Department of Crop Sciences

Detailed genetic approach to improve frost tolerance of German winter faba beans

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Doctoral Dissertation

Submitted for the Ph. D. degree in the Faculty of Agricultural Sciences,

Georg-August-Universität Göttingen,

Germany

by

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Göttingen, May 2014

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Date of dissertation: 13.05.2014

Gedruckt mit Unterstützung des Deutschen Akademischen Austauschdienstes

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Dedicated to

my Parents and my Family

General introduction

Faba bean (*Vicia faba* L.) is one of the most important grain legumes crops, partly due to its relatively high protein content. In developing countries, faba bean is mainly used as human food. In industrialized countries, it is mainly used as feed for e.g. pigs, horses, poultry and pigeons. Abiotic stresses such as frost threaten faba bean growth, mainly affecting survival rate and grain yield. Abiotic stresses limit faba bean production worldwide (Maqbool et al., 2010).

Marked frost during the winter season of North and Central Europe is a major abiotic stress. In such countries, faba bean is mainly grown as spring crop due to insufficient winter hardiness of the germplasm in use. Therefore, breeding to improve frost tolerance in winter faba bean is urgently needed. Many different methods have been employed to quantify the effect of frost stress on faba bean, to screen the symptoms and to select the most tolerant genotypes. These screenings focused on the morphological traits of faba bean shoots such as freezing resistance (FR) and determination of shoot dry matter content (Herzog, 1988), frost-induced loss of leaf turgidity and loss of leaf color (Araboui and Link, 2008), and regrowth after frost and disposition to survive (Roth and Link, 2010). Understanding the physiology associated with frost resistance may shed light on the mechanisms and changes that would allow the plants to survive during and after frost. Changes in leaf fatty acid composition, root fatty acid composition, proline accumulation, and membrane stability have been reported as physiological parameters associated with frost tolerance in faba bean (Hoffmann-Bahnsen and Herzog, 2001; Rizza et al., 2001; Croser et al., 2003; Arbaoui et al., 2008b; Soenke, 2012). The correlation between both types of traits (morphological and physiological) is very important to identify the most promising faba bean winter genotypes. Most of the aforementioned approaches have been used in frost growth chambers and green houses.

Studying and improving winter hardiness is very important, since winter hardiness shows significant correlation with grain yield for most winter crops. Frost tolerance is a major part of winter hardiness (Rizza et al., 1994; Arbaoui, 2007). Testing the frost tolerance and winter hardiness of winter faba bean genotypes should take place in the field in order to identify the

'true' winter hardiness of the most tolerant genotypes. To identify such winter faba bean genotypes, many traits associated with frost tolerance and winter hardiness should be scored.

Traditional plant breeding methods to improve resistance to an abiotic stress take a lot of time and effort. Instead, marker assist selection (MAS) is considered a powerful tool for accelerating breeding programs to improve such traits. Among the various methods to prepare for MAS, association mapping (AM) is considered an effective method to identify the quantitative trait loci (QTL) underpinning trait variation. Furthermore, useful information on the genetic architecture of extant variation in germplasm populations can be gained through the AM approach (Oraguzie and Wilcox, 2007). Unfortunately, only few studies have been conducted on faba bean to detect the important QTLs for frost tolerance, such as that by Araboui et al. (2008b) who successfully constructed a genetic map in faba bean using RAPD markers.

The **first chapter** of the present study aims (1) to assess the genetic variation for frost tolerance in shoots and roots of hardened, juvenile faba bean plants, (2) to analyze leaf fatty acid composition and water content as promising physiological parameters after hardening, and (3) to discover any possible phenotypic and/or genetic correlation between morphological and physiological traits. The objectives of the **second chapter** are (1) to assess the winter-hardiness of winter faba bean genotypes in field trials under natural frost stress, (2) to identify the most promising winter faba bean genotypes with high frost tolerance, and (3) to study the possible correlations between field traits and frost growth chamber traits. Finally, the **third chapter** sets out (1) to analyze the structure of linkage disequilibrium in the current population, (2) to find marker-QTL associations for morphological and physiological traits scored under frost stress and after hardening, and (3) to identify marker alleles associated with favorable QTL alleles which improve frost tolerance in faba bean and which can be integrated in breeding programs for genetic improvement of frost tolerance.

Literature review

1- New trends in breeding research of faba beans

Basically, high yield and high yield quality are main objectives in any crop breeding program. Since the adverse effects of climate change are expected to increase, adequate resistance/ tolerance to abiotic stresses are included as important objectives in such programs. Frost stress is one of the abiotic stresses that may significantly and markedly reduce faba bean yield.

In Europa, two types of beans can be sown: spring and winter beans. Winter beans have several advantages compared to spring types, including higher yielding ability, better use of available moisture in the soil, higher tolerance against drought and against some pests (Link et al., 2010). However, the main drawback of winter faba bean is the risk of winter-kill due to insufficient winter hardiness of faba bean genotypes presently sown in autumn (Arbaoui, 2007). Therefore, in cool-temperate regions, faba bean is often grown as a spring crop. Hence, improving faba bean resistance to frost tolerance is needed. Recently, some studies suggested (Kalo et al., 2004; Stracke et al., 2004; Mudge et al., 2005; Nelson et al., 2006; Cannon et al., 2006) that research on model legumes such as *Medicago truncatula* or *Lotus japonicas* might lead to new perspectives and hence enhance faba bean breeding efforts and results.

Studying model species will allow us to better understand many processes such as plant development (van Hengel et al., 2004; De Smet and Jürgens, 2007), plant response to biotic and abiotic stresses (Jones and Dangl, 2006; Swindell et al., 2007; Ma et al., 2008), and physiological adaptations of plants to threatening stress. A model legume such as *Medicago truncatula* is also interesting for researchers due to its small genome (*M. truncatula* around 500 Mb; Gnanasambandam et al., 2012), which is much better suited to genetic and genomic research than large genomes such as that of *Vicia faba* (around 13,000 Mb, Ellwood et al., 2008). Quantitative traits loci (QTL) for important traits can be mapped and used in selection programs. Moreover, candidate genes involved in stress tolerance processes and/or quality traits may be useful in developing transgenic lines which show tolerance to such stresses (Rispail et al., 2010). The *M. truncatula* seed represents a good model for identifying genes controlling seed composition in grain legumes. These genes can be used to study fatty acid and sugar compositions in seeds of grain legumes, such as pea and faba bean (Duc, 2004; Djemel et al.,

2005). Burstin et al. (2007) mapped some putative QTLs for traits controlling vegetative plant development and seed yield and protein content in pea. They revealed that faba bean presents homologous loci with shared properties, and hence these could be used in selection schemes. Interestingly, some pathogens and pests which limit faba bean yield also can affect *M. truncatula* and *L. japonicus*. Therefore, studying their genome and identifying effective resistance genes will provide a great chance to improve the knowledge in resistance mechanisms against faba bean pathogens (Rispail et al., 2010). Although Brandsaeter et al. (2002) reported that *M. truncatula* displays a poor freezing tolerance, when compared to other annual legumes. QTLs for frost tolerance traits were currently mapped by Avia et al. (2013) in *M. truncatula*. This could actually help in identifying resistance genes to frost stress. Some studies contain promising results for low temperature legume breeding in alfalfa using transgenic expression of an iron-superoxide dismutase, resulting in an enhanced winter survival (McKersie et al., 1993).

2- Winter hardiness and frost tolerance in faba bean

Frost tolerance is most often used to describe the response of such plants to freezing temperatures, which show marked and significant effects of frost. Many plant species have the potential to tolerate frost, developing an enhanced tolerance to freezing temperature when exposed to hardening conditions in which the temperature is low but positive. Trunova (1965) found that exposing plants to even negative but non-injurious temperatures, as a kind of hardening, can confer an increased tolerance to severe frost in the plants. According to Arbaoui (2007), three factors are required for winter hardiness of a genotype, i.e. (1) its degree of frost tolerance, (2) its ability to resist biotic stresses such as snow mold, and (3) its resistance to abiotic stresses such as high level of soil saturation with water. On the other hand, growth stage, hardening conditions, length of cold spells, sequences of frost and thawing, snow cover, maximum level of tolerance, speed of hardening, and the ability to maintain tolerance are considered as vital factors that affect the acquired frost tolerance of a genotype (Lecomte et al., 2003). Meanwhile, Stone et al. (1993) concluded in their study on *Solanum* species that unhardened frost tolerance and hardening response are primary reasons for the frost tolerance of a plant. Both hardening and dehardening were found to be inherited separately. Frost tolerance itself is considered a main component of winter hardiness in cereal as well as in legumes (Petcu and Terbea, 1995).

Faba beans can be grown under different ranges of low temperatures. For instance, Mediterranean faba bean types can tolerate temperatures below -8ºC when they are sown in the autumn and have adaptability to mild winter without a true dormancy phase. European faba beans can survive under temperatures down to -15ºC. Japanese cultivars tolerant to snow cover developed their flowers much rather later than susceptible ones (Fukuta and Yukawa, 1998). Under experiments in controlled conditions, Stoddard et al. (2006) reported about two faba beans, Côte d'Or (French landrace) and BPL4628 (Chinese inbred line), that they are tolerant to frost.

Frost tolerance can be determined through field experiments or through controlled growth chamber experiments (further approaches are possible as well). With regard to field trials, the low temperatures in the field play an important role in this kind of experiment. At the Experimental Field Station in Göttingen University, for example, faba beans in winter 2002/2003 were exposed to harsh frost (below -10ºC). A period of five days occurred with the lowest temperature at -16ºC, strong frost throughout the days, and snow cover <1 cm. In winter 2004/2005, the faba beans were exposed to only one such spell of a single night at -17ºC and 7 cm of a snow cover. This caused barely any winter-kill except among spring bean types (Link et al., 2008). However, the winter of 2012/2013 was too severe in Göttingen for almost all winter beans (nearly zero survival). There were twelve days of freezing temperatures below -11ºC, with the lowest temperature at -19.5ºC and no snow coverage.

On the other hand, frost tolerance can be intensively studied under controlled experimental conditions. In such experiments, many screening methods and tools have been suggested and developed in order to improve the assessment of frost tolerance in crops. In such controlled conditions, plants can be exposed to a wide range of low and freezing temperatures according to the objectives of the study. Visual scoring of leaves after freezing temperature (Herzog, 1989; Duc and Petitjean, 1995; Badaruddin and Meyer, 2001), measuring leaf conductivity (Herzog, 1987a) and measuring chlorophyll content (Taulavuori et al., 2000; Hoffmann-Bahnsen and Herzog, 2001) have been proven to be reliable tests for determining frost tolerance in faba beans. Young potted plants were used by Gehriger and Vullioud (1982) to test their frost tolerance. They exposed plants to hardening during 34 days at 5ºC in the greenhouse followed by actual testing. They gradually reduced temperatures from 5ºC to -15ºC. In other studies, frost

tolerance under controlled conditions was studied by determining the lethal temperature at which 50% of the plants were killed (LT50; Herzog, 1987a; Herzog, 1989; Dörffling et al., 1997). In a study by Herzog, (1987a) on faba beans, he found that plants were killed with an average of 50% at -4.8ºC if unhardened and at -12ºC if hardened.

An ideal frost-tolerant faba bean should present the following characteristics at the vegetative stage: slow rate of leaf development, short habit, small, thick leaves, sufficient dry matter accumulation in tissues, low water content, and high freezing resistance (Herzog, 1988). Winter cultivars should also show resistance against biotic stresses and adverse abiotic conditions such as high levels of saturation of soils with water (Stoddard et al., 2006; Sillero et al., 2010), besides its ability to tolerate frost.

3- Screening methods used for frost test

Several methods, approaches and tools have been utilized to determine the frost tolerance of winter faba bean. Two approaches shall be considered here in more detail: analysis of morphological traits and of physiological traits.

Morphological trait assessment is considered the easiest way to discriminate between genotypes in terms of their response to frost tolerance. Examples are visual scoring of freezing injuries in leaves under controlled conditions (Herzog, 1987b and 1989; Badaruddin and Meyer, 2001) and loss of turgidity and loss of color of both leaves and stems (Arbaoui and Link, 2008). Visual scoring of leaves was carried out on juvenile plants in the field (Eujayl et al., 1999). Freezing resistance (FR), determination of dry matter (DM), and leaf area were assessed in nine European varieties of winter faba bean by Herzog (1988). He concluded that both FR and DM were significantly increased as a response to hardening at 2-10ºC. Roth and Link (2010) calculated disposition to survive (DS), survival rate (SR) and measured shoot regrowth after frost (RG) to asses frost tolerance of 35 inbred faba bean lines. They found high genetic variation between genotypes. The highest significant correlation was between DS and SR (r = 0.97**). Moreover, cell membrane stability was measured in 31 winter faba bean genotypes by (Arbaoui et al., 2008a). The authors found very high significant variation among winter beans at -11ºC, indicating that the structure and function of cell membranes are important; the assessment of membrane damage could help in understanding the tolerance of cells and tissues to stress

exposure. Fresh matter (FM) content, dry matter (DM) content, specific water content, water/DM were measured in leaves of nine European varieties of winter faba bean (Herzog, 1988) through hardening experiments. The author reported that among these traits, water/DM ratio showed high significant correlation with FR.

On the other hand, assessing physiological parameters gives much information about frost tolerance mechanisms in plants. Arbaoui and Link (2008) studied the fatty acid composition of leaves and stems in 12 European faba bean genotypes after hardening and unhardening. They reported that hardening increased C18:3 in leaves and decreased C18:1 in leaves and stems. Furthermore, changes in C18:3 content were negatively and significantly correlated with changes in C18:1 content. The increase of C18:3 content, C18:3 being a polyunsaturated fatty acid, favors frost tolerance because it gives leaf membrane more fluidity by introducing bends or kinks in the fatty acids chains, thereby inhibiting tight packing of adjacent lipid molecules (Lehninger, 1977; Vigh et al., 1998) and hence enhancing frost tolerance (Cyril et al., 2002). Soenke (2012) studied fatty acid composition in the roots of 48 winter faba beans. These materials were exposed to a hardening phase in order analyze fatty acid composition in roots and were scored for frost tolerance traits such as regrowth and disposition to survive after exposure to frost stress. He didn't find promising correlations between root fatty compositions and frost tolerant traits. A significant proline accumulation during hardening was observed in 24 winter faba bean genotypes (Arbaoui et al., 2008a). In addition to proline accumulation, the same genotypes showed differences in their membrane stability at -15°C which was determined to discriminate between winter genotypes. Increase of proline content occurred also during cold acclimation in chickpeas, leading to changes in cellular structure and metabolic processes (Croser et al., 2003). Proline accumulation reached a maximum in faba bean before maximum frost tolerance (Balko, unpublished data), indicating a role for proline accumulation which was a precondition for hardening. The chlorophyll fluorescence method was used to identify frost-tolerant white lupin types (Hoffmann-Bahnsen and Herzog, 2001). Rizza et al. (2001) considers that the chlorophyll fluorescence analysis is an effective test because it helps to monitor the capacity of plants to maintain a functional photosystem II after the exposure to freezing temperatures, a trait which may be associated with the acquisition of frost tolerance.

Screening is possible in both field trials and under controlled conditions. For field trials, no specific equipment is required, and the option to easily score thousands of genotypes is an unbeatable argument. However, the best conditions to assess the results of cold in field should be realized via the use of susceptibility and tolerance checks. Erskine et al. (1981) and Singh et al. (1989) recommended sowing a sensitivity check after every nine test genotypes. When these checks showed 100% mortality, discrimination among candidate cultivars was more efficient and appropriate. In addition, daily minimum air temperatures, the amount and importance of snow cover, and the ratings of known lines covering the scale of sensitivity must be taken into consideration in results presentation (Wery, 1990).

On the other hand, screening cold tolerance in controlled conditions has many advantages. It is possible to control e.g. the hardening conditions and the intensity and duration of the frost period, giving a higher reproducibility of the test than field trials. The main limitation is the cost of buying and maintaining cold rooms to be used for the tests.

4- Molecular genetic studies on faba beans

Generally, molecular markers are considered a powerful tool which can be used for indirect selection of target traits. There are many kinds of molecular markers which can be used depending on the aim of study. This new type of tool is greatly expanding our knowledge of the genetic diversity of plant species and is facilitating the mapping of important genomic regions which may be related to traits of interest.

In faba bean breeding programs, improving seed yield, resistance/tolerance to biotic and abiotic stresses, and adaptation to the target environment, appropriate phenology, plant growth habit, seed quality and traits to enhance crop management are major objectives for dealing with chronic problems such as climate change. Traditional breeding takes many years to accomplish these objectives in the form of a commercial cultivar. However, plant molecular biology techniques and genomic tools could help in accelerating the development of faba bean cultivars (Gnanasambandam et al., 2012). Therefore, many efforts have been undertaken to comprehensively study the genetics and genomic features of the faba bean.

Faba bean is a diploid plant with $2n = 2x = 12$ chromosomes and has one of the largest genomes among crops (~13,000 Mb), similar with the hexaploid genome of wheat (Alghamdi et al., 2012).

This could complicate the development of genetic and physical maps, as well as map- based cloning (Cruz-Izquierdo et al., 2012). However, many researchers have brought about a massive progress. In early studies, RAPDs and RFLPs were used to construct meaningful genetic linkage maps of faba beans using F_2 populations (Torres et al., 1993). Afterwards, SSRs were employed and used for mapping purposes (Pozarkova et al., 2002). These markers were used to develop genetic maps and to identify loci which have a relation to genetically complex traits such as flowering time, pod length, ovlues/pod, and seeds/pod (Cruz-Izquierdo et al., 2012), biotic stresses such as resistance to crenate broomrape (*Orobanche crenata* Forsk), Ascochyta blight (Vaz Patto et al., 1999; Roman et al., 2002 and 2003; Avila et al., 2004; Diaz-Ruiz et al., 2009; Diaz et al., 2010) and rust resistance (Sillero et al., 2006; Sillero et al., 2010). With regared to abiotic stresses, Jayashree et al. (2005) and Buhariwallam et al. (2005) identified expressed sequence tagged sites (ESTs) which are putatively involved in drought avoidance in chickpea, pea and *Medicago truncatula*.

Few studies have been conducted to identify QTLs for frost tolerance in faba bean. Twelve putative QTLs for frost tolerant traits were mapped, for the first time, by Arbaoui et al. (2008b) using RAPD markers. They used 101 faba bean recombinant inbred lines derived from the cross between two frost tolerant lines (Côte d'Or/ 1 x BeanPureLine/ 4628). Frost tolerance and leaf fatty acid composition were analyzed before and after hardening of these lines. They successfully mapped five QTLs for hardened and unhardened frost tolerance and eight QTLs for hardened and unhardened fatty acid composition. In addition, these traits show very high heritabilities, ranging from h^2= 0.53 for C18:3 to h^2= 0.91 for C18:0. Sallam and Link, (2012, unpublished) selected six RAPD-bands which were strongly associated with more than one QTL from the aforementioned study (Arbaoui et al., 2008b). Then they verified them on different, independent materials which consisted of 187 winter faba beans (cf. Material and methods chapter below) lines. A number of morphological traits were scored after frost stress. In addition, leaf fatty acid composition was analyzed after hardening of these lines. Sallam and Link, (2012) successfully verified three RAPD bands (I06-803, F15-476, and I10-661) which showed significant associations with frost tolerant traits and leaf fatty acid compositions after hardening. Recently, QTLs for freezing tolerance and related traits were mapped in a set of 15 *Medicago truncatula* accessions (Avia et al., 2013) using 111 SSR, 57 AFLP, 12 RAPD and 2 EST-CAPS. The authors mapped five QTLs for frost damage after one and eight days of frost, four QTLs for frost

damage after two weeks, three QTLs for number of leaves after cold acclimation, two QTLs for leaf area, three QTLs for electrolyte leakage, five QTLs for chlorophyll content index, two QTLs for shoot dry weight, and five QTLs for root dry weight.

Since climate change is expected to accelerate, more research should be done especially on drought and frost tolerance to cover putative QTLs for drought and frost tolerant traits. Accordingly, these traits should possess a high genetic variation and a high correlation with yield and its attributes.

Materials and methods

Plant materials

The plant materials consisted of N=208 homozygous lines (HLs, see appendix)

1- N= 11 founder lines. These highly inbred winter bean lines originated from different regions in Europe: Germany (Webo, Wibo, Hiverna/1, L79/79, L977/88, and L979/S1), France (Côte d'Or/1 and Arrissot), and UK (Banner, Bourdon, and Bulldog).

2- N= 189 single seed (SSD) descent lines were named as association set (A-set, used for association analysis). Initially, the 11 founder lines were mixed in order to produce a freely recombining population, in isolation by space under open pollination conditions and natural selection. After eight generations of ongoing maintenance of this population (named Göttingen Winter Bean Population, GWBP), > 400 SSD were drawn and inbred to generation $F_{>9}$ and beyond (Gasim, 2003; Stelling, 1989, unpublished). N= 189 of these lines were randomly selected to study their variation with regard to frost tolerance. The population structure of this material was expected to be ideal for the proposed approach. The expected gamete phase disequilibrium between QTL and markers was expected to be neither extremely high nor extremely low.

3- N= 4 further European winter beans; two inbred lines were derived from a cross between two frost tolerant parents (Côte d'Or/ 1 and BeanPureLine 4628), 29H (Ascochyta resistant), and Hiverna/2 (German line)

4- N= 4 spring beans; Limbo/ 7, Ḿelodie/ 7, Hedin/ 2, and Minica were used as checks.

These lines were divided into three classes namely, the original set (O-set) which includes all genotypes (N=208), the winter bean set (WB-set) which includes only winter bean genotypes (N=196), and the association set (A-set) which includes SSD lines (N =189). With regard to the WB-set, eight of the founder lines were excluded; Wibo, L79/79, L977/88, L979/S1, Arrissot, Banner, Bourdon, and Bulldog.

Morphological traits assessments

Artificial frost

The O-set HLs were evaluated through two successive seasons 2011/2012 and 2012/2013 in ten experiments with two replications (as a final, r=20). The experiments were conducted in a 4m² **Vötsch** frost growth chamber (FGCh, size of 2x2x2 m^3, VB4018 extra; allowing frost down to minus 20°C). One use of 4m^2 area of this chamber corresponded to one replication. Each such replication held 216 entries (eight HLs were used twice), with three insulated pots (17cm²) as one incomplete block (12 x18 alpha lattice design; 12 x 18 = 216). The genotypes were sown in such pots containing four different genotypes with two seeds from each. When the juvenile plants reached to two expanded leaves, all pots were transferred to the FGCh. The frost chamber was programmed to give light at 200 µmol s^{-1} m^{-2} during 10 hours per day. The air humidity fluctuated freely within a range of about 80-90% based on temperature fluctuation. All seedlings inside the frost chamber were first exposed to a hardening phase (temperatures of 4˚C days/ 0˚C nights for ten continuous days). Subsequently, the true frost test took place for three days under -16˚, -18˚, and -19˚C during the night and thawing during the artificial day (Figure 1). The soil in pots was a mixture of compost soil and sand (3:1, respectively). Pots were irrigated to keep them at approx. 70% of soil water capacity. The irrigation was done during the hardening phase and was stopped as first frost occurred.

Traits scoring

Before exposing the seedlings to frost, plant height (PH, cm) was scored on each plant. After each such frost step and ten hours in addition, loss of leaf turgidity (LT-16, LT-18, and LT-19) were individually scored on each entry using a scale from 1 (fully turgid) to 9 (not turgid). Likewise, loss of leaf color (LC-16, LC-18, and LC-19) was scored using the same scale (1= green, 9= black). After the final (i.e. third) frost step, all plants were given a break of four days at room temperature. All genotypes were scored again for their loss of turgidity after frost (LTAF) and loss of color after frost (LCAF). All scores for loss of turgidity and loss of color were summed up to yield one trait called LT+LC. The scale of this accumulated trait ranged from 8 (fully turgid and no color loss) to 72 (not turgid and full of color loss). Afterwards (following the four-day break), the main stem at the second internode and tillers of the juvenile plants were chopped off in order to provoke them to regrow. During the next four weeks, number of days

until death was assessed for those plants that died in this period. Number of days until death, or, if the plant survived, ultimate survival was transformed into disposition to survive (DS) [°; from 0° to 90°] according to Roth and Link (2010).

$$[1] \quad DS = arctan \frac{X_i}{\mu_x}$$

x_i = number of days until death and μ_x=mean number of days until death of those plants that actually died. Surviving plants were scored 90°.

At the end of four weeks all surviving, and hence re-growing juvenile plants were chopped off again (Figure 2) to measure this regrowth (REG, g). Thus, not only dead *vs.* surviving plant were noted but also the differences in vigor and health status of surviving plants after frost. Any regrowth of course proves survival of the individual in question. Then, the roots of each genotype were carefully removed from the soil and washed to score root frost susceptibility (RFS). Root frost susceptibility was visually and individually scored using a scale extending from 1 (healthy root) to 9 (dead roots).

Optimum selection indices (Falconer and Mackay, 1996) were calculated to work towards a genuine frost tolerance trait. Index 1(I_1) was used to better describe DS (X_1) using two auxiliary traits; PH (X_2) and LT+LC (X_3) as:

$$[2] \; I_1 = b_1X_1+b_2X_2+b_3X_3.$$

where $b_1 = 0.746$, $b_2 = -0.574$ and $b_3 = -0.245$.

Index 2 (I_2) was used to better describe REG (X_1) using only one auxiliary trait, i.e. relative shoot water content after frost (RWCAF %, X_2, see details below) as:

$$[3] \; I_2 = b_1X_1+b_2X_2.$$

where, $b_1 = 0.553$ and $b_2 = 0.015$.

A Frost tolerance index (FTI) was calculated from I_1 and I_2 as follows:

$$[4] \; FTI= ½ [(I_1/SD_1) + (I_2/SD_2)];$$

where SD is the phenotypic standard deviation of the corresponding index, meanwhile, b_1, b_2, and b_3 are the index coefficients. The vector of Smith-Hazel index coefficient b was calculated as $b= P^{-1} G$ (Baker, 1986), where P^{-1} is the inverse of the phenotypic variance-covariance matrix for the traits; G is a matrix including the estimates of genotypic and covariance.

Physiological traits assessments

Leaf fatty acid composition

The fatty acid composition was analyzed in the WB-set of materials in two additional experiments with two replications in each. All plants were only exposed to a hardening phase in FGCh using the aforementioned regime. After the hardening phase, all shoots were cut, dried at 50˚C for 48 hours and milled. Total lipid extraction from leaves followed the experimental protocol of Thies (1971) with few modifications to avoid polyunsaturated fatty acids oxidation. Fatty acid was analyzed using gas chromatography (Perkin Elmer 8600). Three-microliter samples were injected into the columns (Permabond FFAP-0.25 µm, 25 m x 0.25 mm). The temperatures used were 215°C for column temperature, 280°C for injector temperature, and 280°C for detector temperature. Hydrogen at a pressure of 100 kPa was used as the carrier gas. The individual peaks were identified based on comparisons to a standard sample. Each fatty acid quantity was shown as a percentage of the total fatty acid composition. Six fatty acids were estimated including palmitic acid (C:D=16:0), stearic acid (C:D=18:0), oleic acid (C:D=18:1), linoleic acid (C:D=18:2), linolenic acid (C:D=18:3), stearidonic acid (C:D=18:4); C:D is the ratio between the number of carbon atoms to the number of double bonds in the fatty acid; respectively. Saturated fatty acid content (SFA) was calculated as a sum of 16:0 and 18:0 contents. Likewise, unsaturated fatty acid content (USFA) was calculated as a sum of 18:1, 18:2, and 18:3 contents.

