cat# 15558-026) using a microwave for 2 minutes to help complete the dissolving of the agarose. The agarose solution was cooled down to around 50 ˚C before adding 7 µL of SYBR safe dye (Fisher #VX33102) the solution which then mixed well and immediately poured into the gel tray to solidify for a minimum of 20 min at room temperature. 10X TAE buffer was then added to completely submerge the gel. The 25 µL samples were loaded in the wells with reference ladder volume of 10 µL of the molecular weight markers of 1kb (Fisher #BPE2581-200). The gel was run at 90 V until the bromophenol blue was about 3/4 through the gel (less than 1 h). The tray with the agarose gel was carefully removed and taken to the UV trans illuminator and the gel was examined under UV light in a Bio Rad universal Hood II (Gel-Doc XR: 170- 8170).

- **Real-Time quantitative PCR (qPCR)**

The relative transcript abundance of the gene of interest was tested using the Real-Time quantitative PCR (qPCR) from Applied Bio system (StepOne Plus). The protocol used was according to the manufacturer's instructions (SYBR® Green Jump Start™ Taq Ready Mix™, sigma:cat# S4438). Sample cDNA volume of 2 µL was mixed with a mixture (master mix) consisting of (for each sample):

- 7.2 µL Syber green master mix.
- 0.3 µL reference dye.
- 0.15 µL Forward primer.
- 0.15 µL Revers primer.
- 5.2 µL H_2O (Molecular grade water, Sigma cat, w1754).

The mixture (master mix + cDNA) of three replicates was prepared in a 1.5 mL PCR tube, then a volume of 15 µL for each replicate was transferred to a 96 well plate (Applied Bio system: MicrAmp cat #: 4360954). The procedure applied for the gene of interest (*BN115*) was also applied for the endogenous (the house keeping gene *Actin 1*). The plate was sealed with optical adhesive cover (Applied Bio system) and put in the qPCR machine and subjected to the following thermal cycle:

1. Holding stage at 94˚C for 2 minutes.
2. Cycling stage (40 cycles) of 94˚C for 30 sec followed by 60˚C for 1 minute and then 72˚C for 1 minute also.
3. Melt curve stage at 95˚C for 15 sec followed by 60˚C for 1 minute and then 95˚C for 15 sec.

Finally, results were analysed to find out the relative quantitation of the expression of the gene of interest relative to the endogenous gene.

Chapter 3

The interrelationship between frost tolerance and brown heart disorder in swede

3.1 Introduction:

Swede is considered as a “cool season” crop, however, recently some swede germplasm grown in the UK has demonstrated symptoms of frost susceptibility and there is anecdotal evidence that this is associated with the germplasm demonstrating susceptibility to BH (Elsoms Seeds Ltd, pers. Comm.). This led to the investigation reported here which was to investigate a possible linkage between these two traits (Frost susceptibility and BH) with a view to determining the possibility of using frost tolerance screening as a surrogate method to breeding resistance to BH in swedes.

In order to investigate this possible linkage, a frost tolerance screen was carried out in two parts, first with seedlings and the second with more mature plants. Secondly, Elsoms Seeds made available data from its field trials on BH appearance over the past ten years (2000 – 2010) and this data was interrogated for trends and linkages.

3.2 Frost tolerance screening

Part one - Investigation of frost hardiness of swede seedlings.

Aim: To evaluate swede genotypes for their ability to tolerate frost and to investigate varietal variations.

3.2.1 Materials and Methods

3.2.1.1 Plant materials

Eleven swede genotypes (Table 3.1) were obtained from the Elsoms Seeds Ltd., (Spalding, Lincolnshire, UK) gene bank. Genotypes were picked by Elsoms as the main breeding lines in their swede breeding programme produced by single seed decent for at least 6 generations.

Table 3.1: The eleven genotypes used for seedling frost screening.

No.	Genotype
1	Li713
2	Gr19
3	Or13
4	E20y
5	We19
6	Di06
7	Ly01
8	Me77c
9	Ro107
10	Yn139
11	Sh107

The experiment was carried out at Plymouth University (PL4 8AA, UK) during May 2010 in a complete randomized design with four replicates.

3.2.1.2 Seedling Frost Test

The selected genotypes were sown in plastic seedling trays (37 × 23 × 5 cm) in the glasshouse. Each tray contained four rows of different genotypes (10 plants per row) allocated randomly to each tray, this means 4 genotypes per tray and 10 plants per genotype in each tray. Plants were watered at two-day intervals according to need. Six weeks after planting plants had reached the 3-4 leaf stage. Plants were then moved to the controlled environment cold room at 4˚C for acclimation for 14 day. Then genotypes were subjected to frost treatments (0, -2, -4, -6, -8 or -10˚C) after which plants were immediately moved to a cold room at 4˚C overnight then to the glasshouse for two weeks to recover. The plant survival percentage was estimated by counting surviving plants. LT_{50} was assessed using a logistic regression differential equation for curve fitting by SPSS statistical software V.18:

$$S\% = 100/ (1+e^{a-rt}).$$

Where (S) is the percentage of survival, (a) and (r) are constants, (t) the temperature (Robert, 1974).

The data were analysed by using Minitab software v.15.

3.2.2 Results

Analysis of variance indicated clearly that low temperature, plant genotype and their interaction had highly significant impacts on seedling ability to recover after freezing (Table 3.2). Recovery decreased as the temperature declined to -10 ˚C. At 0˚C and -2˚C, all genotypes responded equally and all completely recovered whereas freezing temperatures of -4 ˚C, -6 ˚C and -8 ˚C caused variable levels of damage whilst at -10˚C all seedlings of all genotypes were all killed (Figure 3.1, Plate 3.1).

Table3.2. Analysis of variance results for seedling recovery % (* = highly significant)**

Source of variance	$L.S.D_{0.05}$	*P* value
Genotypes	**3.68**	**< 0.001*** **
Temperature	**3.01**	**< 0.001*** **
Genotypes × Temperature	**9.02**	**< 0.001*** **

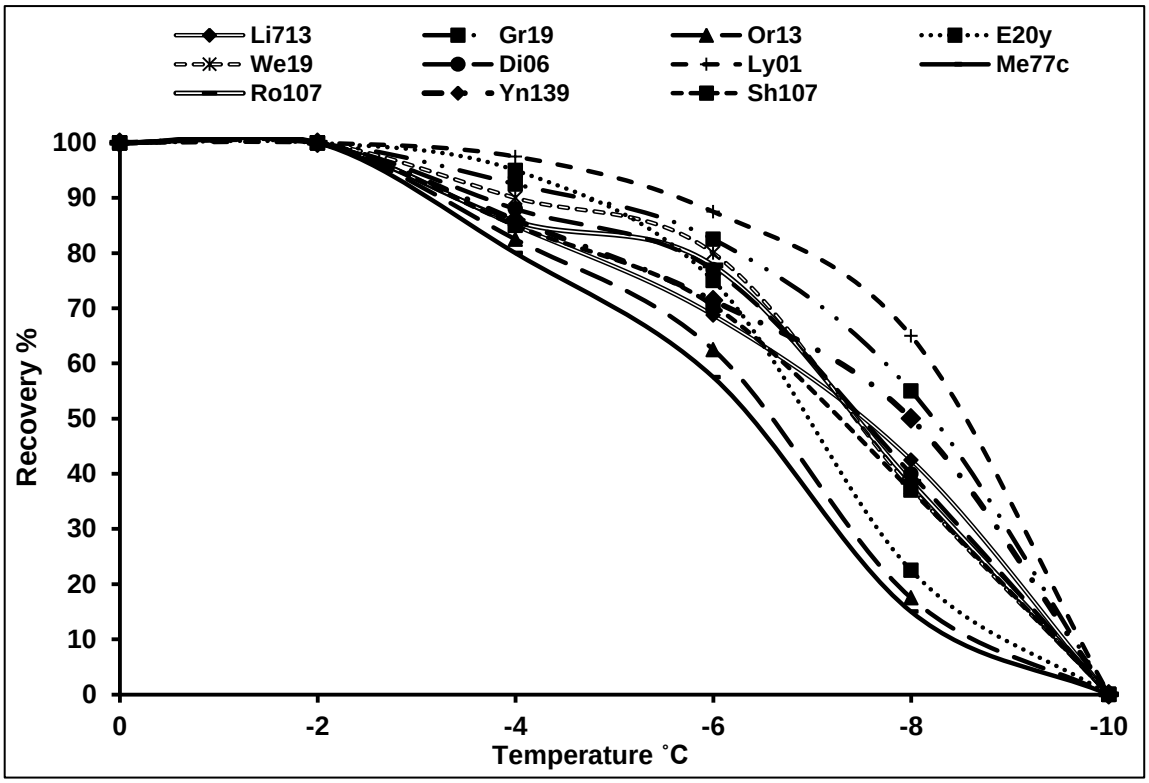
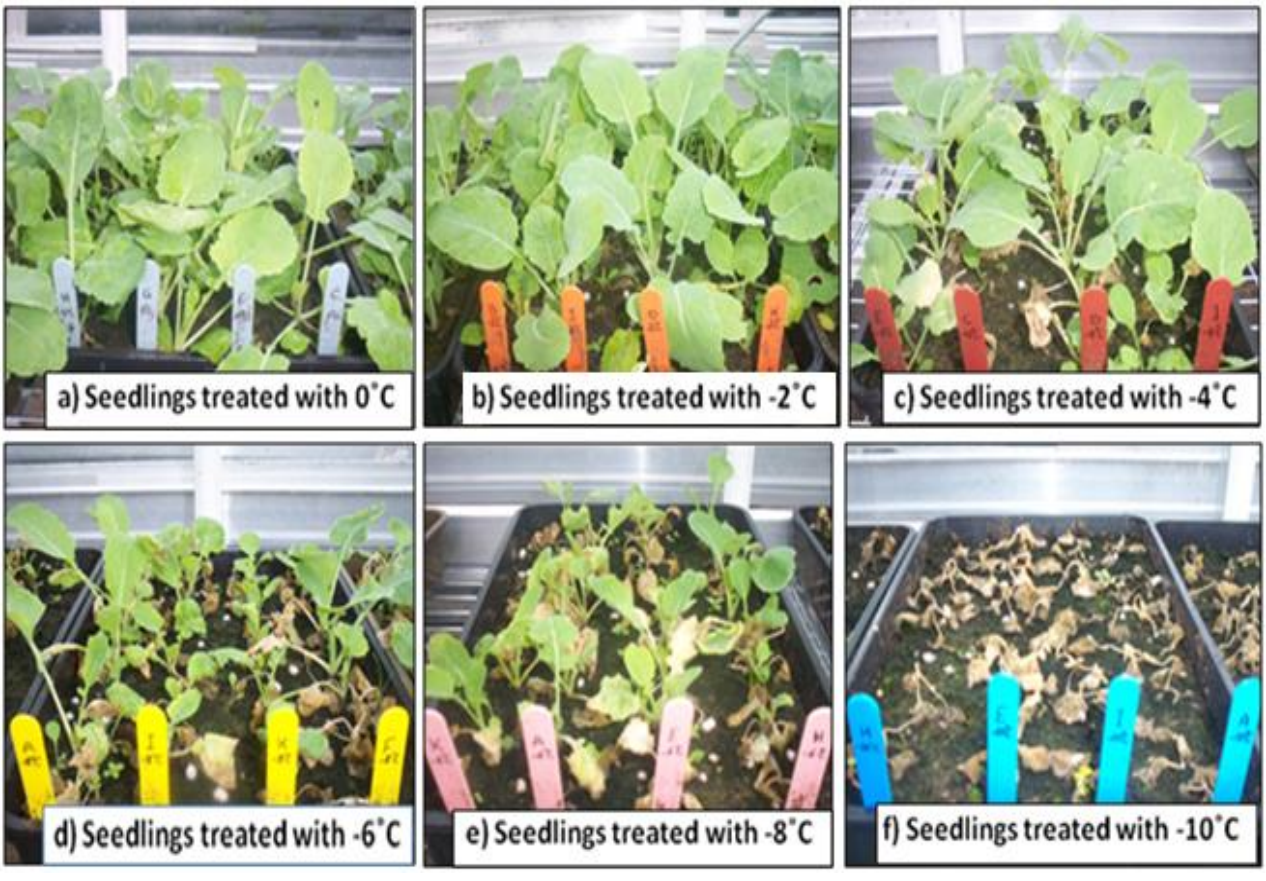


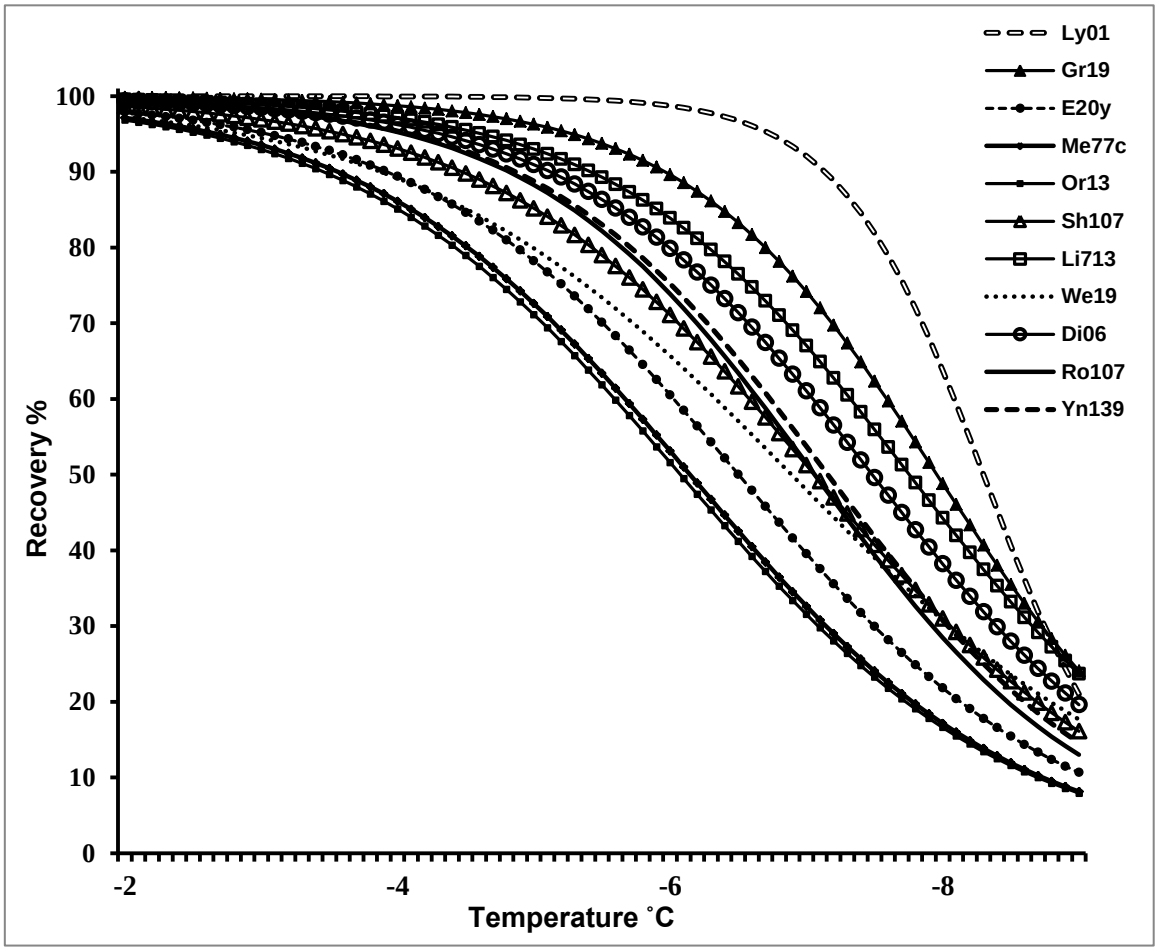
Figure 3.1: Plants survival of swede genotypes following frost treatment.

Plate 3.1: Swede seedlings following 14d recovery after frost treatment.



Logistic curve fitting to the recovery data (Figure 3.2) showed significant (*P*<0.000) variations between genotypes in seedling ability to recover after frosts treatment of -4 ˚C, -6 ˚C, and -8 ˚C and enabled the calculation of LT_{50} temperatures (Table 3.3).

Figure 3.2: Logistic curves fitted to survival data of swede genotypes after frost treatments.



Genotypes significantly (*P* < 0.000) varied in their LT_{50} values. Me77c and Or13 had the highest LT_{50}, -6.05 ˚C and -6.15 ˚C respectively. Ly01 had the lowest LT_{50} at - 8.30 ˚C.

Table 3.3: LT50 Values for seedlings of swede genotypes (means with different letters are significantly different).

Genotypes	LT_{50}	SE
Ly01	-8.3 [a]	0.16
Gr19	-7.7 [b]	0.17
Li713	-7.6 [b]	0.03
Di06	-7.4 [c]	0.02
Yn139	-7.2 [c]	0.28
We19	-6.9 [d]	0.19
Sh107	-6.9 [d]	0.24
Ro107	-6.9 [d]	0.05
E20y	-6.5 [e]	0.19
Me77c	-6.2 [f]	0.19
Or13	-6.0 [g]	0.27
Mean	-6.9	

In addition to LT_{50}, a comparison was made between the genotypes in respect of their ability to survive the - 8 ˚C frost treatment (Figure 3.3) since this was the lowest temperature at which all genotypes' seedlings showed some ability to recover. Generally, Or13 and Me77c showed the lowest level of plant survival at -8 ˚C (15% and 17.5% respectively) with no significant differences between them. This makes them the most frost sensitive genotypes. E20y had 22.5 % and differed significantly from Or13 and Me77c but still poor in terms of tolerance. A aroup of genotypes were in the middle (Li713, Di06, Ro107, We19, and Sh107) with no significant differences among them and showed recovery percent ranged from 37% to 42.5%. Genotypes Ly01, Gr19 and Yn139 showed the best ability to survive -8 ˚C temperature with recovery percent in excess of 50% (65%, 55% and 50%) respectively. This makes them the most frost tolerant genotypes. This data significantly ($P < 0.000$) correlated ($r = - 0.95$) with the LT_{50} values.

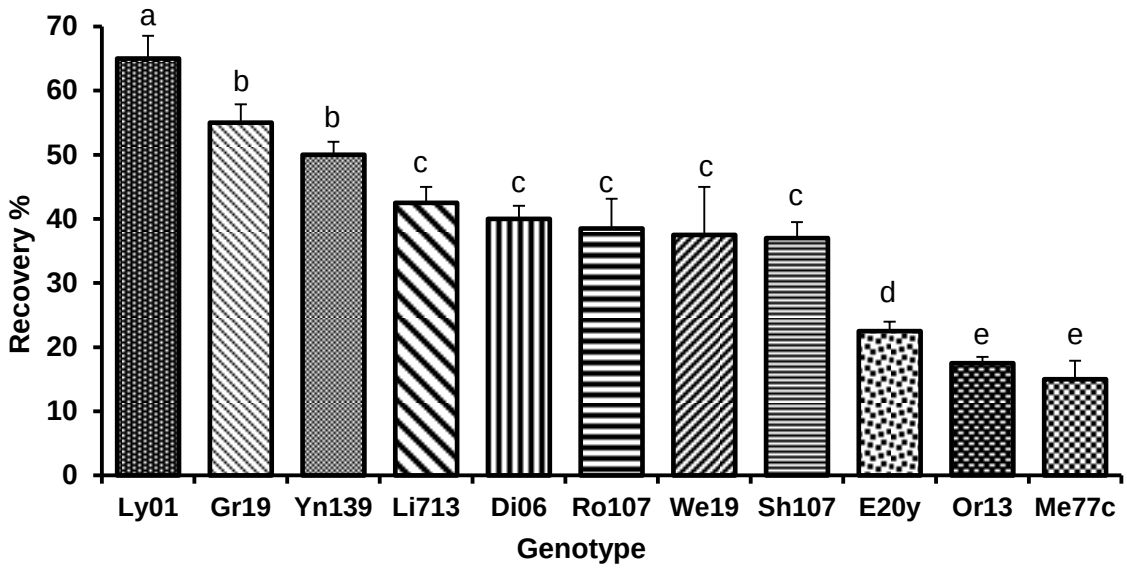


Figure 3.3: Genotype seedling recovery at -8 ˚C. Vertical bars represent ± S.E.M. Different letters above the standard error bars indicate significant differences between genotypes (*P* < 0.001).

The results placed the 11 genotypes into 3 groups (high, mid and low) according to their ability to tolerate frost (Table 3.4).

Table 3.4: Swede genotypes ability to tolerate frost

Recovery %	Group
More than 50%	High Frost Tolerance
30% - 50%	Mid Frost Tolerance
Less than 30%	Low Frost Tolerance

Part two - The Relative Electrical Conductivity (REC %) assessment of tolerance of swede to frost

Aim: To determine the frost hardiness of mature swede plants tissues and whether or not genotypes differences were the same as those found with seedling plants.

3.3.1 Materials and Methods

Plants of Gr19, Or13, E20y, Ly01, and Me77c were grown outside in 12 cm pots to the late vegetative growth stage. Three replicate pots of each genotype were grown, and arranged in completely randomized design late on July 2010 and grown on to the 10 to 12 leaf stage. On 13 November 2010 the plants were randomly divided into 3 groups and subjected to different treatments for the 14 days prior to testing for frost damage:

1. Left in the open-air area, where they were exposed to the natural field environment (FE).
2. Transferred to the glasshouse at a mean temperature of 20 ˚C (non-hardening environment) (GH).
3. Transferred to the Cold Room at a temperature of 4˚C (cold acclimation environment) (CA).

Leaf disc samples (1 cm diameter) were then collected (3 replications of 5 discs per plant per temperature) and placed into 75 mL boiling tubes and exposed to freezing at 0, -2, -4 ,-6, -8 or -10˚C with a 2 hour at each temperature to equilibrate freezing. Ice was added to all tubes at 0 ˚C to facilitate ice nucleation in the plant material being tested and to prevent complicating effects of supercooling. Samples were taken by removing tubes from the frost chamber and placing them immediately at 4 ˚C overnight to defrost.

3.3.2 Results

It was observed that pre-treatment conditions, genotypes and their interaction had a highly significant effect on REC% results (Table 3.5). The 14 days growing conditions prior to frost treatment clearly affected swede response to frost (Figure 3.4). Plants grown in CA conditions showed the lowest REC% (range 12% at 0˚C to 70% at -10˚C) indicating less cell damage due to frost. GH conditions reduced swede hardiness and showed the highest REC % values (range between 14% at 0˚C to 87% at -10˚C). The effect of the field environment (FE) was intermediate between the GH and the CA.

Table 3.5: Analysis of variance results for REC%, (* = highly significant).**

Source of variance	*P* - value
Genotypes	< 0.000***
Pre-treatment conditions	< 0.000***
Genotypes x Pre-treatment conditions	< 0.005**

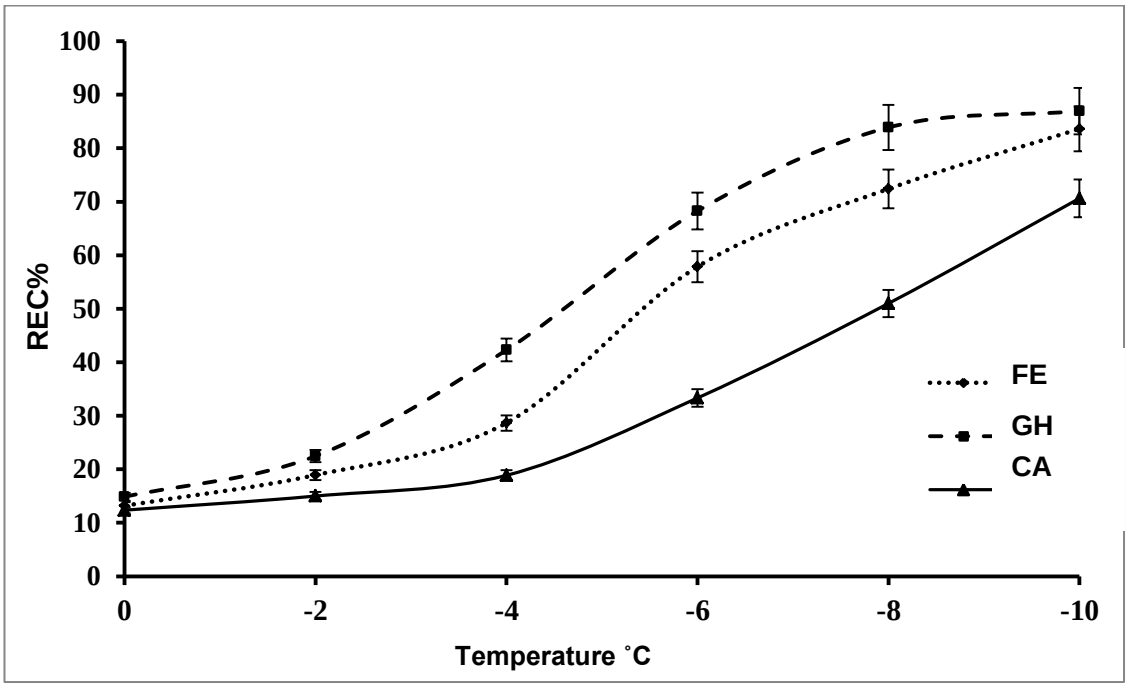


Figure 3.4: Relative Electrical Conductivity (REC %) of swede plants pre-treated in different growing conditions prior to freezing (means of genotypes). Vertical bars represent ± S.E.M.