Relative shoot water content

After frost treatment and first chopping off, the shoot fresh matter and dry matter after frost (SFMAF and SDMAF, respectively) were measured in order to estimate relative water content of shoot after frost % (RWCAF) as follow:

$$[5]\quad RWCAF = \frac{SFMAF - SDMAF}{SFMAF} x100$$

Moreover, relative water content before frost % (RWCBF) was estimated in the experiments used for fatty acid assessment, i.e. for shoots after hardening phase and before milling the samples to analyze fatty acid composition analysis ('before frost' here means literally without frost). The shoot fresh matter before frost (SFMBF) and shoot dry matter before frost (SDMBF) were measured directly after hardening phase using the above formula. The reduction in water content (RIW) due to frost stress was calculated for each genotype as follow

$$[6]\quad RIW = \frac{RWCAF - RWCBF}{RWCBF} x100$$

Field experiments

This research was carried out at the Experimental Field Station at Georg-August-Universität Göttingen, Reinshof, Göttingen, Germany (51º29'51.68 " N, 9º55'47.76" E). The Experimental design was alpha lattice (13x15) with two replications. A number of 50 seeds from each genotype were sown in a two-row plot and at density of 25 seeds/m^2 (i.e. 2m^2 per genotype). Planting distance was 37.5 cm between rows and each row was three meters in length. The minimum daily temperatures at the experimental site from sowing date (October, 2012) until the end of the frost period (April, 2013) are illustrated in Figure 3. This period coincided with the occurrence of the frost stress which plant development was exposed. The temperatures fluctuated between 0ºC in late October 2012 and -13.20ºC in late January 2013. Apparently, the lowest range of temperature which all plants were exposed to was from the end of December 2012 to the end of January 2013. Afterwards, the fluctuation in temperature was around -5ºC, with waves that reached about -12ºC in March 2013.

Ten characters were recorded in this experiment

1- Plant development 1 (PD1) was visually scored on shoots of faba bean plants one month after sowing and before frost start. The visual score (one score per plot) ranged from 1 = weak shoots to 9 = strong shoots.
2- Plant development 2 (PD2) was scored using the scale as in PD1 on April 22, 2013, i.e. after the frost period, which was from November, 2012 to March, 2013 (see Figure 3) to see the effect of frost on shoot development.
3- Days to flowering (DTF, days) were counted from sowing day to the day when 50% of plants flowered in a plot.

4- Leaf frost susceptibility (LFS) was scored as a loss of turgidity and color in a scale ranging from 1 (full turgor + green leaves) to 9 (no turgor + black leaves).
5- Field plant height (FPH, cm) was assessed after all genotypes flowered (one score per plot).
6- Winter survival rate (WSR, %) was calculated by dividing the number of surviving plants after the frost period by the number of emerged plants after sowing (on November 12, 2012).
7- Days to maturity (DTM, days) were visually determined as the number of days from sowing to when 50% of the plants in a plot completely lost green color.
8- Seed fill duration (SFD, days) was calculated as the difference between days to maturity and days of flowering.
9- Seed yield was taken as combined harvested yield ($g/2m^2$)
10- 1000 seed weight (g, 1000-SW) was weighted and calculated from 100 air-dried seeds per plot.

Statistical analyses of phenotypic data

Each experiment was laid out with r=2, analyzed as a lattice design. The Frost Growth Chamber experiments were thereafter, based on the lattice-adjusted means, combined as a series of experiments. This analysis of variance was performed with PLABSTAT software (Utz, 1997) using the following equation:

$$Y_{ij} = \mu + g_i + e_j + ge_{ij}$$

where Y_{ij} is observation of genotype *i* in experiment *j*, μ is the general mean; g_i, e_j are the main effects of genotypes and experiments, respectively; *geij* is genotype × experiment interaction of genotype *i* with experiment *j*. Pooled single entry error of the ten lattice experiments was transferred to the series of the experiments with the ERROR command of PLABSTAT. Genotypes were considered as random effects, while, experiments were considered fixed effects. Repeatability of the genotypes for each trait was calculated as (h^2 = genotypic variance / phenotypic variance). The Spearman rank correlation coefficient was calculated for the phenotypic correlation between traits. The variance-covariance analysis with the lattice-adjusted entry means across the ten experiments was carried out using GENOT-command with PLABSTAT software to estimate the genetic correlation coefficient and to allow the building of the optimum selection indices. Microsoft Office Excel 2010 was used for the graphical analyses.

Part of the findings is displayed as scatter plots, and several prominent genotypes' identity is given.

Molecular genetic analysis

Genetic data

DNA was extracted from founder (N= 11) lines and A-set (N=188, one line was excluded due to technical problems using illustra Nucleon Phytopure Genomic DNA Extraction kits (GE Health UK Limited). The DNA of each sample was analyzed by KBiosciences, Cambridge, UK using 189 SNP markers which promised polymorphism between the 11 founder lines based on earlier studies (Cottage et al., 2012). N= 156 out of these 189 SNP markers indeed showed polymorphism with a minimum allele frequency of 10% between the A-set lines.

Association mapping (AM) and linkage disequilibrium (LD)

Association analysis among markers and morphological as well as physiological traits was carried out using TASSEL version 3.0 (Bradbury et al., 2007). The general linear model procedure of TASSEL was used. A False Discovery Rate of 20% (FDR=0.20) was used to test the statistical significance of marker-trait associations (Benjamini and Hochberg, 1995). Phenotypic effects at the marker loci were calculated as differences between the means of the marker classes. The positive values indicates that the specified marker allele increase the trait, while a negative value indicates this allele is associated with a decrease in the trait. The phenotypic variance explained (R^2) by the significant makers was determined using TASSEL 3.0. The final set of 156 SNP markers was used to perform linkage disequilibrium (LD) investigation and association mapping. Most of these markers were mapped in the so-called consensus map which was recently constructed using 643 polymorphic-SNP markers by Donal O'Sullivan (Cottage et al., 2012). The r^2 values of linkage disequilibrium (LD) for all pairwise marker combination and corresponding significance levels (*P* values) were calculated with TASSEL 3.0. A Bonferroni correction ($P = 4.1 \times 10^{-6}$) with a global α-level of 5% was used to establish the significance threshold of LD between each two markers. In order to clearly visualize the LD decay, the entire linked marker pairs were grouped according to the genetic distances between the two markers per pair, collected into classes in steps of 5 cM, and LD means were calculated per class. Hence, each class contains a group of linked marker pairs; the

average LD of the linked marker pairs per class was plotted against their distance. Microsoft Office Excel 2010 was used for the graphical analyses.

Principal coordinate analysis (PCoA)

In order to answer the question of whether there is any marked population structure in the A-set, PCoA on the genetic distances between the lines was used to reveal whether or not there are subgroups. The simple matching coefficient was used to estimate the genetic distance between lines using R-package 'ade4' (Pene, 2013). The analysis was performed with the help of R software (R Development Core Team , 2013) using the 156 SNP markers. Two plots are presented to show the pattern of the genetic distances among A-set lines; via (PCoA 1 vs PCoA 2) and (PCoA3 vs PCoA 4).

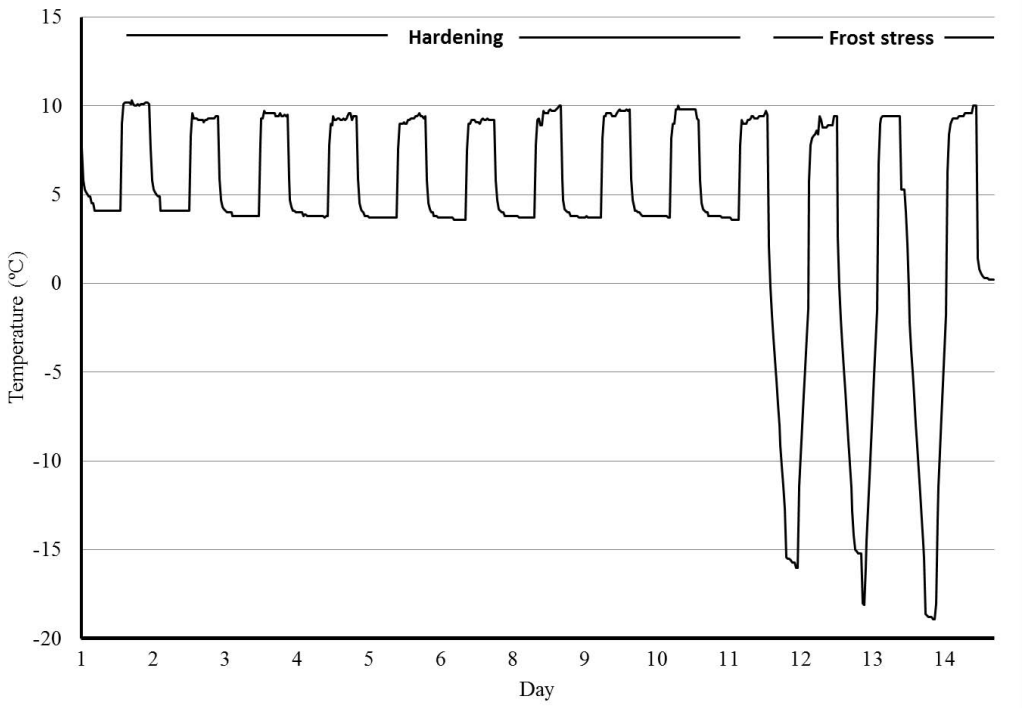

Figure.1 Example of temperatures of the artificial frost test.

Directly after frost | After shoot chop-off | Regrowth or death

Figure 2. Steps of test (left-right): Symptoms after three frost ‚nights', plants after shoot chop-off, several weeks later with regrowth or death. Each pot contains four different genotypes.

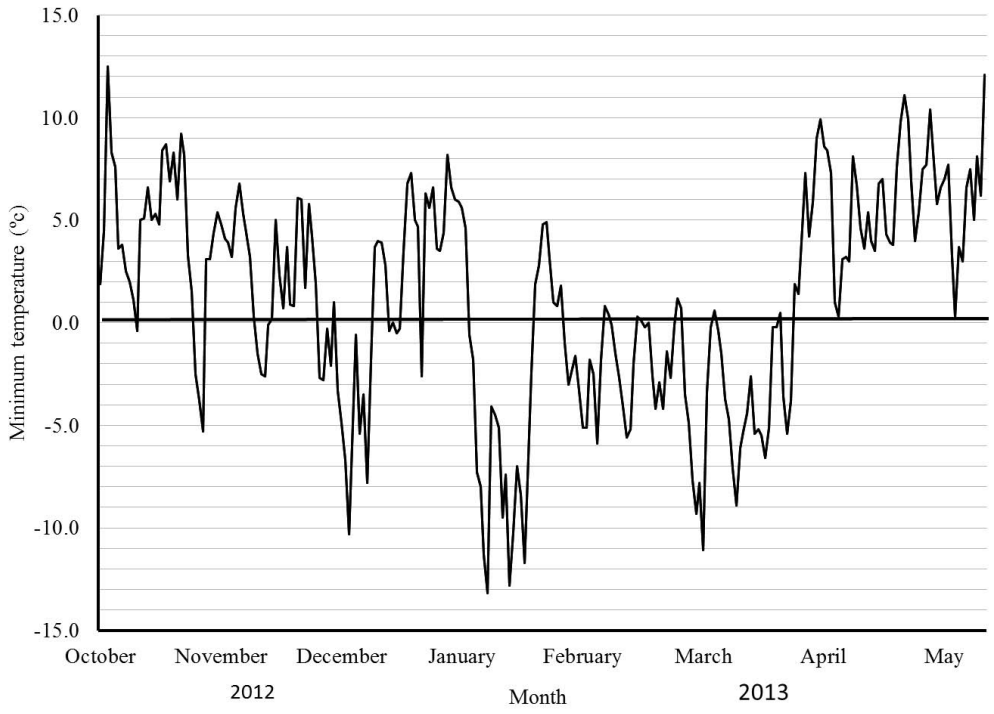

Figure 3. Minimum daily temperatures in field from the sowing date until the end of frost.

Results and discussion

I. Genetic variation in morpho-physiological traits associated with frost tolerance in faba bean

Results

The analysis of variance for the morphological traits, leaf fatty acid composition, and relative shoot water content are presented in Tables I-1, I-2, and I-3, respectively. Mean values, LSD, phenotypic standard deviations (SD), ranges, and repeatability estimates (h^2) for all traits scored under and after frost stress and all traits scored after hardening are presented in Tables I-4, and I-5 respectively. The phenotypic and genetic correlations among morphological traits and among leaf fatty acid composition are presented in Table I-6 and I-7. Table I-8 represents the correlation between relative shoot water content, shoot fresh matter and shoot dry matter after and before frost. The correlation between morphological traits and both leaf fatty acid composition and relative shoot water content are presented in Table I-9 and I-10.

Morphological traits (frost experiments)

The analysis of variance for all morphological traits is presented in Table I-1. The results of the experiments (E) were significantly different from each other for all measured traits. The analysis of variance revealed highly significant ($P < 0.01$) mean squares for genotypes (G), indicating the existence of considerable differences among genotypes for all traits under and after frost stress. The E x G variance showed significance in all traits scored during and after frost. All traits exhibited a wide range of variation (Table I-4). Regrowth after frost (REG) showed very wide range between genotypes under frost stress. The repeatability (h^2) estimates ranged (Table I-4) from 59.45 for root frost susceptibility (RFS) to 94.71 for loss of leaf turgidity + loss of leaf color (LT+LC). The highest repeatability among indices was realized for index 1 (including disposition to survive, I_1).

Physiological traits (hardening experiments)

Fatty acid composition

Gas chromatography of leaves produced results on 11 different fatty acids. Out of these 11, six fatty acids were used in the analysis due to their sum exceeding 95% of the total fatty acid. Those fatty acids were palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and stearidonic acid (C18-4). These fatty acids are divided into two groups: saturated fatty acids (SFA - C16:0 and C18:0) and unsaturated fatty acids (USFA - C18:1, C18:2, and C18:3). C18:4 (as an unsaturated fatty acid) was excluded due to its very low h^2. The analysis of variance for fatty acid composition is shown in Table I-2. The variance due to the experiments was significant for all fatty acids. High significant variation was found among genotypes in all fatty acids analyzed in leaves after hardening. The effect of G x E was insignificant in all fatty acids analyzed. The means, ranges, and repeatability estimates of fatty acids are represented in Table I-5. Lines showed very wide variation in stearic acid (C18:1, C.V. = 25.69%) extending from 2.12 to 9.46%. High similar h^2 estimates were obtained (h^2 > 60.00) for all fatty acids except stearidonic acid (C18:4) (h^2 = 36.52). The highest repeatability was found for C18:0 (h^2=89.04).

Relative shoot water content before and after frost

The analysis of variance (Table I-3) revealed high significant differences between experiments for relative shoot water content before frost (RWCBF) and after frost (RWCAF), shoot fresh matter before frost (SFMBF) and after frost (SFMAF), and shoot dry matter before frost (SDMBF) and after frost (SDMAF) in both treatments. Genotypes showed remarkable and significant differences in all traits before and after frost stress. The E x G was significant for all traits under frost stress. The reduction in shoot water content, fresh matter, and dry matter due to frost stress was 8.19%, 51.65%, and 34.88 % respectively. The h^2 values for relative water content, fresh matter, and dry matter were much higher ($88.00 < h^2 > 100.00$) after frost stress than those after hardening conditions ($67.90 < h^2 < 77.31$). The reduction in water content (%) due to frost stress for all genotypes ranged from 1.39 to 30.94%, indicating to dramatic differences among genotypes in losing water. The frequency distribution of genotypes for water loss due to frost is illustrated in Figure I-1.

Correlation analyses

The Spearman's rank correlation and the genetic correlation among morphological traits and physiological traits are presented in Tables I-6, I-7, and I-8.

To begin with morphological traits (Table I-6), O-set was used to study phenotypic and genetic correlation between traits. Very high, significant phenotypic and genetic correlations were found between traits measured under and after frost stress. Plant height (PH) as the only trait scored before frost showed negative and significant correlation with DS, regrowth (REG), and selection indices. REG showed significant phenotypic (r_p) and genetic (r_g) correlation with disposition to survive (DS) (r_p = 0.77**, r_g = 0.85). Root frost susceptibility was significantly correlated with REG (r_p = -0.83**, r_g = -0.96) and with DS (r_p = -0.68**, r_g = -0.83). For selection indices, frost tolerance index (FTI, including DS and REG) showed significant correlation with all morphological traits. High significant negative correlation was found between FTI and both root frost susceptibility (RFS) and loss of leaf turgidity and loss of leaf color (LT+LC). Selection indices showed high significant phenotypic and genetic correlation among each other.

WB-set was used to study the phenotypic and genetic correlation between leaf fatty acid compositions (Table I-7) after hardening conditions. Spring beans were excluded in order to avoid bias and inflation of the correlation coefficient. C16:0 content was negatively correlated with C18:1 content and C18:3 content. C18:0 content showed negative and significant correlation with C18:1 content. A high significant negative correlation was found between C18:2 content and C18:3 content. SFA content was highly and negatively correlated with USFA content (r_p = -0.64**, r_g = -0.84). Remarkably, among saturated fatty acids, C16:0 content showed very high positive correlation with SFA content (r_p = 0.97**, r_g = 0.97). In contrast, C18:3 content among unsaturated fatty acids showed high significantly positive correlation with USFA content (r_p = 0.54**, r_g = 0.41).

WB-set was also used to study the correlation between morphological traits and both leaf fatty acid composition and relative water content (Tables I-9 and I-10). In the first place, the Spearman rank's correlation between fatty acid composition in leaves and morphological traits (Table I-9) indicates the role of fatty acids and its relation to frost tolerance. LT+LC showed significant positive correlation with C18:0 content, C18:2 content and SFA content, while it

showed significant negative correlation with C18:3 content and USFA content. C16:0 showed similar significant negative correlations (r = -0.17*) with REG, I_2 and FTI. USFA content was positively and significantly correlated with DS, REG, index 1(I_1), index (I_2), and FTI. On the other hand, SFA was negatively correlated with DS, I_1, and FTI. The highest correlation was found between USFA content and PH (r = -0.29**). A significant positive correlation was found between DS and both C18:2 content, and C18:3 content.

The Spearman's rank correlation between morphological traits and changes in relative water content, shoot fresh matter, and shoot dry matter is shown in Table I-10. Notably, RWCAF and RWCBF showed high significant correlations with all morphological traits scored under and after frost stress. RWCAF showed high positive correlations with DS, REG, I_1, I_2, and FTI, while it showed high significant negative correlations with LT+LC and RFS. RWCBF, compared with RWCAF, showed inverse correlations with morphological traits. This is predictable due to a significant negative correlation between RWCAF and RWCBF (r = -0.20**; Table I-8). Among morphological traits, PH only showed significant positive correlation with RWCAF and RWCBF (Table I-8). Unlike the correlation between RWCAF and RWCBF, SFMAF showed significant positive correlation with SFMBF (r = 0.43**). Likewise, a high, significant positive correlation was found between SDMAF and SDMBF (r = 0.64**; Table I-8).

Discussion

Morphological traits (frost experiments)

Scoring morphological traits in a suitable frost test is a rapid and reliable tool for distinguishing faba bean genotypes for their ability to tolerate frost. This can be concluded from the considerable differences between genotypes (P > 0.01) after exposing them to freezing temperature for three nights (-16°, -18°, and -19° C; Table I-1). Among all morphological traits, REG demonstrated a very wide range of differences between genotypes after exposure to freezing (Table I-4). The preparation period of hardening (5°C days/ 0 °C for 10 continuous days) increases the level of frost tolerance of all genotypes. Compared to non-harded seedlings, exposing faba bean seedlings to the hardening conditions of low non-freezing temperatures (2.5°C days/ 0°C for one week) dramatically increased the frost tolerance of all genotypes (Arbaoui et al., 2008a). Looking at selection indices, index 1 (including DS) showed dramatic differences between genotypes ranging from -3.38 to 53.47. FTI (including DS and REG) ranged

from 1.75 to 6.94. Selection based on FTI may be more useful for improving frost tolerance since it was estimated to better describe two important traits: DS and REG. A selection index for frost tolerance was used also in a previous study in with *Pisum sativum* cultivars (Voican et al., 1995). They developed a relative damage index calculated from membrane permeability in order to show differences between genotypes for frost resistance. Despite the complexity of calculations and efforts required to estimate and then determine the frost tolerant index, such index provides a very powerful tool for discriminating the winter hardiness of cultivars (Sãulescu and Brawn, 2001). Among the O-set, the most susceptible genotypes were those of the spring beans group for all traits scored in the frost growth chamber. On the other hand, the most superior frost tolerant genotype differed by traits: S_120 (RFS), S_271 (DS), S_145 (REG), Côte d`Or/1 × BPL4628-95 (LT+LC and $I_1$), and S_028 ($I_2$ and FTI). Côte d`Or/1 × BPL4628-95 was also reported as a frost tolerant line by Arbaoui (2007).

High repeatability (h^2) estimates for all morphological traits were obtained ranging from 59.45 (RFS) to 94.71 (LT+LC). Similar findings were obtained by Arbaoui and Link (2008) for frost tolerant traits such as 'area under symptom progress curve' (AUSPC, h^2 = 89.00) in winter faba beans. Regarding selection indices, the highest h^2 was accounted for by I_1 (90.64) followed by FTI (85.49) and I_2 (72.31). Such high h^2 makes selection under frost stress probably very effective and feasible for improving winter faba beans. Generally, frost tolerance in faba bean if assessed under controlled conditions, is a highly heritable trait due to the large additive effects involved (Duc and Petitjean, 1995).

On average, the genetic correlation between morphological traits was much higher than the phenotypic correlation. The high significant phenotypic and genetic correlation between all morphological traits under frost stress (Table I-6) indicates the possibility of improving frost tolerance in faba bean through a selection process in breeding programs. Notably, the highest phenotypic and genetic correlation among morphological traits was between RFS and REG (r_p = -0.83**, r_g = -0.96). RFS was also significantly correlated with DS (r_p = -0.68**, r_g = -0.83). The root characters have been suggested as remarkable morphological traits that have a relation to frost tolerance in e.g. wheat (Perras and Sarhan, 1989) and corn (Hund et al., 2004). That is because the roots play a vital role after frost stress in regulating plant growth. Furthermore, they are the suppliers of water, minerals and growth substances. In addition to the previous point, RFS

as a trait, reflects the frost injury and allows us to distinguish among surviving genotypes (after regrowth; Figure I-7). Thus, the healthier the root the more promising it is for shoots regrowth, and hence the better is survival after frost. Equally important, DS was significantly correlated with REG (r_p = 0.77**, r_g = 0.85) and LT+LC (r = -0.61**). High significant correlation between disposition to survive and both survival rate (r = 0.97**) and loss of leaf turgidity (r = -0.60**) were found by Roth and Link (2010) in 36 winter faba bean lines. The highest significant correlation between morphological traits and selection indices was found for the association between I_1 (including DS) and DS (r_p = 0.99**, r_g = 0.99). FTI also showed strong significant correlation with all morphological traits (r_p > 0.50**, r_g > 0.60) and other selection indices as well, indicating that considerable genetic gain in each trait could be obtained by selection based on index values (Iqbal et al., 2007) rather than based on single-trait selection (Wells and Kofoid, 1985, Gebre-Mariam and Larter, 1996). Plant height (PH) showed high and significant negative correlations with DS (r =-0.48**), REG (r = -0.39**), and FTI (r =0.52**). These correlations are informative because plant height compared to DS and REG was not affected by frost stress. Therefore, PH can be used to predict frost tolerance.

Physiological traits (hardening experiments)

Fatty acid composition (hardening experiments)

Leaf fatty acid composition after hardening was found to be associated with frost tolerance in faba bean (Aabaoui et al., 2008a). The analysis of variation revealed high significant differences between genotypes in their fatty acid composition after hardening (Table I-2). No fatty acids were significantly affected by the G x E. The largest variation between genotypes was in C18:1 content, while USFA content and SFA content showed a similar range (Table I-5). In several crops including faba bean, a perceptible increase in C18:3 and decrease in C16:0, C18:0, C18:1, C18:2, and C18:4 during hardening have been reported (Samala et al., 1998; Cyril et al., 2002; Falcone et al., 2004; Arbaoui, 2007). High h^2 estimates were obtained for all fatty acids except C18:4, indicating that selection for fatty acid contents could be a possible way to improve frost tolerance in faba beans.

Desaturase enzyme activity plays an important role in fatty acid metabolism under low temperature. Acyl-[acyl carrying protein]-desaturase (acyl-CAP-desaturase) forms the first

double bond in the fatty acid chain, converting C18:0 to C18:1 (Los and Murata, 1998; Shanklin and Cahoon, 1998). This can be illustrated from the significant negative correlation between C18:0 and C18:1 (r_p = -0.23**, r_g = -0.20). Subsequently, C18:1 can be transported to membranes of chloroplasts and the endoplasmic reticulum for further desaturation with the formation of C18:2 (two double bonds) and then C18:3 (three double bonds) by acyl-CAP-desaturase (Shanklin and Cahoon, 1998). Our results are in agreement with aforementioned relationships between fatty acids after hardening. C18:1 content showed negative significant correlation with C18:2 content (r_p = -0.22**, r_g = -0.26). Moreover, a high, significant correlation was found between C18:2 and C18:3 content (r_p = -0.74**, r_g = -0.75). Arbaoui (2007) found high, significant positive correlation between C18:1 content in unhardened leaves and changes in C18:2+C18:3 (r = 0.55**). USFA content was significantly correlated with SFA content (r_p = -0.64**, r_g = -0.84). The function of cis double bonds is to create a kink in the fatty acid chain which works as obstacle to prevent the rigid packing of the phospholipids in the bilayer (Taiz and Zeiger, 2010). As a consequence, the fluidity of the membrane is increased under frost stress. Interestingly, C16:0 content, among saturated fatty acids, showed very high significant correlation with SFA content (r_p = -0.97**, r_g = 0.97). On the other hand, C18:3, among unsaturated fatty acids, was highly correlated with USFA (r_p = 0.54**, r_g = 0.41). This indicated that C16:0 and C18:3 are main components in saturated fatty acid content and unsaturated fatty acid content, respectively.