Genotypes varied significantly ($P < 0.000$) in their response to frost after the different growing conditions (Figure 3.5). Genotypes responses to frost affected by preconditioning treatments are presented clearly in logistic curves (Figure 3.6). Or13 showed the highest REC% at all conditions and was considered the most susceptible to frost and Me77c was next. Or13 had 60%, 54% and 43% at GH, FE, and CA respectively, while Me77c showed values of 57%, 50%, and 42% at GH, FE, and CA respectively. Ly01 and Gr19 had the lowest REC% and were the most tolerant to frost.

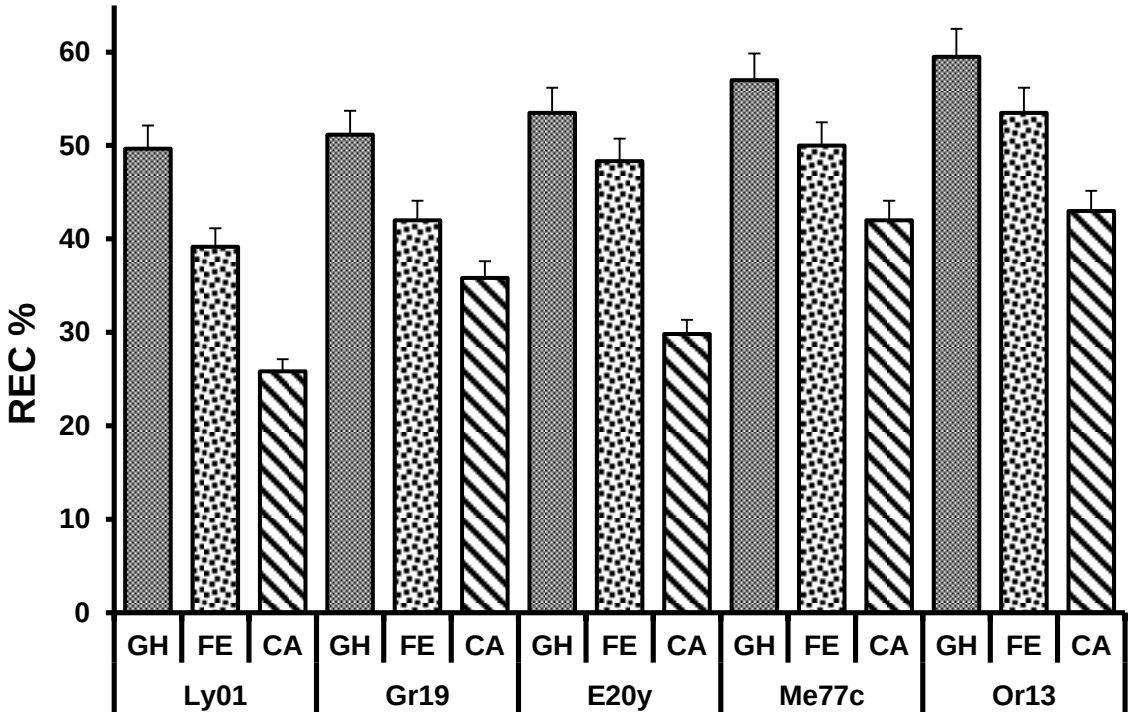


Figure 3.5: The Relative Electric Conductivity (REC %) of swede genotypes grown in different environmental conditions (GH = Glasshouse, FE = Field Environment, and CA = Cold Acclimated) prior to exposure to frost. Vertical bars represent ± S.E.M.

Calculated EL_{50} temperatures confirmed the results of REC% with EL_{50}'s lowest after CA preconditioning and highest under GH (Table 3.6). Using multiple comparisons, one-way ANOVA showed no significant differences ($P < 0.243$) for the EL_{50} between genotypes pre-treated under GH conditions while the difference was significant ($P < 0.007$) after CA preconditioning. After FE conditions the difference was still significant but at a lower level ($P < 0.024$). Overall, Ly01 was rated the most hardy and Me77c the least. The range of EL_{50}'s differed for each growing condition and widened after exposure for 14 day at 4˚C in the cold room giving more varietal discrimination after acclimation in comparison with plants grown 14 day in GH (Figure 3.6).

Table3.6: EL_{50} of studied swede genotypes treated differently prior to frost treatment (means with different letters are significantly different and means without are not significantly different).

50% Electrolyte Leakage (EL_{50})				Mean
Genotypes	**FE**	**GH**	**CA**	
Ly01	-7.5 **a**	-6.1	-10.7 **a**	-8.1
Gr19	-6.7 **b**	-5.4	-7.8 **b**	-6.6
E20y	-6.4 **b**	-5.7	-9.4 **a**	-7.2
Me77c	-5.8 **c**	-5.7	-7.3 **c**	-6.3
Or13	-6.1 **c**	-5.7	-7.2 **c**	-6.3
Mean	**-6.5**	**-5.7**	**-8.4**	

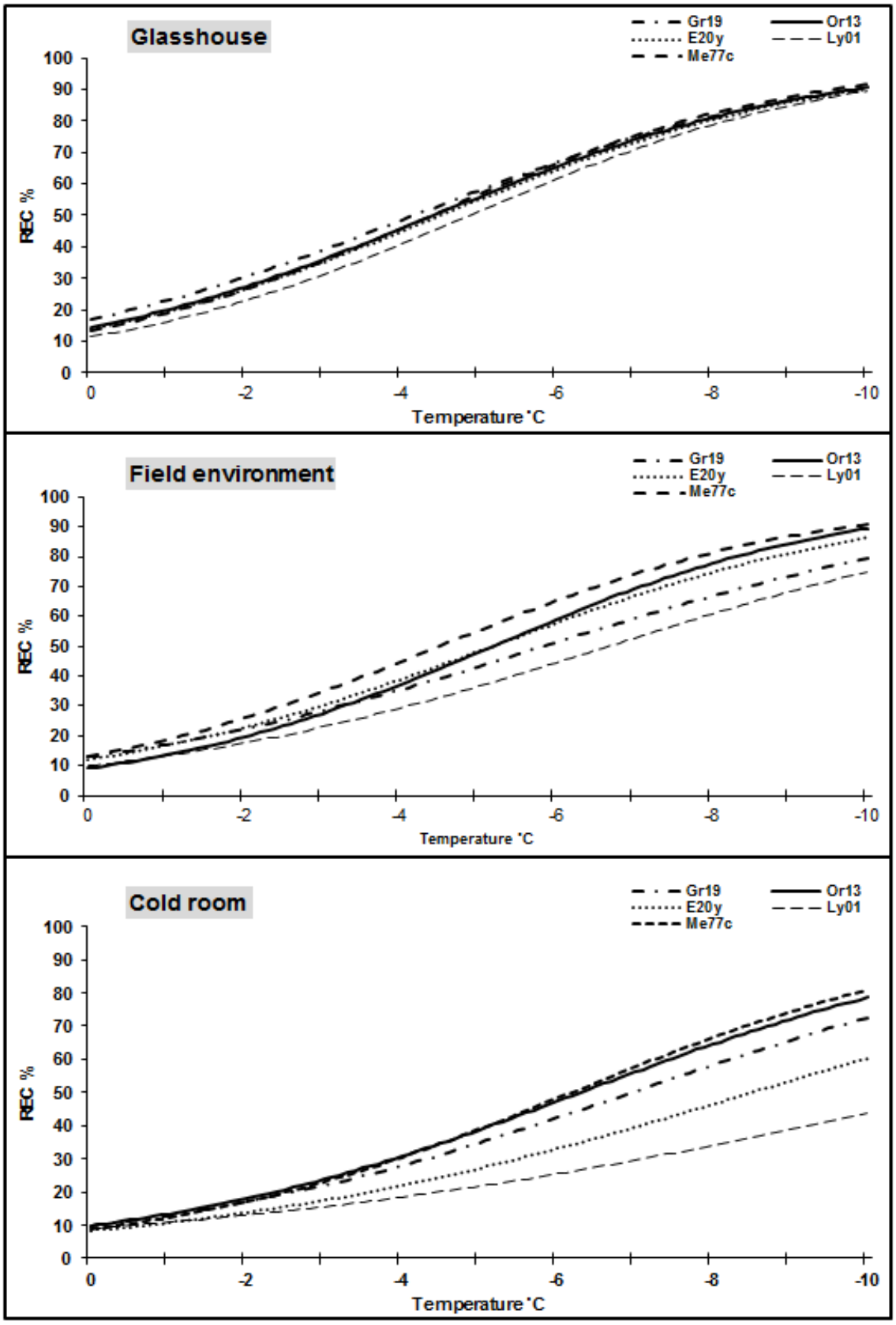


Figure 3.6: Logistic curves of REC% for swede genotypes exposed to different pre-freezing growing conditions; glasshouse (GH), natural field environment (FE) and cold acclimation (CA).

3.4 Seedling frost hardiness and relative electrical conductivity: correlation between LT_{50} and EL_{50}.

The correlation between measured LT_{50} values to swede genotypes in seedling stage and EL_{50} to plant tissues in advanced growth stage was calculated using Minitab 15. The correlation was positive between LT_{50} and EL_{50}s in all growing environments but was only statistically significant with EL_{50} at FE (Table 3.7).

Table 3.7: Correlations between LT_{50} and EL_{50} (= significant correlation, N.S = non-significant correlation).**

Pre-treatment conditions	r	*P* – value	Sig.
FE	0.623	0.013	**
GH	0.092	0.745	N.S
CA	0.379	0.164	N.S

It was clear (Table 3.8) that the EL_{50} discriminated differently between the genotypes within each pre-treatment condition. The EL_{50} of genotypes clearly varied between the three growing conditions and the most similar to the LT_{50} results was EL_{50} at FE. Means comparison using t test (Table 3.9) showed no significant differences ($P < 0.298$) between the TL_{50} and EL_{50} following FE conditions. LT_{50} correlation was not significant with EL_{50} after CA and t test showed no significant differences ($P < 0.129$) between the means whilst it was significant ($P < 0.018$) with EL_{50} after GH preconditioning.

Table 3.8: Correlation between LT50 and EL50 for swede genotypes at different pre-conditioning treatments, CA= cold room at 4˚C, GH= glasshouse and FE= field environment (means with different letters are significantly different and means without are not significantly different).

Genotypes	CA		GH		FE		LT_{50}	Rank
	EL_{50}	Rank	EL_{50}	Rank	EL_{50}	Rank		
Ly01	-10.7 **a**	1	-6.1	1	-7.5 **a**	1	-8.3 **a**	1
Gr19	-7.8 **b**	3	-5.4	3	-6.7 **b**	2	-8.0 **a**	2
E20y	-9.4 **a**	2	-5.7	2	-6.4 **b**	3	-6.9 **b**	3
Me77c	-7.3 **c**	5	-5.7	2	-5.8 **c**	5	-6.2 **c**	4
Or13	-7.2 **c**	4	-5.7	2	-6.1 **c**	4	-6.1 **c**	5
Mean	-8.4		-5.7		-6.5		-7.1	
Range	3.5		0.7		1.7		2.2	

Table 3.9: Means comparison (t test) between LT_{50} and EL_{50} at different pre-treatment condition FE, GH and CA.

Comparison	t	P – value	Sig.
LT_{50} / $EL_{50,}$ FE	-1.114	0.297	N.S
LT_{50} / $EL_{50,}$ GH	-2.959	0.018	*
LT_{50} / $EL_{50,}$ CA	1.688	0.129	N.S

Rankings however were very similar between the two tests (LT_{50} and EL_{50}) except the rank at GH. The most parallel ranking to the LT_{50} was the rank of EL_{50} at FE which was the only treatment significantly correlated to LT_{50}. Gr19 which was ranked 2nd hardiest in LT_{50} and EL_{50} at FE, was the 3rd hardiest in EL_{50} at CA, and E20y which was 3rd hardiest in LT_{50} and EL_{50} at FE, was the 2nd hardiest in EL_{50} at CA. Nevertheless, the extremes were consistently upheld in both tests with Ly01 as the 1st hardiest genotype and Or13 and Me77c being the most susceptible, Or13 being 4th in EL_{50} and Me77c the 5th at CA and FE, while it was the other way around in LT_{50}.

3.5 Brown Heart (BH) symptom appearance in the field:

Aim: To screen field recorded data to investigate whether there is any relation of BH symptoms appearance with specific genotypes or locations.

3.5.1 Data availability

Breeder's data for the frequency of appearance of BH symptom in the field were supplied by swede breeders Elsoms Seeds Ltd. The data included recorded observations from 14 breeding field trials for 353 genotypes over a 10 year period (2000 – 2010) in two different locations in the UK (North Berwick; 55°56′ N, 02°44′ W, 100 m asl) and Spalding; 52°47′ N, 00°10′ W, 4 m asl) and one location in Germany (North Hamburg; 53°39′ N, 10°02′ E, 22 m asl). These data were analysed to investigate the frequency of BH appearance in relation to genotype and location.

3.5.2 Results:

There were 46-recorded instances of internal browning of BH in the field records of the breeder's trials. Whilst incidence of symptoms (Plate 3.2) was distributed between many genotypes, those showing some relationship to three particular genotypes in their pedigrees appeared more commonly than others (Figure 3.7). The data supplied were not always from replicated trials and the number of samples also varied over time so a statistical analysis of the entire data set was not possible. BH was recorded 17 times in genotypes that had Ag31 in their pedigree; genotypes including Or13 in their pedigree were recorded with BH 13 times, and genotypes including Me77c in their pedigree 9 times. There were a further seven appearance of BH across other different genotypes (Table 3.10).

Plate 3.2: Internal browning symptoms of brown heart inside swedes root (courtesy of Elsoms Seeds).

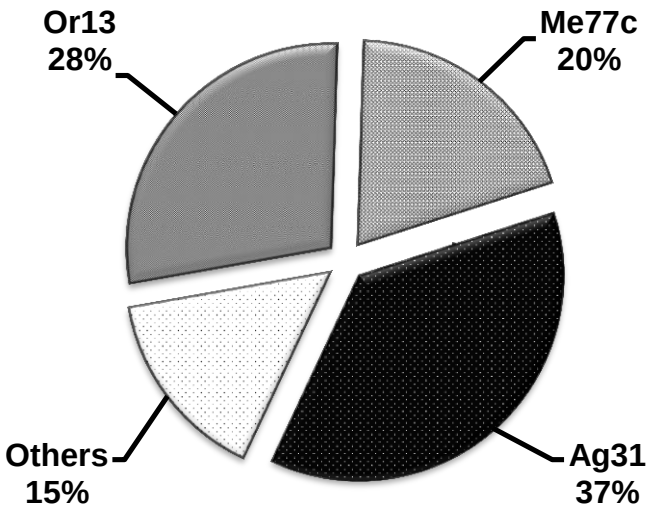


Figure 3.7: Frequency chart of genotypic background in plots with the appearance of brown heart in breeders field trials data (2000-2010) (n = 353).

Results showed that the frequency of BH appearance in the UK trials (total of 27) was more than that recorded in trials in Germany (total of 19).

Table 3.10: Incidence of the genotypic background of plants showing Brown Heart in breeders field trials data (2000-2010) (n = 353)

Location	Genotype				Total
	Ag31	Or13	Me77c	others	
UK	9	7	6	5	27
Germany	8	6	3	2	19
Total	17	13	9	7	46

3.6 Discussion

Generally, the scientific literature contains no information regarding the frost hardiness level of swedes and therefore, the results presented here are new information. Seedlings were used in the first part of this investigation since it is generally considered this is the most susceptible stage in plant growth to frost (Pragya et al., 2005). The second part used much bigger plants and for logistical reasons needed to use the relative electrical conductivity (REC%) method is to measure the amount of the leaked electrolytes from damaged tissues to distilled water, assuming that there is a correlation between the degree of injury and the leakage of electrolytes (Prášil and Zámečník, 1998). As the cytoplasmic membrane is the first cellular part that is damaged during freezing, it is generally accepted that it is possible to specify the level of injury through the electrolyte leakage of damaged tissues.

Seedling recovery results showed that all swede genotypes used in this experiment were equal in their response to 0 ˚C and -2 ˚C with 100% plant recovery two weeks after exposure. Therefore, temperatures between 0 ˚C and -2 ˚C can be considered as non-injurious or to cause reversible injuries to swedes. Reversible injury is considered to be a dysfunction in the plant due to low temperature but the plant can continue to function normally if brought back into a non-frost temperature (Pragya et al., 2005). This indicates that swede is chilling resistant.

Damage to plants was progressively increased as they were exposed to lower and lower freezing temperatures down to -10 ˚C. Differences in the genetic background of the swede genotypes clearly affected plant response to frost and was reflected by the clear variances in their ability to recover after frost treatments of -4, -6 and -8 ˚C. Some genotypes were classified as having low frost tolerance and some demonstrated higher ability to withstand frost, while others could be considered intermediate. Differences in genotypic ability to tolerate frost in many species was mentioned by Thomashow et al. (2001) but the results presented in this chapter are the first to demonstrate that clear quantitative variation of frost tolerance exists within the swede germplasm.

Frost treatment to -10 ˚C caused irreversible damage to all genotypes. Clear appearance of morphological symptoms of frost damage like surface lesions, the appearance of water-soaked tissues, loss of turgor, discoloration; tissue damage; and rapid decay were found after treatment at -10 ˚C and this appears to be the limit of swede seedling frost tolerance. Xin and Browse (2000) revealed that freezing damage is mainly caused by the dehydration of the cell, and it is estimated that -10 ˚C causes -11.6 MPa water potential which is sufficient to remove 80% of the osmotically active water from the cytoplasm in acclimated plants. Such dehydrative stress could be sufficient reason for the complete death of swede seedlings.

The REC% results clearly revealed the effect of the preceding growing environment on swede response to sub-zero temperatures. Results demonstrated that swede genotypes have the ability to be acclimated and how acclimation is necessary for plant tissues to fully cope with freezing. The results for the plants pre-treated in the natural field environment (FE) was better than those in the glasshouse (GH) indicating a degree of acclimation in the field. Plants however did not achieve the same level of freezing tolerance as attained by exposure to constant low temperature (CA) indicating that the natural environment used here did not acclimate the plants to their full frost tolerance capability. Temperatures in the FE during this time averaged 7.5 ˚C (Appendix 3) according to Meteorological Data Archive of Plymouth University weather station (www.plymouth.ac.uk/metnet).

The results clearly showed that low temperature is up-regulating frost hardiness (Russell 2006). The results were consistent with Pan et al., (1994) where they found that there is often an interaction between the environmental factors (day length, water status, wind etc.) and low temperature. Also Gusta et al., (2001) proved that non-acclimated winter plants tolerate much lower temperatures after they have been hardened in controlled environments in comparison to field conditions.

Sub-zero temperature significantly affected the REC%. Electrolyte leakage from injured cells was exacerbated as temperatures were lowered to -10 ˚C and similar results were achieved by Nezami et al. (2010) who found that leaf REC% was extremely affected by the different freezing temperatures, especially in the range -4 ˚C to -8 ˚C.

The REC% at -10 ˚C exceeded 80% which is a high value for the amount of leaked electrolytes from damaged tissues and is usually considered to be lethal. Freezing damage is mainly caused by the dehydration of the cell, around 90% of the osmotically active water from the cytoplasm in acclimated plants was found to be removed at a temperature of -10 ˚C (Xin and Browse, 2000). This dehydration stress is strong enough to cause membrane disruption or rupture leading to the catastrophic loss of integrity and the release of ions as well as the

remaining water in the cell. This could be enough to explain the complete death of swede plants at this temperature.

Plant response to freezing was clearly affected by genotype and was reflected by the clear variation in the values of REC% for mature plants after frost treatments. Me77c and Or13 could be classified as having low frost tolerance, while Ly01 and Gr19 demonstrated higher ability to withstand frost. Differences in genotypic ability to tolerate frost was explained by Thomashow et al. (2001) as a quantitative genetically inherited trait in the ability to remain undamaged despite cellular dehydration caused by extracellular freezing.

EL_{50} values confirmed that swede is a cold season plant with good adaptation to moderate to severe low temperature. The overall mean of EL_{50} for all genotypes in the field and glasshouse was -5.8 ˚C and -5.7 ˚C respectively, whilst EL_{50} for cold acclimated plants was -8.4 ˚C. These results are consistent with Jaglo et al., (2001) who achieved EL_{50} of -4.7 ˚C for non-acclimated *Brassica napus* (oilseed rape) and EL_{50} -8.1 ˚C with cold acclimated plants.

The overall pattern of response to frost was demonstrated by each of the genotypes tested with different values of EL_{50} for each growth condition. Ly01 presented the lowest EL_{50} value under all conditions and reached an EL_{50} of -10.7˚C when cold acclimated. Me77c and Or13 were the most susceptible with the highest EL_{50} (-7.3 and -7.2 ˚C respectively) when cold acclimated and -5.7 ˚C for both when grown in the GH. Beyrami (2006) also reported that susceptible genotypes are expected to have higher EL_{50} than resistant ones.

Differences between LT_{50} and EL_{50} values were not big, but the differences could be due to the difference in growth stage or prior conditioning to frost treatment. LT_{50} was measured for cold acclimated plants in the seedling stage while EL_{50} was measured for plants in a more mature growth stage treated differently prior to frost testing. This is possibly explained by the seedling stage being the most susceptible growth stage to frost (Pragya et al., 2005) as highlighted earlier. However, LT_{50} was significantly correlated to EL_{50} following FE conditions for plants in the advanced growth stage and their means showed no significant differences. This suggests that frost tolerance screening with cold acclimated plants at the seedling stage is a reasonably representative

method to assess ability of swede genotypes to tolerate frost in the natural field environment in more advanced growth stage when BH symptoms are expected to appear.

Genetic variation for ability to cope with frost or not was reflected by the appearance of the same genotypes as tolerant or susceptible in both methods of measurement. It can be confidently confirmed that Ly01 and Gr19 are more tolerant and Me77c and Or13 are the most susceptible to frost. To this end, the aim of this screening was achieved.

After knowing the genotypes ability to cope with frost, then the next step was to find out if this was related to genotypic susceptibility to BH symptoms in the field.

The recorded field observations of BH frequency over a 10-year period revealed that the occurrence of this disorder was associated with certain genotypes and not others. To the authors knowledge this was the first investigation of BH appearance in breeder's data over such a long period. All previous studies about BH disorder appearance in swede roots were comparison studies between not more than three genotypes and all were concerned about the differences between swede genotypes for their ability to resist field BH under different boron fertilizer conditions (Sanderson et al., 2002a, Shelp and Shattuck, 1987, Cutcliffe and Gupta, 1987, Umesh and Cutcliffe, 1978, Beauchamp and Hussain, 1974).

It can be concluded from the results presented in this chapter, that swede is a cool season plant with moderate frost tolerance and capable of increasing this tolerance by cold acclimation. Differences in the genetic backgrounds of the tested genotypes affected plant response to frost but all swede genotypes showed some ability to tolerate frost. The ability of swede to tolerate frost after cold acclimation at 4˚C for 14 days suggests that swede may have the common cold-response pathway, which makes it like *Arabidopsis thaliana* and *B. napus* (oilseed rape). This was studied in more detail in Chapter 5.

The results from the two screening methods showed swede's ability to cope with sub-zero temperatures between -6 ˚C in the seedling stage and -10 ˚C for

larger plants and this is independent new information. This study is the first documented evidence concerning the ability of swede to tolerate frost.

The ten-year breeders' data revealed that some genotypes appear to show a higher susceptibility to field BH than other genotypes. Wherever the frost susceptible breeding lines Me77c and Or13 were included in the pedigree of the genotypes; they were highly susceptible to BH in the field. Unfortunately, Ag31 was not included in the frost screening due to lack of seed stocks.

Overall, results showed that the initial hypothesis associating frost tolerance and BH susceptibility is applicable at least for the tested genotypes. This could mean that the two traits are genetically closely linked. This in turn raises some questions viz:

- Is the association between BH and response to frost a physiologically based association?
- If it is physiologically based, is there a molecular linkage between frost and BH susceptibility?
- If yes, then what is the molecular basis of this linkage?

Moreover, will breeding for frost tolerance produce genotypes able to resist BH disorder?