Relative shoot water content (hardening and frost experiments)

As a rule of thumb, shoot water content tends to decrease during the early stage of cold acclimation (Pellett and White, 1969). Thus, assessing water content has been used successfully to predict the frost tolerance of shoots in the early stages (Rosvall-Åhnebrink, 1985; Calmé et al., 1993). The analysis of variance for relative water content, shoot fresh matter and shoot dry matter revealed significant difference between genotypes (Table I-3). The F values of genotypes for the three traits under frost stress were twice those under hardening conditions. Relative water content, shoot fresh matter, and shoot dry matter showed higher h^2 under frost stress than under hardening conditions, indicating that a selection to improve these traits would be fruitful if it is after frost stress.

The significant negative correlation between RWCAF and RWCBF frost (r = -0.20**) points to a relationship between shoot water content and frost tolerance. It seems that genotypes holding somewhat less water before frost in their shoots form ice crystals under frost stress which were too small to cause mechanical damage. This ice formation was not lethal and the tissue recovered fully when warmed for four days after frost (see Materials and methods). If, on the other hand, genotypes holding high shoot water content before frost are exposed to freezing temperatures for an extended period, the ice crystals may become quite large. Subsequently, the growths of extracellular ice crystals result in a flow of liquid water from the protoplasm to the extracellular ice, inducing extreme dehydration (Taiz and Zeiger, 2010). The lower the water content after hardening conditions the higher the water content after frost and *vice versa*.

Correlation analyses between morphological and physiological traits

The correlation analyses were divided into two categories: correlation analysis between morphological traits and fatty acid composition (Table I-9) and correlation analysis between morphological traits and changes in shoot water content (Table I-10).

Firstly, the correlation between morphological traits and fatty acid composition indicated that unsaturated fatty acids play a role in keeping cell membrane under frost stress more fluid and thus promise higher frost tolerance. This can be observed from the significant correlation between LT+LC with USFA content (r = -0.19**) and C18:3 (r = -0.22**). The fluidity of the membrane, which is caused by high amount of unsaturated fatty acids plays a vital role in many membrane functions. Membrane fluidity is found to be strongly affected by temperature. Plants normally face the problem of maintaining membrane fluidity under conditions of low temperature, which tends to decrease membrane fluidity. Thus, a high percentage of unsaturated fatty acids, such as oleic acid, linoleic acid and α-linolenic acid which are in plant phospholipids can increase the fluidity of their membranes (Taiz and Zeiger, 2010). USFA content was found to be significantly correlated with survival traits such as DS (r = 0.19**) (Figure I-5), REG (r = 0.18*), and FTI (r = 0.22**). Similarly, DS showed significant correlation with C18:2 content (r = 0.22**) and C18:3 content (r = 0.19**). Williams et al. (1988) and Palta et al. (1993) reported that due to desaturase enzyme activity a high proportion of unsaturated fatty acids was observed in the membrane lipids of chilling-resistant plants during acclimation to cool temperatures, which significantly increased the amount of unsaturated lipids. Thus, desaturation of fatty acids

gives some protection against leaf injury from frost and therefore increases the frost tolerance. On the other hand, SFA content was negatively correlated with DS (r = -0.15*) and index 1 (r = -0.16*). C18:0 content showed significant correlation with DS (r = -0.22**), REG (-0.14*), and FTI (r = -0.20). In the same study by Williams et al. (1988) and Palta et al. (1993), it was also found that chilling-sensitive plants contained a great proportion of saturated fatty acid chains the bilayer. As a result, the membranes become less fluid, causing acute damage in the leaf. This can be tentatively confirmed from the positive correlation between C18:0 and LT+LC (r = 0.17*).

Secondly, water content in shoots and its correlation to morphological traits (Table I-10) reflects the importance of shoot water content as a frost tolerant trait. Freezing tolerance can be defined as the ability of plants to resist ice formation in extracellular tissue without significant injury to membranes or other cell components and to avoid water loss. Compared with fatty acid composition, RWCAF and RWCBF showed highly significant correlations with morphological traits in two different ways. RWCBF was negatively correlated with DS (r = -0.36**), REG (r = -0.24**) and FTI (r = -0.33**). Water content in plants after hardening was found to be correlated with winter survival. During hardening, hardier genotypes lose much more water than less hardy genotypes (Fowler et al., 1981). After a frost period, the hardier genotypes keep more water in their shoots than other genotypes. This can be illustrated from the positive correlation between RWCAF and survival traits after frost: DS (r = 0.54**), REG (r = 0.45**), and FTI (r = 0.63**). Moreover, LT+LC showed positive correlation with RWCBF (r = 0.34**) and very high negative correlation with RWCAF (r = -0.85**). Kramer and Boyer (1995) stated that leaf water status is intimately related to several leaf physiological variables, such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration. The reduction in water content due to frost stress was calculated in order to identify the genotypes that lose least water under frost stress (Figure I-5). A reduction in water content was highly correlated with LT+LC (r = 0.87**) (Figure I-6). Increasing freezing tolerance can be obtained without much reduction in water content (Yoshida et al., 1997).

Despite the low water content of some genotypes, they showed low freezing tolerance and *vice versa*. This may to some extent explain the low correlation between RWCAF and RWCBF (r = -0.20**) (Figure I-4). For example, S_242 showed low water after hardening conditions (84.88%) and after frost (74.56%) and therefore it was considered as a susceptible genotype due

to its low FTI (3.57) (Figure 1-3). However, S_028 showed high FTI (6.94) and RWCAF (84.89%) despite its higher RWCBF (86.75%). Similar results were reported by Yoshida et al. (1997), who reported that two important points were proposed for cold hardiness of a variety: (I) its ability to decrease water content in tissues initially and (II) its ability to increase the amount of tightly and moderately bound water in the later stages, preventing ice formation during freezing temperatures. Therefore, the possible reason for a low correlation between RWCAF and RWCBF could be that the strength of water binding in tissues is not strong enough to hold the critical water content needed for cell survival under the sub-zero temperatures at which extracellular ice is easily formed from the weakly bound water in a cell (Yoshida et al., 1997).

Table I-1. Analysis of variance (F-values) of morphological traits measured in the O-set (frost experiments) during seasons 2011/2012 and 2012/2013.

Source of variation	DF[(1)]	PH	DS	REG	LT+LC	RFS[(2)]	Index1	Index2	FTI
Experiments (E)	9	100.84**	37.55**	71.72**	177.83**	40.52**	45.78**	75.95**	69.53**
Genotypes (G)	207	10.64**	8.40**	2.76**	18.90**	2.47**	10.68**	3.61**	6.89**
E x G	1819	1.40**	1.13**	1.20**	1.29**	1.12*	-[(3)]	-	-

[(1)] Degrees of freedom.

[(2)] Root frost susceptibly (RFS) was scored in seven experiments. DF = 6

[(3)] F-values of calculated traits for G x E are not available.

*, ** significant at the 0.05 and 0.01 level of the probability, respectively.

Table I-2. Analysis of variance (F-values) of fatty acid composition measured in the WB-set (hardening experiments) during season 2011/2012.

Source of variation	DF	C16:0	C18:0	C18:1	C18:2	C18:3	C18:4	USFA	SFA
Experiments (E)	1	296.88**	5.13*	34.40**	167.69**	10.59**	161.61**	194.76**	251.11**
Genotypes (G)	195	5.57**	8.64**	5.96**	4.32**	2.95**	1.56**	2.74**	5.05**
E x G	195	1.05	0.85	0.69	1.07	1.22	1.13	-[(1)]	-

[(1)] F-values of calculated traits for G x E are not available.

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

Table I-3. Analysis of variance (F-values) of relative water content in shoots, shoots fresh matter, and dry matter before frost stress (hardening experiments, season 2011/2012) and after frost stress (frost experiments, seasons 2011/2012 and 2012/2013) measured in the WB-set.

Source of variation	DF	RWCBF	SFMBF	SDMBF	DF	RWCAF	SFMAF	SDMAF
Experiments (E)	1	24.99**	127.23**	149.57**	9	176.16**	441.60**	381.24**
Genotypes (G)	195	4.15**	3.17**	3.01**	207	8.80**	9.16**	8.76**
E x G	195	1.12	1.23*	1.19	1819	1.32**	1.33**	1.36**

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

Table I-4. Mean values (Mean), LSD, phenotypic standard deviations (SD), coefficient of variation (COV; %), ranges, and repeatability estimates (h^2) for 11 morphological traits measured in the O-set (frost experiments) during seasons 2011/2012 and 2012/2013.

Trait	Mean	$LSD_{0.05}$	SD	COV[(2)]	Minimum	Maximum	h^2
PH (cm)	5.79	0.71	0.83	14.34	4.14	8.06	90.60
DS (º)	65.13	11.05	11.45	17.58	21.42	83.81	88.09
REG (g)	0.64	0.59	0.35	54.69	0.04	1.66	63.78
LT+LC (1-9)	36.05	4.34	6.80	18.86	17.75	63.42	94.71
RFS (1-9)	7.61	1.37	0.77	10.12	5.11	9.25	59.45
I_1 [(1)] DS	36.48	8.66	10.20	27.96	-3.38	53.47	90.64
I_2 [(1)] REG	1.53	0.34	0.23	15.03	0.90	2.15	72.31
FTI[(1)]	5.02	1.00	0.95	18.92	1.75	6.94	85.49
RWCAF (%)	78.23	4.17	4.46	5.70	59.39	84.89	88.63
SFMAF (g)	1.44	0.26	0.29	20.14	0.64	2.21	89.08
SDMAF (g)	0.28	0.04	0.04	14.29	0.18	0.47	88.58

[(1)] Arbitrary units,

[(1)] COV= (SD/Mean) x100.

Table I-5. Mean values (Mean), LSD, phenotypic standard deviations (SD), coefficient of variation (COV; %), ranges, and repeatability estimates (h^2) for 12 traits measured in the WB-set (hardening experiments) during 2011/2012.

Trait	Mean	$LSD_{0.05}$	SD	COV(1)	Minimum	Maximum	h^2
C16:0 (%)	10.40	0.69	0.58	5.58	9.11	12.17	81.83
C18:0 (%)	1.66	0.14	0.15	9.04	1.30	2.12	89.04
C18:1 (%)	3.62	1.04	0.93	25.69	2.12	9.46	84.09
C18:2 (%)	19.23	1.72	1.25	6.50	15.26	24.51	75.64
C18:3 (%)	55.27	2.53	1.57	2.84	50.78	59.01	66.69
C18:4 (%)	5.53	0.91	0.41	7.41	4.00	6.57	36.52
USFA (%)	78.12	1.40	0.82	1.05	75.64	80.18	62.89
SFA (%)	12.07	0.77	0.63	5.22	12.51	16.67	80.21
RWCBF (%)	85.70	0.93	0.70	0.82	83.77	88.41	77.31
SFMBF (g)	3.00	0.90	0.57	19.00	1.43	4.55	67.90
SDMBF (g)	0.43	0.13	0.08	18.60	0.22	0.64	66.94

(1) COV= (SD/Mean) x100.

Table I-6. Spearman's rank correlation (**bold font**) and genetic correlation coefficients (normal font) among morphological traits measured in the O-set (frost experiments) during seasons 2011/2012 and 2012/2013.

Trait	PH	DS	REG	LT+LC	RFS	Index1	Index2	FTI
PH (cm)	-	-0.57++	-0.57++	0.61++	0.58++	-0.63++	-0.72++	-0.63++
DS (º)	**-0.48****	-	0.85++	-0.81++	-0.83++	0.99++	0.90++	0.97++
REG (g)	**-0.39****	**0.77****	-	-0.68++	-0.96++	0.85++	0.97++	0.93++
LT+LC (1-9)	**0.59****	**-0.61****	**-0.50****	-	0.69++	-0.87++	-0.82++	-0.86++
RFS (1-9)	**0.35****	**-0.68****	**-0.83****	**0.45****	-	-0.83++	-0.93++	-0.88++
Index1(1) DS	**-0.55****	**0.99****	**0.76****	**-0.71****	**0.67****	-	0.92++	0.98++
Index2 (1) REG	**-0.45****	**0.79****	**0.98****	**-0.62****	**0.81****	**0.80****	-	0.98++
FTI (1)	**-0.52****	**0.92****	**0.92****	**-0.69****	**-0.79****	**0.93****	**0.96****	-

(1) Arbitrary units

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

+, ++ coefficient is larger than one time and two times the standard error, respectively.

Table I-7. Spearman's rank correlation (**bold font**) and genetic correlation coefficients (normal font) among fatty acid compositions measured in the WB-set (hardening experiments).

Trait	C16:0	C18:0	C18:1	C18:2	C18:3	C18:4	PUFA	SFA
C16:0 (%)	-	0.08+	-0.28++	0.31++	-0.46++	-0.09	-0.78++	0.97++
C18:0 (%)	**0.05**	-	-0.20++	0.17+	0.22+	0.02	-0.40++	0.36++
C18:1 (%)	**-0.24****	**-0.23****	-	0.27++	-0.25++	0.02	0.35++	-0.30++
C18:2 (%)	**0.35****	**0.19****	**-0.22****	-	0.75++	-0.48++	-0.16+	0.32++
C18:3 (%)	**-0.48****	**-0.23****	**-0.07**	**-0.74****	-	0.22++	0.41++	-0.52++
C18:4 (%)	**-0.12**	**-0.03**	**-0.27****	**-0.28****	**0.04**	-	0.39++	0.05
USFA (%)	**-0.57****	**-0.32****	**0.39****	**-0.19****	**0.54****	**-0.45****	-	-0.84++
SFA (%)	**0.97****	**0.28****	**-0.28****	**0.38****	**-0.52****	**-0.12**	**-0.64****	-

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

+, ++ coefficient is larger than one time and two times the standard error, respectively.

Table I-8. Spearman's rank correlation among relative water content in shoots, shoot fresh matter, and shoot dry matter before frost stress (hardening experiments) and after frost stress (frost experiments) frost in WB-set.

Trait	RWCAF (%)	SFMAF (g)	SDMAF (g)
RWCBF (%)	-0.20**	-0.16*	-0.11
SFMBF (g)	-0.19**	0.43**	0.60**
SDMBF (g)	-0.15*	0.48**	0.64**

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

Table I-9. Spearman's rank correlation between morphological traits measured in the frost experiments and fatty acid composition measured in the hardening experiments in the WB-set.

FrTi[2] / FAC[1]	PH (cm)	DS (º)	REG (g)	LT+LC (1-9)	Index1[3] DS	Index2[3] REG	FTI[3]
C16:0 (%)	0.16**	-0.09	-0.02	0.08	-0.10	-0.04	-0.08
C18:0 (%)	0.17*	-0.22**	-0.14*	0.17*	-0.23**	-0.15*	-0.20**
C18:1 (%)	-0.05	0.18*	0.07	-0.09	-0.17*	0.09	0.13
C18:2 (%)	0.19**	0.22**	-0.03	0.19**	-0.23**	-0.05	0.14*
C18:3 (%)	-0.26**	0.19**	0.1	-0.22**	0.22**	0.11	0.17*
C18:4 (%)	-0.02	-0.01	-0.04	-0.031	0.00	-0.04	-0.02
USFA (%)	- 0.29**	0.19**	0.18*	-0.19**	0.21**	0.20**	0.22**
SFA (%)	0.20**	-0.15*	-0.07	0.13	-0.16*	-0.12	-0.14*

[1] Fatty acid composition.

[2] Morphological traits measured in frost experiments during seasons 2011/2012 and 2012/2013.

[3] Arbitrary units.

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

Table I-10. Spearman's rank correlation between morphological traits measured in the frost experiments and relative water content measured in frost and hardening experiments in the WB-set.

Trait	PH (cm)	DS (º)	REG (g)	LT+LC (1-9)	RFS (1-9)	Index1[2] DS	Index2[2] REG	FTI[2]
RWCAF (%)	-0.52**	0.54**	0.45**	-0.85**	-0.40**	0.62**	0.61**	0.63**
SFMAF (%)	-0.13	0.28**	0.28**	-0.35**	-0.22**	0.30**	0.35**	0.34**
SDMAF (%)	0.28**	-0.13	-0.07	0.25**	0.09	-0.16*	-0.10	-0.13
RWCBF (%)	0.18*	-0.36**	-0.24**	0.34**	0.19**	-0.38**	-0.26**	-0.33**
SFMBF (%)	0.28**	-0.22**	-0.11	0.31**	-0.12	-0.25**	-0.15*	-0.20**
SDMBF (%)	0.27**	-0.15*	-0.01	0.23**	-0.11	-0.18*	-0.01	-0.13
RIW[1] (%)	0.52**	-0.58**	-0.42**	0.87**	0.42**	-0.66**	-0.65**	-0.67**

[1] Reduction in water content due to frost stress (%).

[2] Arbitrary units.

*, ** significant at the 0.05 and 0.01 level of the probability, respectively.

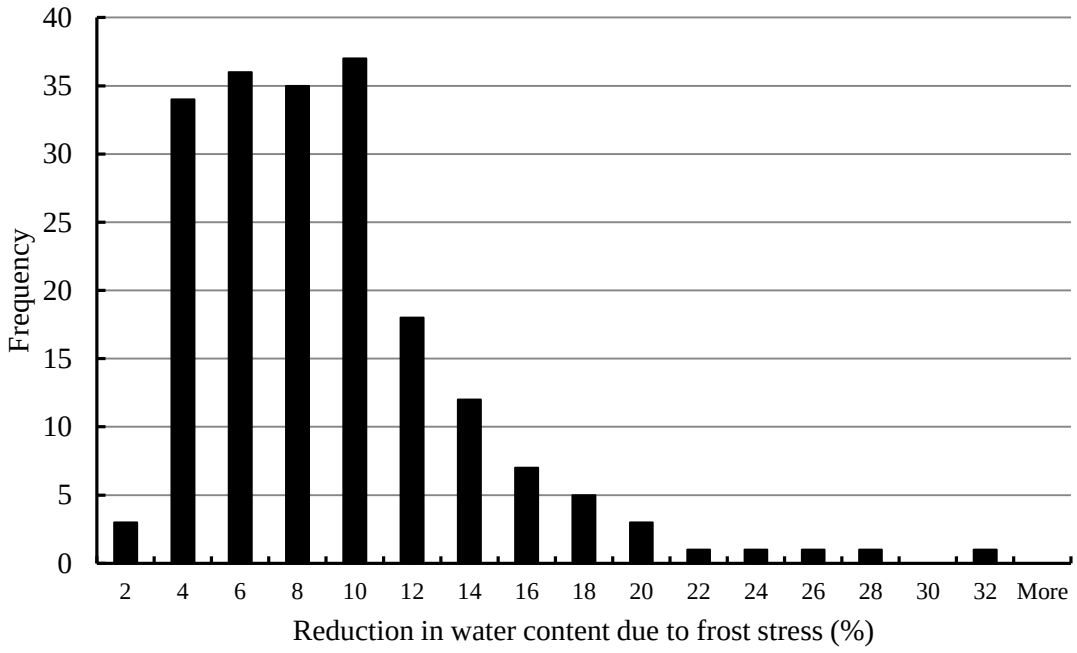

Figure I- 1. Frequency of genotypes according to their reduction in water content due to frost stress (%).

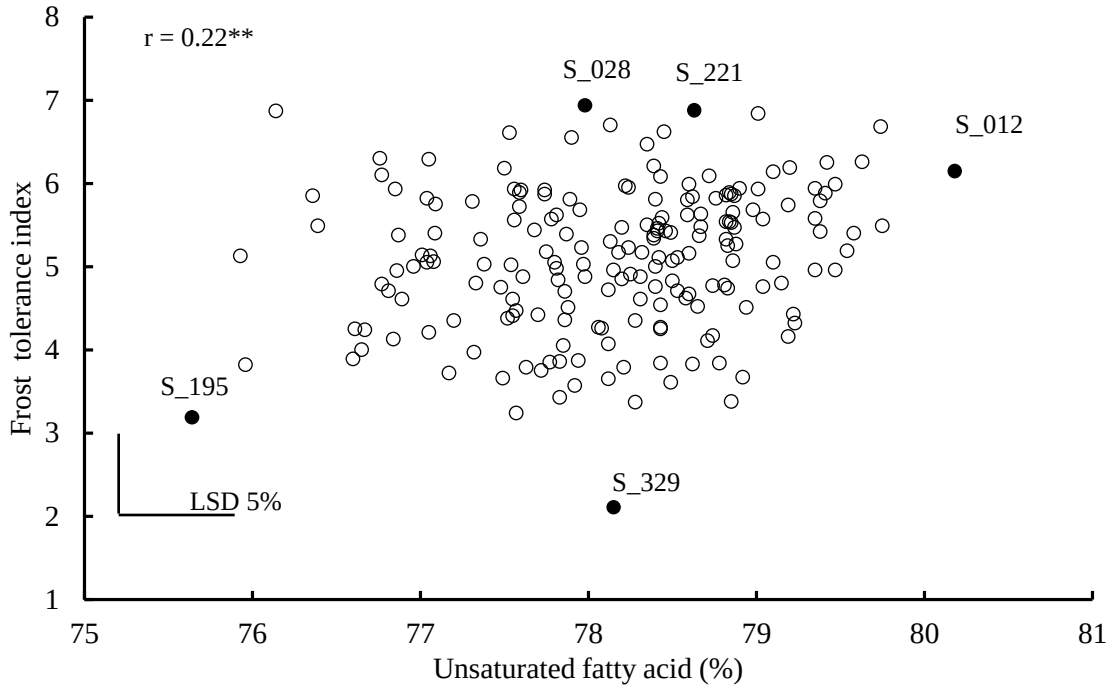

Figure I- 2. Frost tolerance index (frost experiments) and unsaturated fatty acid (hardening experiments).

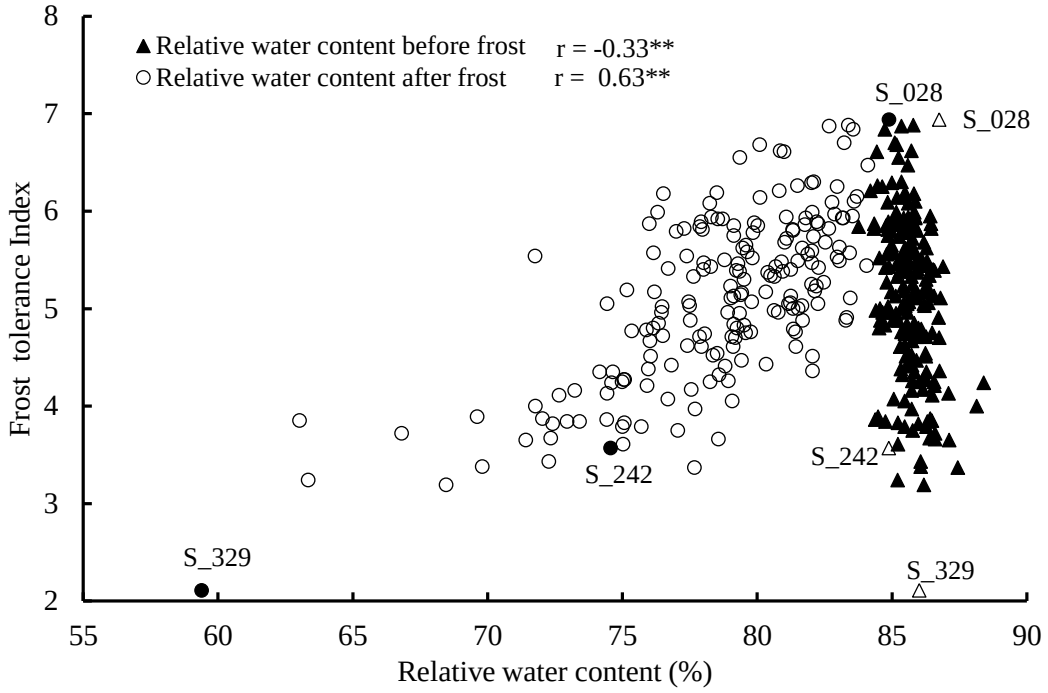

Figure I- 3. Relative water content in shoots (before frost - LSD 5% = 0.93, and frost experiments – LSD 5% = 4.17) and frost tolerance index (frost experiments, LSD 5% = 1.00).

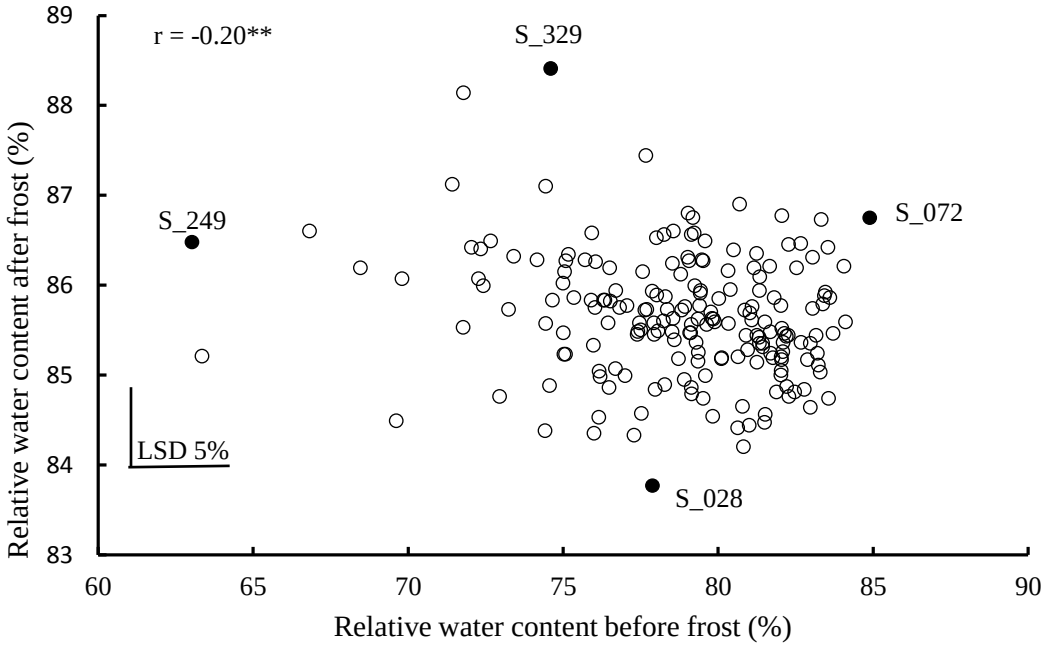

Figure I- 4. Relative water content in shoots after (frost experiments) and before frost (hardening experiments).

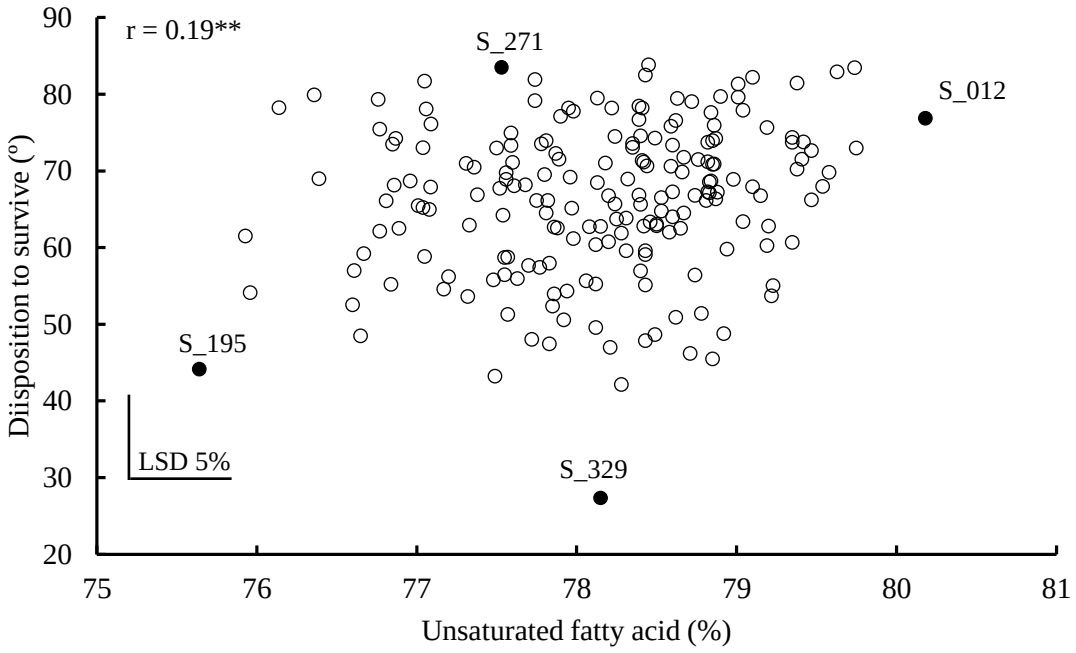

Figure I- 5. Disposition to survive (frost experiments) and unsaturated fatty acid (hardening experiments).

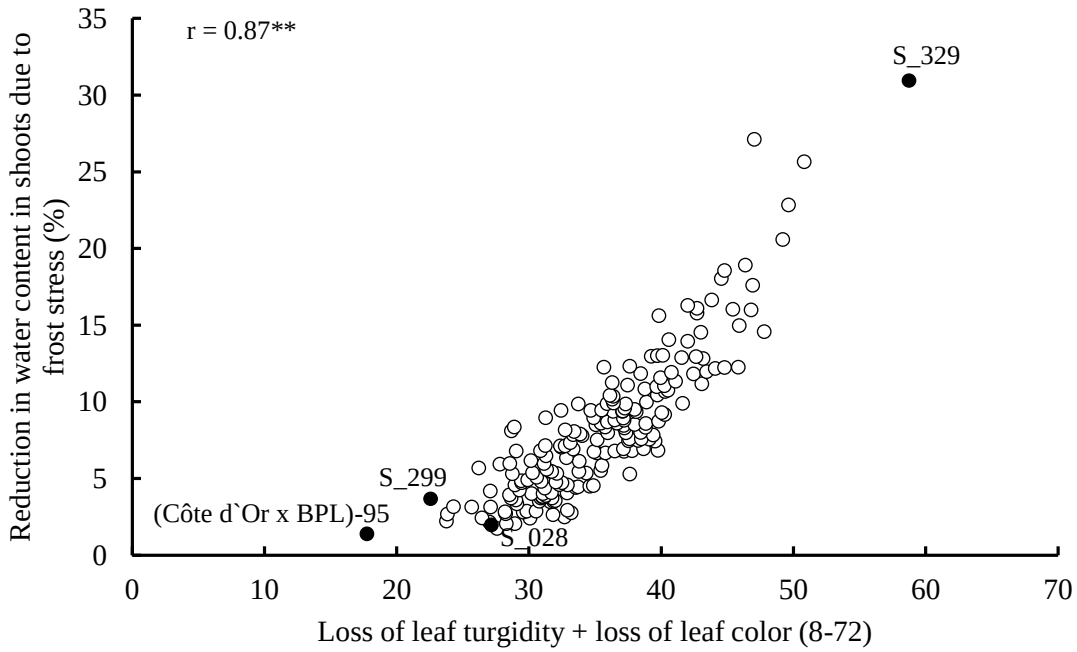

Figure I- 6. Reduction in water content in shoots due to frost stress and loss of leaf turgidity + loss of leaf color.

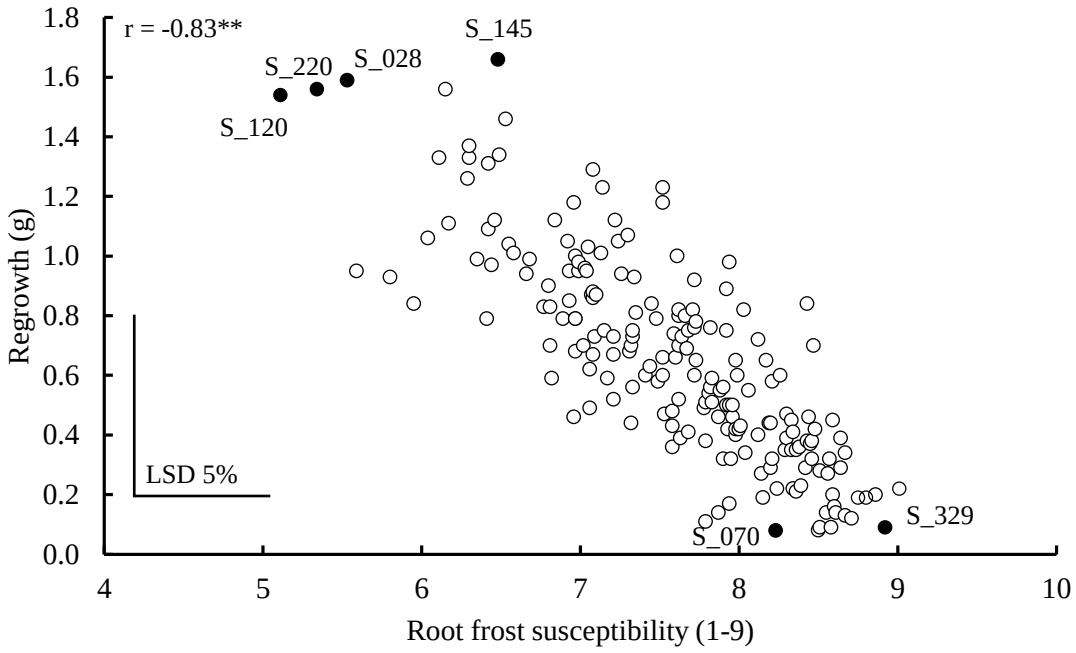

Figure I- 7. Regrowth (frost experiments) and root frost susceptibility (frost experiments).

II. Genetic variation in winter hardiness in faba bean

Results

An analysis of variance for field traits is presented in Table II-1a and II-1b. The mean values, least significant differences (LSD), phenotypic standard deviations (SD), coefficients of variation (C.V.), and repeatability estimates (h^2) are presented in Table II-2. The correlation among field traits and between field traits and Frost Growth Chamber traits are presented in Table II-3 and II-4, respectively.

Analysis of variance

The analysis of variance for all traits scored in the field is presented in Tables (II-1a and II-1b). The analysis showed high significant differences between genotypes for all traits. The variance due to replications was only significant for field plant height (FPH), days to maturity (DTM), 1000 seed weight (1000-SW), seed yield, and seed fill duration (SFD). Highly significant incomplete blocks were found in all traits. All traits showed high repeatability estimates (h^2) extending from 62.06 for seed yield to 92.03 for days to flowering (DTF). For frost tolerant traits, the highest h^2 was for winter the survival trait (WSR, h^2 = 81.58), followed by leaf frost susceptibility (LFS, h^2 = 78.11) and plant development 2 (PD2, h^2 = 68.87). All traits exhibited a wide range of variation. Seed yield varied widely from 0 to 1632.32 g/2m^2 with an average of 967.79 g/2m^2 (Table II-2). Maturity varied from 294.98 to 312.58 days in the experimental lines. For days to flowering (DTF), the genotypes ranged from 131 to 166 days.

Correlation analyses

Correlations between field traits

The Spearman's rank correlations for each pair of traits are shown in Table II-3. Field plant height (FPH) was significantly correlated with all traits except seed fill duration (SFD). Plant development 2 (DP2) showed positive and significant correlations with seed yield(r = 0.45**) and survival rate (WSR, r = 0.43**) and it negatively and significantly correlated with leaf frost susceptibility (LFS) (r = -0.52**). A high positive significant correlation was found between WSR and seed yield (r = 0.44**). WSR was negatively and significantly correlated with LFS (r = -0.51**). A similar correlation was found between 1000-SW and both seed yield and SFD (r =

0.29**). Seed yield showed negative significant correlation with LFS (r = -0.28**) and positive significant correlation with days to flowering (DTF) and days to maturity (DTM). LFS was negatively as well as significantly correlated with DTF (r = -0.15*) and SFD (r = -0.51**). DTM showed significant correlation with DTF (r = 0.31**) and SFD (r = 0.46**).

Correlations between field traits and Frost Growth Chamber (FGCh) traits

The Spearman's rank correlation coefficients between field traits and FGCh traits are presented in Table II-4. Disposition to survive (DS) was negatively correlated with LFS (r = -0.29**) and positively correlated with both WSR (r = 0.36**) and seed yield (r = 0.27**). Likewise, regrowth (REG) showed similar significant correlation with LFS, WSR, and seed yield. Loss of leaf turgidity + loss of leaf color (LT+LC) was positively and significantly correlated with LFS (r = 0.27**) and negatively correlated with seed yield (r = -0.21**). Root frost susceptibility (RFS) showed negative significant correlation with PD2, WSR, and seed yield. On the other hand, RFS showed positive significant correlation with LFS (r = 0.22**). Selection indices showed significant correlations with almost all field traits. DS showed similar significant correlation with index 1 (I_1, including DS), index 2 (I_2, including REG), and frost tolerance index (FTI, including REG and DS). Similarly, LFS showed correlations with the three selection indices. Selection indices also showed positive significant correlations with seed yield. Compared with PD1, selection indices showed higher significant correlation with PD2.

Discussion

Variation in winter hardiness

Winter hardiness is considered a complex trait compared to resistance against flooding, mechanical stress, fungal disease, freeze drying and freezing temperature (Herzog, 1988). The symptoms of frost stress were observed between genotypes after the frost period (Figure 3, Material and method). The main traits of winter hardiness are PD2, LFS, and WSR. The superior winter hardy genotypes differed by trait; S_169 (PD2), CôteD`Or/1 (LFS) and S_309 (WSR). On the other hand, S_329 was the most susceptible genotype for all of these three traits. The line CôteD`Or/1 was reported to be a superior frost tolerant genotype in previous studies (Herzog, 1988 and Arbaoui, 2007). Remarkably, none of aforementioned lines was reported to be superior in frost tolerance for any trait scored in Frost Growth Chamber (FGCh) experiments, while

S_329 showed susceptibility frost for the most of traits scored in FGCh. This may be due to the difference in freezing temperature regimes (field and FGCh) which WB-set were exposed to (Figure 1 and 3, Materials and methods). Artificial freezing tests are valuable for predicting the frost tolerance and for discriminating between spring and winter types in fully-hardened pea or faba bean material (Herzog, 1988; Lejeune-Henaut and Wery, 1994). The considerable genetic variability of genotypes examined is evident for days to flowering (DTF), days to maturity (DTM), seed fill duration (SFD), seed yield and 1000 seed weight (1000-SW). All traits showed high repeatability estimates, indicating that selection could be fruitful for improving these traits after testing in many locations and years under field winter conditions. For winter hardiness traits, the highest h^2 was accounted for by WSR (81.58), followed by LFS (78.11) and then PD2 (79.44). Arbaoui (2007) reported high heritability estimates for overwintering (h^2 = 90.00) in faba bean lines. Moreover, narrow heritability estimates for winter hardiness in lentils ranged from 32.00 to 71.00 over five locations (Ali and Johnson, 2000). The inheritance and heritability studies of lentil winter hardiness under field conditions suggested that probably many genes are involved in controlling winter survival and their effects are highly affected by environment (Kahraman et al., 2004). In the Frost Growth Chamber (FGCh), disposition to survive (DS), tregrowth (REG), and loss of leaf turgidity + loss of leaf color (LT+LC) showed higher repeatability than WRS, PD2, and LFS as scored in field trial, indicating that selection to improve frost tolerance in winter faba beans would be more efficient under controlled environments. Using controlled environments, line selection can be effectively achieved due to the consistency of the environment (Ali and Johnson, 2000).

The best 20 genotypes for field traits (LFS, WSR, seed yield, 1000-seed weight) and for FGCh traits (RFS, LT+LC, DS, REG, and FTI) were selected to identify the frost tolerant genotypes with high yield attributes. As an example, S_299 was among the best 20 genotypes for all FGCh traits and for some field traits such as LFS and 1000-SW. In addition, this genotype showed high WSR (92.12 %) with high seed yield (1207.38 g\2m^2). Most of the best frost tolerant genotypes in FGCh experiments showed high winter survival rates in the field. This indicates the possibility of selecting frost tolerant genotypes with high yield attributes and introducing them in future breeding programs. In contrast, some best genotypes for WSR (90 % < WSR < 100%) showed low DS (FGCh experiments) after artificial frost. For instance, S_038 (WSR = 99.00 %, DS = 58.68º), S_186 (WSR = 97.12 %, DS = 48.47º), and S_077 (WSR = 96.54%, DS = 46.98º).

The cold chamber conditions were preferable for selecting the best genotypes that had displayed an acceptable performance in field trials, but the contrary was not true. Therefore, choosing the best genotypes to cold resistance should be based on their performances in the cold chamber and then among these the best frost tolerant genotypes in the field can be chosen (Ordás et al., 2006)

Correlation analyses

The correlation analyses were divided into two categories: correlation analysis between field traits (Table II-3) and correlation analysis between field traits and traits scored in FGCh (Table II-4).

Firstly, field plant height (FPH) showed positive significant correlation with WSR (r = 0.25**). Both traits were scored after frost which affected them by killing some plants that belong to the same genotype. Therefore, this association may not be helpful because it depends on the temperatures that occur in the winter season. For example, if both traits are evaluated after exposure mild season, the correlation between these two traits will be different. A similar significant positive correlation also was found between the ratio of plant height to number of leaves and winter survival (r = 0.47*) by Annicchiarico and Iannucci (2007). Moreover, a positive relationship between plant height and winter hardiness in oat was reported by (Marshall, 1976). In contrast, no significant association was found between plant height and frost tolerance in 48 frost-tolerant (r = 0.13) and 57 non-frost-tolerant (r = -0.21) durum wheat lines tested in the field across four locations (Longin et al., 2013). In the case of FGCh experiments, plant height (PH) showed high and significant negative correlations with DS (r =-0.48**) and REG (r = -0.39**). These correlations are informative because plant height was scored before frost (see Materials and methods), whereas, DS and REG were scored after frost. Therefore, PH can be used to predict frost tolerance.

Compared to plant development 1 (PD1), PD2 showed stronger significant correlations with WSR (r = 0.43**) and LFS (r = -0.52**). PD2 is found to be significantly correlated with seed yield (r = 0.45**). This indicates that a good status of shoots after frost promises a high survival rate and therefore high seed yield. WSR was negatively and significantly correlated with LFS (r = -0.52**) and positively correlated with seed yield (r = 0.44**). The winter survival of plants is considered to be a meaningful test for cold tolerance in the field (Fowler et al., 1981). In FGCh, a

similar correlation was found between LT+LC and both disposition to survive (r = -0.61**) and regrowth (r = -0.50**) (Table I-6, Chapter I). DTF showed positive and significant correlation with WSR (r = 0.17*) and negative significant correlation with LFS (r = -0.15*), indicating that winter hardy genotypes tended to flower slightly late. Time of flowering influences plant tolerance after frost stress periods. Rout and Senapti (2013) reported that regulation of flowering generally is found to be associated with cold and frost conditions since it is considered as an adaptation mechanism to avoid abiotic stresses such as cold and frost. Various strategies have been suggested for conducting the selection of materials with a longer vegetative growth phase, i.e. flowering only after frost period (Guedes-Pinto et al., 1966). A significant negative correlation was found between LFS and seed fill duration (SFD, r = -0.51**). Late flowering and long fill duration are good characteristics for winter hardy genotypes. The correlation between DTF and SFD was positive and significant (r = 0.33**). 1000-SW showed positive correlation with DTM (r = 0.23**) and SFD (r = 0.26**). On the other hand, no correlation was found between 1000-SW and winter hardiness traits. Since winter hardy genotypes tend to show late maturity, seed size as scored in 1000-SW may show some association with other winter hardy traits (different from PD2, WSR, and LFS).

Secondly, the correlations between field traits and traits scored in the Frost Growth Chamber (FGCh) (Table II-4) promise an improvement of frost tolerance in faba beans. Many previous studies strongly recommended conducting experiments in growth chambers (Newman and Bailey, 1987; Frencel et al., 1987; Rimmer and van den Berg, 1992). The advantage of conducting experiments in growth chambers is the ability to test and evaluate seedlings of many genotypes in a short time and thus develop suitable resistant cultivars (Cargeeg and Thurling, 1980). Plant height (PH, FGCh experiments) was negatively and significantly correlated with WSR (r = -0.16*) and LFS (r = -0.14*). This points to the aforementioned association between plant height, as a trait scored before frost, and frost tolerance prediction. In its vegetative stage, a shorter seedling may enhance winter survival through its correlations with lower relative plant water content (Herzog, 1988). Such correlations between PH and relative water content after frost (r = -0.52**) and before frost (r =0.18*) were obtained in FGCh experiments (Table I-10, Chapter I). WSR showed similar positive significant correlation with DS (r = 0.35**) and REG (r = 0.36**). A high significant correlation (r = 0.97**) was reported also between survival rate and disposition to survive (Roth and Link, 2010). Furthermore, Hu et al., (2010) observed the

ability of faba bean leaves to regrow after the damage caused by low temperature. They concluded that this trait (regrowth) could be used as one of the tools to test for winter hardiness in faba beans. Loss of leaf turgidity + loss of leaf color (LT+LC, FGCh experiments) showed significant correlation with LFS (r = 0.27**) and WRS (r = -0.16*), indicating the reliability of visual scoring in both field and controlled environments in assessing frost injury in faba bean leaves. Visual scoring of frost injury is feasible because it allows large number of plants to be screened with little efforts and time. A significant negative correlation was found between overwintering (scored in field) and leaf frost symptoms (r = -0.43*) as assessed in the Frost Growth Chamber (Arbaoui, 2007). Root frost susceptibility (RFS, FGCh experiments) verified its contribution to frost tolerance through its significant correlations with WSR (r = -0.28**) and LFS (0.22**) in the field trials. It offers additional confirmation to the reliability of visual scoring in the evaluation to improve frost tolerance in faba beans.

Remarkably, the frost tolerance index (FTI, including DS and REG, FGCh experiments) showed significant correlations with traits scored in the field experiments except 1000-SW, indicating that artificial freezing tests may provide valuable information to improve frost tolerance. Equally important, seed yield showed significant correlation with all traits except PH scored in the FGCh experiments. This indicates that FGCh traits may be used as predictors for high yield. However, a single season is not enough to select for high seed yield combined with frost tolerance. WB-set needs to be tested in many different locations and over several years to select for the most desirable agronomic features.

Although high significant correlations were found between field traits and traits scored in FGCh, the size of these correlations are small. Poor correlations between seedlings and field reactions were reported in earlier studies (Newman and Bailey, 1987; Gugel et al., 1990; Gugel et al., 1991; Ordás et al., 2006). In our study, this low correlation may be due to the maximum freezing temperature to which all genotypes in the WB-set were exposed in FGCh (-19ºC) and in field (-12ºC) experiments. The differences between genotypes increased with the increase in freezing temperatures. Another reason for the low correlation might be that, plants in the field are exposed to various biotic and abiotic stresses, unlike to those tested in the FGCh experiments. All genotypes in the WB-set were scored in field trials for their susceptibility to naturally occurring powdery mildew and ascochyta blight (data not shown). Winter faba bean cultivars

should show some resistance to biotic stress in addition to frost tolerance (Stoddard et al., 2006; Sillero et al., 2010). Although significant differences between genotypes were observed with regards to their susceptibility to powdery mildew and ascochyta blight, no correlation was found between disease susceptibility and frost tolerance or winter hardiness traits.

Table II-1a. Analysis of variance (F-values) of traits measured in the WB-set, Experiment Field Station at Georg-August-Universität Göttingen during 2012–2013.

Source of variation [(1)]	DF	PD1 (1-9)	PD2 (1-9)	LFS (1-9)	DTF (Days)	FPH (cm)
Replications (R)	1	0.02	2.08	0.17	1.08	17.54 **
Genotypes (G)	195	5.79 **	5.46 **	7.97 **	93.99 **	11.59 **
Incomplete bocks	28	2.88 **	2.54 **	1.56 *	1.81 *	6.61 **
Intra block error	160					

(1) Lattice design

*, ** significant at the 0.05 and 0.01 level of the probability, respectively.

Table II-1b. Analysis of variance (F-values) of traits measured in the WB-set, Experiment Field Station at Georg-August-Universität Göttingen during 2012–2013.

Source of variation**(1)**	DF	DTM (Days)	1000SW (g)	seed yield	SFD (Days)	WSR (%)
Replications (R)	1	10.11 **	0.00	46.17 **	5.36 *	0.20
Genotypes (G)	195	6.74 **	10.19 **	4.31 **	7.90 **	9.85 **
Incomplete bocks	28	3.43 **	2.04 **	2.54 **	1.79 *	2.11 **
Intra block error	160					

(1) Lattice design

*,** significant at the 0.05 and 0.01 level of the probability, respectively.

Table II-2. Mean values (Mean), least significant difference (LSD), phenotypic standard deviations (SD), coefficient of variation (COV), and repeatability estimates (h^2) for 11 traits measured in the WB-set, Experiment Field Station at Georg-August-Universität Göttingen during 2012–2013.

Trait	Mean	$LSD_{0.05}$	SD[(1)]	C.V [(2)]	h^2
PD1(1-9)	6.81	1.01	0.88	12.92	70.18
PD2 (1-9)	6.40	1.08	0.90	14.06	68.87
LFS (1-9)	3.13	1.23	1.27	40.58	78.11
DTF (Days)	142.02	3.44	11.88	8.37	92.03
FPH (cm)	136.22	12.95	15.51	11.39	74.32
DTM (Days)	303.92	5.51	5.06	1.66	73.63
1000SW (g)	549.16	65.46	75.06	13.67	82.19
Seed yield g/2m^2	967.79	323.69	237.65	24.56	61.98
SFD (Days)	161.16	6.67	6.73	4.18	77.74
WSR (%)	90.13	13.77	15.47	17.16	81.58

(1) Standard deviation among adjusted-genotype mean according to a lattice design. [(2)]COV = (SD/Mean) x100.

Table II-3. Spearman's Rank correlation among traits measured in the WB-set, Experiment Field Station at Georg-August-Universität Göttingen during 2012–2013.

Trait	FPH	PD1	PD2	WSR	1000SW	seed yield	LFS	DTF	DTM
PD1 (1-9)	0.14*	-							
PD2 (1-9)	0.24**	0.16*	-						
WSR (%)	0.25**	-0.10	0.43**	-					
1000SW (g)	0.23**	0.29**	0.29**	-0.10	-				
seed yield (g/m^2)	0.48**	0.12	0.45**	0.44**	0.29**	-			
LFS (1-9)	-0.13	0.21**	-0.52**	-0.51*	0.01	-0.28**	-		
DTF(days)	0.33**	-0.04	-0.04	0.17*	-0. 10	0.32**	-0.15*	-	
DTM (days)	0.46**	-0.06	0.09	0.05	0.22**	0.37**	-0.13	0.31**	-
SFD (days)	0.06	-0.01	0.14	-0.09	0.29**	0.01	-0.51**	-0.66**	0.46**

*,** significant at the 0.05 and 0.01 level of the probability, respectively.
+, ++ coefficient is larger than one time and two times the standard error, respectively.

Table II-4. Spearman's rank correlation between field traits (season 2012/2013) and Frost Growth chamber traits during (seasons 2011/2012 and 2012/2013) scored for WB-set.

FiTr[1] \ FrTr[2]	PH (cm)	DS (º)	REG (g)	LT+LC (1-9)	RFS (1-9)	Index1 (DS)	Index2 (REG)	FTI
FPH (cm)	0.10	0.18*	0.18*	-0.02	-0.09	0.13	0.15*	0.16*
PD1 (1-9)	0.23**	-0.13	-0.11	0.22**	0.11	-0.16*	-0.15*	-0.15*
PD2 (1-9)	-0.03	0.24**	0.29**	-0.07	-0.21**	0.19**	0.25**	0.24**
WSR (%)	-0.18*	0.38**	0.39**	-0.16*	-0.30**	0.35**	0.36**	0.37**
LFS (1-9)	0.15*	-0.30**	-0.31**	0.27**	0.23**	-0.30**	-0.33**	-0.33**
1000SW (g)	0.04	-0.08	0.02	0.05	0.07	-0.08	-0.01	-0.05
seed yield (g/m^2)	-0.13	0.30**	0.37**	-0.21**	-0.22**	0.27**	0.32**	0.33**

[1] Traits measured in Experiment Field Station Georg-August-Universität Göttingen during season 2012/2013.
[2] Traits measured in Frost Growth Chamber (frost experiments) during seasons 2011/2012 and 2012/2013.
*,** significant at the 0.05 and 0.01 level of the probability, respectively.