Chapter 4:

Genotypic resistance to brown heart incidence in swede parent lines and F1 hybrids and the influence of applied boron

4.1 Introduction:

Brown heart (BH) is a recurring problem that has plagued the industry for some time and makes swede roots unsalable as they are deemed to be unfit for human consumption. The incidence of BH has been reported worldwide particularly in northern Europe, Australia, the US., Canada and New Zealand (Umesh et al., 1969). The occurrence of BH in swede was fairly extensive in the south-east of Scotland during 1949 (Smith and Anderson, 1955). It is also known as water-core, raan and various other local names. Soft and water-soaked discoloured areas appear in infected roots with BH and these areas may increase in size until almost the whole swede root is affected (Cutcliffe and Gupta, 1987). The discoloration varies from light to dark brown, and can be single area or several smaller areas scattered throughout the centre part of the root. BH has been attributed to an abiotic stress disorder related to localised boron deficiency in the developing root and the first connection between boron and BH was reported in 1936 by Hurst and MacLeod (Sanderson et al., 2002).

BH may occur even when there is an adequate boron concentration in the soil and when applications of boron are correctly timed. When the root is just starting to swell at the 7 – 10 leaves growth stage is the first stage that boron application is required. If BH has already developed by this stage, it is usually too late to correct it with boron applications. Swede grown in soil with a soluble boron content of 0.5 ppm or less is more likely to get affected by BH and it would appear that 0.7 ppm of boron is sufficient to give unaffected crops on medium and heavy soils, whereas on sandy and light soils a level of 1.0 ppm or more is required (Smith and Anderson, 1955).

Many studies have been carried out with boron to determine its effect in *Brassica napus* and have shown that *B. napus* (both oilseed rape and swede) is sensitive to boron deficiency (Shelp and Shattuck, 1987, Du et al., 2002, Kyoko, 2010). It has been found that BH symptoms did not appear when the boron content of the root was more than 27 µg g^{-1} (Shelp and Shattuck, 1987), but when it fell to the range between 10 to 18 µg g^{-1} it gave severe, moderate or slight internal signs of brown discoloration (Umesh et al., 1969, Beauchamp and Hussain, 1974, Shelp and Shattuck, 1987).

There appear to be variations between genotypes within a species in their response to boron deficiency (Xu et al., 2001, Fangsen and Yunhua, 1998). Shlep and Shattuck (1987) maintained that swede genotypes that have a good capacity for translocation of boron to developing roots from their leaves always showed less sensitivity to boron deficiency and BH disorders. Xu and Wang (1998) have shown that plant boron utilization efficiency is a dominant trait. However, there are no recent studies on this and nothing in the literature examining boron effects on the new generation of F1 hybrids being produced in the UK.

As reported in the literature, BH is unpredictable and cannot be reliably detected in the field (Shelp and Shattuck, 1987). This means that growers can only assess the marketability of a crop after it has been grown and as a consequence the over application of exogenous boron in an attempt to minimise the risk of BH is practiced. This means that it is crucial that the breeders understand and select for BH resistance and present only resistant varieties to the market.

4.2. Field investigation and chemical analysis of symptomatic roots:

4.2.1 Aims and Objectives:

This investigation was partly to establish the laboratory techniques required for boron analysis and partly as an initial attempt to investigate potential differences in boron in affected plants and to investigate BH syndrome and severity in grower's fields in preparation for a large field experiment. Moreover, it was also to investigate if there is any trait associated with BH appearance.

4.2.2 Materials and Methods:

An investigation was carried out in commercial fields planted with three different swede cultivars V1, V2 and V3 in winter 2010/11 (courtesy of Coles Ltd. of Wellington, Somerset). Roots (n = 5 – 10) were randomly sampled from each variety and examined for BH. Roots were then cut transversely to investigate any internal discolouration of BH. Symptoms such as mottling and tinting of foliage, death of the growing point and rough skin that in the literature are sometimes related to boron deficiency were recorded. Information was collected

from the grower concerning field management and fertilizer application especially levels of applied boron, and its application method and timing.

4.2.2.1 Boron concentration analysis:

The whole root of affected and unaffected swedes were taken from the field to the laboratory at Plymouth University. Roots were cleaned and chopped (3 – 5 cm) then frozen at -20 ˚C before being subjected to a boron analysis protocol. Roots were freeze-dried for 3 - 5 days to constant weight. A dried weight sample of 0.25 g was then digested in concentrated nitric acid (16 Molar) at 80 ˚C using the hot block digestion system. Digested samples were cooled then diluted to 25 mL using 2 % nitric acid. Boron content was determined using plasma spectrometer (X-Series ICP-MS).

4.2.2.2 Microscopy section:

Subsamples of affected and unaffected roots were cut into small pieces (0.2 – 0.3 cm) and fixed in 37% (saturated) formaldehyde for 7 to 10 days prior to carrying out a methacrylate resin method to embed samples for microscopy. Two solutions were used in this method; solution A consisted of; 2-Hydroxyethyl Methacrylate (80 mL), 2-Butoxy ethanol (15 mL) and benzoyl peroxide (0.5 gm). Solution B which consist of polyethylene glycol 400 (10 mL) and N, n-dimethylnine (1 mL). To embed; resin (a) was mixed with its catalyst resin (b) in the proportions 50:1 A:B. The specimen was allocated in moulds then filled with the embedding mixture, after 4 - 10 hours later blocks were ready for sectioning. Sections of 2 µm were placed on a slide and stained using Methylene Blue Chloride. Pictures were taken under light microscopy to investigate any differences between samples due to BH infection.

4.2.3 Results:

4.2.3.1 Visually obtained results:

Symptoms of potential boron deficiency such as mottling and tinting of foliage and rough skin were noticed in the investigated swede fields; the tinting of the foliage and skin roughness were the most frequent symptom (Plate 4.1). However the appearance of these symptoms in the field was not necessarily accompanied by internal discoloration of BH inside the root (Plate 4.2, a, and b) and most of the BH infected plants showed healthy roots with no signs of boron

deficiency symptoms (Plate 4.2, d and e). In a few roots there was internal discoloration of BH disorder in association with tinting of foliage and/or rough skin (Plate 4.2, c and f). The incidence of BH in the field appeared sporadic and unpredictable and affected roots could be next to healthy roots in the same row (Plate 4.2, e). No sharp distinction could be drawn in the field between affected and non-affected roots.

There was no constant level of BH severity. Slight and moderate internal discoloration was the most frequently recorded level in the field. Although internal discoloration usually appears in the middle of the root slicing roots vertically proved better than cutting them in two halves transversely where the brown spot could be missed.

Plate 4.1: Potential boron deficiency symptoms in field crops of swede.

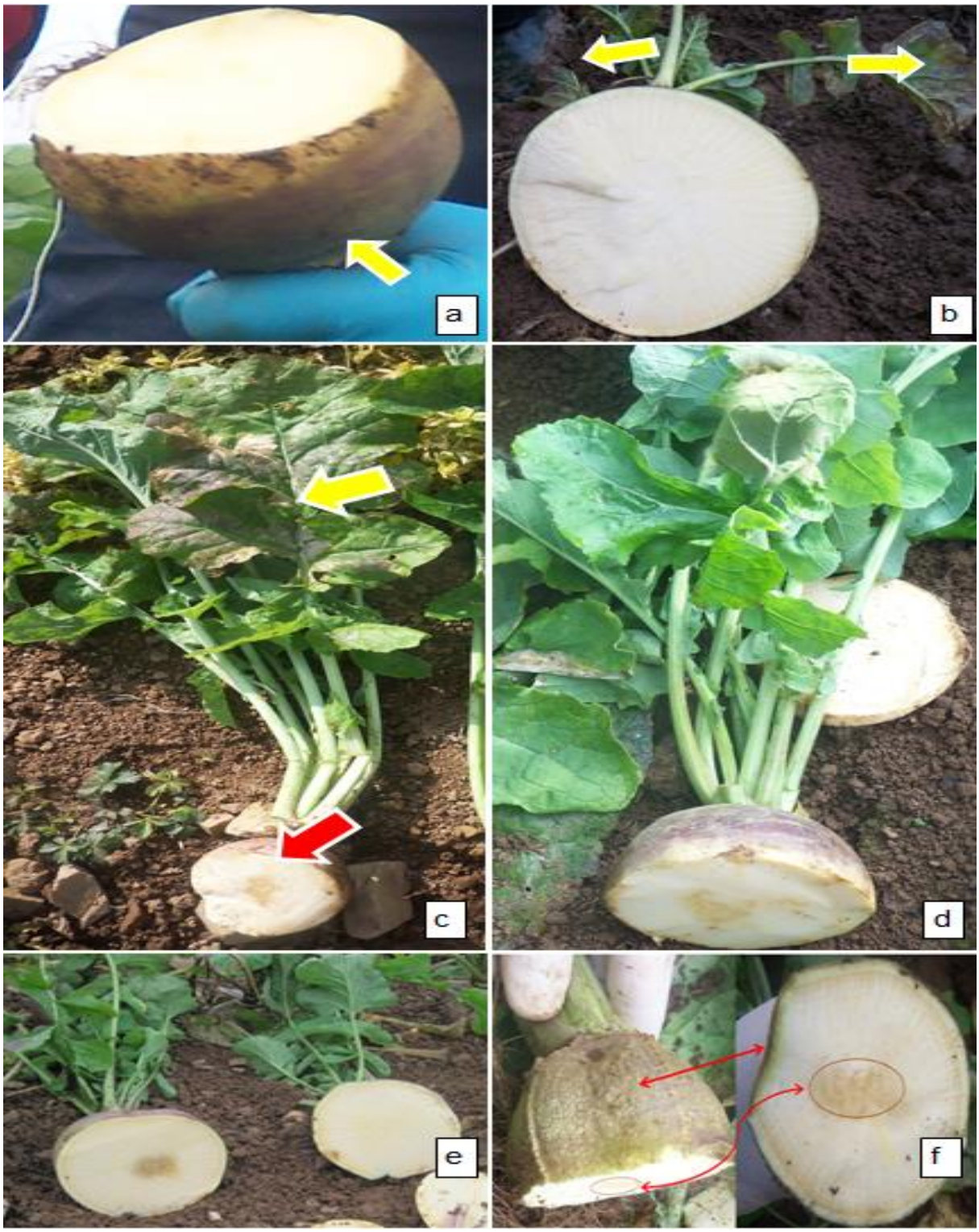


Plate 4.2: Boron deficiency symptoms and root internal discoloration of brown heart, a and b) mottling and tinting of foliage and rough skin with no root discoloration, d and e) brown heart with no any boron deficiency sing, c and f) mottling and tinting of foliage and rough skin with brown heart, e) brown heart affected root with intact one next to it.

4.2.3.2 Chemical analysis of scored roots:

Swede roots with internal browning symptoms had lower boron content (average of 15.18 µg g^{-1}) compared with non-symptomatic roots (average of 17.34 µg g^{-1}) for all varieties (Table 4.1). Differences were not big but ranged from a 7 to 16% reduction compared to unaffected roots.

Table 4.1: Boron content (µg g-1) in swede roots with and without internal browning of brown heart (BH).

Variety	with BH	without BH	reduction in BH affected roots
V1	**15.99**	**17.22**	**7.69 %**
V2	**15.02**	**17.59**	**17.11 %**
V3	**14.52**	**17.22**	**18.60 %**
Mean	**15.18**	**17.34**	**14.23 %**

Information collected from farmers regarding crop fertilization management revealed that the quantities of boron applied were typically 0.75 – 0.90 kg B ha^{-1} worked into the soil pre drilling, then 0.30 – 0.45 kg B ha^{-1} as a foliar application 6 - 8 weeks after drilling, then 0.30 kg B ha^{-1} 8 - 10 weeks after drilling and finally 0.15 kg B ha^{-1} 10-12 weeks after drilling. The timing and quantities of boron used were dependent on the boron level ascertained from soil sampling moderated by variety, and if the crop was to be early, mid or late harvested. Farmers felt that low soil boron levels was boosted by using prilled fertiliser with an inclusion of boron but this is less specific due to the fact that the quantities applied vary greatly.

4.2.3.3: Microscopy sectioning:

Light microscopy sectioning failed to show any definitive differences between BH affected and non-affected roots (Plate 4.3).

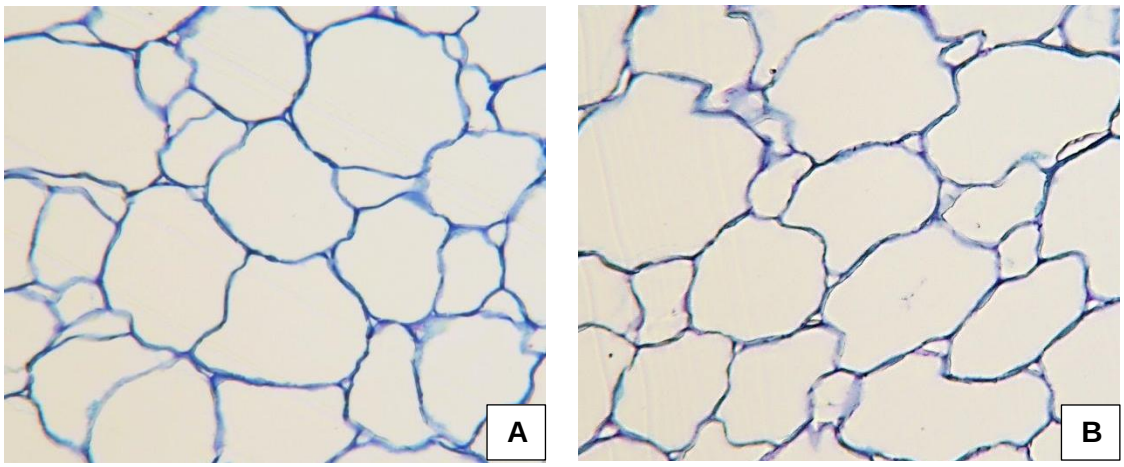


Plate 4.3: Sections of swede roots (A) healthy root and (B) brown heart affected root. Light microscopy x400.

4.2.4 Discussion:

BH was first linked to boron deficiency by Hurst and Macleod (1936) (Sanderson et al., 2002) but attempts to link BH disorder appearance in the field with any unusual growth sign due to boron deficiency failed. This is confirmed here where no differences between plants were visible in the field and mottling and tinting of foliage, and rough skin were not found to be appropriate indicators for BH disorder. The disorder appearance in the field was also sporadic and unpredictable and it was concluded that the symptoms of BH in swede generally cannot be detected from the exterior examination of the roots or by a visual examination of the foliage. These observations were in agreement with Smith and Anderson (1955) and Cutcliffe and Gupta (1987) who also found no clear differences between healthy and affected roots. Brown and Jones (1971) reported that some boron deficiency disorders appear to be physiological in nature and occur even when boron is in ample supply and these disorders are thought to be related to peculiarities in boron transport and distribution.

The amount of boron applied by farmers during the growing season ranged between 1.5 kg B ha^{-1} to 1.8 kg B ha^{-1}. Although there is no firm recommendation in the literature, studies on BH appearance in swede roots in the field showed no BH incidence when boron application was 2 kg ha^{-1} or

more as a foliar application (Sanderson et al., 2002) and Umesh and Cutcliffe (1978) recommended that a band application of 1.12 kg ha^{-1} was sufficient to control BH. Interestingly farmers believe that they are applying lots of boron but they still complain of BH appearance in the field. Boron application timing by farmers seems to not be precise as there was about a two week period of flexibility for each application time where the first foliar application 6 – 8 weeks after drilling then 2nd application 8 – 10 weeks after drilling and finally last application 10 – 12 weeks after drilling. This is in contrast with Umesh and Cutcliffe (1978), Cutcliffe and Gupta (1987) and Sanderson et al. (2002) who emphasized the importance of adding boron to swede 28 – 30 day after seeding and then second application after 42 – 45 day after seeding. They also reported lower boron concentration in swede tissues due to late applications and also pointed out that calendar date boron application did not provide acceptable control of BH. Cutcliffe and Gupta (1987) found that foliar application of boron at 40 days and repeated at 52 – 55 days did not provide acceptable control of BH at three of five locations of their study and showed that boron uptake is critical at about the five-leaf stage when the root starts to swell which for swede is approximately 28 days after seeding.

Analysing swedes root for boron concentration was based on the information that BH disorder incidence in the field is mainly due to boron deficiency and first connection between boron deficiency and BH was reported by Hurst and MacLeod (1936) (Sanderson et al., 2002). Results of this “preliminary” investigation showed that affected roots had lower boron content in comparison with healthy ones, results which agreed with findings of Umesh et al. (1969), Beauchamp and Hussain (1974) and Shelp and Shattuck (1987).

Overall the observations and results in this field investigation raised an important question, “can BH disorder be counted as one of boron deficiency symptoms in swede. Is it an advanced stage of boron deficiency or is it the first sign and then the other symptoms following?” Furthermore if BH incidence is affected by boron availability in the field then why is that BH affected roots are not always showing clear symptoms of boron deficiency or are there some other factors that may affect BH disorder appearance. Is it environmental and/or genetic factors that are related to swede genotypes and the genetic background of the parents used in F_1 hybrid production? To answer these questions a large

field trial was carried out to study the role of boron in BH incidence and severity, and the influence of genotype and environmental (different levels of boron) impacts on BH appearance in the field.

4.3 Genotype and environmental resistance to brown heart (BH) incidence in swede.

4.3.1 Aims and Objectives:

This experiment was designed to examine swede response to boron application levels in the field and to examine the role of boron on the incidence of BH syndrome in UK bred lines and F_1 hybrids.

4.3.2 Materials and Methods:

Following results obtained in Chapter 3 and the outcome of the field observations and chemical analysis of symptomatic roots, 12 swede genotypes were selected including 7 parent lines (2 carrying cytoplasmic male sterility - cms) and a selection of five of their F_1 hybrids (Table 4.1), not all reciprocal F1 hybrids were available. The trial was conducted within a commercial swede crop in Treburley / East Cornwall, Devon, UK (Grid Reference: SX 355785). The previous crop sown in this field was winter wheat. The soil type was a silty-clay loam of the Denbigh series with a pH of 6.7. Soil samples were collected prior to sowing to measure the concentration of boron. Soil sampling was carried out from the surface horizon (0 to 5 cm) and the root zone (5 to 10 cm). Samples were collected from each replicate block mixed thoroughly then dried in the shade. Boron concentration in soil was measured using the Hot Water Soluble test method (HWS) (Mulvaney and Bartels, 1996). Representative soil sampling showed that soil mineral content was 0.7 µg g^{-1} B, 23.0 µg g^{-1} Ca, 11.4 µg g^{-1} K, 6.6 µg g^{-1} P and 11.0 µg g^{-1} Mg.

Plots were established in raised beds according to current commercial practice in rows with an inter-row spacing of 0.35 m and an intra-row of 0.15 m giving a field plant population of approximately 17 plants per square metre. There were 3 replicate blocks and the 12 genotypes formed the plots (Table 4.2) and were fully randomised within each replicate (Appendix 5). The beds were covered with Enviromesh® (Plate 4.4) to raise early season temperature and to prevent cabbage root fly infestation. Three datalogers were fixed in the field (Plate 4.5); one under the Enviromesh® to measure the air temperature under the mesh. The second had a probe was buried in the soil (root zone depth) to measure the soil temperature. The third was fixed at a height of 1.5 m at the field edge to measure the air temperature. All dataloggers were covered with silver foil to prevent the impact of the direct radiation and to protect them from rain. Data

from the loggers were integrated to provide a mean daily temperature reading and these summarised as a mean monthly reading (Appendix 4).

Four levels of foliar application of boron were applied (Table 4.3) including the growers' commercial rate (1.80 kg B ha^{-1}). A non-selective herbicide and liquid fertilizer (04:10:12 N:P:K) were applied before sowing and a soil residual broadleaf herbicide (Treflan) was also applied.

Table 4.2: Swede genotypes used in the field trial (ms = male sterile).

Genotype	
Parent lines	**F1 Hybrids**
Gr19	ms.Gr19 X Ly01
ms .Gr19	ms.Gr19 X Or13
Ly01	ms.Ag31 X Gr19
Or13	ms.Ag31 X Or13
Ag31	ms.Or13 X Me77c
ms.Ag31	___
Me77c	___

Table 4.3: Boron (B) treatments applied in the field trial (kg B ha-1).

No.	Treatment	Pre-sowing	Post-emergence 1 (28 DAS*)	Post-emergence 2 (42 DAS*)	Total B applied
1	No B	0.000	0.000	0.000	0.000
2	Low B	0.900	0.225	0.225	1.350
3	Mid B	0.900	0.450	0.450	1.800
4	High B	0.900	0.900	0.900	2.700

*DAS = days after sowing.

Plate 4.4: Field trial covered with the Enviromesh®.

Plate 4.5: Dataloggers fixed in the field. Two under the Enviromesh® one to measure the air temperature under the mesh, the second to measure the soil temperature (A). The third was fixed at a height of 1.5 m to measure the air temperature (B).

Scoring for BH symptoms was carried out just prior to harvest. This entailed working beneath the Enviromesh. Ten roots per plot were randomly selected, cut in half transversely and scored for internal browning discoloration using a 4 point scale (Table 4.4):

Table 4.4: Brown heart scoring categories.

Score	Severity	Description
0	CLEAN	No discolouration
1	SLIGHT	Very slight browning
2	MODERATE	Obvious browning
3	SEVERE	Dark brown and water

Sub-samples of root tissue were taken for chemical analysis, placed in labelled plastic bags and placed in a Coolbox with ice blocks for transportation to the laboratory where they were digested using concentrated nitric acid and analysed for boron content.

4.3.3 Results:

4.3.3.1 Brown heart appearance and severity:

Results of BH incidence clearly divided the genotypes into two groups: resistant genotypes where the percentage of BH incidence was 0% at all levels of boron treatment including no boron applied treatment (0.00 kg B ha^{-1}), and included the parent lines Gr19, ms.Gr19, Ly01 and the hybrids ms.Gr19 X Ly01, ms.Gr19 X Or13 and ms.Ag31 X Gr19; and susceptible genotypes, which had two subgroups; very susceptible genotypes including those that showed BH even at the highest level of boron applied and comprised the parents Ag31, ms.Ag31and the hybrid ms.Ag31 X Or13; and intermediate susceptibility, which showed BH only at the lowest levels of applied boron and this included the parent lines Me77c and Or13 and the hybrid ms.Or13 X Me77c.

Generally the percentage of roots with BH appearance in susceptible genotypes (the very susceptible and the intermediate subgroups) was significantly ($P<0.001$) ameliorated by boron application (Figure 4.1). The overall frequency of BH incidence (scores 1 – 3 combined) in susceptible genotypes decreased from 55.9% with 0.00 kg B ha^{-1} boron applied to 11.7 % at 2.70 kg B ha^{-1} applied. The application of boron decreased the overall incidence of BH disorder incrementally with each increase in boron applied.

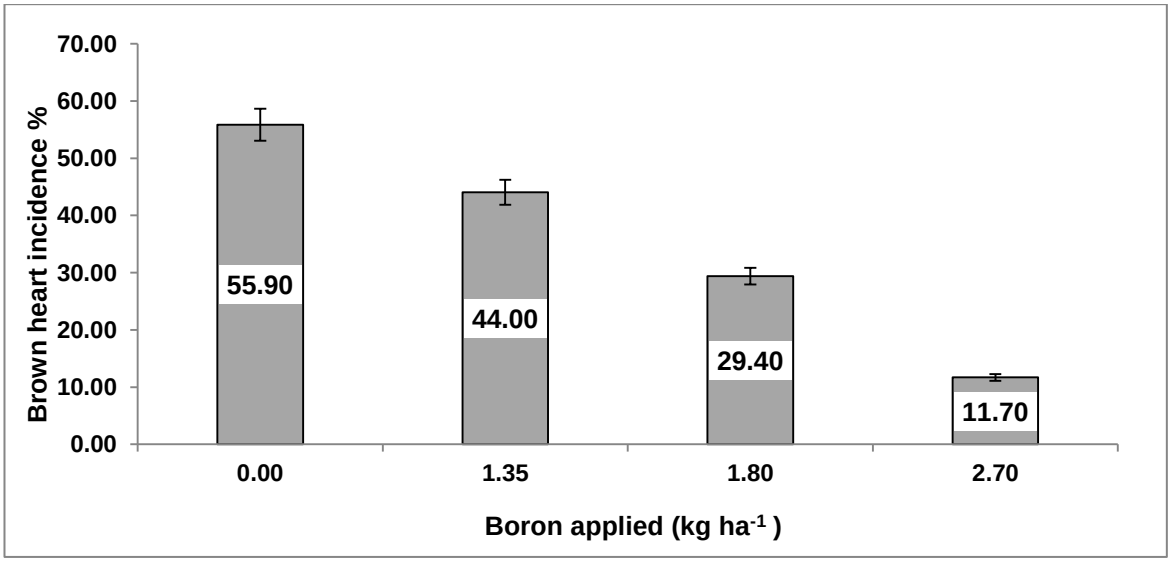


Figure 4.1: Overall incidence of brown heart in susceptible swede genotypes at different boron application levels.