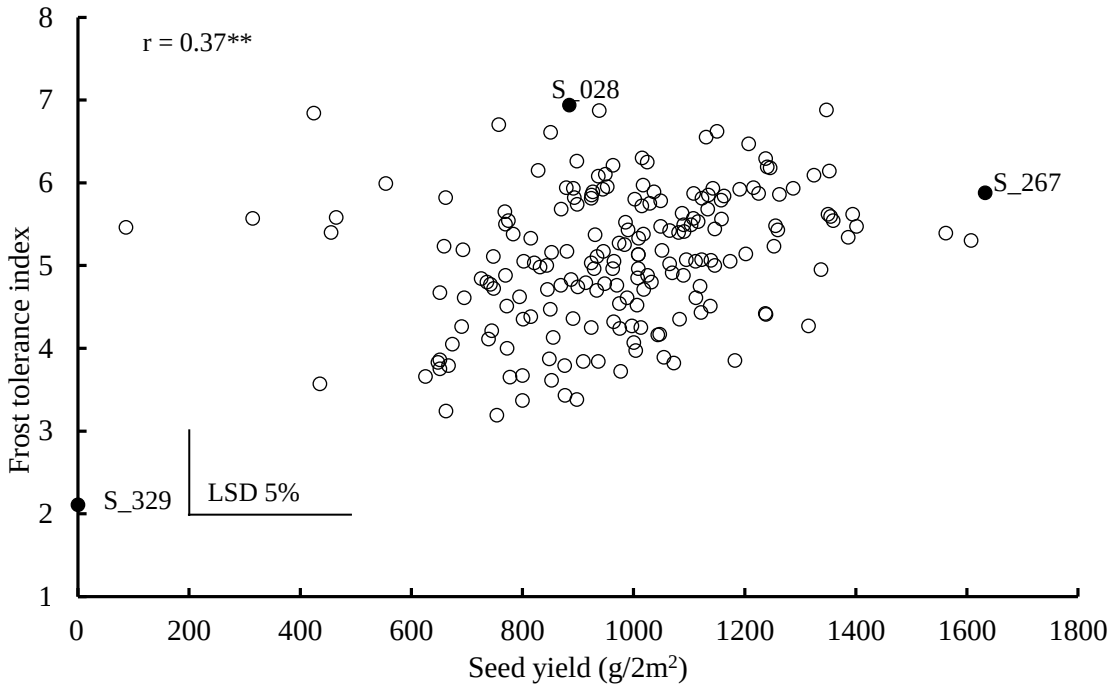

Figure II-1. Frost tolerance index measured in the FGCh experiments and seed yield measured in the field trial.

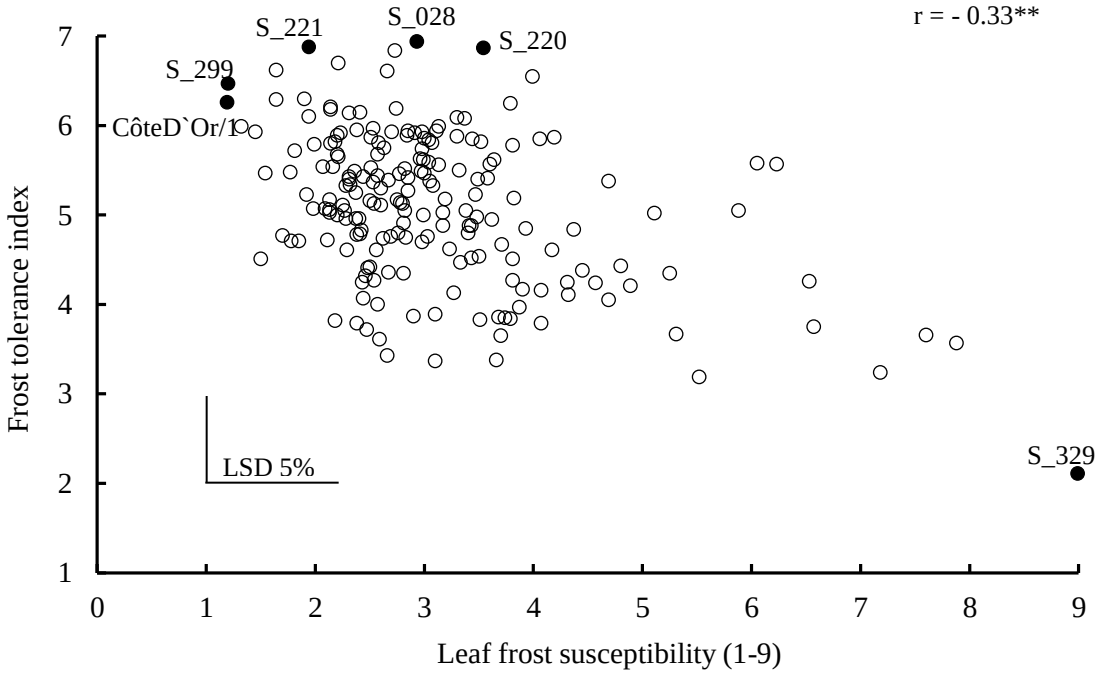

Figure II-2. Frost tolerance index measured in the FGCh experiments and leaf frost susceptibility measured in the field trial.

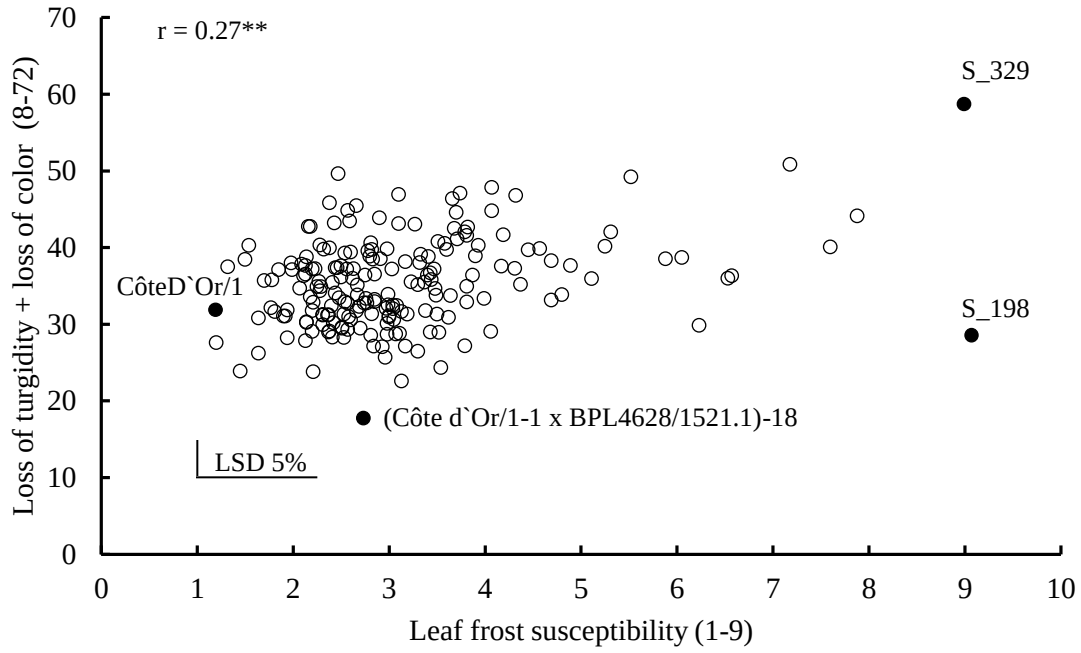

Figure II-3. Loss of leaf turgidity + loss of leaf color measured the FGCh experiments and leaf frost susceptibility measured in the field trial.

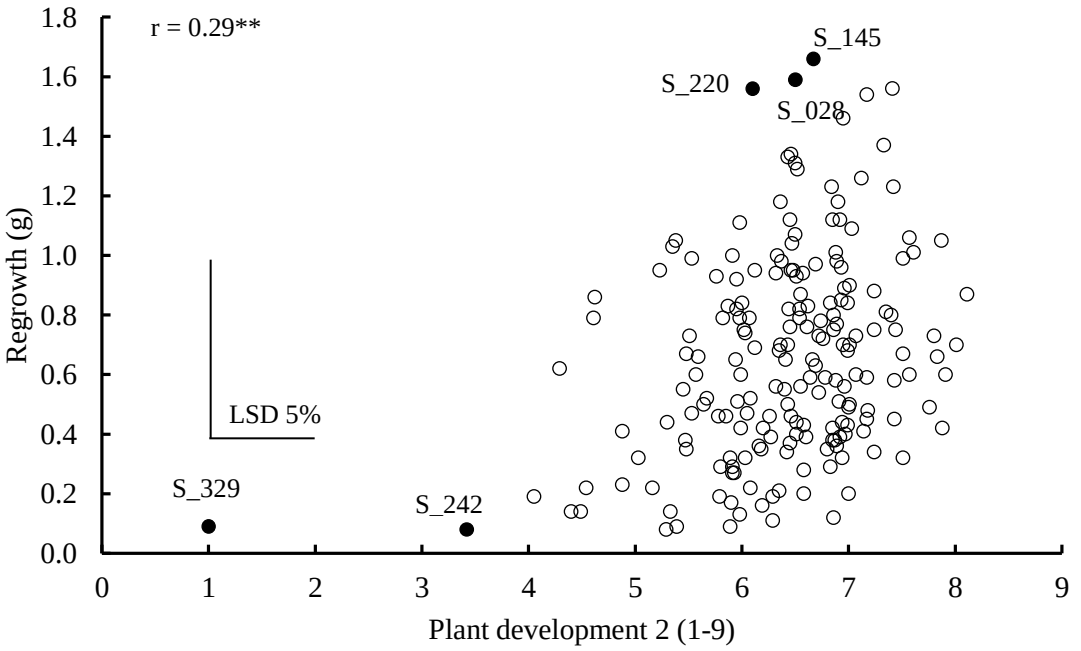

Figure II-4. Regrowth measured in the FGCh experiments and Plant development 2 measured in the field trial.

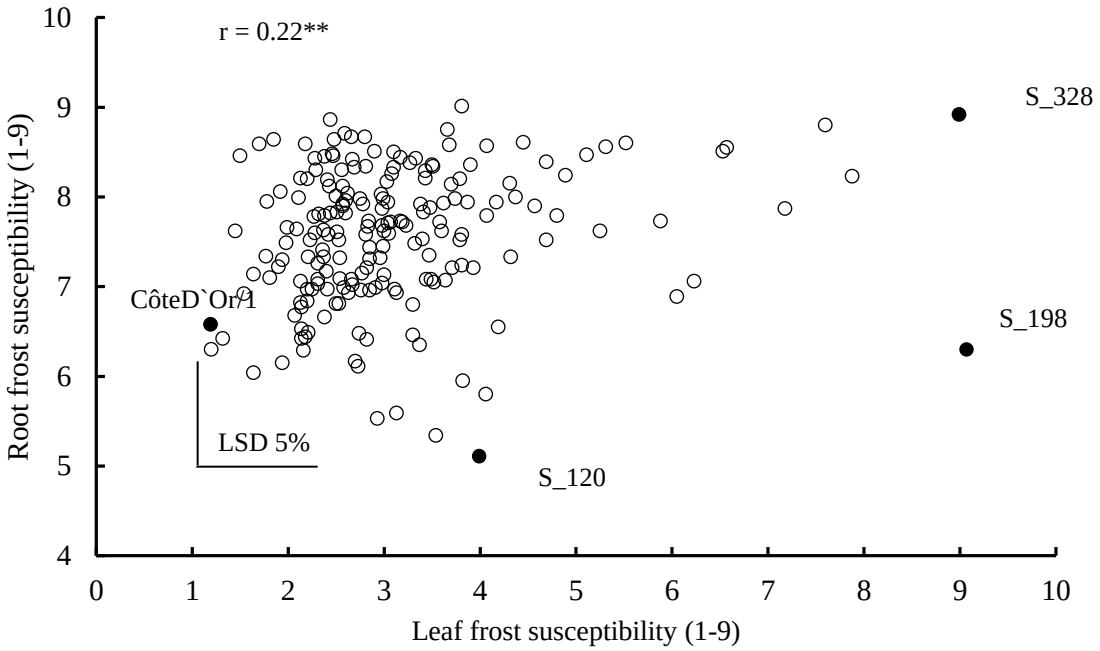

Figure II-5. Root frost susceptibility measured in the FGCh experiments and leaf frost susceptibility measured in the field.

III. Association analysis of frost tolerance in faba bean

Results

The summary of the consensus map (Cottage et al., 2012) with SNP markers used in the association analysis is presented in Table III-1. The number of marker pairs and average level of LD (mean r^2) in different classes of marker pairs is presented in Table III-2. The association analyses for all traits are presented in Tables from III-3a to III-3k. Table III-4 contains markers showing significant association with more than one morphological and/or physiological trait.

Analysis of linkage disequilibrium (LD):

The number of markers which were used in the association analysis is presented in Table III-1. Out of 189 SNP markers, 156 markers showed polymorphism at ≥10 % allele frequency. Among these 156 markers, 113 markers were found to be mapped in the consensus map of O'Sullivan (Cottage et al., 2012) which includes 643 SNP markers distributed across six linkage groups. The r^2 coefficient was used to calculate pairwise linkage disequilibrium (Table III-2). As a result, 12,090 possible pair combinations were obtained with an LD average of r^2 = 0.0075. Out of these pairs, N =1,066 marker pairs were found to be linked (both markers of each pair belong to the same linkage group) with an LD average of r^2 =0.0121. These marker pairs ranged from 27 marker pairs located in LG02 to 12 marker pairs mapped in LG05. Furthermore, the number of unlinked marker pairs was 11,024 with an average LD of r^2 = 0.0070.

The linkage disequilibrium (r^2) between each pair of linked markers (N = 1,066) was plotted against the genetic distance of their two markers (in cM) as obtained from the consensus map in order to determine LD decay (Figure III-1). The LD decayed below r^2 = 0.091 at about 18 cM. All linked marker pairs were grouped according to their distances (Figure III-2) and group means were plotted against the LD averages of each (see Material and method) to give a clearer visualization of LD decay. A group of 47 marker pairs showed a decay of LD = 0.015 at 20 cM. The majority of marker pairs (N = 900) showed an LD (r^2) of between 0.00 and 0.02 (Figure III-3). Using a Bonferroni correction (p = 4.1 x 10^{-6}), only 21 (0.17%) of the 12,090 pairwise combinations showed a significant LD with an r^2 average of 0.206. Furthermore, among linked marker pairs, 1.22% showed a significant LD with an LD average of r^2 = 0.219 (Table III-2).

A share of 0.07% of the unlinked marker pairs showed a significant LD with an LD average of r^2 = 0.186. Linked as well as unlinked markers pairs in significant LDs are illustrated in Figure III-4. A distinct relationship was found between LD and genetic distance in the linked marker group.

Principal coordinates analysis (PCoA)

Using the genetic distance values (simple matching coefficient), the two dimensional principle coordinates analysis (PCoA) is illustrated in Figure III-5, III-6. PCoA 1 and PCoA 2 account for 5.18% and 3.96 % of total variation, respectively. Notably, no specific group was found and the points were rather evenly distributed in the plot. The 10 most tolerant as well as susceptible genotypes were labeled based on FTI (frost experiments). Obviously, the genotypes in both groups look randomly distributed and there was no specific grouping detected for either tolerant or susceptible genotypes. The diagram PCoA 3 versus PCoA 4 confirms the even distribution of the inbred lines, covering 3.53% and 3.45% of the total variation.

Association Mapping (AM)

The association analysis for morphological as well as physiological traits described in Chapter I are presented in Tables from III-3a to III-3k. A total number of putative 70 QTLs were found for six morphological traits (disposition to survive, loss of leaf turgidity + loss of leaf color, plant height, frost tolerance index, index 1, index 2) and for seven physiological traits (saturated fatty acid content, unsaturated fatty acid content, water content before frost, water content after frost, reduction in water content due to frost stress, C16:0, and shoot fresh mater after frost). For all traits presented here, no significant LD (the correlation between alleles at two loci) was found between any two markers mapped in the same linkage group.

1- Disposition to survive (DS):

A number of 11 markers were found to be significantly associated with DS (Table III-3a). These markers were mapped in four linkage groups (based on the consensus map of O'Sullivan et al., 2012); five in LG01, three in LG02, two in LG03, and one in LG04. All markers showed allele effects ranging from 3.67° for allele G in marker Vf_Mt5g075540 to 9.92° for allele G in marker Vf_Mt3g086600. The frequency of the alleles associated with increased DS (better survival after frost) ranged from 0.26 to 0.89. The percentage of phenotypic variation explained by the 11

SNPs for DS extended from 3.36% in the case of Vf_Mt5g015280 (LG01) to 9.57% (Vf_Mt3g086600 - LG02).

2- Loss of leaf turgidity + loss of leaf color (LT+LC):

The result of the association analysis for LT+LC is shown in Table III-3b. A total of seven markers showed significant association with LT+LC. These markers were mapped in three LGs; two markers in LG01, three markers in LG02, and two markers in LG03. The allele effects of those markers ranged from 2.40 for allele C in marker Vf_Mt5g075540 to 6.04 for allele T in marker Vf_Mt3g086600. The allele frequency associated with increased LT+LC (high frost symptoms) ranged from 0.11 to 0.71. The minimum phenotypic variation (3.91%) was explained by one marker in LG02 (Vf_Mt4g007030) and one marker in LG03 (Vf_Mt1g056180). In contrast, the maximum phenotypic variation of 11.49% was explained by one marker mapped in LG01 (Vf_Mt5g046030).

3- Plant height (PH)

A total of four SNP markers were found to be significantly associated with plant height (Table III-3c). Three markers showed a significant association with PH at 0.20 of FDR, and one additional marker showed significant association at 0.22 of FDR. Out of these four markers, two markers were distributed across two linkage groups; one marker each in LG02 and LG04. The minimum effect (0.38 cm) was found for allele A in marker Vf_Mt4g007030, whereas, the maximum effect (0.68 cm) was found for allele T in marker Vf_Mt4g101130. The frequency of the allele associated with increased PH ranged from 0.12 for to 0.66. The phenotypic variation explained by these markers ranged from 4.42% for GLIP265SNP (unmapped) to 6.94% for Vf_Mt4g101130 (LG04).

4- Frost tolerance index (FTI, including disposition to survive and regrowth)

The association analysis results of FTI are shown in Table III-3d. Four markers showed significant association with FTI. The four markers were distributed through three linkage groups with two markers in LG01 and only one each in LG02 and LG03. The phenotypic variation explained by these markers extended from 4.75% for marker Vf_Mt2g086880 (LG01) to 9.21% for marker Vf_Mt5g046030 (LG01). The allele frequency which was associated with increased FTI ranged from 0.37 for allele T in marker Vf_Mt2g086880 (LG01) to 0.89 for allele G in

marker Vf_Mt3g086600 (LG02). All markers presented effect sizes for FTI with a range extending from 0.39 to 0.84.

5- Index 1 (including disposition to survive)

Out of 156 SNP markers used in the association analysis, 11 markers were found to be significantly associated with index 1 (Table III-3e). All markers were attached in four linkage groups: five markers in LG01, three markers in LG02, two markers in LG03, and one marker in LG04. A minimum phenotypic variation of 3.93 % was given by marker Vf_Mt5g015280 (LG01). On the other hand, marker Vf_Mt3g086600 (LG02) showed a maximum phenotypic variation of 10.58 %. A range of allele frequency associated with increased index 1 was found from 0.26 for allele T in marker Vf_Mt4g127690 (LG04) to 0.89 for allele G in marker Vf_Mt3g086600 (LG02). The allele effects ranged from 3.43 for allele G in marker Vf_Mt5g075540 to 9.16 for allele G in marker Vf_Mt3g086600.

6- Index 2 (including regrowth):

Only two markers showed significant association with index 2 (Table III-3f). These two markers were mapped in two different linkage groups: LG02 and LG03. The phenotypic variation explained by Vf_Mt3g086600 was 6.46 %, while Vf_Mt1g105040 explained a phenotypic variation of 6.98%. The allele G in both markers was found to be significantly associated with increased index 2 with a high frequency of > 0.80.

7- Relative water content after frost (RWCAF):

Four markers were found to be significantly associated with RWCAF in shoots (Table III-3g). Two of these markers were presented in LG02, while, one marker was presented each in LG01 and LG03. The phenotypic variation explained by these markers extended from 4.40% for Vf_Mt3g086600 (LG02) to 8.24% for Vf_Mt5g046030 (LG01). The frequency of alleles associated with increased RWCAF ranged from 0.53 to 0.89. The allele effects extended from 1.69 for allele G in marker Vf_Mt5g075540 to 3.85 for allele G in marker Vf_Mt3g086600.

8- Reduction in water content due to frost stress (RIW):

Six significant markers were the results of association analysis of RIW (Table III-2h). All of these markers were mapped in three different linkage groups: two markers in each LG of 01, 02, and 03. The phenotypic variation explained by this marker ranged from 3.83% for

Vf_Mt5g015280 (LG01) to 9.49% for Vf_Mt5g046030 (LG01). A wide frequency of the alleles associated with increased RIW ranged from 0.11 % to 0.66. The allele effects ranged from 1.20 to 4.71%.

9- Unsaturated fatty acid content (USFA):

A total of nine markers showed significant association with USF (Table III-3i). Among these markers, one marker (LG01), four markers (LG02), one marker (LG04), and two markers (LG06) were mapped. A minimum phenotypic variation of 3.48% was explained by marker Vf_Mt5g075540, while marker HYPTE3SNP explained the maximum phenotypic variation of 9.41 %. Allele G in marker RBPC_0SNP showed the highest positive effect (0.55%) with 0.45 of allele frequency, whereas, allele G in marker Vf_Mt5g075540 showed the lowest positive effect of 0.31% with 0.49 of allele frequency.

10- Association analysis of palmitic acid (C16:0):

Eight markers were found to be highly and significantly associated with C16:0 (Table III-3j). Only one marker showed a significant association with C16:0 at 0.21 of FDR. Out of these eight, seven markers were attached in four different linkage groups: two markers in LG02, three markers in LG04, and one marker each in LG05 and LG06. The variance explained by markers ranged from 3.64% for marker Vf_Mt8g086470 to 9.03% for markerVf_Mt3g061590. The positive effects of alleles ranged from 0.24% for allele T in marker Vf_Mt3g114780 and allele A in marker Vf_Mt3g117800 to 0.49% for allele C in marker Vf_Mt3g061590. The frequency of those associated with increased C16:0 extended from 0.15 to 0.85.

11- Association analysis of saturated fatty acid content (SFA), relative water content before frost (RWCBF), and shoot fresh matter after frost (SFMAF):

Only three different markers showed as significant association with three different traits (Table III-3k). These three markers were mapped in two different linkage groups. In LG02, one marker showed association with SFA and one with SFMAF, while in LG01, one marker was significantly associated with RWCBF. Allele T in marker Vf_Mt2g027240 and marker Vf_Mt4g014710 was found to be associated with increased RWCBF and SFMAF, respectively. Furthermore, allele C was significantly associated with increased SFA content. Among this

group of traits, the maximum percent of variance (10.54%) was explained by marker Vf_Mt3g061590 in SFA content.

The markers which showed significant association with more than one trait are presented in Table III-4. A total of 12 markers showed a significant association with a group of traits. The markers Vf_Mt3g086600 and Vf_Mt1g105040 showed significant associations with the highest number of traits (seven), while only one marker (Vf_Mt5g026780) showed significant association with only two traits. Out of 12 markers, only two markers showed significant association with fatty acid composition (USFA and SFA contents). The allele T of these two markers was significantly associated with increased USFA content and decreased both C16:0 content and SFA content. Remarkably, all alleles of each marker showed one, and consistent trend with regard to their effects on frost tolerance. For instance, the allele T in marker Vf_Mt5g075540 showed significant association with increased DS, index 1, RWCAF, and USFA. The same allele was significantly associated with decreased LT+LC and RIW.

Discussion

The analysis of LD

A very low level of linkage disequilibrium (LD) was observed with a mean r^2 of 0.0075 for all possible combinations of marker pairs (Table III-2). This notion is strengthened by the observation that only 0.17% of marker pairs are in significant LD ($P_{0.05}$ = 4.1 x 10^{-6}). Ecke et al., (2010) stated that calculating the LD means over marker pairs (linked marker pairs were grouped based on their genetic distances and the LD of marker pairs within each group were averaged) gave a good estimation of the average LD at different distances in the rapeseed genome. Although, with an average r^2 of 0.219 (all linked marker pairs in significant LD), it was still quite low in absolute terms (Table III-2). Among the linked marker pairs (both markers on the same linkage group), 2.16% showed a significant LD which is much higher than the 0.33% of all unlinked marker pairs with a significant LD. This indicates that the major element of LD was due to genetic linkage and the residual LD may be due to selection or genetic drift or other factors which governs LD in this population of inbred lines. The marker pairs were classified based on their LD (Figure III-3). The majority of marker pairs with 900 pairs fell in the r^2 class between 0.00 and 0.02. Moreover, 13 marker pairs showed moderate LD ($0.10 > r^2 > 0.20$) and only one marker pair showed high r^2 of 0.80. This offers more explanation of the low LD in the

population. Low LD has been reported in *M. truncatula* (legume model) and soybean by Branca et al., (2011). The LD in this study was found to markedly decay at about 18 cM, however, only one marker pair in significant LD showed a large distance of 87.24 cM. This can be easily distinguished in Figure III-4, which presents the distance between marker pairs in significant LD, indicating that the extent of LD may differ between different genome regions in the current faba bean population. Some regions in the population's genome showed a high extension of LD across large distance (c.f. Ecke et al., 2010). Furthermore, the rate of LD decay helps the researchers to determine the number of markers that would be needed for Genome Wide Associating Analysis (GWAS) (Fedoruk, 2013). This number of markers will differ between populations as well as between species. In an experiment with lentils, the LD for the breeding material was the highest at around 20 cM (Fedoruk, 2013).

AM analysis for morphological and physiological traits:

Association mapping is considered a powerful approach to reveal variants that are responsible for important traits in different crops (Pritchard and Przeworski 2001; Reich et al., 2001). In this study, we have identified putative QTLs affecting frost tolerance and related traits using association mapping. Among the 156 SNP markers, 28 different markers showed a significant association with 13 morphological and physiological traits controlling frost tolerance in faba bean. Once SNP markers are associated with a target trait, they can be applied by plant breeders for marker assisted selection (MAS) in order to select individual plants having a combination of alleles of interest from large segregating populations (Oraguzie and Wilcox, 2007). Recently, SNP markers have been used in many studies to identify alleles with a relation to frost tolerance in rye (Li et al., 2011), barley (Visioni et al., 2013), and wheat (Zhao et al., 2013). In the present study, out of 28 significant markers, only four markers were found to be unmapped markers. The number of putative QTL resulting from the current association analysis differed between traits, ranging from 11 QTLs for disposition to survive (DS) and index 1 (including DS) to one QTL for saturated fatty acid contest (SFA), shoot water content before frost (WCBF), and shoot fresh matter after frost (SFMAF). The highest number of QTLs was found in both DS and index 1, which are the most important traits for the development of frost tolerance in faba bean (Link and Roth, 2010). This implies that high allelic variation is found in these traits and can be used in breeding programs to improve survival rate after frost in faba bean. Notably, the significant

markers showed stronger association with traits scored after frost stress (N = 8) than with traits scored before actual frost stress (but of course as well after hardening conditions; N=4). This may be due to the fact that freezing temperatures can create significantly large differences between genotypes, making the detection of QTLs easier. On the other hand, no significant markers were observed for regrowth, root frost susceptibility, shoot dry matter after and before frost, shoot fresh matter before frost, C18:0, C18:2, and C18:3. Association mapping has been confirmed to be a powerful tool for analyzing the complexity of quantitative traits in plants (Flint-Garcia et al., 2003; Nordborg and Tavare 2002; Mackay and Powell 2007). The significant variation in the number of QTLs detected in each trait may be explained by the genetic architecture of the traits (Ecke et al., 2010). Bearing in mind the high heritability estimates of morphological traits, phenotyping such traits under frost stress is beneficial for frost tolerance selection (Longin et al., 2013). Five QTLs for morphological traits and seven QTLs for fatty acid composition were reported as frost tolerant traits in faba bean (Arbaoui et al., 2008b). These QTLs were determined using 131 RAPD markers, which are less powerful in QTL detection than the SNPs used in this study (Arbaoui et al., 2008b).

In order to avoid an expected inflation in the number of QTLs detected in association analysis because of rare alleles, we applied a limit for the minor allele frequency of 10% for all 189 SNP markers. As a result, the applied set of 156 polymorphic SNP markers was identified. The allele frequency of the rare allele showed a very wide variation ranging, from 0.11 to 0.49. This offers more support for the reliability of the association analysis used here in this study. The power of AM to detect an association is influenced by allele frequency distribution at the functional polymorphism level. The problem of rare alleles is that they tend to be presented in few individuals and subsequently lack resolution power. The PCoA of genetic distances resulted in no clear group between the 186 lines. This indicates that there is probably no marked population structure which could cause spurious associations (Oraguzie and Wilcox, 2007). The high resolution of association mapping can be obtained by avoiding closely related genotypes samples (Sherry and Buckler, 2013). No significant LD was found between any marker pair in the same linkage group for any trait. This could imply that they mark single QTLs. Moreover, the current AM analysis revealed several putative QTLs (70 QTLs). Most of these QTLs (64) were of minor effect and explained less than 10% of the variance (Mauricio, 2006), and only five QTLs (two

for LT+LC, two for index1 and one for SFA) explained phenotypic variance ranging from 10.14 to 11.49%.

Interestingly, 12 markers were found to be associated with more than one trait. This enhances the prospects of MAS through choosing such most promising markers for improving frost tolerance in faba bean. Many earlier studies in QTL mapping reported the finding that QTLs for various traits sometimes occur at closely linked loci in the genome (Amar et al., 2008; Zhao et al., 2008; Basunanda et al., 2009). This may be due to the fact that these QTLs, which affect more than one trait, have pleiotropic effects at one locus. The pattern of allele effects in different traits reflects the pattern obtained in the phenotypic correlations between the traits (Tables from I-6 to I-10, see chapter 1). Taking the relationship between DS and LT+LC as an example, these two traits shared seven significant markers with opposite allele effects. These seven markers were found to have alleles associated with increased DS and decreased LT+LC. This corroborates the negative phenotypic ($r = -0.61^{**}$) and genetic ($r = -0.81$) correlations between these two traits. The highest number of shared significant markers was found between DS and index 1 (N = 11), which showed the same alleles effects on both traits. This can be expected from the positive correlation between these DS and index 1. Remarkably, two SNP markers showed an association with seven QTLs covering most of the morphological and some of the physiological traits. These two markers may be very useful in future breeding programs for improving frost tolerance. Generally, significant markers associated with QTLs need to be verified in different genetic backgrounds in order to enhance the selection process for rapidly improving frost tolerance in faba beans. Sallam et al. (2013, unpublished) employed the same number of SNP markers (N = 189) to re-construct the map of Arbaoui et al. (2008b) using their 101 lines derived from a cross between two frost tolerant genotypes. The QTL re-mapping was performed using the morphological and physiological data reported by Arbaoui et al. (2008b). As a result, 22 QTLs for morphological and physiological traits were mapped using 102 SNPs (mapped in 13 LGs). Out of the significant markers (markers showing association with QTLs) used in the QTL mapping (N =102) and in the association analysis of the present study (N = 156), 10 SNP markers (data not shown) were found to be associated with frost tolerant traits in these two different genetic backgrounds. These markers are highly recommended for use in a future faba bean breeding program for cultivars that show frost tolerance.

Table III-1. Summary of the consensus map (CM; taken from O'Sullivan., 2012) with SNP markers used in the association analysis.

Marker category	No. of SNP marker in CM (643 markers)	Length of LG in cM	No. of markers in A-set (156 markers)
Unmapped markers	-	-	43
Mapped markers	643	1597.29	113
- LG01	187	442.51	18
- LG02	119	236.99	27
- LG03	81	259.33	18
- LG04	91	218.97	20
- LG05	96	234.38	12
- LG06	69	205.11	18

Table III-2. Number of marker pairs and average level of LD (mean r^2) in different classes of marker pairs.

Class	Number	%	r^2
All marker pairs	12,090	100.00	0.0075
Linked marker pairs	1,066	8.82[(1)]	0.0121
Unlinked marker pairs	11,024	91.18[(1)]	0.0070
Marker pairs in significant LD at *P*= 4.1 x 10-6	21	0.17[(1)]	0.206
Linked marker pairs in significant LD	13	1.22[(2)]	0.219
Unlinked marker pairs in significant LD	8	0.07[(3)]	0.186

[(1)] Percentage from all (12,090) marker pairs. [(2)] Percentage from all linked marker pairs. [(3)] Percentage from all unlinked marker pairs

Table III-3a. The association analysis for disposition to survive (DS) in A-set.

LG	Marker locus	Allele[(1)]	Position	*p*	Marker R^2 (%)	Allele Frequency[(2)]	Allele effects	LD			
								1	2	3	4
LG01	Vf_Mt5g046030	T:C	293.36	0.000186	9.23	0.53	6.17	-			
LG01	Vf_Mt2g086880	T:C	62.66	0.000661	6.46	0.37	5.31	0.01	-		
LG01	Vf_Mt2g027240	C:T	401.92	0.002421	4.94	0.54	4.41	0.00	0.00	-	
LG01	Vf_Mt5g026780	A:C	301.30	0.004903	4.26	0.82	5.36	0.07	0.01	0.03	-
LG01	Vf_Mt5g015280	T:C	325.99	0.010416	3.36	0.34	3.89	0.00	0.00	0.00	0.00
LG02	Vf_Mt3g086600	G:T	103.65	0.000015	9.57	0.89	9.92	-			
LG02	Vf_Mt4g007030	C:A	234.93	0.008793	3.67	0.34	4.02	0.03	-		
LG02	Vf_Mt5g075540	G:C	31.06	0.010962	3.46	0.49	3.67	0.02	0.01	-	
LG03	Vf_Mt1g105040	G:T	97.13	0.002560	5.31	0.85	6.37	-			
LG03	Vf_Mt1g056180	A:G	227.32	0.009538	4.14	0.29	4.15	0.00	-		
LG04	Vf_Mt4g127690	T:C	24.50	0.003313	4.74	0.26	4.76				

[(1)] The 'left' allele increases the trait. [(2)] The allele frequency was calculated for the allele which increases the trait.

Table III-3b. The association analysis for loss of leaf turgidity + loss of leaf color (LT+LC) in A-set.

LG	Marker locus	Allele [(1)]	position	*p*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD	
								1	2
LG01	Vf_Mt5g046030	C:T	293.36	0.000027	11.49	0.47	4.03	-	
LG01	Vf_Mt2g027240	T:C	401.92	0.001230	5.59	0.46	2.78	0.01	-
LG02	Vf_Mt3g086600	T:G	103.65	0.000008	10.14	0.11	6.04	-	
LG02	Vf_Mt5g075540	C:G	31.06	0.005012	4.20	0.51	2.40	0.02	-
LG02	Vf_Mt4g007030	A:C	234.93	0.006812	3.91	0.66	2.46	0.03	0.01
LG03	Vf_Mt1g105040	T:G	97.13	0.001423	5.92	0.15	4.02	-	
LG03	Vf_Mt1g056180	G:A	227.32	0.007097	3.91	0.71	2.52	0.00	-

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.

Table III-3c. The association analysis for plant height (PH) in A-set

LG	Marker locus	Allele[(1)]	position	*P*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects
LG02	Vf_Mt4g007030[(3)]	A:C	234.93	0.002699	4.78	0.66	0.38
LG04	Vf_Mt4g101130	T:C	107.45	0.000270	6.94	0.12	0.68
-	Vf_Mt6g068920	A:T	-	0.003626	4.48	0.26	0.40
-	GLIP265SNP	A:T	-	0.003870	4.42	0.18	0.46

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.[(3)] Marker showed a significant association at FDR = 0.22

Table III-3d. The association analysis for frost tolerance index (FTI) in A-set.

LG	Marker locus	Allele[(1)]	position	*P*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD	
								1	2
LG01	Vf_Mt5g046030	T:C	293.36	0.000327	9.21	0.53	0.51	-	
LG01	Vf_Mt2g086880	T:A	62.66	0.003647	4.75	0.37	0.39	0.007	-
LG02	Vf_Mt3g086600	G:T	103.65	0.000023	8.54	0.89	0.84		
LG03	Vf_Mt1g105040	G:T	97.13	0.000477	7.07	0.85	0.63		

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.

Table III-3e. The association analysis for index 1 (I_1) in A-set.

LG	Marker locus	Allele[(1)]	position	*p*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD			
								1	2	3	4
LG01	Vf_Mt5g046030	T:C	293.36	0.000071	10.34	0.46	5.71	-			
LG01	Vf_Mt2g027240	T:C	401.92	0.001284	5.57	0.34	4.12	0.00	-		
LG01	Vf_Mt5g026780	A:C	301.3	0.006018	4.07	0.82	4.60	0.03	0.00	-	
LG01	Vf_Mt5g015280	T:C	325.99	0.006474	3.93	0.34	3.63	0.01	0.00	0.07	-
LG01	Vf_Mt2g086880	T:A	62.66	0.000692	6.42	0.37	6.62	0.01	0.00	0.01	0.00
LG02	Vf_Mt3g086600	G:T	103.65	0.000005	10.58	0.89	9.16	-			
LG02	Vf_Mt4g007030	C:A	234.93	0.004462	4.31	0.34	3.82	0.03	-		
LG02	Vf_Mt5g075540	G:C	31.06	0.006604	3.94	0.49	3.43	0.02	0.01	-	
LG03	Vf_Mt1g105040	G:T	97.13	0.002061	5.54	0.85	5.71	-			
LG03	Vf_Mt1g056180	A:G	227.32	0.006189	4.05	0.29	3.83	0.00	-		
LG04	Vf_Mt4g127690	T:C	24.5	0.004145	4.52	0.26	4.06				

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.

Table III-3f. The association analysis for index 2 (I_2) in A-set.

LG	Marker locus	Allele[(1)]	position	*P*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects
LG02	Vf_Mt3g086600	G:T	103.65	0.000415	6.46	0.89	0.18
LG03	Vf_Mt1g105040	G:T	97.13	0.000494	6.98	0.85	0.16

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.

Table III-3g. The association analysis for relative water content after frost (RWCAF) in A-set

LG	Marker locus	Allele[(1)]	position	*P*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD	
								1	2
LG01	Vf_Mt5g046030	T:C	293.36	0.000421	8.24	0.53	2.38		
LG02	Vf_Mt3g086600	G:T	103.65	0.000029	4.40	0.89	3.85	-	
LG02	Vf_Mt5g075540	G:C	31.06	0.003381	4.54	0.49	1.69	0.02	-
LG03	Vf_Mt1g105040	G:T	97.13	0.000345	7.40	0.85	3.05		

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.

Table III-3h. The association analysis for reduction in water content due to frost stress (RIW) in A-set.

LG	Marker locus	Allele[(1)]	position	*p*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD	
								1	2
LG01	Vf_Mt5g046030	C:T	293.36	0.000147	9.49	0.47	3.10	-	
LG01	Vf_Mt5g015280	C:T	325.99	0.007360	3.83	0.66	1.20	0.01	-
LG02	Vf_Mt3g086600	T:G	103.65	0.000027	9.12	0.11	4.71	-	
LG02	Vf_Mt5g075540	C:G	31.06	0.003519	4.56	0.51	2.05	0.02	-
LG03	Vf_Mt1g105040	T:G	97.13	0.000155	8.28	0.18	3.92	-	
LG03	Vf_Mt1g056180	G:A	227.32	0.006820	3.97	0.29	2.12	0.00	-

(1) The 'left' allele increases the trait. **(2)** The allele frequency was calculated for the allele which increases the trait.

Table III-3i. The association analysis for unsaturated fatty acid content (USFA) in A-set.

LG	Marker locus	Allele[(1)]	position	*p*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD			
								1	2	3	4
LG01	Vf_Mt2g086880	T:A	62.66	0.008028	3.99	0.37	0.34				
LG02	RBPC_0SNP	G:A	64.21	0.000032	9.15	0.45	0.55	-			
LG02	Vf_Mt3g076650	T:C	84.27	0.001603	5.28	0.84	0.43	0.01	-		
LG02	Vf_Mt3g061590	T:C	47.37	0.003774	4.49	0.74	0.48	0.02	0.01	-	
LG02	Vf_Mt5g075540	G:C	31.06	0.010972	3.48	0.49	0.31	0.03	0.00	0.00	-
LG04	Vf_Mt4g127690	T:C	24.5	0.002851	4.92	0.26	0.42				
LG06	HYPTE3SNP	T:A	157.01	0.000061	9.41	0.67	0.54	-			
LG06	Vf_Mt4g085900	A:G	87.24	0.005678	4.10	0.48	0.34	0.00	-		
-	Vf_Mt3g090670	A:G	-	0.001292	5.49	0.74	0.44				

[(1)] The 'left' allele increases the trait. [(2)] The allele frequency was calculated for the allele which increases the trait.

Table III-3j. The association analysis for palmitic acid (C16:0) in A-set.

LG	Marker locus	Allele[(1)]	position	*p*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects	LD		
								1	2	3
LG02	Vf_Mt3g061590	C:T	47.37	0.000032	9.03	0.16	0.49			
LG02	Vf_Mt3g114780	T:G	186.85	0.005950	4.02	0.48	0.24	0.03		
LG04	Vf_Mt3g117800	A:T	5.89	0.008238	3.73	0.64	0.24	-		
LG04	Vf_Mt4g125100	T:G	34.92	0.002479	4.89	0.78	0.32	0.00	-	
LG04	Vf_Mt4g127690	C:T	24.5	0.004892	4.39	0.65	0.29	0.02	0.16	-
LG05	Vf_Mt7g051360	A:G[(3)]	77.48	0.003886	4.51	0.27	0.29			
LG06	Vf_Mt8g086470	C:A	76.28	0.009065	3.64	0.85	0.32			
-	Vf_Mt1g030300	C:T	-	0.005864	4.05	0.15	0.33			

[(1)] The 'left' allele increases the trait. [(2)] The allele frequency was calculated for the allele which increases the trait..
[(3)] Marker showed a significant association at FDR = 0.21

Table III-3k. The association analysis for relative water content before frost (RWCBF), saturated fatty acid content (SFA), and shoot fresh matter after frost (SFMAF) in A-set.

Trait	LG	Marker locus	Allele[(1)]	position	*p*	Marker R^2 (%)	Allele frequency[(2)]	Allele effects
RWCBF	LG01	Vf_Mt2g027240	T:C	401.92	0.001066	5.76	0.46	0.34
SFA	LG02	Vf_Mt3g061590	C:T	47.37	0.000006	10.54	0.16	0.56
SFMAF	LG02	Vf_Mt4g014710	T:C	222.09	0.000576	6.26	0.62	0.14

[(1)] The 'left' allele increases the trait. [(2)] The allele frequency was calculated for the allele which increases the trait

Table III-4. Markers showing significant association with more than one morphological and/or physiological trait.

Marker locus	Allele	No. of traits	Morphological traits						Physiological traits					
			DS	LT+LC	PH	FTI	I_1	I_2	RIW	RWCAF	RWCBF	USFA	SFA	C16:0
Vf_Mt5g046030	T:C	6	+	-		+	+		-	+				
Vf_Mt2g086880	T:C	4	+			+	+					+		
Vf_Mt2g027240	C:T	4	+	-			+				+			
Vf_Mt5g026780	A:C	2	+				+							
Vf_Mt5g015280	T:C	3	+				+		-					
Vf_Mt3g086600	G:T	7	+	-		+	+	+	-	+				
Vf_Mt4g007030	C:A	4	+	-	-		+							
Vf_Mt5g075540	G:C	6	+	-			+		-	+		+		
Vf_Mt1g105040	G:T	7	+	-		+	+	+	-	+				
Vf_Mt1g056180	A:G	4	+	-			+		-					
Vf_Mt4g127690	T:C	4	+				+					+		-
Vf_Mt3g061590	T:C	3										+	-	-

+ / -: sign indicates positive and negative effects of the ‘left’ allele.

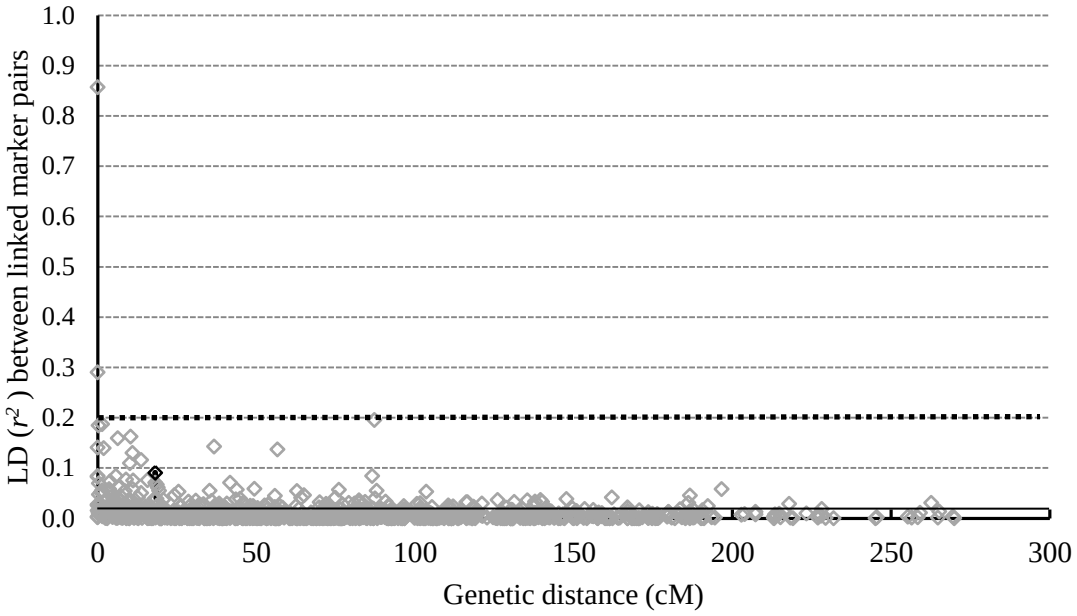

Figure III-1. Linkage disequilibrium decay and genetic distance between each marker pair (N = 1066). Dashed line at $r^2 = 0.206$ refers to Bonferroni correction at 5% . The continuous line at $r^2 = 0.023$ refers to the uncorrected significance level (χ^2) at 5%. The black point refers to LD decay ($r^2 = 0.091$ at 18 cM)

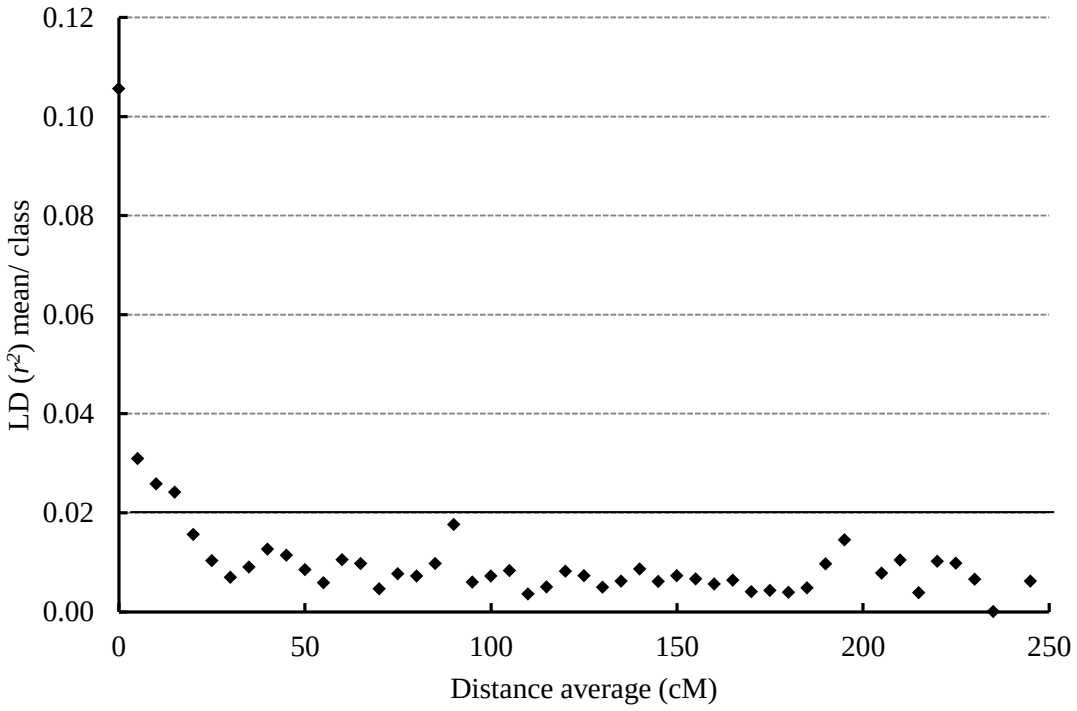

Figure III-2. Average of LD versus genetic distance average of marker pair class taken in steps of 5 cM. The continuous line at $r^2 = 0.023$ refers to the uncorrected significance level (χ^2) at 5%.

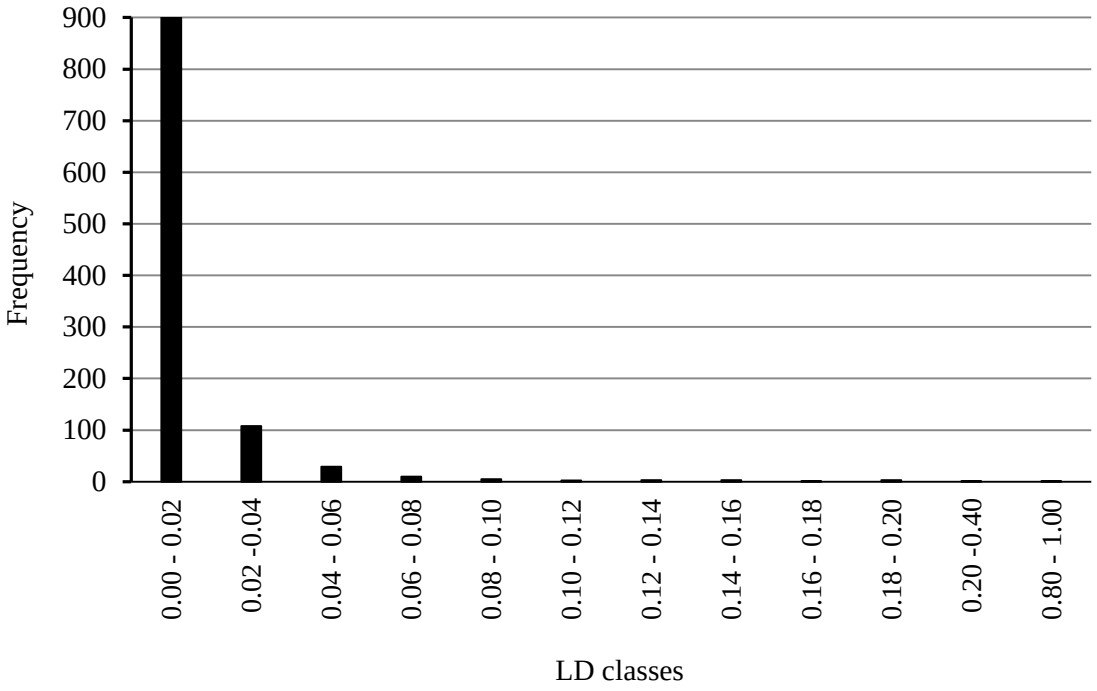

Figure III-3. The frequency of marker pairs based on their LD class.

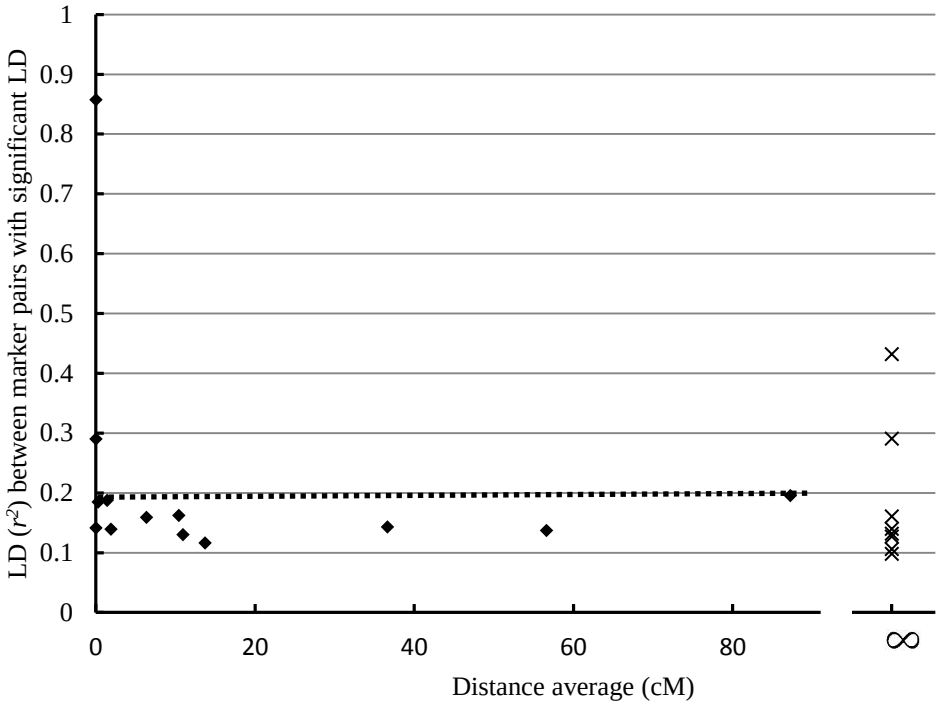

Figure III-4. Significant LD of linked and unlinked marker pairs at different genetic distances. Dashed line at $r^2 = 0.219$ refers to Bonferroni correction at 5%

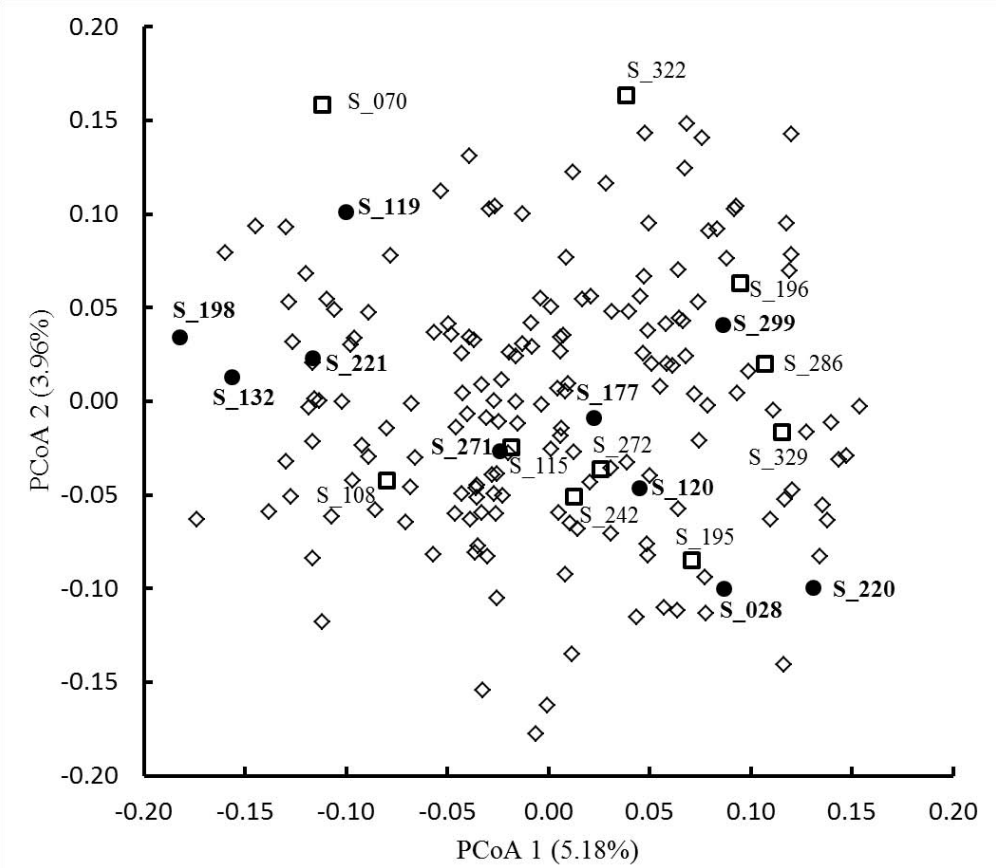

Figure III-5. Results of a principle coordinate analysis (PCoA) on genetic distances between lines in A-set. The explained variance is given in brackets. **Bold and filled** circles refer to the 10 most tolerant genotypes based on FTI, whereas, the unfilled squares refer to the 10 most susceptible genotypes.