The severity level of BH in susceptible genotypes was also affected significantly (P<0.002) by an increased level of applied boron (Figure 4.2) with symptoms of severe BH for all affected genotypes significantly decreasing at 1.80 kg B ha^{-1} and disappearing at 2.70 kg B ha^{-1} applied. Moderate internal browning dropped from 31.00 % at 0.00 kg B ha^{-1} to 1.50 % at 2.70 kg B ha^{-1}. However the incidence of slight BH symptoms increased from 10.2 % at 1.35 kg B ha^{-1} to 23.0 % at 1.80 kg B ha^{-1} because symptoms in some genotypes that showed moderate infection at lower levels of applied boron were reduced to slight internal browning at 1.80 kg B ha^{-1} applied.

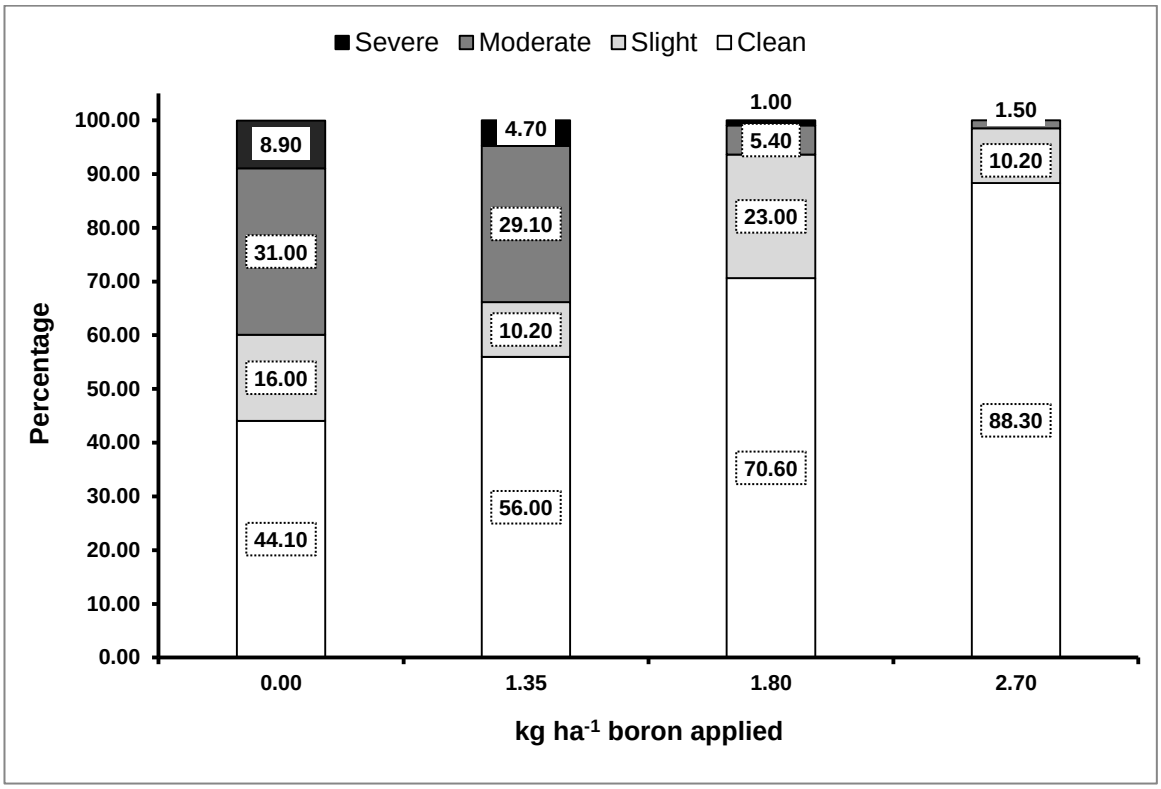


Figure 4.2: Percentage of brown heart severity in susceptible genotypes of swede at different boron application levels.

Genotypes demonstrated clear significant (P<0.01) differences in incidence of BH in their response to boron application (Figure 4.3). Parent lines Gr19, ms.Gr19 and Ly01 showed plants 100% clean of BH at all boron treatments including 0.00 kg B ha^{-1} whilst in complete contrast Ag31 and ms.Ag31 showed internal discoloration at all boron treatments even at the highest level (2.70 kg B ha^{-1}) but with a reduced level of severity.

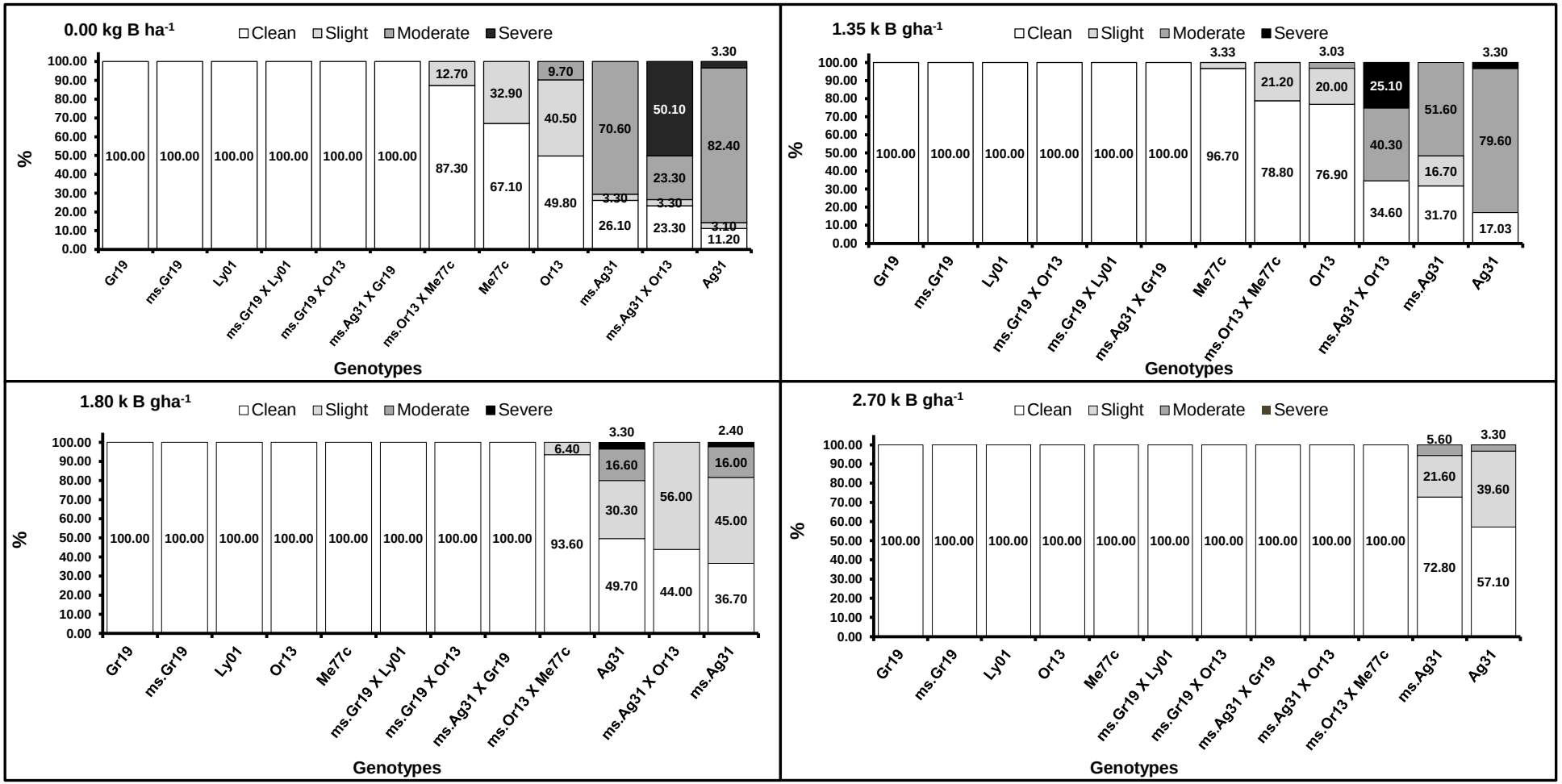


Figure 4.3: The percentage incidence of brown heart in swede genotypes at different levels of applied boron, arranged by severity from right to left.

Increasing the level of boron applied reduced the frequency of BH incidence. Between parent lines; the highest percentage of BH recorded at 0.00 kg B ha^{-1} was 88.7% and 73.9% with Ag31 and ms.Ag31 respectively and they were the only genotypes that showed any BH infection symptoms at 2.70 kg B ha^{-1} (42.9% and 27.2% for Ag31 and ms.Ag31 respectively). In contrast, parent lines Gr19, ms.Gr19 and Ly01 had no affected roots at all boron levels including when no boron was applied. Me77c and Or13 were intermediate in their response, at 0.00 kg B ha^{-1} Me77c showed 32.9% root with BH disorder; while Or13 had 50.2% affected roots. At 1.35 kg B ha^{-1} applied, BH decreased to 3.3% for Me77c and 23.3% for Or13. Both Or13 and Me77c were 100% clean of root internal browning of BH at 1.80 and 2.70 kg B ha^{-1} applied.

Influenced by their parent lines; ms.Or13 X Me77c and ms.Ag31 X Or13 were the only hybrids to show BH symptoms and were the only hybrids which were combined from susceptible parent genotypes. In contrast ms.Gr19 X Ly01, ms.Gr19 X Or13 and ms.Ag31 X Gr19 showed plants 100% clean of BH at all levels of boron applied.

So overall, Gr19, ms.Gr19, Ly01, ms.Gr19 X Ly01, ms.Gr19 X Or13 and ms.Ag31 X Gr19 can be reasonably considered as highly resistant genotypes to BH disorder, ms.Ag31 X Or13, Ag31 and ms.Ag31 were very susceptible whilst Me77c, Or13 and ms.Or13 X Me77c were intermediate. Interestingly genotypes susceptible to BH showed different levels of BH severity at the same level of boron treatment (Plate 4.1).

Plate 4.6: Internal browning severity of brown heart at different levels of boron treatment.

4.3.3.2 Boron concentration in healthy and affected plant roots:

Genotypes differed significantly ($P<0.05$) in their response to the levels of boron applied and all genotypes increased their boron root tissue content with each increasing level of boron treatment (Figure 4.4). The minimum boron concentration in roots not showing BH symptoms at 0 kg B ha^{-1} was 23.3 µg g^{-1} for Gr19 while the highest was 32.2 µg g^{-1} for ms.Ag31. At 2.70 kg B ha^{-1} again Gr19 showed the lowest root content of boron which was 35.6 µg g^{-1} and the highest (42.6 µg g^{-1}) was shown by Or13.

F1 hybrid plants showed a minimum root content of boron of 25.2 µg g^{-1} and that was for ms.Gr19 X Or13 at 0.00 kg B ha^{-1} whilst ms.Ag31 X Or13 had the highest (30.9 µg g^{-1}) at the same level of boron applied. At the highest level of boron applied (2.70 kg B ha^{-1}) ms.Gr19 X Me77c and ms.Gr19 X Or13 had the highest value of boron concentration in the root (40.7 and 40.6 µg g^{-1} respectively). The lowest concentration was 33.8 µg g^{-1} shown by ms.Gr19 X Ly01.

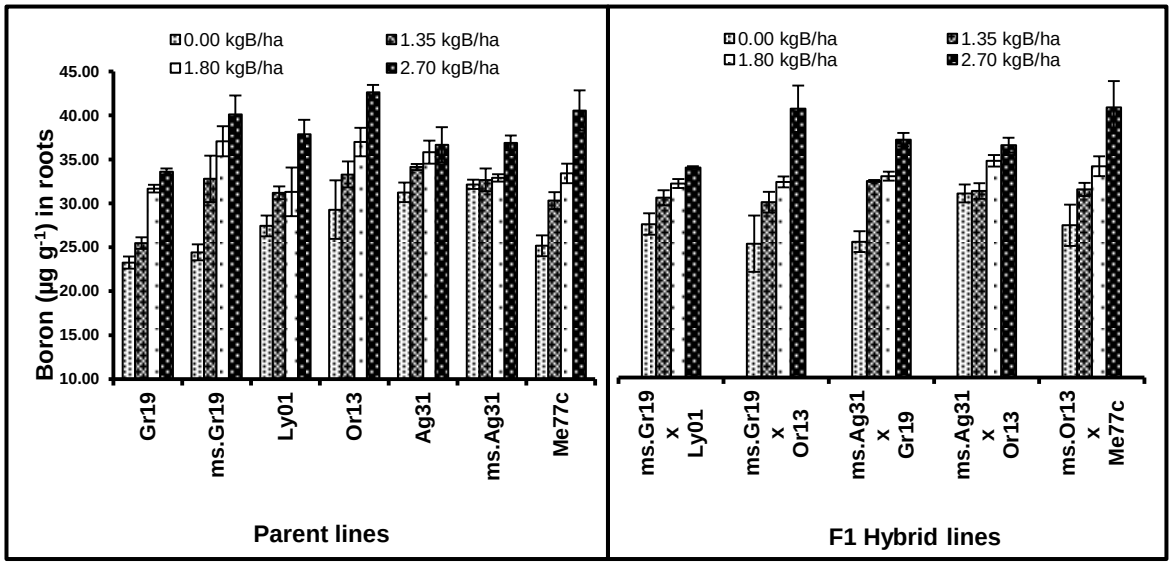


Figure 4.4: Boron concentration in swede roots not showing brown heart symptoms. Vertical bars represent ± S.E.M.

Roots showing BH disorder exhibited lower levels of boron concentration compared to non-affected plants of the same genotype (Table 4.5) and this supports the results presented in the first part of this chapter. Genotypes also differed in their ability to respond to a scarcity of soil boron (Figure 4.5) and not all genotypes showed a propensity to be affected by BH disorder. Boron

concentration in affected roots ranged between a minimum of 12 µg g^{-1} in the parent line ms.Ag31 at 0.00 kg B ha^{-1} to a maximum of 27.16 µg g^{-1} in Ag31 at 2.70 kg B ha^{-1} in contrast to a minimum for unaffected roots of 21.18 µg g^{-1} in Me77c and a maximum of 42.63 µg g^{-1} in Or13 at 0.00 kg B ha^{-1} and 2.70 kg B ha^{-1} respectively. The extent of the range of boron content in roots affected with BH was 19.65 µg g^{-1} compared to 32.88 µg g^{-1} in healthy roots of the same genotypes. Root boron content always increased as the level of applied boron increased.

Table 4.5: Boron (B) concentration ranges in intact and brown heart (BH) affected roots.

Genotype	Sensitivity to BH	B in intact roots (µg g^{-1}) (min-max)	B in BH affected roots (µg g^{-1}) (min-max)
Gr19	Resistant	23.3 – 40.5	Not measurable as no BH
ms.Gr19			
Ly01			
ms.Gr19 X Ly01			
ms.Gr19 X Or13			
ms.Ag31 X Gr19			
Me77c	Intermediate	25.2 – 42.6	14.5 – 19.0
Or13			
ms.Or13 X Me77c			
Ag31	Susceptible	30.9 – 36.9	12.0 – 27.2
ms.Ag31			
ms.Ag31 X Or13			

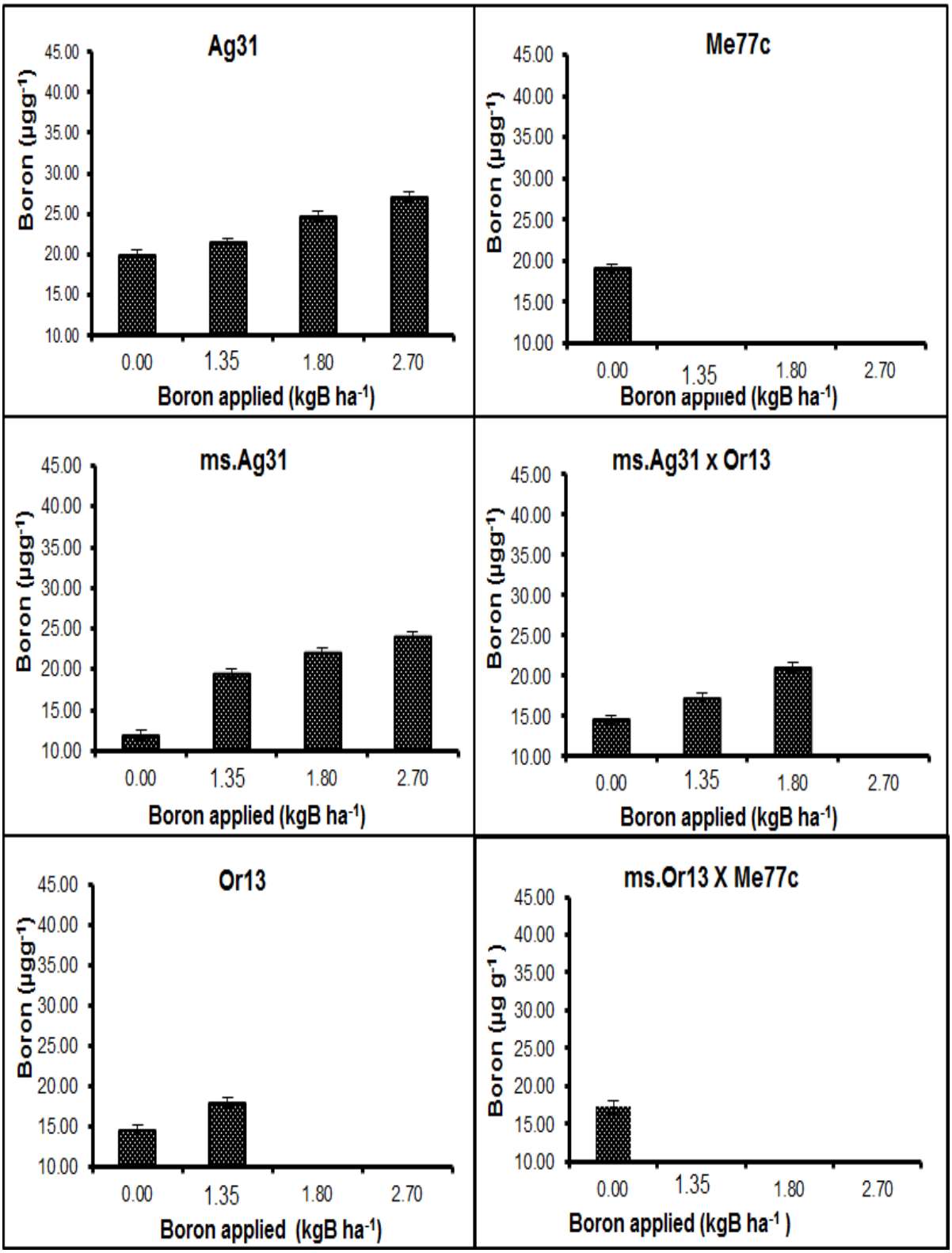


Figure 4.5: Boron concentration in brown heart affected roots for swede genotypes sensitive to brown heart disorder. (Where no columns are shown there was no brown heart disorder). Vertical bars represent ± S.E.M.

4.3.3.3 Brown Heart severity related to root content of boron:

There was an overall significant ($P < 0.001$) negative correlation between BH severity and root content of boron for genotypes which exhibited BH symptoms ($r = -0.632$). This negative correlation was evident for the individual susceptible genotypes (Figure 4.6).

The most susceptible genotypes all had Ag31 in their genetic background and the parent lines Ag31 and ms.Ag31 were BH affected at all levels of boron even though the severity declined at the higher levels of boron applied (Figure 4.6). Roots that were severely affected with BH disorder always showed low boron content (12 – 21.5 µg g^{-1}) but precise values differed between genotypes. Moderate internal browning (score of 1.5 - 2) was also related to low root boron content (19.5 to 24.8 µg g^{-1}). Roots free of BH disorder were associated with boron content in excess of 31 µg g^{-1} for the very susceptible genotypes while others required a minimum of 23 µg g^{-1}.

Or13 showed slight root discoloration at 0.00 kg B ha^{-1} and 1.35 kg B ha^{-1} but none at 1.80 kg B ha^{-1}. Me77c was mostly free of BH at all levels of boron with an average severity score of only 0.3 at 0.00 kg B ha^{-1}. ms.Ag31 X Or13 was free of internal browning at 2.70 kg B ha^{-1}. The hybrid ms.Or13 X Me77c was clean at all boron treatments with only a few incidences of slight discoloration in treatments 0.00 and 1.35 kg B ha^{-1}.

Parent lines Gr19, ms.Gr19 and Ly01 and the hybrids ms.Gr19 X Ly01, ms.Gr19 X Or13 and ms.Ag31 X Gr19 were free of BH at all levels of applied boron even at 0.00 kg B ha^{-1}.

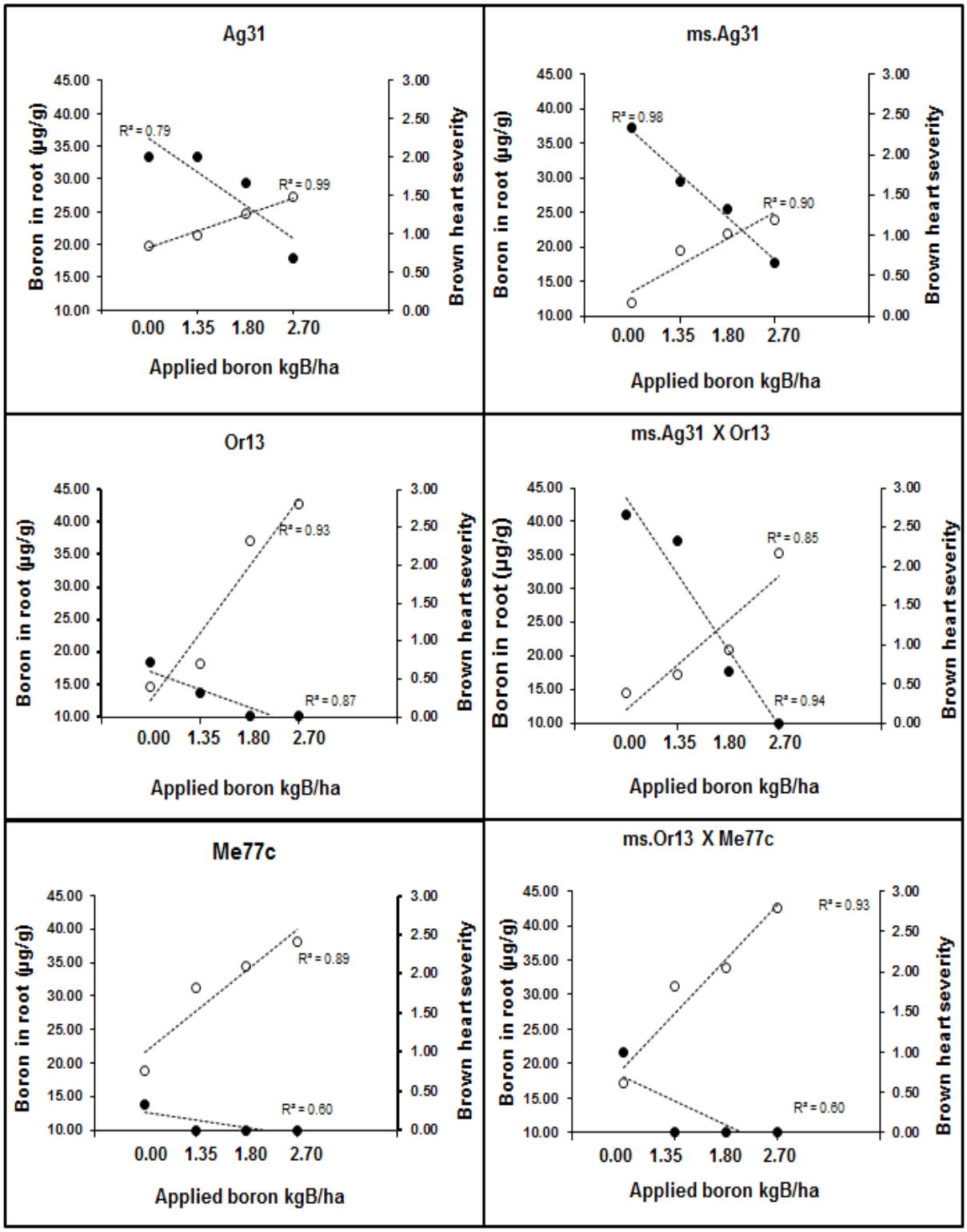


Figure 4.6: The relationship between swede root boron content (open circles) and brown heart severity (closed circles, where 0 clean, 3 severe brown heart) in susceptible genotypes with different levels of boron fertilizer applied.

4.3.3.4 Male sterility and brown heart incidence:

Male sterility in all of the studied genotypes (ms.Gr19, ms.Ag31 and ms.Or13) appeared to have little or no effect on BH symptom appearance. Hybrids having Gr19 or ms.Gr19 as a paternal or maternal parent showed the same resistant phenotype in terms of BH symptoms. Likewise Ag31 and ms.Ag31 and Or13 and ms.Or13 were susceptible to BH disorder irrespective of male sterility and this was also reflected in the F_1 hybrids from these susceptible parents. These findings suggest that the cytoplasmic male sterility used here had no effect on swede susceptibility to BH suggesting that BH susceptibility is probably a nuclear controlled trait and not cytoplasmic.

4.3.4 Discussion:

The field observations of the frequency of BH appearance over a 10-year period in chapter 3 revealed that the occurrence of this disorder was associated with certain genotypes and not others.

Results obtained from the field trial reported here showed that both the applied level of boron and the plant genotype significantly affected BH incidence and severity and root tissue boron content. Xu et al. (2001) referred to the existence of genetic variation for boron deficiency response within the *Brassica napus* germoplasm, and this is confirmed here. The results also agree with the findings of Cutcliffe and Gupta (1987) who reported a higher percentage of BH incidence at low levels of boron application. Umesh and Cutcliffe (1978) recorded a reduction in BH severity from very severe at no boron added to free of BH at 4.48 kgB ha^{-1}. All genotypes in the current study clearly responded to increased levels of boron application in terms of their root boron content which increased incrementally with each boron application, and this agrees with Umesh et al. (1969) who reported a significant positive correlation between applied boron level and swede tissue boron content.

The swede genotypes tested here also exhibited different abilities to withstand low levels of applied boron. Six of the twelve genotypes showed some BH at 0.00 kg B ha^{-1} applied while at 2.70 kg B ha^{-1} just two of them had BH but with both reduced incidence and severity. Roots with BH symptoms had lower concentrations of boron than healthy ones and a root boron content of between 12 µg g^{-1} to 27.9 µg g^{-1} was needed for BH symptoms to appear in susceptible genotypes. These results were supported by those of Shelp and Shattuck (1987) and Beauchamp and Hussain (1974) who also reported BH disorder at this range of boron root contents. In this experiment, the soil concentration of boron was measured at 0.7 µg g^{-1} and this seemed to be enough for some genotypes to be clean of any symptoms of BH. In the field most soils have boron contents typically ranging between 0.5 - 1.0 µg g^{-1} (Shiffler et al., 2003) and the soil contents recorded here prior to the trial. At 0.7 µg g^{-1} this was below that recorded by Gupta and Cutcliffe (1971) who stated that a soil concentration of 0.8 µg g^{-1} or greater soil available boron is required for no BH symptoms to appear in swede indicating that the field site chosen was marginally deficient in

boron. Shelp and Shattuck (1987) however referred to BH occurrence when soil boron ranged between 0.4 and 1.3 μg g^{-1} and our field site was within this range. Our results clearly demonstrated a strong genotypic effect which would influence the classification of “at risk” soil threshold levels of boron and threshold levels or recommendation need to be moderated by cross reference to genotype.

The parent lines Gr19, ms.Gr19 and Ly01 and F_1 hybrids with this genetic background (ms.Gr19 X Ly01, ms.Gr19 X Or13 and ms.Ag31 X Gr19) can be considered as highly resistant genotypes to BH disorder because they did not show any internal browning symptoms of BH even at 0.00 kgB ha^{-1} applied. This is consistent with the findings of Fangsen et al. (2002) who showed that some *Brassica napus* cultivars can grow normally in limited boron conditions. In contrast, Ag31 and ms.Ag31 can be considered as very susceptible genotypes showing BH symptoms at all levels of boron applied whereas genotypes Or13, Me77c showed intermediate susceptibility. Genotype variation within a species in respect of response to nutrient levels in the soil is not uncommon (Kelly and Gabelman, 1960) and two mechanisms have been attributed to these differences:

(1) plants have different abilities to absorb nutrients from the soil (Blarney and Chapman, 1982),

(2) plants have different nutrient utilization and translocation or re-translocation in the plant (Brown et al., 1972).

Good capacity to translocate the boron from leaves to roots is thought to make a plant less sensitive to boron deficiency and BH disorder (Shelp and Shattuck, 1987). Interestingly in the experiment reported here, hybrids that didn’t show any BH disorder symptoms included some hybrid combinations between susceptible and resistant parent lines and the only hybrids that showed BH (ms.Ag31 X Or13 and ms.Or13 X Me77c) were hybrids between two susceptible parent lines and in contrast wherever a resistant genotype was used in the parentage then the hybrid was resistant. These findings suggest BH resistance is a dominant trait that can be ameliorated by boron availability and this is supported by Xu and Wang (1998). It is suggested that the resistant

genotype for this trait can be considered to be BHBH or BHbh while susceptibility is considered recessive bhbh. However there was clearly some quantitative variation in BH susceptibility among genotypes suggesting that resistance was not a single gene effect.

Increasing boron application levels and therefore increasing boron availability, generally increased the root concentration of boron, even in the sensitive genotypes, and assisted in reducing BH incidence, but did not overcome the problem completely. Where resistant genotypes were used, boron application altered boron content but the absence of brown heart symptoms may mean that for these genotypes the application of additional boron in the field may be unnecessary.

Amongst the susceptible genotypes, there was a negative and highly significant correlation between BH severity and root boron concentration. Severe BH symptoms were associated with root boron contents between 12 µg g^{-1} and 21.5 µg g^{-1} at levels of 0.00 and 1.35 kg B ha^{-1} applied. Moderate discoloration was associated with boron concentrations between 19.5 µg g^{-1} to 24.8 µg g^{-1}. The boron content of clean roots differed between susceptible and resistant genotypes, and susceptible genotypes always had a root content in excess of 31 µg g^{-1} whilst resistant genotypes showed a minimum of 23 µg g^{-1} for clean roots. Clearly severity of BH differed between genotypes and their content of boron, and affected genotypes differed in their ability to not show BH at different levels of boron applied. We suggest that it is possible to define the term “boron stress” as the root concentration at which BH symptoms occur but it is important to recognise that the level varies for each particular genotype and is not necessarily the same as for other genotypes.

Results clearly showed that BH incidence and severity was primarily affected by swede genotype and for susceptible genotypes the incidence of BH could be ameliorated by boron application to the crop. It is clear that plant breeders should utilise BH resistance dominant germplasm such as offered by Gr19, Ly01 and Me77c when making new hybrid crosses. It is acknowledged that the susceptible germplasms of Ag31 and Or13 may offer desirable agronomic or quality traits but unless the dominant BH trait is introduced from a resistant

parent any hybrids will be susceptible to BH and risk rejection by the growers. If the quality of the germplasm from susceptible genotypes is highly superior then it would be a breeding goal to introgress the BH resistance trait into these germplasms, but it is acknowledged that this could be a long and time consuming process. To the authors knowledge there is no such breeding work attempting this and the first step would be to establish a backcrossing programme and to identify the gene locations and markers for these. Also it is recommended that screening of hybrids and selections emanating from these should be made at low soil boron levels in order that susceptibilities can be unmasked without the ameliorating effect of high boron availability. Cytoplasmic male sterility did not appear to have an impact on genotype response to BH and is therefore acceptable for swede breeders to use this breeding tool in swede hybrid production programmes without threatening BH resistance/susceptibility. It will become increasingly necessary for breeders to supply more variety information to growers so that when they choose F_1 hybrids with a genetic background from susceptible parents, such hybrids will require a high input of boron.

Finally, it is suggested that commercial growers of swedes are frequently over applying post-emergence boron to genotypes that are highly resistant and whilst this is understandable from a grower's point of view it could be ameliorated if more detailed information on genotypes and soil risk factors were available to them. Before such information is published however, the results reported here would need to the confirmed across various soil types and across seasons as it is acknowledged the results presented here are from a single field trial.

Chapter 5:

The effect of boron application and cold acclimation on the development of frost hardiness in swedes.

5.1 Introduction:

Boron has intermediate properties between metals and non-metals and is a member of the subgroup III of metalloids (Marschner, 1995). Although boron abundance is low in nature, it is widely distributed in both the lithosphere and hydrosphere. Boron concentrations range from 5 - 10 mg kg^{-1} in rocks, 3 - 30 µg kg^{-1} in rivers and ~ 4.5 mg L^{-1} in the ocean (Camacho et al., 2008).

Boron is essential for plants, and its availability in irrigation water can be an important determinate of agricultural production (Tanaka and Fujiwara, 2008). It primarily exists as boric acid (H_3BO_3) in the soil solution and can easily be leached out of the root zone under high rainfall conditions leading to deficiencies in plants. In contrast, in the arid and semiarid regions, boron cannot be leached sufficiently and due to the evaporation of groundwater can accumulate to levels that become toxic to plants (Reid, 2007). Both boric and borate forms of boron are able to form complexes with a wide variety of biological compounds with hydroxyl groups in *cis*-configuration.

The roles of boron in plant nutrition are not yet fully understood and what is known about boron has been the outcome from studies where boron was withheld or resupplied after deficiency. This information shortfall is unexpected, since on a molar basis, the need of dicotyledonous plants is much bigger than that for any other micronutrient. Lack of boron rapidly induces a wide range of distinct metabolic changes in addition to clearly visible symptoms of deficiency in certain plant species.

Several reviews have proposed that boron is implicated in three main cellular processes: keeping cell wall structure, maintaining membrane function, and supporting metabolic activities. In higher plants, one of the primary functions of boron has been reported to be its capacity to form esters with apiose residues of rhamnogalacturonan II (RG-II) (Kobayashi et al., 1996). The formation of the RG-II complex is essential for cell wall structure and function (O'Neill et al., 2004) where it contributes significantly to the control of cell wall porosity and tensile strength. For instance, symptoms like swollen cell walls and a decreased

RG-II dimer formation have been reported to be due to boron deficiency (Ishii et al., 2001).

It is well known that boron deficiency can influence different diverse processes in vascular plants such as IAA oxidase activity, sugar translocation, root elongation, carbohydrate metabolism, nucleic acid synthesis, and pollen tube growth (Blevins and Lukaszewski, 1998, Goldbach and Wimmer, 2007). It has also been reported that boron inhibits one step of *in-vitro* pre-mRNA splicing reaction (Shomron and Ast, 2003). Reid (2007) suggested that boron toxicity is primarily due to disruption of RNA splicing. Recently, several studies have reported that boron deficiency affects the expression level of genes related to nitrogen metabolism (Camacho-Cristóbal and González-Fontes, 2007), oxidative stress, boron uptake, and cell wall development (Camacho-Cristóbal et al., 2008). However, no direct evidence is available to explain how the signal as a result of boron deficiency could be transferred to the nucleus. It has been proposed by Kobayashi et al. (2004) that a quick signal transfer from the cell wall to the cytoplasm could be involved for gene induction after the cellular redox imbalance imposed by boron deficiency. Changes in boron concentrations may lead to a cascade of signals extending into the cytoplasm via the cell wall-plasma membrane-cytoskeleton continuum, with the possible involvement of some surface proteins that are attached to the membrane via a glycosyl-phosphatidyl-inositol anchor like arabidogalactan proteins (AGPs) which were suggested to be putative B-binding structures (Goldbach and Wimmer, 2007). A putative role of boron was suggested by González-Fontes et al. (2008) as a cellular signal capable of interacting with transcription factors, which could explain the rapid alteration in gene expression of several genes that are involved in different physiological processes when vascular plants are subjected to boron deficiency. It is possible boron could be affecting calcium-mediated signalling (González-Fontes et al., 2008) as it is emerging that this is a common signalling pathway under abiotic stress.

Brassica napus is sensitive to boron deficiency, but there are significant differences in the response of various cultivars to boron deficiency (Fangsen X et al., 2002). The genetic and molecular basis for differences in boron efficiency

remains to be clarified even though two relevant genes (*BOR1* and *NIP5:1*) have been identified in *Arabidopsis* (Zeng et al., 2008).

The main objective of the present experiment was to identify the genetic basis of natural variation in the physiological and genetic response of swede to boron stress and low temperature. It is believed that this is the first study in this area regarding swede and will be the first to identify boron molecular influence on plant response to low temperature.

Temperature is one of the main environmental factors that not only affects plant growth but also affects plant responses to boron supply. However, the interaction of low boron supply with low temperature varies between plant species as they differ in their response to low temperature and their cold tolerance (Moraghan and Mascagni, 1991). It has been reported in several studies (Braekke, 1983, Hanson and Breen, 1985, Blevins et al., 1996, Subedi et al., 1998) that plant growth disruption appeared in fruit trees, forest trees, and field crops under low boron in association with cold weather. Also, damage symptoms observed due to low boron and low temperature stress include greater pollen sterility in wheat (Subedi et al., 1998), young leaves and shoot tip death in forest trees and injuries to leaves in sugar beet and oilseed rape (Ye et al., 1997). Low leaf boron concentration has been found to be closely related to frost injuries and shoot dieback and boron fertilization often reduced such symptoms (Braekke, 1983).

In contrast to field experiments, controlled environment experiments have shown how to prevent the effect of multiple factors of boron and temperature treatments and their interaction with plant growth. Numerous reports and experiments in controlled environments have dealt with plant response to low temperature, but few of these have simultaneously dealt with boron deficiency. In order to improve the physiological and molecular basis knowledge of frost hardiness induced by boron application, the present experiment studied frost damage to swede in relation to *BN115* (the cold-regulated downstream gene in *Brassica napus* which is homologous to *Arabidopsis COR15a* (Jaglo et al., 2001)) expression in response to boron applied and low temperature.

5.2 Materials and Methods:

Following results obtained in Chapter 4, the two extreme genotypes in their response to boron application and BH appearance, Gr19 and Ag31, were chosen to compare for their ability to tolerate frost under different boron fertilization levels.

5.2.1 Pilot study (Physiological impact of boron in relation to frost tolerance)

Prior to the main experiment, a pilot study was carried out in order to verify the ability of boron to enhance plant hardiness to frost with and without cold acclimation. In this experiment, the Relative Electrical Conductivity (REC %) was measured as an indicator of frost damage. The activity of the antioxidant enzymes catalase (CAT) and superoxide dismutase (SOD) were assessed as the most likely to be active and efficient protective mechanism against oxidative stress caused by chilling. Three Hoaglands hydroponic solutions were prepared, one with no boron added (0B), the second with 30 µM B L^{-1} (30B) (Noguchi et al., 2000b) and the third standard Hoagland (sB). The source of boron used was boric acid (H_3BO_3). One genotype (Gr19) was used in this pilot experiment and was sown in seedling module trays (24 cell, cell dimension 5 x 5 x 5 cm) in the glasshouse with a maximum air temperature of 20 ˚C and minimum 10 ˚C. Sowing media used was standard grade horticultural perlite (William Sinclair Horticulture Ltd) (Plate 5.1). Before germination and during very early growth stages up to 2 leaves, sB was used for watering plants to make sure that plants received all the required nutrients they need for initial growth before subjecting them to the varying boron treatments. Two to three weeks after germination (about 2-leaf stage), sB application was stopped and boron treatments (0B and 30B) were started. When plants had reached the growth stage of 3 - 4 leaves, plants within each boron treatment were divided into two halves, one kept in the glasshouse (NA), and the other moved to the cold room at 4 ˚C for cold acclimation (CA). After 14 day in cold acclimation, the REC % and the activity of CAT and SOD were assessed.

Plate 2.1: Swede plants sown in standard grade horticultural perlite in module trays.

5.2.2 Main Experiment. Swede cold tolerance and cold responsive gene (*BN115*) up-regulation affected by Boron application and cold acclimation.

With the exception of the boron treatments, the same growing conditions as in the pilot experiment were followed. Two swede genotypes (Ag31 and Gr13) were used in this experiment. Boron treatments were 0B (no boron added), 30 µM B L^{-1} (30B), and 60 µM B L^{-1} (60B) (Noguchi et al., 2000b). In contrast to the pilot experiment, plants received their respective boron treatment from sowing to sampling stage, so that plants irrigated with 0B Hoagland received no boron for the whole period of growth and relied only on boron carried over in the seeds.

The REC % was measured after cold acclimation (14 days). Leaf samples were collected to examine the impact of boron on the up-regulation of the cold responsive gene *BN115* at three different times during cold acclimation, time 0h (before plants were moved to the cold room), 24h of acclimation, and at the end of acclimation after 14 days. Samples were kept at -80 ˚C for later molecular analysis.

5.2.3 Frost tolerance evaluation.

The Relative Electric Conductivity (REC %) was estimated as an indicator of frost damage (see chapter 2). Leaf discs of 1 – 1.5 cm were cut and put into 75 mL boiling tube. Sub-zero temperature treatments (0, -2, -4, -6, -8, and -10˚C) were applied. Samples were held 2 hours at each temperature after which samples were removed. Tubes were inoculated with a piece of ice at 0 ˚C to prevent supercooling.

5.2.4 Antioxidant enzyme activity determination

This was a “look-see” investigation as it was not the main aim for this experiment. Plants remaining after collecting samples for the frost tolerance test were subjected to antioxidant enzyme analysis of Catalase (CAT) and superoxide dismutase (SOD).

5.2.4.1 Protein extraction and purification

Protein extraction was prepared as described by Ratkevicius et al. (2003). Each sample (weight of 1.5 g) was ground in a mortar and pestle containing liquid nitrogen. A solution containing 100 mM potassium phosphate buffer (pH 7) with 5 mM 2-mercaptoethanol was added in a ratio of 1 g: 3 mL. The mixture was filtered through Miracloth paper (Millipore, UK) and centrifuged at 13,000 rpm for 10 min at 4 ˚C. The supernatant was moved into a new microfuge tube (1.5 mL) and centrifuged again for 10 minutes then the clear supernatant was distributed in aliquots and stored at -80 ˚C.

5.2.4.2 Catalase (CAT) assay.

Volumes of 50 µL of each sample were added to each well of a 96-well plate, followed by 25 µL of H_2O_2 (40 µM) for each well. Then plates were incubated at 37 ˚C for 30 min. After incubation, a volume of 50 µL of reaction buffer containing: 100 µM Amplex Red reagent + 0.4 U ml-1 of HRP (Sigma-Aldrich, Poole, UK) was added to each well. Using a CytoFluor II fluorescence microplate reader (PerSeptive Biosystems, Framingham, USA) with excitation 540 nm and emission 590 nm, the resulting fluorescence was measured. CAT activity was calculated from a standard curve prepared from purified catalase (0-2000 U mL-1, Sigma, Poole, UK).

5.2.4.3 Superoxide dismutase (SOD) assay.

The assay mixture consisting of three reagents (xanthine, xanthine oxidase and cytochrome c) was dissolved in potassium buffer and stored at room temperature during use and at 4 ˚C between uses. 50 µL of each sample added to the each well of a 96-well plate. Some wells were also used as controls either by replacing the sample with SOD or with buffer. The assay mixture (250 µL) without xanthine was pipetted into each well and mixed carefully. Xanthine oxidase solution (100 µL) was added immediately before measurement. The wells were loaded as appropriate and readings started within 1 min of this addition of xanthine. The plate was immediately transferred to a VersaMax plate reader (Molecular Devices, Sunnyvale, CA, USA), and the increase in absorbance monitored at 550 nm using a kinetic program for at least 5 min. All data are expressed as unit mL^{-1}.

5.2.5 mRNA to cDNA, PCR and Real-Time quantitative PCR (qPCR).

Extracted RNA (see chapter 2) was quantified using the Nano-drop 1000 technique in order to estimate its concentration and then stored at -80 ˚C until further analysis. Using the High capacity RNA-to-cDNA kit (Applied Biosystems, PN 4352604), the first cDNA strand was obtained at the end of the reverse transcription process in a volume of 20 µL. cDNA was amplified using either ordinary PCR or qPCR. Forward and reverse primers for the cold responsive downstream gene in *Brassica napus* (*BN115*) (Jaglo et al., 2001, White et al., 1994) were designed with gene sequences obtained from Blast software. *Actin 1*, a constitutively expressed gene, was used as a housekeeping gene and to reduce variations due to gel loading pipetting in subsequent analysis. *BN115* gene expression was normalised against *Actin 1* within a lane.

5.2.6 cDNA amplification using ordinary PCR.

cDNA was amplified following the instructions provided by the kit manufacturer (Sigma-Aldridge) and following an ordinary PCR protocol. The Master-mix was prepared as below (for one sample):

- 1 µL Red taq polymerase.
- 2.5 µL Red taq polymerase buffer.
- 0.5 µL forward primer.
- 0.5 µL reverse primer.
- 0.5 µL dNTPs.
- 18 µL H_2O (molecular water, Sigma cat, w1754).
    - Total volume 23 µL.

The mixture was prepared for all samples at one time. 23 µL from the master mix was then added to 2 µL cDNA from each sample and mixed in a 1.5 mL nuclease-free PCR tube. The total volume of the reaction mixture (25 µL) was run in an Applied Biosystems, Veriti PCR machine. The final product was loaded in a 2 % agarose gel and run for less than 1 hour then examined under UV light (for more details see chapter 2 section 2.6.3).

5.2.7 cDNA amplification using qPCR.

A reaction mixture consisting of 2 µL cDNA and 13 µL master mix (7.2 µL Syber green, 0.3 µL reference dye, 0.15 µL of each forward and reverse primer, 5.2 µL molecular grade water) for each sample was prepared in a 96-well plate and run

using the Applied Biosystem StepOne Plus Real-Time quantitative PCR (qPCR). The same procedure was also applied for the endogenous gene Actin 1. The relative quantification was obtained for the expression of *BN115* against *Actin 1*.

5.3 Results

5.3.1 Pilot study: Physiological impact of boron and cold acclimation in relation to frost tolerance

Frost damage (REC %) was significantly ($P < 0.001$) affected by both boron application and cold acclimation (Figure 5.1). Applying boron resulted in an enhanced ability of swede to tolerate frost for both cold acclimated and non-acclimated plants. Plants that were either B-deficient or un-acclimated had the highest REC value indicating lower frost tolerance. Cold acclimated and boron application gave plants the highest tolerance to frost and these showed the lowest REC value indicating less damage. For non-acclimated plants, treatment with boron gave plants that were as tolerant (Average REC % of 58.2) as those that are cold acclimated but with no boron added (REC % of 55.5).

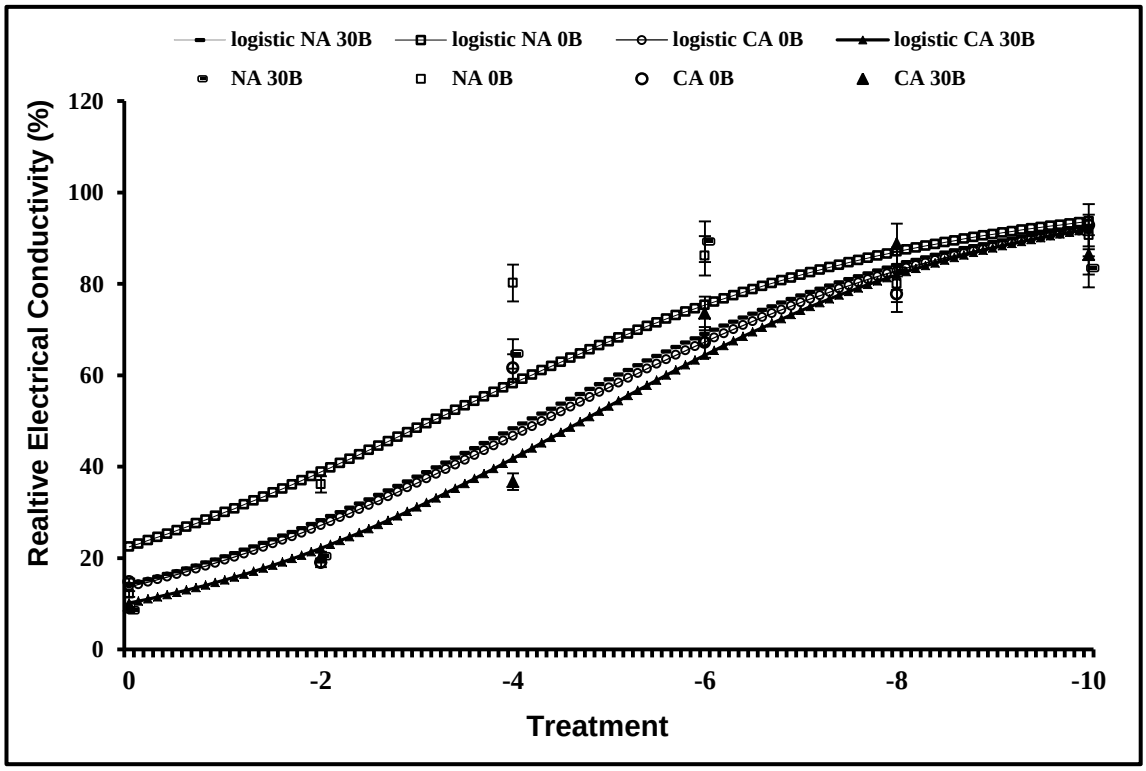


Figure 5.1: The relative electrical conductivity (REC) (logistic and actual values) for swede plants treated with 0 and 30 μML^{-1} boron under acclimating and non-acclimating 14 days growing conditions.