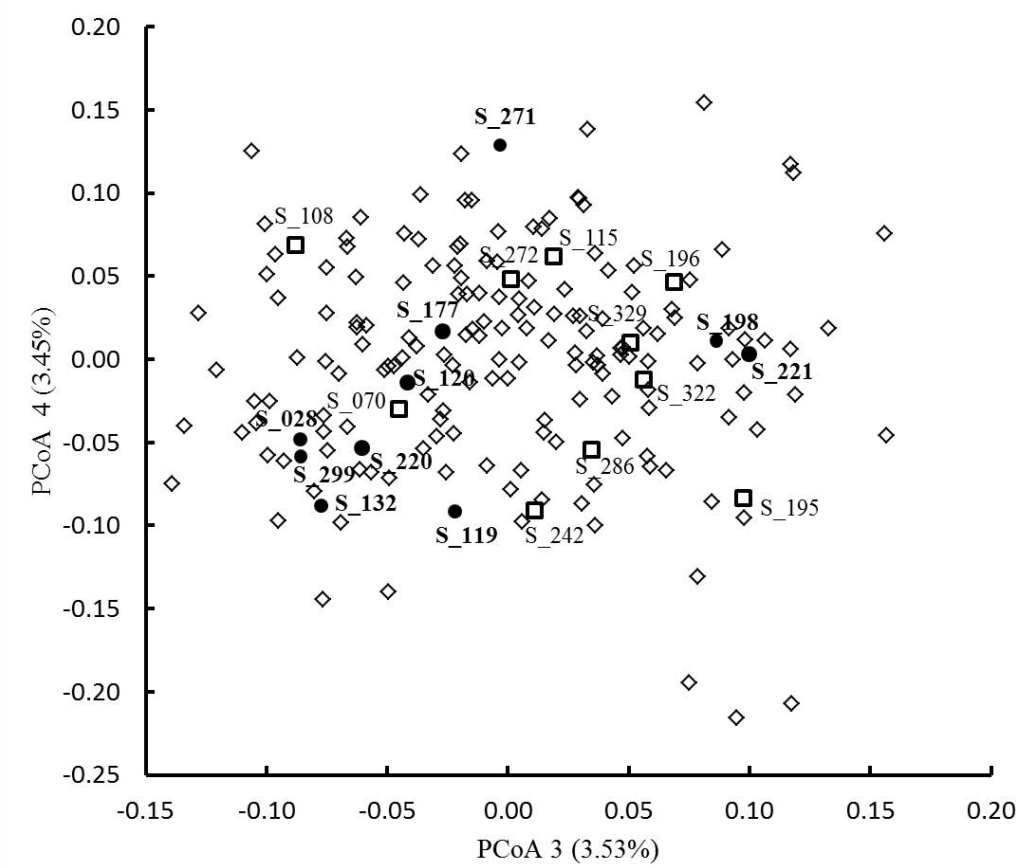

Figure III-6. Further results of the principle coordinate analysis (PCoA) of the genetic distances between lines in A-set; PCoA3 and PCoA 4 are displayed. The explained variance is given in brackets. **Bold and filled circles** refer to the 10 most tolerant genotypes based on FTI, whereas, the unfilled squares refer to the 10 most susceptible genotypes.

General discussion

As a result of this study, a very high genetic variation can be reported in all traits scored in the Frost Growth Chamber (FGCh), green houses, and field experiments. Such genetic variation is of high value for breeders who can use this information on diversity among faba bean lines in response to frost stress in improvement programs. The frost tolerance index (FTI) is especially useful because it reflects two important traits: disposition to survive (DS) and regrowth (REG). Both traits were scored after frost, so they are considered as decisive indicators (alive *vs* dead) to frost tolerance in faba bean. Furthermore, both traits showed significant phenotypic (r_p = 0.77**) and genetic (r_g = 0.85) correlations. Oberländer et al. (2012) studied the relationship between DS and REG in a series of six frost experiments which were conducted in FGCh. Their phenotypic correlations ranged from r = 0.63** to r = 0.82**. The high repeatability estimates for DS (h^2 = 88.09) and REG (h^2 = 63.78) supports the notion that they be included in one index to allow improvement of both traits in parallel. The FTI (including DS and REG) is a good indicator of frost tolerance and can be used in future breeding program to improve tolerance to frost since it shows high repeatability estimates (h^2 = 85.49). Generally, the selection index approach has been thought to be more efficient in increasing genotypic aggregates when compared with single-trait selection (Wells and Kofoid, 1985, Gebre-Mariam and Larter, 1996).

Most of earlier studies assessed the symptoms of frost stress on faba bean shoots (Herzog, 1988; Arbaoui and Link., 2008; Roth and Link, 2010). In the present study, root traits such as root frost susceptibility (RFS), root fresh matter, root dry matter, water content in root, and root length were included to study the effect of frost stress on faba bean. Among these traits, RFS was the most indicative trait for observing the symptoms of frost injury. After frost, all surviving plants showed high genetic variation in their root health. RFS showed high strong genetic (r_g = -0.88) and phenotypic (r_p = -0.79**) correlations with FTI. We concluded that it is important to look at the root health of the surviving genotypes because death of a initially surviving genotype may occur even after a period of survival; the fewer frost symptoms in the roots directly after frost, the more likely is ultimate survival and shoot production (regrowth). S_120 showed fewest frost symptoms on roots among all genotypes through all experiments.

Fatty acid composition (after hardening) and relative shoot water content (before and after frost) explained and reflected an important part of frost tolerance in faba bean. Previous studies

reported that the increase of unsaturated fatty acid content (USFA) and the decrease of saturated fatty acid (SFA) are associated with frost tolerance in faba bean (Arbaoui, 2007). The main point is that the USFA content increases the membrane's fluidity under frost stress, alleviating the frost symptoms on leaves and thus increasing frost tolerance. This can be tentatively observed from the significant correlation between USFA and both loss of leaf turgidity + loss of leaf color (r_p = -0.19**) and FTI (r_p = 0.22**). In the case of shoot water content, few studies have examined the role of this in the frost tolerance of faba bean. We concluded that the amount of shoot water content before frost plays a significant role in faba bean frost tolerance. The genotypes which have little water content in their shoots may form fewer ice crystals during frost stress. Fewer ice crystals are less harmful for the membrane and therefore, the genotypes with lower water content (before frost) may not lose that much water due to frost. The significant negative correlation (r = -0.20**) between water content before and after frost nicely points to this. Seemingly, the smaller the water content in shoots, the better is survival after frost. This can be seen from the correlation between shoot water content before frost and LT+LC (r_p = 0.34**) and FTI (r_p = -0.33**). The reduction in water due to frost stress strongly underlines its role as an additional symptom from this stress through its strong significant correlation with LT+LC (r = 0.87**) and FTI (r_p = -0.67**). Fatty acid composition and water content before frost can be used as a predictors for frost tolerance. Studying such physiological parameter is worthwhile in order to understand the mechanisms that the plants use to tolerate to stress and hence to better guide the selection process.

The artificial frost in FGCh is very useful because it allows us to test a fair number of plants simultaneously under controlled conditions and to identify those with the best frost tolerance. However, winter field experiments should be used in research programs to study winter hardiness. It is informative to test these genotypes as screened in FGCh experiments which have the best frost tolerance under filed conditions where frost happens naturally. This not only tests their survival rate but also provides information on their agronomic features such seed yield, days to flowering, 1000 seed weight, and maturity. A good faba bean cultivar should show a good combination of frost tolerance and seed high yield. Furthermore, a series of experiments should be repeated over different locations and several years to obtain stable cultivars with superior frost tolerance as well as high yield attributes.

Significant correlations were established between field traits and FGCh traits, supporting the idea of using a FGCh to improve frost tolerance in faba bean. The most favorable correlation was found between winter survival rate (WSR, field experiments) with both DS (r_p = 0.39**, FGCh experiments) and REG (r_p = 0.38**, FGCh experiments). Moreover, seed yield, being correlated by r_p = 0.44** with WSR, showed significant correlation with FTI (r_p = 0.33**). These significant correlations indicate that FGCh traits could be used as predictors for winter hardiness and high seed yield. No promising correlations were found between winter hardiness (plant development 2, leaf frost susceptibility, and WSR) traits and both leaf fatty acid composition and relative shoot water content as physiological parameters (data not shown). Among fatty acids, only C18:0 content (saturated fatty acid) showed positive correlation with leaf frost susceptibility (r = 0.19**) and negative correlation with WSR (r = -0.19**).

Two important factors should be taken into account in order to identify the genotypes with superior frost tolerance; (1) the number of traits should be as high as possible. The more traits that are scored, the more precise the identification of the superior genotype can be, and (2) the evaluation should be in artificial, controlled conditions as well as in the field. To address these two points, in this study, the best genotypes (those promising high frost tolerance, high winter hardiness and high seed yield) were labeled according to their frost tolerance and according to correlated features (Table 1). The 20 best genotypes were determined for each trait that showed a relation to frost tolerance (FTI or WSR). For seed yield and winter survival rate, an absolute threshold was applied > 90% of WSR and > 1000 g\2m^2. Accordingly, 12 genotypes showed a combination of high frost tolerance and high seed yield. Remarkably, most of the 12 genotypes showed high frost tolerance in all traits scored in FGCh experiments. These genotypes also presented high WSR in the field. This supports the idea that the frost tolerance test might be done first in FGCh and then the genotypes should be tested in the field. Ordás et al. (2006) reported that the best cold-tolerant genotypes that showed an acceptable performance under field conditions were those tested first under cold chamber conditions but that the contrary was not true. Therefore, the initial selection for frost tolerance could be done via cold chamber experiments and then among these the best genotypes should be tested in the field trials.

The association mapping approach was used in this study to detect putative QTLs which promise association with frost tolerance aspects. To catch as many QTLs as possible for frost tolerance,

the phenotypic scores of all traits (field traits were excluded) were combined with 156 polymorphic SNP markers. The outcome is a set of 70 putative QTLs for six morphological traits and seven physiological traits. Four QTLs were detected for FTI (including DS and REG). Interestingly, 12 markers showed a significant association with more than one trait. These promising markers are QTLs for different traits that can significantly expand our understanding of the genetic architecture of complex traits such as frost tolerance, and can increase the efficiency of selection by integrating it into future MAS selection programs (Ibrahim, 2012).

Two important issues that enhanced the power of association mapping were: (1) the low level of linkage disequilibrium between makers, and (2) our not finding any clear population structure. Using marker assisted selection to improve frost tolerance can significantly accelerate the breeding program. Unfortunately, few studies have been conducted to improve frost tolerance using MAS. Arbaoui et al. (2008b) constructed an initial map for winter faba beans and detected some important QTLs for frost tolerance in faba bean using RAPD markers. Clearly, further studies are needed to pave the way for an efficient employment of MAS in improving frost tolerance in the faba bean.

Table 1. The most promising winter faba bean genotypes with high frost tolerance, high winter hardiness, and seed yield.

Trait / Genotype	Morphological traits							Field traits				Physiological traits			
	LT+LC	DS	RFS	REG	Index1 (DS)	Index 2 (REG)	FTI	Seed yield	WSR	LFS	PD2	RWCAF	RIW [2]	USFA	SFA[2]
S_299	+ [1]		+	+		+	+	+	+	+	+	+	+		
(Côte x BPL)-18	+	+	+	+	+	+	+		+						
S_028	+		+	+	+	+	+		+			+	+		
S_004				+		+	+	+	+						
S_220	+	+	+	+	+	+	+								
S_221	+	+	+	+	+	+	+	+	+	+	+	+	+		
S_177		+		+	+	+	+	+	+	+					
S_119	+	+		+	+	+	+		+			+	+		
S_145			+	+		+	+	+	+						
S_120			+	+		+	+	+	+						
S_198	+	+	+	+	+	+	+		+					+	+
S_048		+	+		+	+	+	+	+		+				

[1] +: refers that the genotype is among the best 20 genotypes in the trait. [2] The low values are preferable.

Summary

A number of experiments were conducted to genetically analyze frost tolerance in faba bean (*Vicia faba* L.) based on a set of 208 highly homozygous lines. The lines were divided into three groups namely the original set (N=208, O-set), the winter bean set (N=196, WB-set, part of O-set), and association set (N=186, A-set, part of WB-set). All experiments took place in Frost Growth Chamber, Green House, Molecular Genetic Lab, and Field Experimental Station at the Georg-August-Universität Göttingen, Germany. A total of 65 morphological (seasons 2011/2012 and 2012/2013), physiological (season 2011/2012), and field (season 2012/2013) traits were scored. Out of these, 25 traits were included in the present study. Association mapping was conducted on the A-set using 156 polymorphic SNP markers. The aim of this research has been to prepare a marker-assisted improvement of frost tolerance in faba bean genotypes, and thus to detect QTLs for frost tolerance in order to use them in future breeding programs. The results are summarized as follows:

1- A very high genetic variation between all lines (original set, O-set) was observed in all morphological traits (eight traits) scored on shoots and roots of juvenile faba beans genotypes. All plants were exposed to hardening conditions for 10 days (5ºC) then to frost for three nights (-16, -18, and -19ºC). All traits showed very high repeatability (h^2), estimates ranging from h^2 = 59.45 for root frost susceptibility (RFS) to h^2 = 94.71 for loss of leaf turgidity + loss of leaf color (LT+LC). Furthermore, highly significant phenotypic and genetic correlations were discovered between morphological traits under frost stress. The genotypic correlations between traits were higher than the phenotypic correlations. The strongest genetic (r_g = -0.96) and phenotypic (r_p= -0.83**) correlation was found between regrowth (REG) and RFS. Three selection indices were calculated to characterize frost tolerance in faba bean. The most important was the frost tolerance index (FTI) because it combines two key frost tolerant aspects, namely, disposition to survive (DS) and REG. Such high genetic variation, phenotypic correlation, genetic correlation, and high repeatability estimates promise the selection process to genetically improve frost tolerance to be fruitful and effective.

2- Fatty acid composition (after hardening, 5ºC) and relative shoot water content (before and after frost) were studied as physiological parameters associated with frost tolerance in the faba

bean. The WB-set was used to study these physiological parameters. The genetic variations in detectable fatty acids and relative shoot water content between lines were noted. All physiological parameters showed very high repeatability estimates ($62.78 < h^2 < 89.04$). As expected, the unsaturated fatty acid (USFA) content was negatively correlated with saturated fatty acid (SFA) content ($r_p = -0.62$, $r_g = -0.84$**). Increasing USFA content and decreasing SFA were reported to be associated with increasing frost tolerance in faba bean. Similar negative significant correlation (r = -0.20**) was found between water content after frost (RWCAF) and before frost (RWCBF, after hardening). It is current knowledge that frost tolerance in plants is associated with the plant's ability to modulate ice crystal formation in shoot and root tissue during frost. Significant correlations were established between morphological and physiological traits. USFA content showed significant positive correlation with FTI (r = 0.22**). Moreover, reduction in water content due to frost stress showed strongly significant correlations with all morphological traits. Such correlations offer a better description of the physiological operations that the plants use to resist frost.

3- The WB-set was also used to test the genetic variation in the winter hardiness of the genotypes in a one-season field trial. A high, significant variation was found in all traits scored in the field. The repeatability estimates ranged from 61.98 for seed yield ($g/2m^2$) to 92.03 for days of flowering. S_299 and S_271 combined high winter survival rate (WSR), low leaf frost susceptibility, and high seed yield under field conditions. The same genotypes ranked among the best frost tolerant genotypes in the Frost Growth Chamber experiments. Interestingly, several field traits showed significant correlations with traits scored in FGCH. The highest correlation (r = 0.39**) was found between REG (FGCh experiments) and WSR (field experiments).

4- In order to enhance the improvement of frost tolerance in the faba bean, an association mapping approach was used in the A-set. As a result, 70 putative QTLs affecting seven morphological traits (DS, LT+LC, FTI, plant height, index 1, and index 2, shoot fresh matter after frost) and six physiological traits (SFA, USFA, RWCBF, RWCAF, reduction in water content due to frost stress, and C16:0 content - palmitic acid) were identified. The linkage disequilibrium among marker loci showed a very low average level ($r^2 = 0.00075$). Furthermore, the principal coordinate analysis for genetic distance between A-set lines revealed no obvious structure in this population. A number of significant markers showed significant association with

more than one trait, seriously raising hope that the marker-assisted selection approach can be used to improve frost tolerance in the faba bean.

Zusammenfassung

Es wurden mehrere Experimente durchgeführt, um - basierend auf 208 hochgradig homozygoten Inzuchtlinien - genetisch die Frosttoleranz von Ackerbohnen (*Vicia faba* L.) zu analysieren. Die Inzuchtlinien sind in drei Sätzen organisiert: Original-Satz (N=208, O-Satz), Winterbohnen-Satz (N=196, WB-Satz; eine Untermenge des O-Satzes), und Assoziations-Satz (N=186, A-Satz; eine Untermenge des WB-Satzes). Alle Experimente fanden statt: in der sog. Frost-Kammer (Pflanzenwuchskammer), im Gewächshaus, im Molekulargenetischen Laboratorium, oder im Zuchtgarten (Versuchsstation Reinshof der Georg-August-Universität Göttingen). Insgesamt wurden 65 morphologische Merkmale (Saison 2011/2012 und 2012/2013), physiologische Merkmale (Saison 2011/2012), und Feld-Merkmale (Saison 2012/2013) erfasst. Daraus werden 25 Merkmale für diese Studie hier verwendet. Mittels des A-Satzes und mit 156 polymorphen SNP-Markern wurde eine Assoziationsanalyse durchgeführt. Das Ziel dieser Untersuchung war, eine markergestützte Züchtung auf Frosttoleranz bei der Ackerbohne vorzubereiten, und also QTLs für Frosttoleranz für zukünftige Zuchtprogramme zu detektieren. Die Ergebnisse können wie folgt zusammengefasst werden.

1- Es wurde eine hohe genetische Variation zwischen allen Linien (Original-Satz) beobachtet, für alle acht morphologische Merkmale, die an Spross und Wurzel von Ackerbohnen-Jungpflanzen erfasst wurden. Die Pflanzen wurden über 10 Tage (5°C) gehärtet und dann während dreier Nächte einem Frost ausgesetzt (-16°C, -18°C, -19°C). Alle Merkmale zeigten eine hohe Wiederholbarkeit (h^2), die Schätzwerte reichten von h^2=59,45 für Wurzel-Frostempfindlichkeit bis zu h^2=94,71 für den Blattturgor- und Blattfarbverlust. Darüber hinaus wurden hochsignifikante phänotypische und genotypische Korrelationen zwischen den morphologischen Merkmalen unter Froststress entdeckt. Die genotypischen Korrelationen zwischen den Merkmalen waren höher als die phänotypischen Korrelationen. Die höchste genetische (r_g=-0,96) und phänotypische (r_p= -0,83**) Korrelation wurde zwischen dem Wiederaufwuchs und der Wurzel-Frostempfindlichkeit gefunden. Zur Charakterisierung der Frosttoleranz von Ackerbohnen wurden drei Selektionsindices berechnet. Der wichtigste war der Frosttoleranz-Index, weil er zwei Schlüsselaspekte kombiniert: die Überlebensneigung

und den Wiederaufwuchs. Diese hohe genetische Variation, phänotypische Korrelation, genotypische Korrelation, und hohe Wiederholbarkeit versprechen, dass ein Ausleseprozess zur erblichen Verbesserung der Frosttoleranz fruchtbar und effektiv wäre.

2- Als physiologische Parameter mit Beziehung zur Frosttoleranz der Ackerbohne wurde die Fettsäurezusammensetzung nach Härtung (5°C) und der relative Sproßwassergehalt (vor und nach Frost) studiert. Für diese physiologischen Parameter wurde der WB-Satz benutzt. Die genetische Variation in den aufgefundenen Fettsäure-Gehalten und im relativen Sproßwassergehalt wurde festgehalten. Alle physiologischen Kennwerte zeigten sehr hohe Wiederholbarkeitsschätzwerte ($62,78 < h^2 < 89,04$). Wir erwartet war die Korrelation zwischen den ungesättigten Fettsäuren und den gesättigten Fettsäuren negativ (r_p = -0,62, r_g = -0,84**). Eine Erhöhung der ungesättigten Fettsäuren geht in Ackerbohnen bekanntermaßen mit einer Verringerung der gesättigten Fettsäuren einher. Eine ähnlich negative und signifikante Korrelationen (r = -0,20**) wurde zwischen dem Wassergehalt nach Frost und dem Wassergehalt vor Frost (nach Härtung) gefunden. Es ist gängiges Wissen, dass die Frosttoleranz von Pflanzen mit der Fähigkeit der Pflanzen assoziiert ist, die Eiskristallbildung während des Frosts im Spross- und Wurzelgewebe zu modulieren. Es wurden signifikante Korrelationen zwischen morphologischen und physiologischen Merkmalen gefunden. Der Gehalt an ungesättigten Fettsäuren war mit dem Frosttoleranz-Index positiv korreliert (r = 0,22**). Darüber hinaus war die Verringerung des Wassergehaltes durch Froststress hochsignifikant korreliert mit allen morphologischen Merkmalen. Solche Korrelationen bieten eine verbesserte Beschreibung des physiologischen Verhaltens, welches die Pflanzen dem Frost gegenüber zeigen.

3- Der WB-Satz wurde auch benutzt, um die genetische Variation der Winterhärte der Genotypen in einem einjährigen Feldversuch zu überprüfen. Eine hohe, signifikante Variation wurde für alle Feldmerkmale gefunden. Die Wiederholbarkeitsschätzwerte reichten von 61,98 für Kornertrag ($g/2m^2$) bis zu 92,03 für Blühbeginn. Die Linien S_299 und S_271 kombinierten hohe Winter-Überlebensraten mit niedriger Frostanfälligkeit des Blattes und mit hohem Kornertrag unter Feldbedingungen. Dieselben Genotypen rangierten bei den Besten für Frosttoleranz in den Frostkammer-Experimenten. Interessanterweise zeigten mehrere Feldmerkmale signifikante Korrelationen mit

Merkmale aus der Frostkammer. Die höchste Korrelation (r=0,39**) war die zwischen Wiederaufwuchs (Frostkammer) und Winter-Überlebensrate (Feld).

4- Um die Verbesserung der Frosttoleranz der Ackerbohne weiter nach vorne zu bringen, wurde ein Assoziationsanalyse-Ansatz mit dem A-Satz gefahren. Als Ergebnis liegen nun 70 mutmaßliche QLTs vor, die sieben morphologische Merkmale (Überlebensneigung, Blattturgor- und Blattfarbverlust, Frosttoleranz-Index, Wuchshöhe, Index1, Index2, und Sproß-Frischmasse nach Frost) und sechs physiologische Merkmale (Gehalt an gesättigten Fettsäuren, Gehalt an ungesättigten Fettsäuren, relativer Wassergehalt vor Frost, relativer Wassergehalt nach Frost, Verringerung des Wassergehaltes durch Froststress, und C16:0-Gehalt, also Palmitinsäuregehalt) betreffen. Das Gametenphasenungleichgewicht zwischen Marker-Loci zeigte ein sehr niedriges Niveau (r^2=0,00075). Darüber hinaus zeigt die Hauptkoordinatenanalyse für genetische Distanzen zwischen den A-Satz-Linien keine offensichtliche Struktur in dieser Linienpopulation. Mehrere Marker zeigten eine signifikante Assoziation mit mehr als einem Merkmal, was ernstlich die Hoffnung erhöht, dass der markergestützte Ausleseansatz für die Verbesserung der Frosttoleranz bei der Ackerbohne genutzt werden kann.

الملخص العربى

تم إجراء عدد من التجارب على محصول الفول البلدي (*Vicia faba* L.) وذلك لغرض تحسين تحمّل النبات للصقيع حيث استخدمت 208 سلالة عالية التماثل. قسمت هذه السلالات إلى ثلاث مجموعات هي: مجموعة تشمل كل السلالات المستخدمة في هذه الدراسة، وعددها 208 سلالة يرمز لها بالرمز(Original set, O-set) ومجموعة الفول الشتوية، وعددها 196 سلالة، ويرمز لها بالرمز (Winter bean set, WB-set) وهي جزء من مجموعة الـO-set . وأخيرًا المجموعة المستخدمة في تحليل خرائط الارتباط، وعددها 186 سلالة ويرمز لها بالرمز (Association set, A-set) وهذه المجموعة جزء من الـWB-set. أجريت جميع التجارب بغرفة الصقيع (Frost growth chamber) والصوب الزجاجية ومعمل الهندسة الجزيئية ومحطة البحث العلمي بجامعة جورج أوجست بمدينة جوتنجن بألمانيا. وقد تمّ تسجيل 65 صفة مظهرية وفسيولوجية وحقلية (سجلت الصفات المظهرية في موسم نمو 2011/2012 والصفات الفسيولوجية في موسم نمو 2011/2012 فقط. أما الصفات الحقلية فقد تمّ تسجيلها في موسم نمو 2012/2013). استخدمت 25 صفة من هذه الصفات فقط في هذه الدراسة. تمّ رسم الخرائط الوراثية للمجموعة المرتبطة (A-set) وذلك باستخدام 156 واسمات جزيئية Single nucleotide polymorphism (SNP). تهدف هذه الدراسة إلى تحسين صفة تحمّل الصقيع من خلال الانتخاب للوسمات الجزئية ، وتحديد المواقع الكمية المتحكمة في هذه الصفة؛ وذلك بغرض استخدامها في برامج التربية بغرض تحسين صفة تحمل الصقيع في الفول البلدي.