Boron application and cold acclimation also induced anti-oxidant enzyme system activity (Figures 5.2 and 5.3) which is one of the plant defence tools against chilling. CAT and SOD were significantly affected by both boron and acclimation and their interaction (Table 5.1).

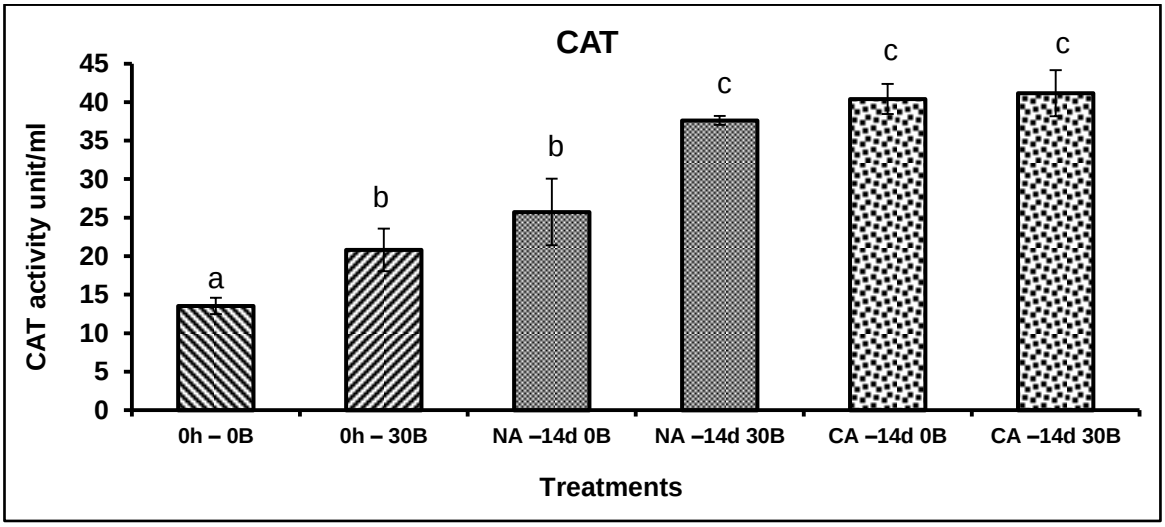


Figure 5.2: Antioxidant enzyme, Catalase (CAT) affected by boron treatments and cold acclimation in swede (*Brassica napus var. napobrassica*) seedlings and plants aged 7 – 8 weeks. Vertical bars represent ± S.E.M. Different letters above the standard error bars indicate significant differences between treatments.

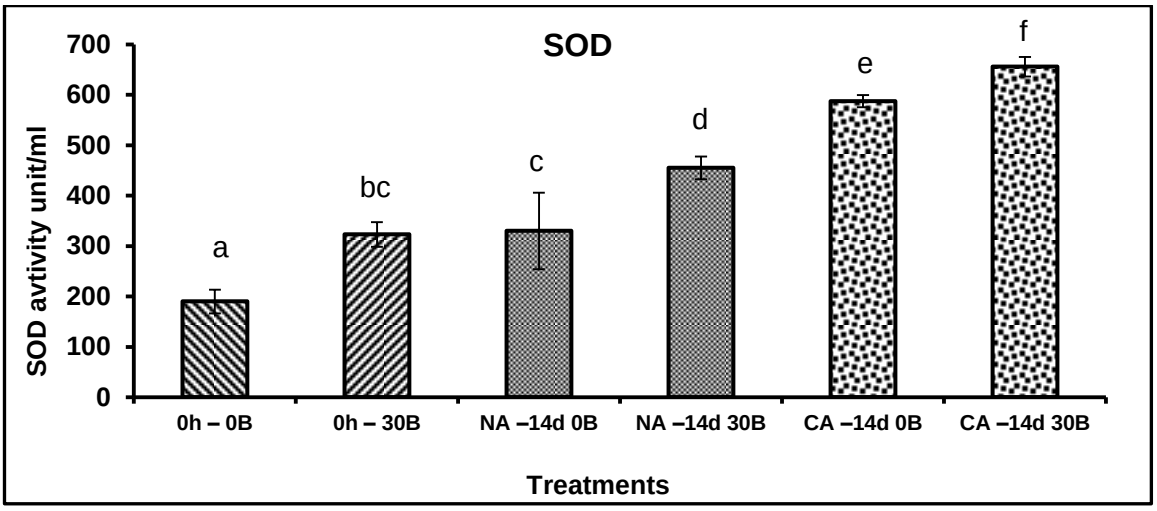


Figure 5.3: Antioxidant enzyme, Superoxide dismutase (SOD) affected by boron treatments and cold acclimation in swede (*Brassica napus* var. *napobrassica*) seedlings and plants aged 7 – 8 weeks. Vertical bars represent ± S.E.M. Different letters above the standard error bars indicate significant differences between treatments.

Table 5.1: Analysis of variance (S.O.V) results for catalase (CAT) and superoxide dismutase (SOD) affected by boron (B) application and cold acclimation (CA).

S.O.V	P value	
	SOD	CAT
B	0.000	0.001
CA	0.000	0.000
B x CA	0.000	0.011

There was a clear significant influence of boron alone and cold acclimation on swede plants ability to tolerate frost in the pilot experiment. This was interesting and motivated the investigation to determine more about swede response to boron with and without cold acclimation, and the role of boron in plant responses to low temperature.

5.3.2 Main experiment. Swede cold tolerance and cold responsive gene (*BN115*) up-regulation affected by boron application and cold acclimation.

In this experiment, both cold acclimation and boron application improved swede plants ability to tolerate frost. Cold acclimation significantly ($P < 0.02$) reduced the relative electrical conductivity (REC %) compared to non-acclimated plants under all levels of boron application (Figure 5.4). EL_{50} results (Table 5.2) showed an average difference of -1.5 ˚C between cold acclimated and non-acclimated plants. Ag31 and Gr19 both responded to boron application, and cold acclimation significantly ($P < 0.001$) improved their frost tolerance. Overall, Ag31 was more sensitive to frost and always had the highest REC % value compared to Gr13 under all treatments.

Analysis of variance for the REC % of both CA and NA plants showed highly significant differences ($P < 0.000$) between applied boron concentrations (Figure 5.5). Increasing boron application significantly decreased swede susceptibility to low temperature. Using multiple comparisons, One-way ANOVA revealed that under acclimation conditions, 60 µmB L^{-1} had no significant influence ($P < 0.090$) compared to 30 µmB L^{-1}, EL_{50} showed difference of -0.8

˚C between EL_{50} at 30B and EL_{50} at 60B for Ag31 and -0.1˚C for Gr19. For non-acclimated plants, 60 µm B L^{-1} significantly ($P < 0.034$) decreased frost damage compared to that which occurred for plants treated with Hoagland of 30 µmB L^{-1}. EL_{50} was reduced by -2.3 ˚C for Ag31 and -1.1 for Gr19.

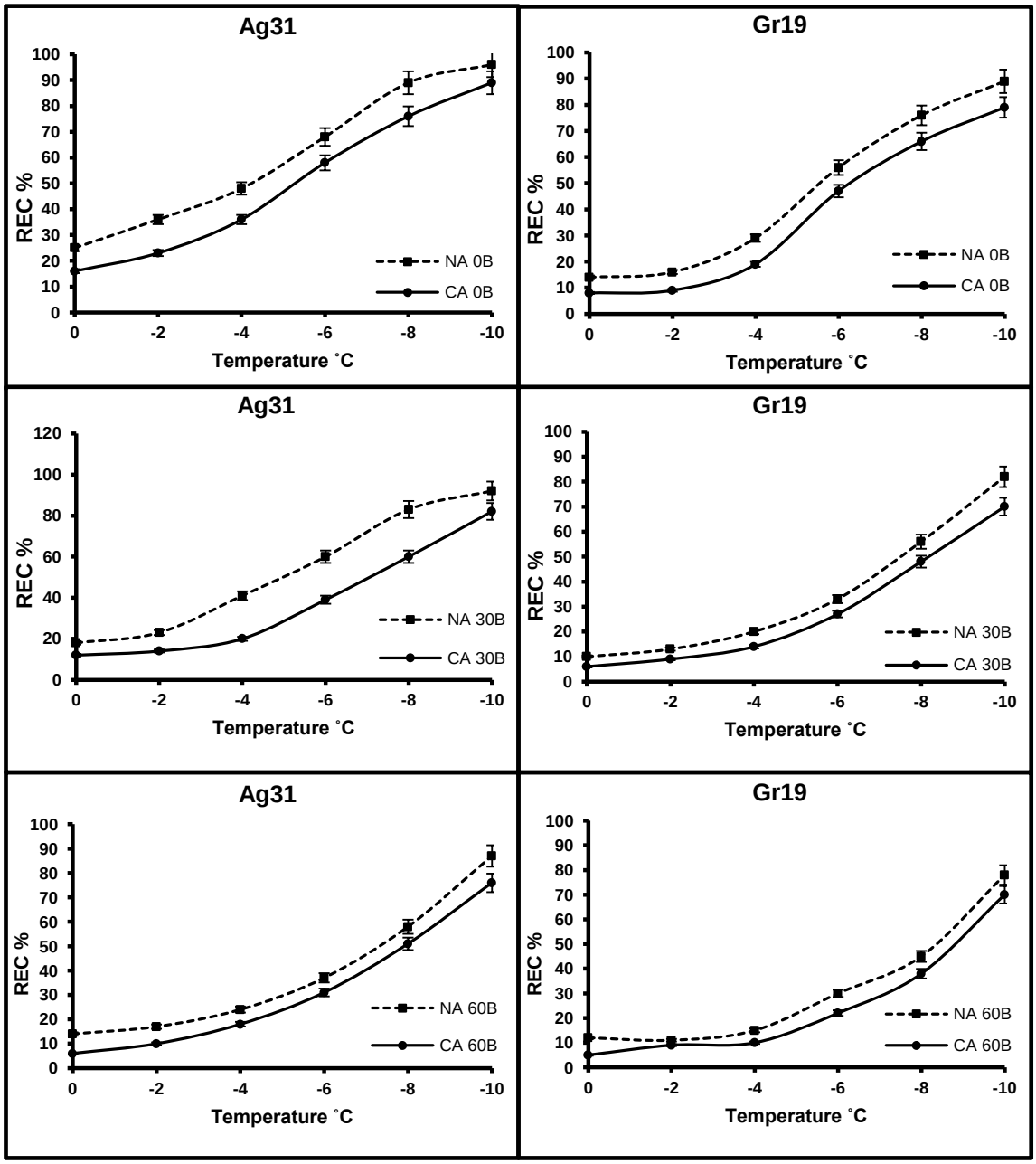


Figure 5.4: Cold acclimation impact on swedes plants response to frost under different levels of boron (0, 30, and 60 µmL^{-1}) in modified Hoagland solution. (Dotted line is for non-acclimated NA, the sold line is for cold acclimated CA).

Table 5.2: EL_{50} values affected by boron treatments and cold acclimation.

Genotypes	Boron treatment	EL_{50} NA	EL_{50} CA	ΔEL_{50} after acclimation	ΔEL_{50} affected by boron application		
					Compression sources	EL_{50} NA	EL_{50} CA
Ag31	0B	-4.3	-5.8	-1.5	0B vs. 30B	-1.2	-1.2
	30B	-5.0	-6.9	-1.9	0B vs. 60B	-3.0	-2.2
	60B	-7.3	-7.7	-0.4	30B vs. 60B	-2.3	-0.8
Gr19	0B	-5.4	-7.5	-2.1	0B vs. 30B	-2.3	-1.4
	30B	-7.0	-8.9	-1.9	0B vs. 60B	-3.1	-1.5
	60B	-8.0	-9.0	-1.0	30B vs. 60B	-1.0	-0.1
Mean		-6.1	-7.6	-1.5		-2.2	-1.2

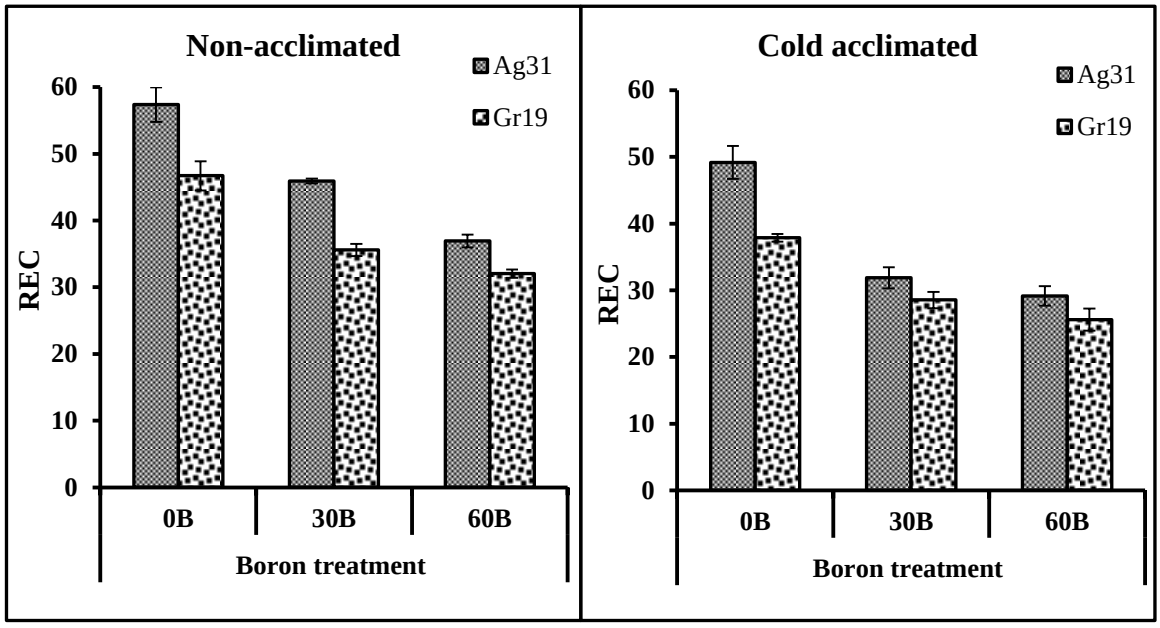


Figure 5.5: The relative electrical conductivity (REC %) affected by boron application under cold acclimated and non-acclimated conditions. Vertical bars represent ± S.E.M.

The REC for non-acclimated plants ranged between 57.0 % at 0B to 36.0 % at 60B for Ag31 and between 46 % at 0B to 32 % at 60B for Gr19. For cold acclimated swede, the REC % reduced from 43 % at 0B to 29 % at 60B for Ag31, and from 37 % to 25 % for Gr19 at 0B and 60B respectively (Figure 5.6).

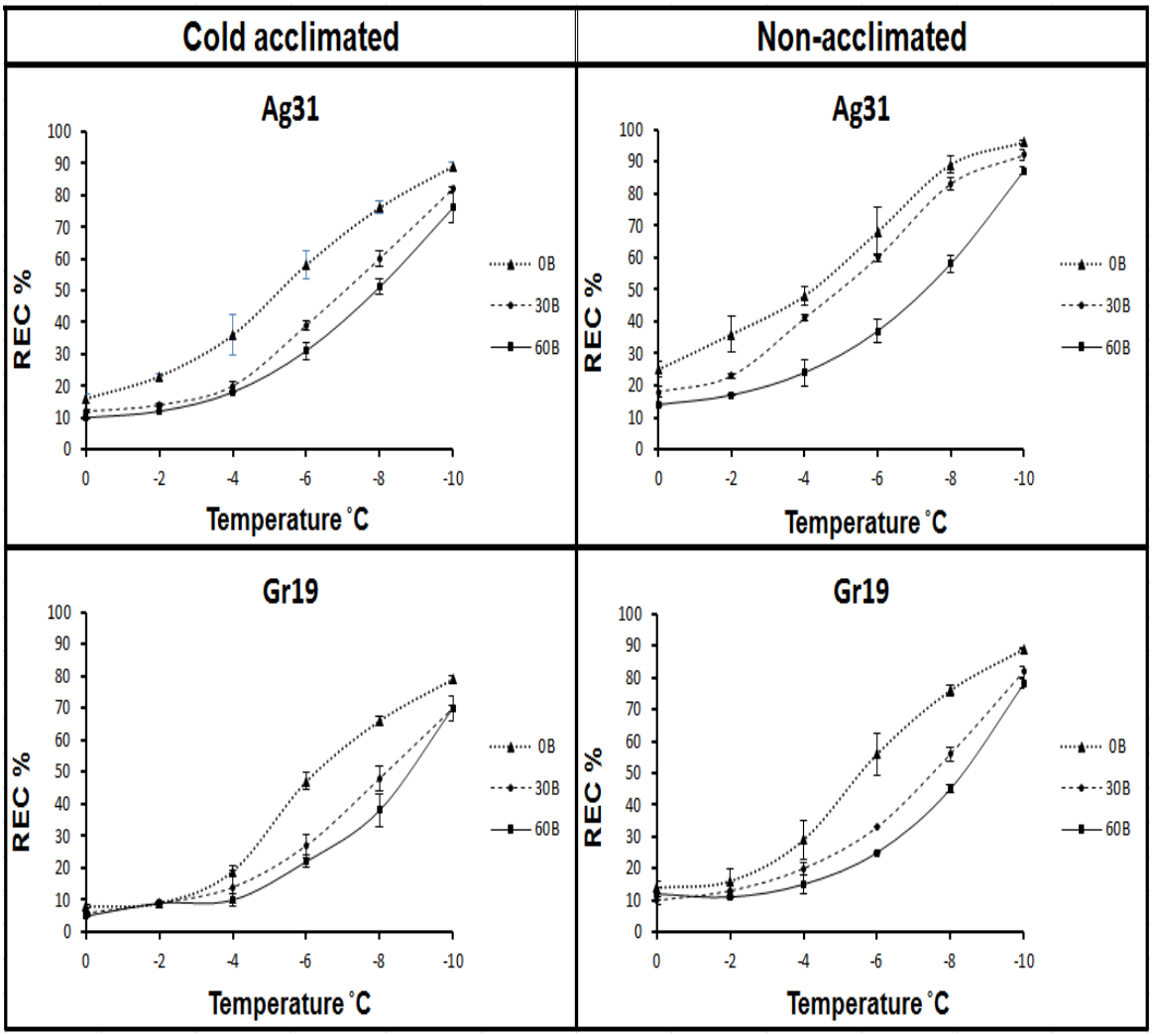


Figure 5.6: Frost damage measured as REC %, affected by different boron concentration (0, 30, and 60 µML^{-1}) for cold acclimated and non-acclimated plants of two of swede genotypes. Vertical bars represent ± S.E.M.

The physiological response of swede to low temperature was affected by cold acclimation and boron application and the differences between the genotypes used (Ag31 and Gr19) was evident and consistent. Molecular analysis results of the ordinary PCR (Plate 5.2) showed significant impact of the two factors on *BN115.*

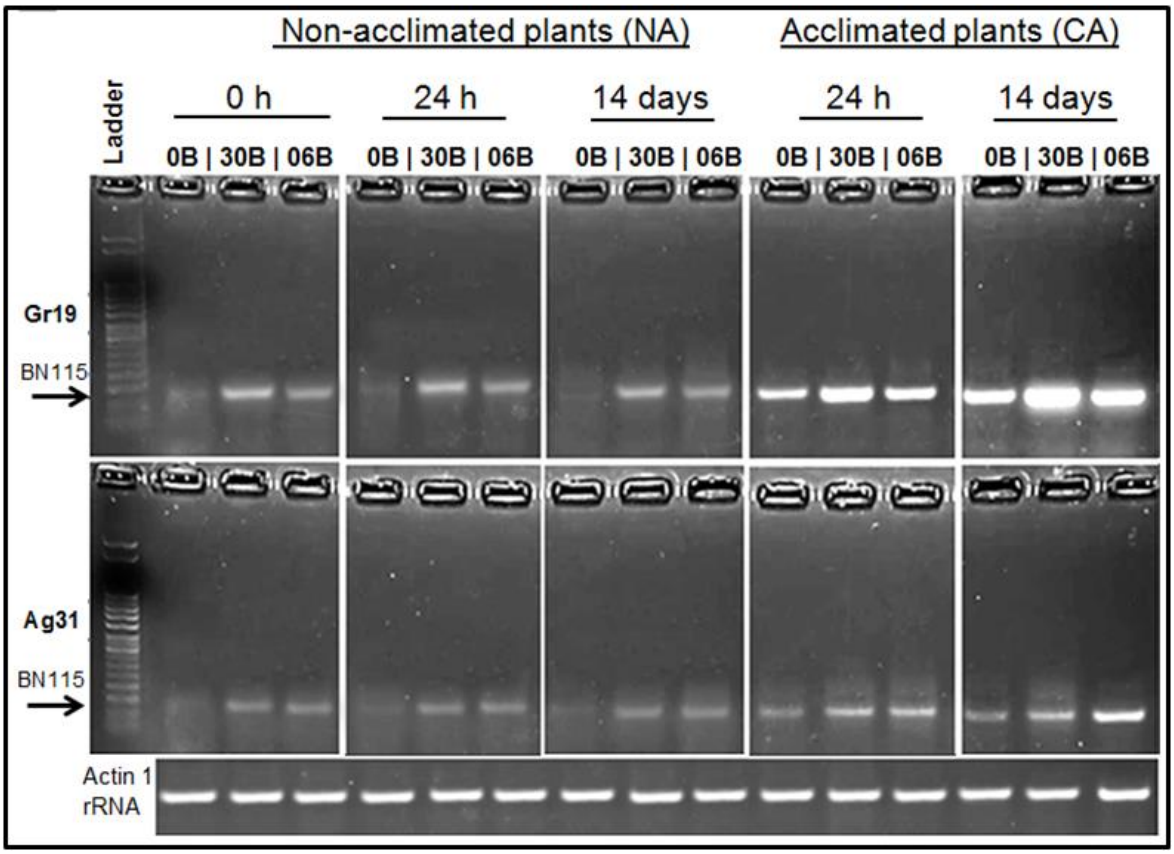


Plate 5.2: The expression of Brassica napus cold responsive gene (BN115) in leaves of cold acclimated and non-acclimated young plants of two swede genotypes (Ag31 and Gr13) grown hydroponically under three different levels of boron (0, 30, and 60 µMB L-1) applied using modified Hoagland's solution.

It was found that boron treatments significantly affected *BN115* gene up-regulation in both genotypes (Gr19 and Ag31) in both cold acclimated and non-acclimated plants. With no boron included in the hydroponic solution, non-acclimated swedes had a very weak barely noticeable band but 30B and 60B enhanced *BN115* up-regulation even in non-acclimated plants. Gr19 demonstrated stronger expression of *BN115* than Ag31. Gr19 responded differently and significantly ($P < 0.020$) to boron treatments and interestingly bands at 60 µM B L^{-1} were weaker than those in the 30 µM B L^{-1} treatment. Ag31 showed no clear differences between 30B and 60B in non-acclimated conditions.

Cold acclimation significantly ($P < 0.000$) up-regulated *BN115*. By the end of the cold acclimation period (14 days) gene bands were noticeably stronger than

those after 24 hour of acclimation. Comparing between boron treatments in cold acclimated plants, again Gr19 showed stronger band intensity at 30B compared to 60B and 0B whereas for Ag31 the band intensity progressively increased from weak at 0B to strong at 30B and then stronger still at 60B, however, it was always weaker than the Gr19 bands. Overall, the two genotypes differed in their response to boron treatments and cold acclimation, and Gr19 showed noticeably higher expression than Ag31.

Real time quantitative PCR (qPCR) (Figure 5.7) confirmed that *BN115* transcription levels increased markedly with cold acclimation and boron application. There was significantly high transcript abundance due to boron treatments and cold acclimation in both Gr19 and Ag31 and the response in Gr19 was greater than that in Ag31. Gene transcription level in acclimated Gr19 increased significantly ($P < 0.009$) by increasing acclimation time from less than one fold at 0 hour under 0B to 2.5 fold after 14 days acclimation. At 30B, it increased from 1.5 fold to 4.5 folds, while 60B was between these levels. Overall Ag31 showed non-significant ($P < 0.664$) differences between transcription levels with increased time of acclimation and overall it was between 0.5 to 1.5 fold. At 60B applied, *BN115* up-regulation level in Gr19 was significantly lower than that at 30B but both were significantly ($P < 0.020$) higher than 0B applied. In Ag31, 30B and 60B applied appeared at the same level however and both differed significantly ($P < 0.006$) from 0B applied. Non-acclimated Ag31 treated with 30B showed the same level of expression of *BN115* as 0B treatment with acclimation.

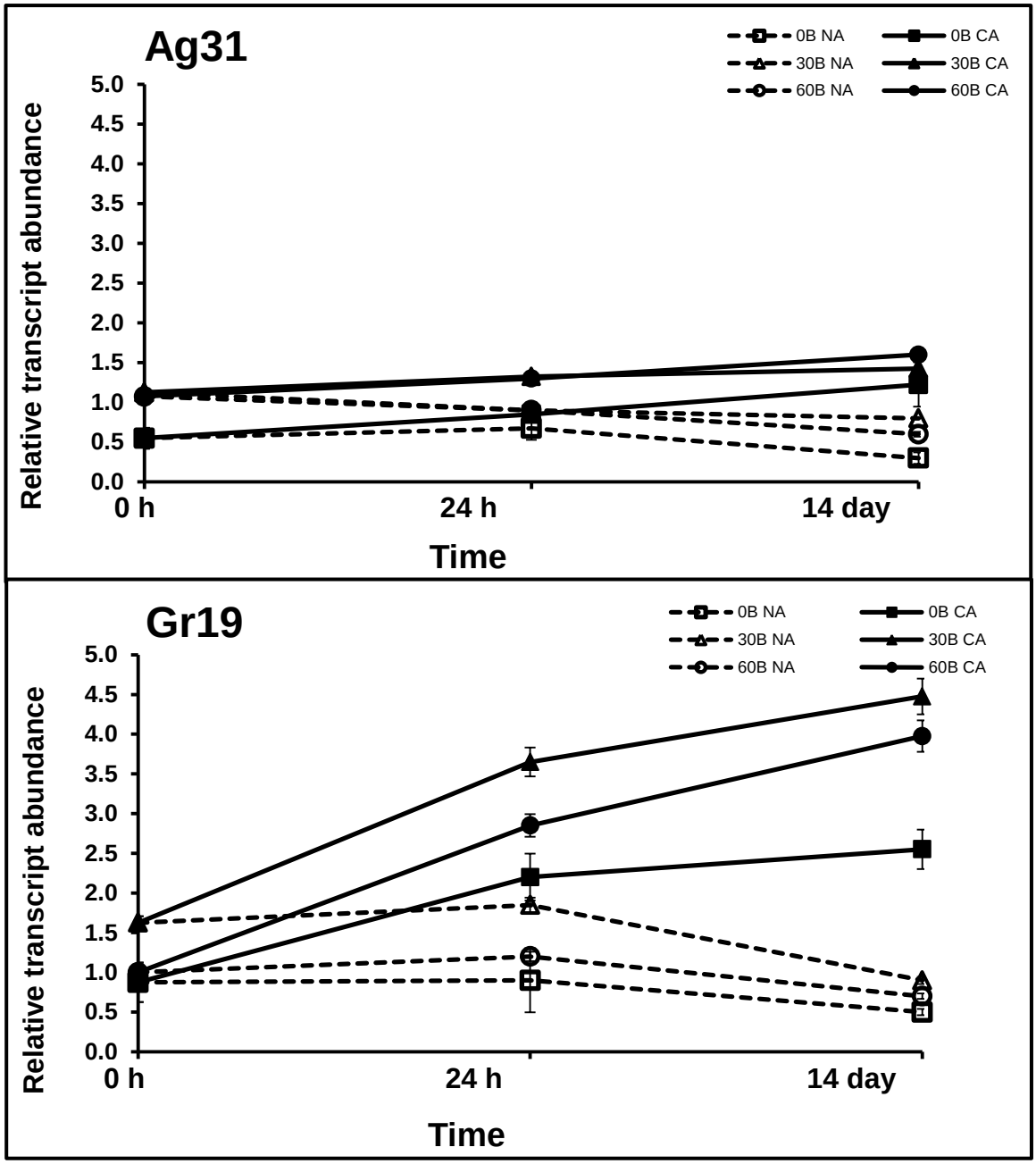


Figure 5.7: The effect of boron on *BN115* transcription abundance under acclimation (solid lines) and non-acclimation (dotted lines) in two genotypes of swede Ag31 and Gr19. Vertical bars represent ± S.E.M.

5.4 Discussion

Results obtained in chapter three linked BH appearance with low temperature susceptibility, and in chapter four the role of boron on BH incidence in the field was clearly demonstrated and these results were the motivation behind this investigation of the link between the two factors (boron and Low temperature) which might explain how they have a shared mechanism. A few published studies have mentioned the association between boron and low temperature and boron importance in improving freezing tolerance in plants. However, mechanisms responsible for such interaction and association between boron and low temperature are yet to be determined. In this study the Boron:low-temperature association was looked at in a different way than other studies previously have done

It is well known that the REC method is an indicator test for cell damage caused by sub-zero temperature. Compared to non-acclimated swedes, plants incubated for 14 day in a cold room at 4 ˚C showed higher ability to tolerate freezing treatments, EL_{50} significantly reduced by average of -1.5 ˚C, and this corresponds with Al-Issawi (2013) and Hadi (2010) who found the same in wheat and cauliflower respectively using the same assessment procedures. These results proved that the cold acclimation pathway exists in swede plants and this is the first study that has reported this. Acclimating plants improves tolerance since it alters the gene expression (Thomashow et al., 2001, Hughes and Dunn, 1990) and that was clearly evident in results obtained here. Swede genotypes responded differently and Gr19 showed better frost tolerance than Ag31 in both cold acclimated and non-acclimated conditions corresponding to earlier results presented in Chapter 3. Al-Issawi (2013), Donoso Ñanculao et al. (2013), Fahleson et al.(1994) and Guy (1990) also referred to the differences between genotypes in their susceptibility to frost and such quantitative responses are not uncommon in crop plants. Applying boron increased swedes hardiness and EL_{50} reduced by -2.3 ˚C for Ag31 and -3.1 ˚C for Gr19 with the treatment of 60B compared to 0B. (Han et al., 2008, Eaton et al., 2007) also studied frost damage for B-deficient plants, in citrus and blueberry respectively, and reported their susceptibility to frost compared to B-sufficient plants. It is claimed that an important role in maintaining the integrity of plasma membranes

and to lessen the damage of membranes due to low temperature is controlled by boron (Wang et al., 1999). Stoker and Tolman (1941) were the first to speculate that damage in plant tissues due to frost is associated with low boron status in plants in the field. Such a relation was then mentioned by Cooling and Jones (1970) on forest trees and by Hanson and Breen (1985) on fruit trees. Results presented in this Chapter support these previous studies with acclimation and boron application both increasing cold tolerance as measured by membrane damage. Interestingly boron treated swede plants under non-acclimated conditions were as cold tolerant as B-deficient which had been cold acclimated. This suggests that boron application can have an influence almost equivalent to cold acclimation. This was supported by the preliminary antioxidant results mentioned in the pilot study where both cold acclimation and boron induced and increased catalase and superoxide dismutase antioxidant enzyme levels which also supports previous findings in wheat plants (Baek and Skinner, 2003). Camacho-Cristóbal and González-Fontes (2007) however referred to catalase increasing but not superoxide dismutase due to boron treatments in tobacco plants. The antioxidant results need more investigation but have been mentioned here to support the cold tolerance results and clearly gives a good indication of the importance of boron in plant physiological response to abiotic stress. In all previous physiological studies results regarding the influence of boron on plant response to low temperature have been mentioned but not verified and this has only been proven for the first time in the current study. The unique step taken in this study in investigating the role of boron *in-planta* was the molecular analysis by PCR and real time qPCR which showed firstly that the cold responsive downstream gene in *BN115* was present in the swede genome and that it was clearly affected by Boron. Its expression was up-regulated at 30B and 60B under both cold acclimating and non-acclimating conditions. There was an interesting match between the physiological and molecular analysis, where non-acclimated swedes that were B-sufficient showed transcription levels of *BN115* equivalent to that of cold acclimated B-deficient swedes. However for high expression of *BN115* both boron and cold acclimation were necessary. There was an additive effect of boron application and cold acclimation where increases in transcriptions level were observed with cold acclimated and B-sufficient swedes compared to those

for boron only or for cold acclimation only. The two genotypes studied also clearly differed in their responses. The less frost tolerant genotype, Ag31, showed increasing response to boron from 0 to 30B to 60B applied, while the more frost tolerant Gr19, showed quantitatively higher transcriptions levels than Ag31 but there was lower expression at 60B compared to 30B. Such results could indicate that differences in boron sufficiency/toxicity thresholds exist between genotypes.

Chapter 6:

General Discussion

The study presented here is a part of an ongoing programme of swede breeding in the UK. The commercial partners in this project, seed producers and plant breeders Elsoms Seeds Ltd, and vegetable growers Coles Ltd, were very concerned about BH disorder causing large financial losses. Frequent discussions, meetings and several field investigations in addition to two visits to the breeding company resulted in the following understanding:

Swede growers are losing around ten thousand pounds per field if their crop became affected with BH. Growers are accusing seed producers and plant breeders for occasionally providing unstable varieties that are sensitive to BH disorder. Swede breeders were confused about how to breed against BH. In its current presentation in the field, BH has no exterior-associated symptoms, neither on the vegetative growth nor on the visible part of the root and this is also reported in the historical literature (Gupta and Cutcliffe, 1971). Therefore, there appeared to be no clear phenotypic differences between healthy and affected plants, and BH appearance is sporadic and unpredictable in the field. This has made it difficult for the breeders to select BH resistant plants. Another complicating factor is that breeders must grow their genotypes in the field for approximately 3-4 months (until harvest stage) and then cut the root to check for BH and even if a sampled plant was healthy there is no way to replant it for seed production. The process of field evaluation is therefore expensive and time-consuming and may well eventually fail to produce any significant advance in breeding for BH resistance.

BH disorder has been traditionally attributed to boron availability to swede plants, being firstly reported by Hurst and Macleod (1936) and studied and confirmed later by several researchers across the world (Gupta and Cutcliffe, 1971, Beauchamp and Hussain, 1974, Umesh and Cutcliffe, 1978, Cutcliffe and Gupta, 1987, Sanderson et al., 2002). Chemical analysis of symptomatic roots randomly collected during field investigations showed that there was a reduction of 14% in boron concentration in BH affected roots compared to healthy roots. However, plants that showed vegetative boron deficiency symptoms did not always show BH and mostly their roots were clear. On the other hand, healthy looking plants are reported to show brown discoloration of BH varying from slight to severe. In a more recent study Gupta (2007) concluded that boron

deficiency symptoms can naturally occur even when an adequate amount of boron was supplied. Given such contradictory observations from several academic studies it is unsurprising that the breeders have not been successful in reducing or eliminating BH in their gene stocks.

Although metabolic disorders are associated with the roles controlled by the essential element in plant metabolism and functions, an inadequate supply results in a nutritional disorder reflected by characteristic deficiency symptoms (Du et al., 2002). However, BH disorder and boron appeared to be excluded from this general "fact" or at least it is not 100 % applicable to this case. Based on the author's field investigation notes, and the literature cited here, in combination with the growers and breeders experiences of commercial swede production for more than ten years, there are no external nutrient deficiency symptoms consistently associated with BH. Hence, it is therefore strongly suggested that boron deficiency symptoms are not useful signs for BH incidence in the field.

Relationship between low temperature and BH:

The brassicas are generally considered as "cool season" plants which means they are relatively tolerant to "mild" sub-zero temperatures (Guerena, 2006). Although swedes are well adapted to some severe environmental conditions, following the very hard winter in 2009/10 field grown swedes were anecdotally reported by the breeders to show frost damage accompanied with the internal browning discoloration of BH. It was therefore postulated that if an association (physiological or genetic) can be determined between BH appearance and another more easily assessed trait such as frost susceptibility, then a frost screening test might be developed as a useful surrogate method to screen for BH resistance. Theoretically, there is always a possible link between some plant traits as there are always several genes with different functions located on one chromosome and they are largely moving together from one generation to another depending on the recombination frequencies between the genes. To the author's knowledge, there have been no major gene locations or QTL's identified for BH and no mapping carried to a particular chromosome in the Brassica genome. Furthermore it has been clearly documented in studies

carried out by the plant breeders, as well as molecular biologists that plant responses towards stresses are generally multigenic in nature (Vaishali et al., 2010) and genes are often distributed throughout the genome.

To the best of the author's knowledge, the current study was the first study conducted to test swede's ability for frost tolerance. This was done by the screening of 11 genotypes at the seedling stage for their ability to recover after freezing. Results showed that -8 ˚C was the lowest temperature that swede seedlings were able to recover from and that -10 ˚C was 100 % lethal at this growth stage. LT_{50}'s for swede were determined to be between -6.1 to -8.3 ˚C.

Cold acclimation is required to induce cold tolerance and alters gene expression to provide protection at all levels and is accompanied with increased levels of osmoprotectant accumulation due to cellular and metabolic changes (Thomashow et al., 2001). Members of the *Brassicaceae* family like *Arabidopsi*s, have been proven to have the CBF cold-responsive pathway that is induced by cold acclimation leading to improved tolerance to frost. That pathway had not been proved to exist in swedes before this study. For cold acclimated plants in more advanced growth stages, the EL_{50} of cold acclimated swedes ranged between -7.0 to -10.0 ˚C while non-acclimated plants ranged between -5.8 ˚C to -7.5 ˚C. This clearly showed swede's ability for acclimation and suggests that that the CBF cold-responsive pathway is closely related to that characterised in *Arabidopsis*. Swede genotypes clearly varied in their ability to tolerate frost and could be divided into susceptible and tolerant. Following the postulated hypothesis of linking susceptibility to low temperature with the susceptibility to BH, frost susceptible genotypes were clearly of interest in order to prove an association between the two traits.

Out of 11 genotypes, Me77c and Or13 were classified as the most cold sensitive by showing the highest LT_{50} (- 6.15 ˚C and - 6.08 ˚C respectively). Me77c and Or13 also had the highest EL_{50} of -7 ˚C. It was therefore planned to test these genotypes for their susceptibility to BH in the field.

Before starting the field trial, historical records for BH were provided by the plant breeders for scrutiny. Data (n = 353) for a ten year period were collected for BH appearance in the field in three different locations (two in the UK and one in

Germany). Searching this data and looking at the genetic history of the BH affected genotypes, it was found that 48 % of the genotypes which were frequently showing BH had Me77c and/or Or13 in their genetic background. The remaining 52 % was allocated for genotypes that are progeny of Ag31 (37%) and the rest (15%) were spread across a mixture of genotypes with different genetic backgrounds. Although Ag31 was not included in the initial frost screening, it was tested in the last experiment of this project (Chapter 5) and was confirmed to be frost susceptible. Although there were differences between growing conditions in the first frost test (Chapter 3) and the last test (Chapter 5), Ag31 showed EL_{50} of -6.9 ˚C in cold acclimation conditions, and -5.0 ˚C for non-acclimated plants. These frost tolerance scores were for Ag31 grown at 30 µM B L^{-1} (0 µM B L^{-1} and 60 µM B L^{-1} considered as extreme boron treatments). In the first frost test, cold acclimated Me77c and Or13 had an EL_{50} of -7.2 ˚C and -7.3 ˚C respectively while it was -5.8 ˚C and -6.5 ˚C respectively in non-acclimated conditions. This clearly demonstrated that, by comparison, Ag31 is among the 'sensitive to freezing temperatures' group of swede genotypes. Thus the three genotypes Ag31, Or13, and Me77c are the most susceptible to frost, and these between them comprised the most common pedigrees that had BH in their roots during the 10 year period of the breeders' trials. Thus the hypothesis that frost susceptibility and BH are associated appears to be applicable.

Genotypic BH response affected by boron application in the field:

Outcomes from the field trial provided some new information that has not been reported before regarding BH appearance in the field. Results clearly demonstrated the impact of boron availability on BH incidence and severity. A reduction of 44.2 % in the incidence of BH was recorded due to increased levels of boron applied from (0 kg B ha^{-1} to 2.7 kg B ha^{-1}). The severity of the browning discoloration was reduced and in some genotypes disappeared entirely when boron application level was increased. In this study, it was reported for the first time, that BH severity was significantly negatively correlated to root concentration of boron but that the relationship differed in different genotypes. According to these results, it is suggested that 'boron stress' is the boron concentration level in plant tissue at which a physiological disorder will appear

but it is important to recognise that the precise level varies for each particular genotype.

The anatomy of BH brown spots have not been investigated in detail in this study but light microscopy sections showed no clear differences between the BH affected part of the root and non-affected parts. So far, there is still no scientifically-based explanation of why this internal browning is happening. The author suggests, as BH incidence and severity was affected by boron, it might be possible to explain BH appearance as a result of a metabolic disruption due to the lack of boron in affected parts. It is known that boron deficiency induces the formation of callose and might block the sieve plate pores and result in the impairment of sugar transportation (Taiz and Zeiger, 2002). Another postulated role of boron is in cell wall synthesis and membrane maintenance and it has been suggested that boron deficiency might result in a 'cascade effect' due to interruption of a critical and central cellular process (Marschner, 2012). An inhibition of root growth and reduction in dry weight occurs due to boron deficiency, Han et al., (2008) and Loomis and Durst (1992) reported abnormalities in the cell wall and organisation of the middle lamella as the first symptoms of boron deficiency.

The impact of genetic factors on BH symptoms was clearly evidenced by the phenotypic variation between the genotypes used in the field trial. Out of 12 genotypes (7 parent lines and their 5 F_1 hybrids) used in the field trial, only six showed BH affected roots, the others were completely healthy. Of the six showing BH; Ag31, Or13, and Me77c were confidently demonstrated to be the main genetic source of BH susceptibility by pedigree analysis. These three parent lines clearly passed their susceptibility to BH to their F_1 hybrids that were combinations of these parents (that is: ms.Or13 X Me77c and ms.Ag31 X Or13). Furthermore it was interesting to note that susceptibility to BH and boron deficiency was clearly associated, in the same lines, with susceptibility to frost. Indeed, Ag31 had the highest EL_{50} (most sensitive to frost) among the three susceptible genotypes, and it was the only one that kept showing BH under all boron application levels despite the reduced severity at the highest level 2.70 kg B ha^{-1}. Added to this, Ag31, ms.Ag31 and ms.Ag31 X Or13 were the only three genotypes that showed the severe discoloration category of BH.

Furthermore, Me77c which had a lower EL_{50} than Or13 (Chapter 3) always showed a lower percentage of BH affected roots than Or13 and never showed more than slight internal browning while Or13 showed some moderate browning and even some severe BH when it was in combination with Ag31 in the F_1 hybrid (ms.Ag13 X Or13).

It was also interesting to note that genotypes that were shown to be more frost tolerant like Gr13 (Chapter 3) had no BH at all in the field trial and if they were in any F_1 hybrid combination together or with any of the frost susceptible genotypes (Ag31, Or13, and Me77c) their hybrid also showed resistance to BH. These findings were also supportive of the postulated hypothesis reflecting the other side of the hypothesis, that is, that frost tolerant genotypes are resistant to BH. It can be confirmed that, the hypothesis is applicable and screening for frost tolerance can be used as a surrogate method to screen against BH susceptibility.

As discussed above susceptible genotypes passed their susceptibility to their F_1 hybrids only if crossed with another susceptible genotype but the resistant genotypes passed their resistance to their F_1 hybrids and were able to mask susceptibility in hybrids between resistant and susceptible types. These findings demonstrate that BH resistance is a dominant trait and the resistant genotype for this trait can be considered as BHBH or BHbh while susceptibility can be considered recessive bhbh. This is a classical breeding view of resistance but it is important to note that resistance is probably controlled by more than one gene as it showed quantitative variation. That clearly shows the importance of genotypic impact on BH appearance in the field but also highlights other factors (especially boron availability) that can affect BH appearance and its severity.

BH association with Low temperature and boron refers to an association between the two abiotic factors

BH appearance and plant susceptibility to freezing temperatures was shown to be associated and BH clearly appeared as a genetic trait affected by boron application level. This raised the important question that, if boron improved swede resistance to BH, will boron affect and improve swede tolerance to freezing? And if so how is this achieved?

Across all the results obtained in this study regarding the tolerance of swedes to low temperature, it was clearly shown that the ability to tolerate frost was associated with its ability to be cold acclimated. In the literature whilst cold tolerance induced by cold acclimation has been studied in many plant species and investigated in detail, the study of the impact of mineral nutrition on plant response to low temperature is very rare. Al-Issawi et al (2013) recently reported a study of the impact of molybdenum on the response to frost and found a positive impact in wheat. In the current study, results clarified that B-sufficient plants were more tolerant to frost than B-deficient plants. As such, boron application improved EL_{50} values in both non-acclimated and cold acclimated plants. Improved plant response to low temperature by boron has also been reported in some of the literature (Han et al., 2008, Eaton et al., 2007) but none has suggested an explanation for this observation.

For any plant to be tolerant to freezing there is a metabolic mechanism involved that makes plant cells hardened against frost. The exposure to cold but non-freezing temperatures was found as a promoter for this defence mechanism, and swede was shown here for the first time to have a cold responsive pathway and the potential to be cold acclimated. Many cellular and metabolic changes accompany cold acclimation including increased levels of accumulation of osmoprotectants and altered gene expression to provide protection at all levels of metabolism (Thomashow et al., 2001). It is suggested, that to understand the influence of minerals on frost tolerance ability then it is useful to find any matches between mineral roles on cell metabolism and cold acclimation influences on plant cells.

The plasma membrane is considered as the primary site of injury during freezing and membrane lipid composition is widely considered to be important in tolerance to low temperature. During cold acclimation, cellular and metabolic changes that occur include increased levels of soluble proteins, proline, sugar, and organic acids and altered lipid membrane composition (Hughes and Dunn, 1990). Boron plays a key role in sugar transport and carbohydrate metabolism, and in membrane stability. Boron is required for membrane integrity and function in addition to its importance for the formation and maintenance of membrane potentials (Taiz and Zeiger, 2002). In relation to this, freezing of plant tissues results in extracellular formation and growth of ice crystals which then inflict a dehydrative stress on cells. It could be suggested that sufficient boron concentration in the cell might make the membrane potential enough to tolerate such dehydrative stress. Injury due to freezing can be caused by lesions in the plasma membrane that result in loss of osmotic responsiveness during subsequent thawing and deficiency in boron was found to alter membrane permeability for ions and other solutes (Wang et al., 1999, Cakmak et al., 1995). The author suggests that this indicates the role of boron as a potential explanation of plant response to low temperature. This requires further investigation but is a working hypothesis given the results presented here.

Another aspect of dehydrative stress is the risk of oxidation, and antioxidant enzyme activity is described as a defence tool against low temperature and furthermore is found to be activated during cold acclimation in several plant species including wheat (Janda et al., 2002, Baek and Skinner, 2003), maize (Hodges et al., 1997), chickpea (Ardic et al., 2009) and oilseed rape (*Brassica napus* L.) (Ashraf and Ali, 2008). The relative membrane permeability (RMP) was found to be associated with antioxidant enzyme activity, and lower permeability was related to the higher activities of antioxidants. In this study, like cold acclimation, boron application increased antioxidant levels and improved cold tolerance and it is possible that the boron impact on cell membranes might be due to its impact on antioxidant concentration. In the pilot study reported here cold acclimation also resulted in a significant increase in antioxidant concentration (SOD and CAT) when in association with boron application.

From a molecular perspective, for the first time, cold acclimation was found to up-regulate the cold responsive pathway in swede, where genes that are responsible for increased cold tolerance were switched on. Thomashow et al (2001) working with *Arabidopsis* was one of the first to demonstrate changes in gene expression in response to low non-freezing temperature (i.e. CBF controlled upregulation of *COR15a*) and subsequently this has been shown to increase freezing tolerance in transgenic *Brassica napus*. The cold responsive gene studied in the current project was *BN115* which is homologous to the *COR15a* in *Arabidopsis* (Jaglo et al., 2001) and was proved to exist in swede for the first time in this study. Studying the expression of this cold responsive gene was necessary to determine if boron influence is just a physiological influence or whether it is a molecular effected response.

It was shown that *BN115* was significantly affected by boron application levels. Ordinary PCR results at 0B showed a very low expression of which could mean that *BN115* is constitutively expressed in swede at a low level that could explain why swede is classified as a 'cool season' plant for its ability to tolerate some freezing even when non-acclimated. As presented earlier, boron fertilized plants were more tolerant to frost and *BN115* expression was higher at 30B and 60B compared to 0B. Several other studies have also described the impact of boron on the expression levels of some plant genes (Camacho et al., 2008) and Redondo-Nieto et al. (2001) and Camacho-Cristóbal and González-Fontes (2007) demonstrated that boron had an influence on genes related to nitrogen metabolism whilst Kobayashi et al. (2004) reported oxidative stress responsive genes were affected by boron nutrition.

An indirect influence of boron on gene expression might also be involved since nucleic acid concentration has been shown to be affected by boron. B-deficient plants contained low levels of DNA and the synthesis of DNA was also decreased and its synthesis inhibition can be attributed to either a primary or secondary effect of boron deficiency (Taiz and Zeiger, 2002). RNA content was also decreased in tomato plants grown under low boron whilst the RNase activity was increased (Dave and Kannan, 1980). In this study, it can be confirmed that boron affected the expression of cold responsive gene in *Brassica napus*.

Interestingly, *BN115* expression in Ag31 was always lower than that in Gr13 which reflects the difference between their ability to tolerate frost. In Gr13 at 60B, *BN115* expression presented in qPCR results showed a lower level than that at 30B while it was higher in Ag31 but Ag31 remained less frost tolerant than Gr13. This might explain the differences in apparent toxicity levels of boron. In some cases, boron application was equivalent to cold acclimation in term of *BN115* expression and had the same influence as cold acclimation in terms of improving plant tolerance to frost.

Apart from BH appearance being affected by boron application, none of the findings described in this thesis have been demonstrated in any previous study on swede. In particular the report here that boron affects the *B. napus* cold responsive gene *BN115* is novel. Furthermore results obtained separately in different experiments done over four years were supportive of each other.

It can be confidently stated therefore that the aim of the project was achieved and BH was proven to be related to low temperature tolerance and boron availability, and that there is a strong genetic component to BH resistance. In other words swede responses to low temperature and boron are physiologically and genetically linked.

Chapter 7:

Conclusions, Limitations, and Future Work

7.1 Conclusions:

The main aim of this study was to investigate the genetics and the physiology of BH abiotic disorder in swede and to investigate a possible strategy to assist plant breeders in the breeding of swedes resistant to BH. This study had the advantage of access to some of the breeding material from Elsoms Seeds Ltd with a diversity of genotypes (n = 18) which covered the main breeding lines in use in the UK and some new F1 hybrids. Elsoms Ltd also made available a wide range of trials data collected over 10 years (2000 – 2010) in three different locations in two countries. Added to this, the researcher was introduced to and had direct contact with a leading swede grower and their fieldsman and consultant agronomist.

The points presented below summarise the conclusions from the study:

1- From the literature and from previous anecdotal field treatments by growers, BH is considered a serious abiotic disorder which appears due to boron deficiency and growers to date apply great amounts of boron in both soil applied fertiliser and post-emergence spray treatments to guard against BH, but still complain about sporadic unpredictable BH appearance in crops. The initial work presented here attempted to link BH disorder appearance in the field with any unusual morphological growth due to boron deficiency but this failed to note any consistent signs or symptoms.

2- In an extensive field investigation boron was shown to be important to reduce BH severity, and the severity of internal browning was significantly correlated with the concentration of boron in roots, but it did not overcome the problem entirely, and it was not the main factor controlling BH *per se*.

3- BH appearance in the field was shown to be much more associated with swede genotype and susceptibility demonstrated classical Mendelian inheritance as a recessive trait moderated by boron availability and resistance as a dominant trait. Thus the genotype for resistance was suggested as BHBH or BHbh and susceptible considered as the recessive bhbh. Some quantitative variation in BH appearance was clear

and so BH resistance/susceptibility could not be considered a single gene effect.

4- It can be recommended that the breeders either eliminate the less frost tolerant and more BH susceptible parent lines from their breeding strategy or, if they are to be used because of other desirable traits then they are only combined with a resistant parent line so that the BH susceptibility is masked in the heterozygous F1.

5- Cytoplasmic male sterility which has been used by the breeders to facilitate the creation of F1 hybrids had no discernible impact on genotype susceptibility to BH and it is confirmed that this is therefore acceptable for swede breeders to use in swede hybrid production programmes without a concern that it may affect BH susceptibility.

6- Frost screening results confirmed that swede is a cold season plant with some freezing tolerance in an un-acclimated state and with good adaptation to moderate to severe freezing ranging between -6.5˚C to -10˚C when acclimated. Growing conditions affected swede response to frost and demonstrated that swede plants have the ability to cold acclimate.

7- Susceptibility to BH in swede was shown to be associated with susceptibility to frost and this was cross-confirmed in 3 separate assessments. Technically, breeding for frost tolerance is easier compared to breeding for BH resistance and this suggests therefore that frost tolerance screening could be a useful surrogate method to breed for BH resistance.

8- Swede was proven to have the cold responsive pathway under acclimation conditions and the cold responsive gene *BN115* isolated in oilseed rape (*Brassica napus)* and homologous to *COR15* in wheat and other cereals, was shown to be present in swede by this study.

9- Under controlled conditions boron application was shown to improve swede frost tolerance and both boron application and cold acclimation induced the molecular cold response mechanism.

7.2 Limitations in the study:

A number of challenges were evident in this study which both limited the scope of the work and present a need for further work to confirm or corroborate the findings. The swede crop is a fairly unusual target for this type of study and good quality published literature on either swede or BH is scarce at present. In addition, as swede is considered a minor crop, gene databases to support crop improvement work are currently lacking. Furthermore the study poses a number of questions which will require further research to elucidate them.

For the frost tolerance screening, it was recognised that it was necessary to start with as large a number of genotypes as possible to have an acceptable screen and to include a wide range of genetic variation. Elsoms Seeds kindly provided seeds of 11 genotype that they had available in their breeding program but unfortunately they either had no or very limited stocks of some of their crosses and parent lines and they were unable to provide a complete factorial set of genotypes. The first frost screening was carried out at the seedling stage using four replicates, and the experiment consisted of 72 seedling trays. It was challenging treating them all with the 6 frost temperatures with a 2 hour hold at each temperature in the frost chamber. It proved impossible to place all of the trays into the frost chamber at the same time, so they were treated in groups by replicate (18 trays) and this proved to be the maximum capacity of the frost chamber. Although this could be construed as a criticism of the consistency of the frost test, it was the only logistical solution, and treatment temperatures were always checked during each run using a datalogger which demonstrated the consistency of the freezing treatments from replicate to replicate.

Unfortunately, limited seeds stock meant that there were not enough seeds of all genotypes for the second frost test at the more advanced growth stage. Furthermore the breeders declared that they were no longer interested in 6 of their breeding lines of the 11 used in first screen, so the second test was carried out only on 5 genotypes. So there was a lack of complete comparability between the 2 tests. In the second test, plants were sown in large pots (12 L) and with different preconditioning treatments before

freezing. It was challenging to manage 75 pots in the glasshouse with limited space available and it was even more difficult to manage 75 pots in the cold room. Added to this, the physical energy required to move watered pots of around 15 kg from field to laboratory was a logistical challenge. It was impossible frost test whole plants in the second test as in first test and so the Relative Electrical Conductivity (REC %) technique was used in this test instead. This again makes the 2 tests not completely comparable but the correlation between the tests especially at the top and bottom of the scale lends credit to the cross-comparability.

The agronomy part (field trial) of the work reported here was the core of the project and it was a big challenge in the field to deal with a hidden disorder (BH) with no related exterior symptom that might indicate its appearance. The field trial was covered an area of 0.5 ha with 144 plots and was covered with Enviromesh to provide a protected environment and to ensure comparability with conventional production techniques. The Enviromesh was laid in 12 m widths and in 180 m runs which meant that the author had to scramble under the mesh to access the plots prior to harvest which meant movement in a very restricted and limited space from the edge of each plot and cutting 10 roots per plot to investigate BH appearance and severity and then recording the data. This had to be repeated to collect samples for boron concentration analysis for healthy and affected roots. It was extremely challenging to collect samples and score for BH appearance for 144 treatments (12 genotype X 4 B treatments X 3 replicates) especially when it was raining.

The acid digestion method used for boron concentration analysis proved to be an extremely time-consuming method. Samples had to be freeze-dried and that took 3 to 4 days per 10 samples. The freeze drier was not big enough to take the all samples at one time, so it had to be repeated many times until all samples were ready, and samples had to be stored in boxes containing silica gel packs until analysis. Also, there was a limit to the number of Teflon bombs used for the hot acid digestion (24) as it was very expensive to purchase more. Lastly each set of 24 samples required 5 to 6 hour for complete digestion. There initially had been ambitions to measure

many more samples for boron concentration, but again compromises were made because of time.

The main experiment undertaken to study boron and cold acclimation on the response of swede to frost failed the first time due to unsuitable growing conditions in the laboratory. The experiment had to be repeated in the glasshouse where it was successful. The time lost to this was about 4 months.

It was planned to include *Arabidopsis* in the last experiment (molecular study) but due to a complete failure of germination two times in a row it had to be abandoned and the project just continued with swede only. This again lost a considerable amount of time.

All of these difficulties and failures resulted in a time cost that prevented the author from investigating more aspects. In particular it led to a cancellation of the last experiment which planned to test low temperature impact on boron transporter genes (*BOR1* and *NIP5;1*) so that had to be left for future work.

7.3 Future work

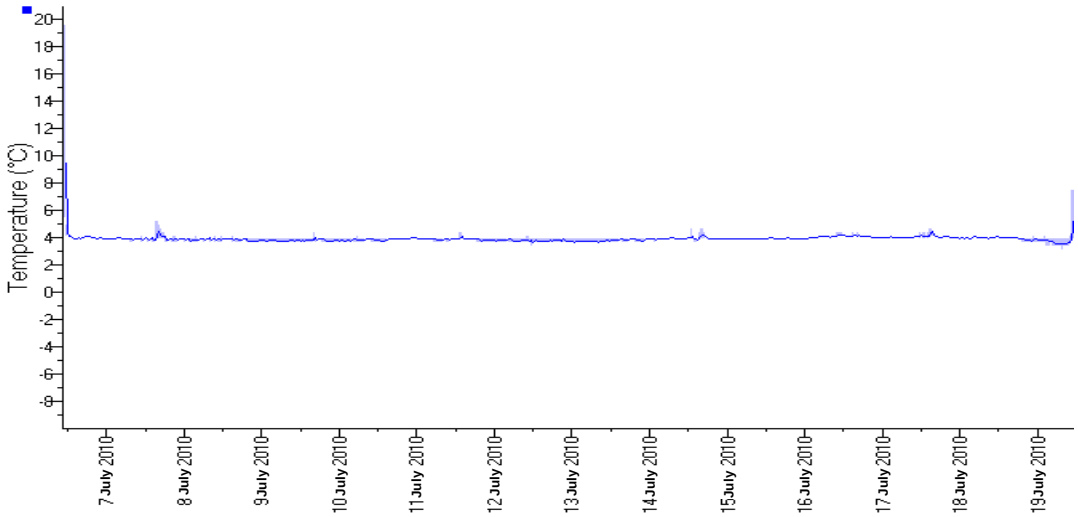
1- As boron affected plant response to freezing temperature and up-regulated the cold responsive gene *BN115*, it is suggested to study the impact of low non-freezing temperature on boron transport channel genes. It has been mentioned that low temperature has a negative physiological impact on boron absorption, and it is suggested to study the molecular effect on *BOR1* and *NIP1;5* genes which are well known as boron transporter genes (Takano et al., 2002, Tanaka and Fujiwara, 2008) .

2- Proteomic analysis is rapidly superseding transcriptomic analysis as equipment and costs improve. Proteomics is beginning to provide better details with regard to what is changing post-translationally in plant cells due to imposed stresses. The author suggests using the 2D DIGE proteomics analysis to test the interaction between low temperature and boron. In the literature it has been recently reported that there are alterations in protein production in *Brassicas* due to temperature stress including a putative myrosinase–associated protein (Castelo-Branco, 2010) and glyceraldehyde-3-phosphate dehydrogenase (Neilson et al., 2010). Interestingly these are the same as those that have been reported to be affected by boron stress in *Brassica napus* roots (Wang et al., 2010, Wang et al., 2011). Despite undertaking proteomics training the author was limited by time otherwise this would have been investigated.

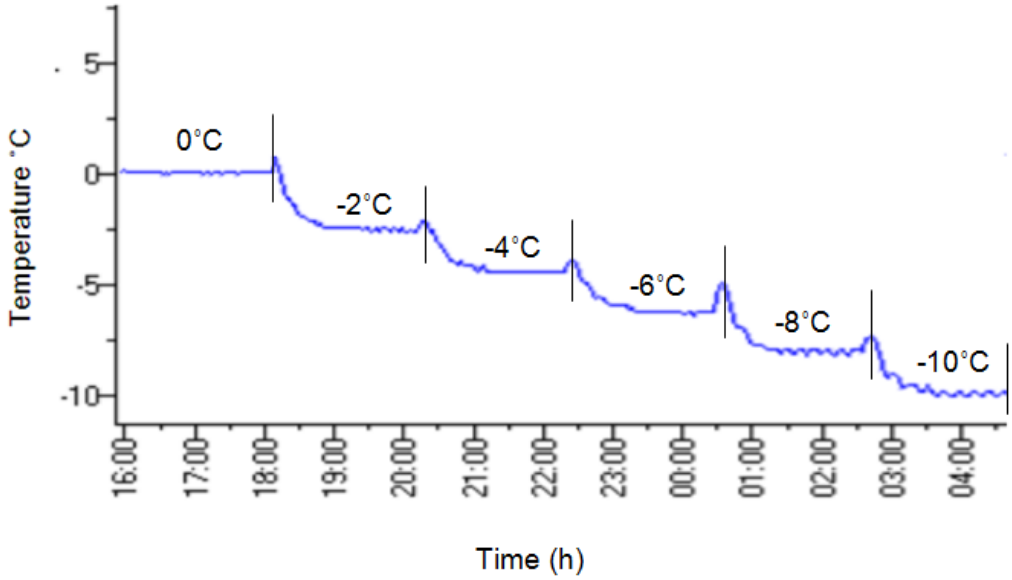
3- It is suggested that swede breeders need to screen a lot more crosses and parent lines to corroborate the results presented in this thesis. Also it would be interesting to set up a segregating population and test these for their susceptibility to frost first and then set up a field test without applying any boron to test for BH susceptibility to test the linkage hypothesis more robustly.

4- Since it is difficult to breed against BH using traditional breeding methods like plant selection or backcross, the marker gene technique appears to offer an appropriate modern breeding method to introduce BH resistance to swede genotypes.

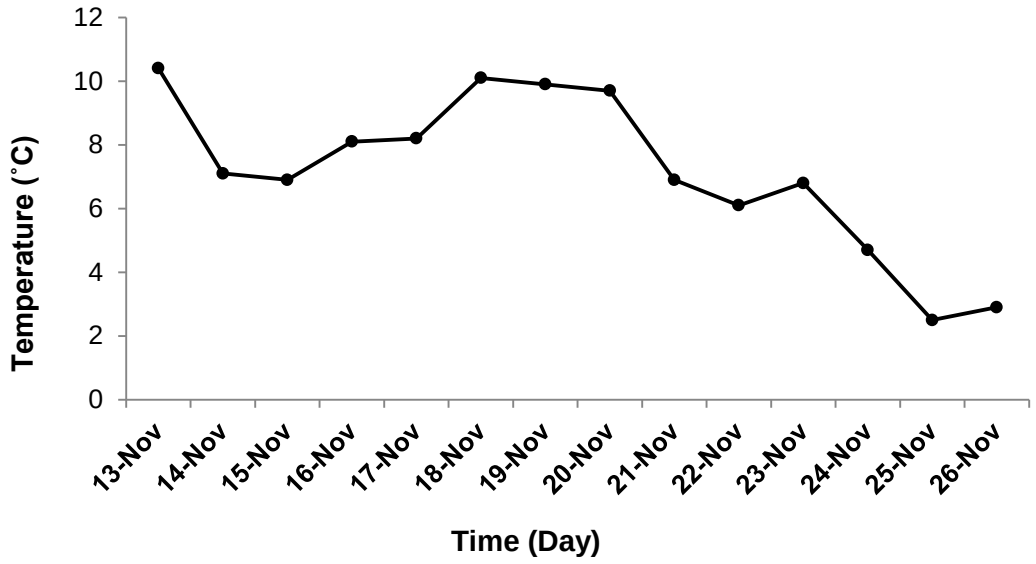
Appendices:



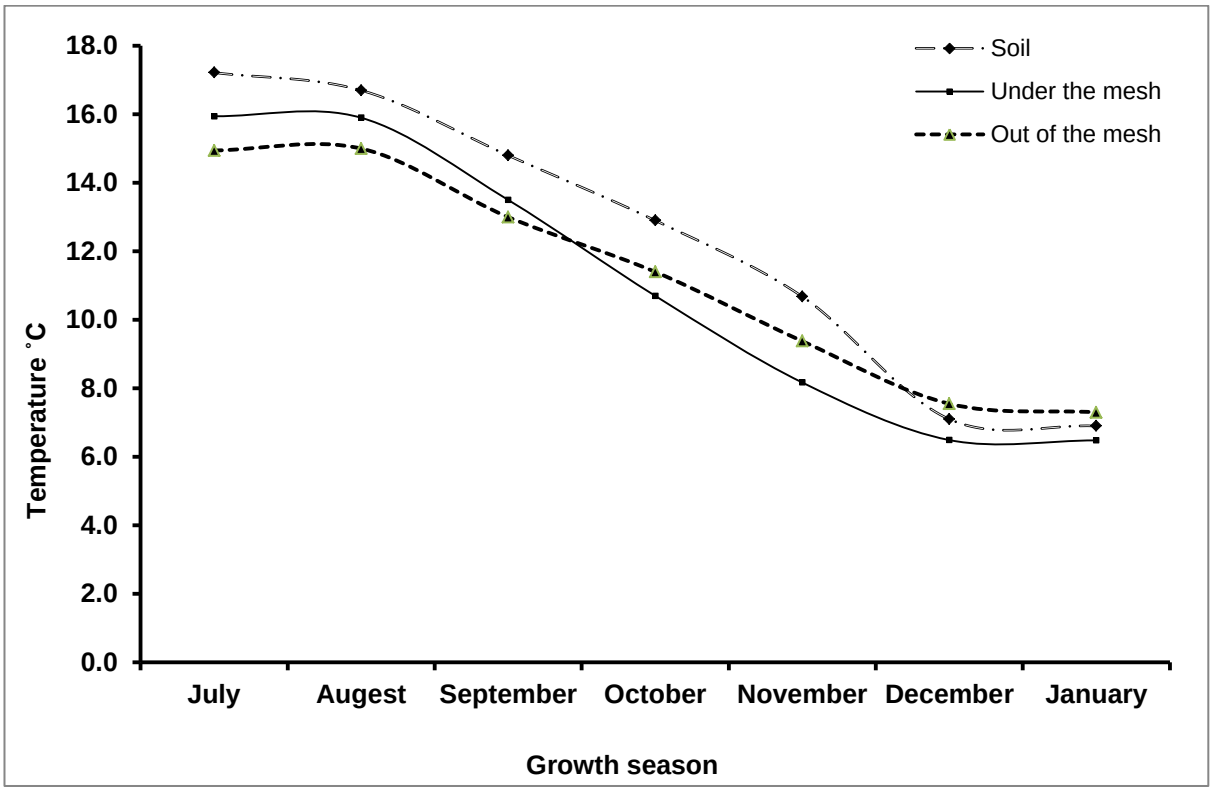
Appendix 1: Example of datalogger recorded temperature in the cold room (4˚C).



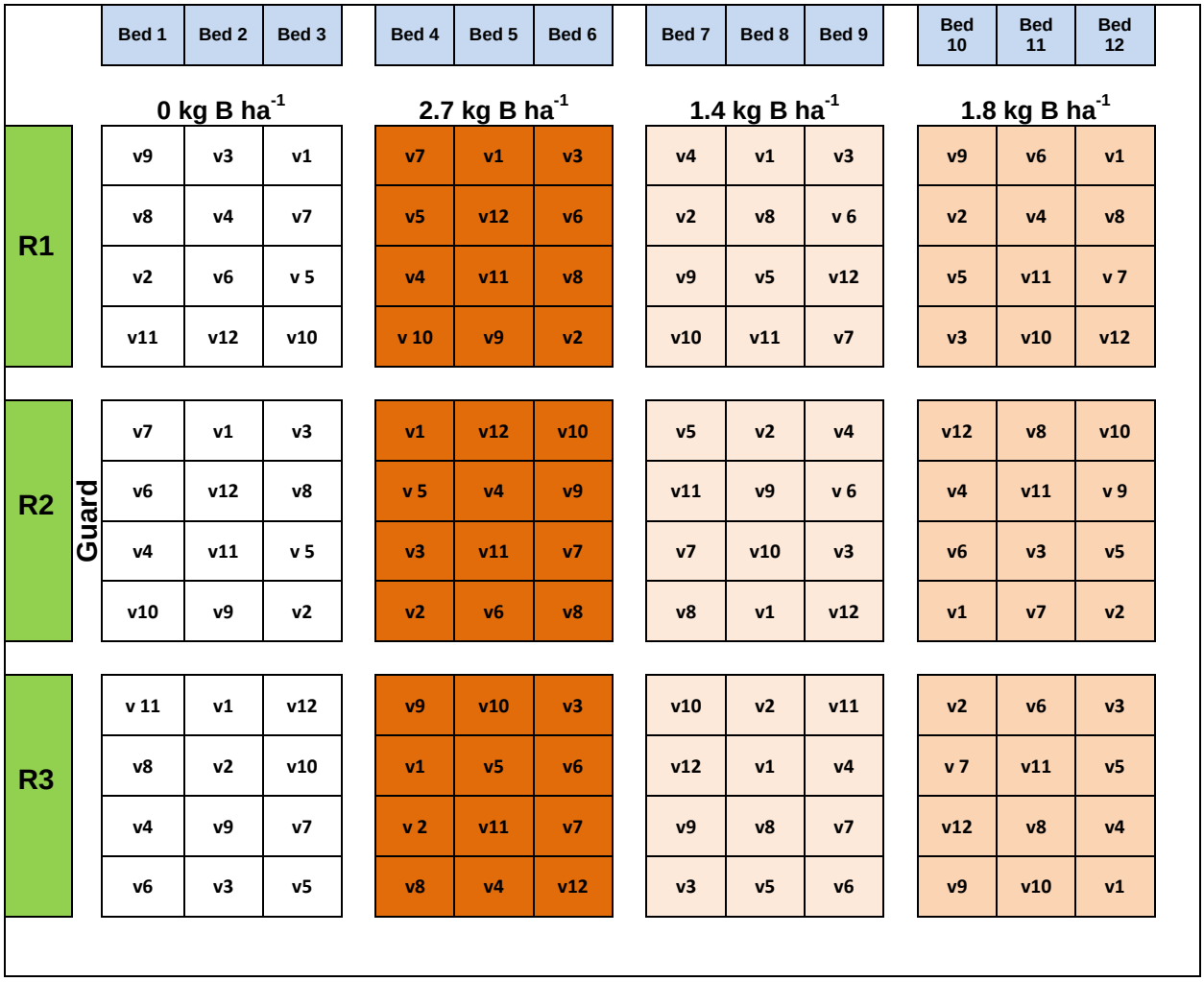
Appendix 2: Example of datalogger recorded temperature in the frost incubator for frost treatments 0,-2,-4,-6,-8,-10˚C. Vertical lines indicate sampling points and are associated with a slight temporary rise in the incubator temperature.



Appendix 3: Average recorded temperature outside between 13th to 26th of November 2010.



Appendix 4: Mean monthly temperatures recorded by dataloggers in the field.



Appendix 5: Field trial plan.

Component	Stock Solution				mL Stock Solution / 1L
Macronutrients					
2M KNO_3	202 g/L				2.5
2M $Ca(NO_3)_2 \cdot 4H_2O$	236 g/0.5L				2.5
Iron (Sprint 138 iron chelate)	15 g/L				1.5
2M $MgSO_4 \cdot 7H_2O$	493 g/L				1
1M NH_4NO_3	80 g/L				1
Micronutrients					
H_3BO_3	0B	Standard (sB)	30B	60B	1
	0	2.86 g/L	3.73 g/L	7.46 g/L	
$MnCl_2 \cdot 4H_2O$	1.81 g/L				1
$ZnSO_4 \cdot 7H_2O$	0.22 g/L				1
$CuSO_4$	0.051 g/L				1
$H_3MoO_4 \cdot H_2O$ or	0.09 g/L				1
$Na_2MoO_4 \cdot {}_2O$	0.12 g/L				1
1M KH_2PO_4 (pH to 6.0)	136 g/L				0.5

Appendix 6: Hoagland solution formulations.

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