يمكن تلخيص النتائج المتحصل عليها من هذه الدراسة إلى ما يلي:

1- تم الحصول على تباين وراثي عالٍ بين جميع سلالات المجموعة الاصلية (O-set) بالنسبة لجميع الصفات المورفولوجية التي تمّ تسجيلها حيث إنه سجلت 8 صفات مورفولوجية على المجموع الخضري والمجموع الجذري للبادرات. عُرِّضت جميع النباتات إلى فترة تقسية (5ºC) لمدة 10 أيام ثمّ إلى ظروف صقيع لمدة 3 ليالٍ بدرجات حرارة (-16, -18, and -19ºC). أظهرت جميع الصفات معدلات تكرار عالية (h^2) تتراوح بين 59.45 بالنسبة لحساسية الجذور للصقيع إلى 94.71 بالنسبة لمجموع صفتي فقدان حيوية الأوراق وفقدان لون الأوراق (LT+LC) بالإضافة إلى ذلك، تمّ الحصول على ارتباط وراثي ومظهري عالى المعنوية بين الصفات المورفولوجية تحت ظروف الصقيع، وكان الارتباط الوراثي أعلى من الارتباط المظهري. فكانت أعلى قيمة للارتباط الوراثي (r_g=-0.96) والارتباط المظهري (r_p=-0.83**) حصل عليها بين صفتي القدرة على النمو بعد الصقيع (Regrowth, REG) وصفة حساسية الجذور للصقيع (Root frost susceptibility, RFS). تم حساب ثلاث معاملات انتخاب بغرض توصيف تحمّل الصقيع في نبات الفول البلدي. فكان أهم معامل انتخاب هو معامل تحمّل الصقيع؛ وذلك لأنه يدمج بين سمتين هامتين لتحمل الصقيع؛ هما: صفة القدرة على العودة إلى النمو (REG) والميل إلى البقاء على قيد الحياة (Disposition to survive). تشير القيم العالية للتباين الوراثي بالإضافة إلى الارتباط المظهري والارتباط الوراثي ودرجة التوريث إلى أن عملية الانتخاب سوف تكون ناجحة وفعالة في تحسين صفة تحمل الصقيع.

2- تم دراسة محتوى الأحماض الدهنية في المجموع الخضري للنبات بعد تعريضه لفترة تقسية على درجات حرارة منخفضة (5ºC) بالإضافة إلى المحتوى المائي للمجموع الخضري قبل الصقيع وبعده كعوامل فسيولوجية مرتبطة

بتحمل نبات الفول للصقيع، وذلك باستخدام مجموعة الفول الشتوي (WB-set). تم ملاحظة الاختلافات الوراثية لهاتين الصفتين بين السلالات المختلفة. أظهرت جميع الصفات المدروسة معامل تكرار عالٍ ($62.78 < h^2 < 89.04$) وكما هو متوقع كان هناك ارتباط سالب بين محتوى الأحماض الدهنية غير المشبعة ومحتوى الأحماض الدهنية المشبعة. زيادة محتوى الأوراق من الأحماض الدهنية غير المشبعة وانخفاض محتواها من الأحماض الدهنية المشبعة كان له علاقة وثيقة بزيادة تحمل النبات للصقيع. ومن المعروف أنَّ هناك ارتباط سالب كذلك بين محتوى النبات من الماء بعد التعرض للصقيع وقبل التعرض له، وأنَّ قدرة النبات على تحمل الصقيع تختلف باختلاف قدرته على تكوين بلورات الثلج في المجموع الجذري والخضري أثناء الصقيع. تم الحصول على ارتباط معنوي بين الصفات المورفولوجية والفسيولوجية. أظهرت صفة محتوى النبات من الأحماض الدهنية غير المشبعة ارتباطًا معنويًا موجبًا مع معامل تحمّل الصقيع .(r=0.22**) وكذلك أظهرت صفة انخفاض محتوى النبات من الماء كنتيجة لإجهاد الصقيع ارتباطًا معنويًا قويًا مع جميع الصفات الموفولوجية. مثل هذه الارتباطات تُظهر لنا قدرة أفضل على فهم العمليات الفسيولوجية التي يستخدمها النبات لمقاومة الصقيع.

3- استخدمت مجموعة الفول الشتوي (WB-set) كذلك لاختبار الاختلافات الوراثية بالنسبة لإجهادات الشتاء في التراكيب الوراثية في موسم واحدٍ من التجارب الحقلية، حيث ظهرت اختلافات عالية المعنوية بالنسبة لجميع الصفات التي تم تسجيلها في الحقل. معامل التكرار تراوح بين 61.98 بالنسبة لصفة محصول البذور (جم/2م2) إلى 92.00 بالنسبة لصفة عدد أيام التزهير. أظهرت سلالتا S_299 و S_271 معدل نجاة شتوي بعد الصقيع عاليًا (Winter survival rate, WSR) وحساسية قليلة للصقيع في الأوراق (Leaf frost susceptibility, LFS) ومحصولًا عاليًا تحت الظروف الحقلية. كانت هذه السلالات من أحسن السلالات المتحملة للصقيع في التجارب التي أجريت داخل غرف الصقيع. من الجدير بالاهتمام أن هناك صفات حقلية عديدة أظهرت ارتباطًا معنويًا مع الصفات التي تم الحصول عليها داخل غرف الصقيع وأعلى معامل ارتباط (r=0.39**) تم الحصول عليه بين صفتي القدرة على العودة إلى النمو (REG) في تجارب الصقيع ومعدل البقاء الشتوي (WSR) في التجارب الحقلية.

4- تم استخدام A-set في تحليل الخرائط المرتبطة (Association mapping) بغرض تعزيز تحسين تحمل الصقيع في الفول البلدي، وتمّ الحصول على 70 QTLs التي تؤثر على سبع صفات موفولوجية؛ هي: الميل إلى البقاء على قيد الحياة (DS)، مجموع صفتي فقدان حيوية الأوراق وفقدان لون الأوراق (LT+LC) ، طول النبات ، index 1، index 2 ، الوزن الرطب للمجموع الخضري بعد الصقيع، وستة صفات فسيولوجية؛ هى: محتوى النبات من الأحماض الدهنية غير المشبعة، محتوى النبات من الأحماض الدهنية المشبعة ، محتوى النبات من الماء بعد التعرض للصقيع وقبل التعرض للصقيع ، انخفاض محتوى الماء في النبات كنتيجة للتعرض للصقيع، ومحتوى حمض البالمتك C16:0. كما أظهر عدم الاتزان الارتباطي بين مواقع الواسمات مستوى منخفضًا جدًا من الارتباط (r2= 0.00075). كذلك أوضح تحليل التنسيق الأساسي للمسافة الوراثية بين سلالات المجموعة المرتبطة (A-set) أنه ليس هناك مجموعات متشابة وراثيًا في هذه العشيرة. أظهرت عدد من الواسمات المعنوية معامل ارتباط معنوي مع أكثر من صفة مما يوضح زيادة الأمل في استخدام تقنية انتخاب الواسمات المرتبطة في تحسين تحمل الصقيع في نبات الفول البلدي.

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Acknowledgments

All praises to Allah for giving me the power, and patience to successfully complete this thesis. I would like to express my special appreciation and thanks to my advisor Prof. Dr. Wolfgang Link for his time, excellent guidance, and for allowing me to express my own ideas. I would also like to fully acknowledge my co-supervisor, Prof. Dr. Maria Finckh who showed very great flexibility and cooperation towards accomplishing this project.

I highly appreciate the financial support of the German Academic Exchange Service (DAAD) and the Ministry of Higher Education in Egypt.

I am grateful to PD Dr. Wolfgang Ecke who has kindly guided me through the Association Mapping and QTL analysis. I thank all staff members in the Division of Plant Breeding, Georg-August-Universität Göttingen; Prof. Dr. H. Becker, Dr. C. Möllers, and Dr. Sabine von Witzke-Ehbrecht. I would like also to thank Prof. Dr. Donal O'Sullivan for providing us with his preliminary consensus map.

I would like to thank Regina Martsch for her full support and help in Frost Growth Chamber, Molecular Genetic Lab and field experiments. Not forgotten, my full thanks go to Uwe Ammermann for his help in the fatty acid composition experiments.

I am thankful to my colleagues Yasser Moursi, Siyang Liu, Edy Suprianto, Nina Behnke, Haiko Brandes, Christopher Hoppe, Gregor Welna, Makis Kampouridis, Sebastian Miersch, Julia Rudloff, Li Shia Teh, Mareile Stever, Ariana Valdés, and Mohamed Ali, none of who have hesitated to help and recommend me.

I cannot find any words that can express my grateful thanks to my wife Amira Mourad for her support, encouragement, and patience. Her tolerance of my being busy most of the time increases my appreciation to her. I also thank all my family members in Egypt especially my father and mother. I cannot forget to thank all the staff members and my colleagues in the Department of Genetics, Assiut University, Egypt.

Finally, I can never forget the kindness and generosity of all the Egyptian community members in Göttingen who have given me their full support, valuable recommendations and advice especially during my hardest times. I ask Allah to keep our friendship forever.

Appendix 1: list of genotypes and their frost tolerance index (FTI)

Serial	Genotype	Original set	Winter bean set	Association set	FTI	Ranking
	Founder lines (N=11)					
1	Arrisot/1-1	+			2.56	203
2	Banner/1-1	+			4.40	159
3	Bourdon/1-5	+			4.76	138
4	Bulldog/1-4	+			3.85	184
5	CôteD`Or/1	+	+		6.26	13
6	Hiverna/1-1-2 EP3-EP01-1	+	+		5.30	90
7	L79/79/1	+			4.51	154
8	L977/88/S1wn	+			5.16	100
9	L979/S1/1/1sn	+			5.31	89
10	Webo/1-1-1 EP 10-1_2	+	+		5.46	74
11	Wibo/1	+			5.03	113
	Göttingen Winter Bean Population (N=189)					
12	S_002-1-1	+	+	+	5.80	47
13	S_003-1-1-1	+	+	+	5.23	94
14	S_004-1	+	+	+	6.18	17
15	S_005-1-1	+	+	+	5.43	77
16	S_008-1-1	+	+	+	4.25	168
17	S_009-1-1	+	+	+	3.82	188
18	S_010-1-1-1	+	+	+	5.27	91
19	S_012-1-1	+	+	+	6.15	18
20	S_013-2	+	+	+	3.97	179
21	S_015-1-1-1	+	+	+	3.85	183
22	S_016-1-1-3	+	+	+	5.94	28
23	S_019-1-1-1	+	+	+	5.19	95
24	S_020-1-2	+	+	+	3.67	193
25	S_021-1	+	+	+	5.62	58
26	S_022-1-1-1	+	+	+	4.51	153
27	S_025-1	+	+	+	5.93	31
28	S_027-1-1	+	+	+	4.71	143
29	S_028-1-3-1-1	+	+	+	6.94	1
30	S_029-1-1-1	+	+	+	5.37	85
31	S_030-2	+	+	+	4.26	167
32	S_033-1-1	+	+	+	4.35	163
33	S_034-1-2	+	+	+	3.75	191
34	S_035-1-1-2	+	+	+	4.07	176
35	S_036-1-1	+	+	+	5.81	46
36	S_038-1-1-1	+	+	+	3.72	192
37	S_039-1-1	+	+	+	4.79	133
38	S_040-1-1-1	+	+	+	4.43	156
39	S_043-1-1	+	+	+	5.38	84
40	S_045-1-1	+	+	+	4.05	177
41	S_046-1-1-1	+	+	+	5.53	66
42	S_048-1	+	+	+	6.29	12
43	S_050-1-1-1	+	+	+	5.57	62
44	S_052-1-1-1	+	+	+	5.16	99
45	S_054-1-3-1	+	+	+	4.54	150

Continuation of table

Serial	Genotype	Original set	Winter bean set	Association set	FTI	Ranking
46	S_055-1-3-1	+	+	+	5.40	81
47	S_059-1-2-2	+	+	+	5.94	27
48	S_060-1-2	+	+	+	5.42	78
49	S_062-2	+	+	+	5.50	68
50	S_064-1-3-1	+	+	+	5.25	92
51	S_065-1-1	+	+	+	4.35	162
52	S_066-1-1-1	+	+	+	5.49	70
53	S_067-2	+	+	+	4.74	140
54	S_069-1-1-1	+	+	+	5.59	59
55	S_070-1-1	+	+	+	3.37	200
56	S_072-1-1	+	+	+	5.84	42
57	S_076-1-1	+	+	+	4.16	173
58	S_077-1-1	+	+	+	3.79	190
59	S_079-1-2-2	+	+	+	3.83	187
60	S_081-2_1-3-	+	+	+	5.92	33
61	S_082-2-2-1-1	+	+	+	5.87	38
62	S_083-1-1-2	+	+	+	5.82	44
63	S_084-2	+	+	+	5.07	107
64	S_085-1-1	+	+	+	6.08	22
65	S_093-1-1-1	+	+	+	6.25	14
66	S_097-1-1-1	+	+	+	4.62	146
67	S_100-1-1	+	+	+	5.54	65
68	S_102-1-1	+	+	+	4.41	158
69	S_104-1-1-1	+	+	+	4.96	122
70	S_106-1-1-2	+	+	+	4.84	129
71	S_108-1-1	+	+	+	3.65	195
72	S_111-1-1-1	+	+	+	3.86	182
73	S_115-1-1	+	+	+	3.61	196
74	S_116-1-1-1	+	+	+	4.27	166
75	S_119-1-1-1	+	+	+	6.70	5
76	S_120-1-1-1	+	+	+	6.55	9
77	S_122-1-1-4-2-1	+	+	+	5.13	103
78	S_123-1-1	+	+	+	5.05	112
79	S_125-1	+	+	+	5.41	79
80	S_126-1-1	+	+	+	5.68	54
81	S_129-1-2	+	+	+	4.27	165
82	S_131-1-1	+	+	+	5.85	41
83	S_132-1-1	+	+	+	6.61	8
84	S_133-1-1	+	+	+	5.58	60
85	S_134-1-2-1	+	+	+	5.05	111
86	S_142-1-1	+	+	+	5.72	52
87	S_145-1-1	+	+	+	6.19	16
88	S_147-1-1	+	+	+	5.05	110
89	S_150-1-2-1	+	+	+	4.95	123
90	S_151-1-1-1-1	+	+	+	6.21	15
91	S_153-1-1-1	+	+	+	4.85	128
92	S_158-1-1-1	+	+	+	6.09	21
93	S_160-1-1-1	+	+	+	4.52	151
94	S_161-2	+	+	+	5.11	105

Continuation of table

Serial	Genotype	Original set	Winter bean set	Association set	FTI	Ranking
95	S_162-1-1-2	+	+	+	5.99	23
96	S_163-1-1	+	+	+	5.40	80
97	S_165-1-1	+	+	+	4.96	121
98	S_166-1-1	+	+	+	4.61	149
99	S_167-2	+	+	+	5.62	57
100	S_168-1-1	+	+	+	5.82	43
101	S_169-2	+	+	+	3.84	186
102	S_170-1-1	+	+	+	5.95	26
103	S_172-1-1-1	+	+	+	3.84	185
104	S_173-1-1	+	+	+	4.76	137
105	S_174-1-1	+	+	+	5.93	30
106	S_175-1-1	+	+	+	5.43	76
107	S_176-1-1	+	+	+	5.74	51
108	S_177-1-1	+	+	+	6.3	11
109	S_181-1-1	+	+	+	4.21	171
110	S_182-1-1	+	+	+	4.75	139
111	S_185-1-1	+	+	+	4.76	136
112	S_186-1-1	+	+	+	4.00	178
113	S_189-1-1-2	+	+	+	4.8	132
114	S_190-1-1	+	+	+	5.23	93
115	S_191-1-3-1-1	+	+	+	5.52	67
116	S_192-1-1	+	+	+	5.54	64
117	S_194-1-1	+	+	+	4.98	119
118	S_195-1-1	+	+	+	3.19	202
119	S_196-1-1-1	+	+	+	3.66	194
120	S_197-1-1-1	+	+	+	5.11	104
121	S_198-1-1-1	+	+	+	6.68	6
122	S_199-1-3-1	+	+	+	5.06	108
123	S_201-1-1-1	+	+	+	5.33	88
124	S_202-1-1	+	+	+	5.56	63
125	S_209-2	+	+	+	5.03	115
126	S_210-1-1-1	+	+	+	5.48	71
127	S_213-1-1-1-7	+	+	+	5.05	109
128	S_217-1-1-	+	+	+	5.07	106
129	S_218-2	+	+	+	4.47	155
130	S_220-1-1	+	+	+	6.87	3
131	S_221-1-1-1	+	+	+	6.88	2
132	S_226-1-1-1	+	+	+	5.86	39
133	S_227-1-1-1-1	+	+	+	4.80	131
134	S_231-1-1-1	+	+	+	4.13	174
135	S_232-1-1-1-1	+	+	+	4.32	164
136	S_233-1-2-1	+	+	+	5.03	114
137	S_235-1-1-2	+	+	+	4.38	160
138	S_236-1-1	+	+	+	3.89	180
139	S_238-1-1	+	+	+	5.57	61
140	S_240-1-1-2	+	+	+	4.61	148
141	S_241-1	+	+	+	5.63	56
142	S_242-1	+	+	+	3.57	197
143	S_243-1-1	+	+	+	5.89	35

Continuation of table

Serial	Genotype	Original set	Winter bean set	Association set	FTI	Ranking
144	S_245-2	+	+	+	5.44	75
145	S_246-1-1-1	+	+	+	4.72	141
146	S_249-1-1-1	+	+	+	4.24	170
147	S_252-1-1-1-1	+	+	+	5.33	87
148	S_253-1-1-1	+	+	+	5.68	53
149	S_254-2-2-1	+	+	+	4.88	127
150	S_258-2	+	+	+	5.47	73
151	S_259-1-1	+	+	+	4.11	175
152	S_264-1-1-1	+	+	+	5.65	55
153	S_265-1-	+	+	+	5.39	82
154	S_267-2	+	+	+	5.88	36
155	S_268-1-1-2	+	+	+	4.70	144
156	S_269-1	+	+	+	5.14	101
157	S_271-1-2-1	+	+	+	6.62	7
158	S_272-1-3-1	+	+	+	3.24	201
159	S_274-2	+	+	+	5.85	40
160	S_275-1-1	+	+	+	6.10	20
161	S_277-1-1	+	+	+	5.81	45
162	S_279-2-1	+	+	+	5.17	98
163	S_280-1-3-2	+	+	+	5.13	102
164	S_281-1-1	+	+	+	4.78	134
165	S_282-1-1-1	+	+	+	4.88	126
166	S_284-1-2	+	+	+	4.96	120
167	S_285-2	+	+	+	4.61	147
168	S_286-1-1	+	+	+	3.38	199
169	S_287-1	+	+	+	4.17	172
170	S_289-1-1-1	+	+	+	5.00	118
171	S_290-1-1	+	+	+	5.38	83
172	S_291-1-1	+	+	+	5.47	72
173	S_295-1-1	+	+	+	5.93	29
174	S_298-1-1-1	+	+	+	5.34	86
175	S_299-1	+	+	+	6.47	10
176	S_300-1-3-1	+	+	+	5.49	69
177	S_301-1-1-1	+	+	+	5.97	25
178	S_302-1-2-1	+	+	+	4.36	161
179	S_303-1	+	+	+	3.79	189
180	S_304-1-3-1	+	+	+	6.14	19
181	S_307-1	+	+	+	5.79	48
182	S_308-1-1-1	+	+	+	4.83	130
183	S_309-2	+	+	+	5.89	34
184	S_310-1-2-1	+	+	+	5.17	97
185	S_312-1	+	+	+	4.88	125
186	S_314-1-1	+	+	+	3.87	181
187	S_315-1	+	+	+	5.87	37
188	S_319-1-1-2	+	+	+	5.02	116
189	S_322-1-1	+	+	+	3.43	198
190	S_326-1-1-1-1	+	+	+	5.00	117
191	S_328-1-1-1	+	+	+	5.18	96
192	S_329-1-1	+	+	+	2.11	206

Continuation of table

Serial	Genotype	Original set	Winter bean set	Association set	FTI	Ranking
193	S_330-1-1	+	+	+	4.67	145
194	S_331-1-1	+	+	+	5.75	50
195	WAB_EP98_21-2-1 EP4-1-1	+	+	+	4.42	157
196	WAB_EP98_267-11-1 EP1-1	+	+	+	5.92	32
197	WAB_EP98_98-3-1 EP4-1-1	+	+	+	5.78	49
198	WAB_EP98_98-4-1-1-1	+	+	+	4.91	124
199	WAB-EP02-Fam/S1_157-1-2	+	+	+	4.71	142
200	WAB-EP02-Fam/S1_159-1-2	+	+	+	4.51	152
	Additional winter beans lines(N=4)					
201	(Cô/1 x BPL...)-95-4-1-1-3	+	+		6.84	4
202	(Cô/1 x BPL…)-18-3-1-1-2	+	+		5.99	24
203	29H(Asc.-resistent)-1-3	+	+		4.77	135
204	Hiverna/2-5 EP1-1-8-1-3-2-1	+	+		4.25	169
	Spring beans lines (N=4)					
205	Hedin/ 2	+			1.75	208
206	Limbo/ 7	+			2.21	205
207	Mélodie / 7	+			1.99	207
208	Minica	+			2.21	204

Appendix 2: list of abbreviations

Abbreviation	Term	Units	Place of scoring
1000-SW	1000 seed weight	g	Field
AM	Association mapping	-	
A-set	Association set	-	
DS	Disposition to survive	^{o}	Frost growth chamber
DTF	Days to flowering	days	Field
DTM	Day to maturity	days	Field
FDR	False discovery rate	-	
FGCh	Frost growth chamber	-	
FPH	Field plant height	cm	Field
RFS	Root frost susceptibility	1-9	Frost growth chamber
FTI	Frost tolerance index (including DS and REG)	-	Frost growth chamber
GWBP	Göttingen winter bean population	-	
I_1	Index 1 (including DS)	-	Frost growth chamber
I_2	Index 2 (including REG)	-	Frost growth chamber
LC	Loss of leaf color	1-9	Frost growth chamber
LCAF	Loss of leaf color after frost	1-9	Frost growth chamber
LD	Linkage disequilibrium	-	
LFS	Leaf frost susceptibility	1-9	Field
LT	Loss of leaf turgidity	1-9	Frost growth chamber
LT+LC	Loss of leaf turgidity + loss of leaf color	8-72	Frost growth chamber
LTAF	Loss of leaf turgidity after frost	1-9	Frost growth chamber
O-set	Original set	-	
PCoA	Principal coordinates analysis	-	
PD1	Plant development 1	1-9	Field
PD2	Plant development 2	1-9	Field
PH	Plant height	cm	Frost growth chamber
REG	Regrowth	g	Frost growth chamber
RIW	Reduction in water content due to frost stress	%	Frost growth chamber
RWCAF	Relative water content after frost	%	Frost growth chamber
RWCBF	Relative water content before frost	%	Frost growth chamber
SDMAF	Shoot dry matter after frost	g	Frost growth chamber
SDMBF	Shoot dry matter before frost	g	Frost growth chamber
SFA	Saturated fatty acid	%	Frost growth chamber
SFD	Seed fill duration	days	Field
SFMAF	Shoot fresh matter after frost	g	Frost growth chamber
SFMBF	Shoot fresh matter before frost	g	Frost growth chamber
USFA	Unsaturated fatty acid	%	Frost growth chamber
WB-set	Winter bean set	-	
WSR	Winter survival rate	%	Field

Curriculum vitae of

Ahmed Mohamed Atef Sallam

Biographical

Name	:	Ahmed Mohamed Atef Sallam
Position	:	Assistant Lecturer in Dept. of Genetics, Faculty of Agriculture, Assiut University. Assiut, Egypt
Date of Birth	:	03.01.1984, Assiut, Egypt
Marital Status	:	Married, one child
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Education

2000-2004	:	B.Sc. in Agricultural Sciences (Genetics), excellent with honor degree, Faculty of Agriculture, Assiut University
2006-2008	:	M.Sc. in Agricultural Sciences (Plant Breeding and Genetics), Faculty of Agriculture, Assiut University *Title thesis* Genetic variation in stem diameter in wheat (*Triticum aestivum* L.) in relation to drought and heat stress tolerance
2011-2014	:	Ph.D. in Agricultural Sciences (Molecular Plant Breeding and Genetics), Division of Plant Breeding, University of Göttingen, Germany. *Title thesis* Detailed genetic approach to improve frost tolerance of German winter faba beans

Academic records

2000-2004	:	Demonstrator (Teaching and Research Assistant), Dept. of Genetics, Faculty of Agriculture, Assiut University.
2008-Now	:	Assistant Lecturer (Teaching and Research Assistant), Dept. of Genetics, Faculty of Agriculture, Assiut University.

Awards

2009-2014	:	DAAD PhD Scholarships, Division of Plant Breeding, Faculty of Agricultural Sciences, University of Gottingen, Germany.

Attended workshops and training courses

11-13.01.2006	:	Workshop on Basic Principles and Laboratory Techniques on DNA Cloning, HEEPF, Genetic Engineering and Molecular Biology Center, Assiut University, Egypt.
26-29.06.2006	:	Workshop on Conventional PCR to Real – Time PCR , HEEPF, Genetic Engineering and Molecular Biology Center, Assiut University, Egypt.
13-15.05.2007	:	Training workshop to support the Egyptian participation in Seventh Program for Scientific Research funded by European Union. Food Technology Center- MEDA GO TO EUROPE project, Assiut University, Egypt.
12-14.04.2010	:	Training Workshop in Application of Biotechnology, Genetics Department, Faculty of Agriculture, University Assiut, Egypt
13-17.02.2012	:	Training course in Statistics and STATISTICA, University of Göttingen, Germany.
02-06.09.2013	:	Workshop on Molecular Techniques in Phytopathology, University of Göttingen, Germany.
07-11.10.2013	:	Training course on Linear Statistical Models with R, University of Göttingen, Germany.

Attending conferences:

28.02-01.03.2012	:	Breeding crops for sustainable agricultural production international conference. Justus-Liebig-Universität Gießen, Germany.
18-20.09.2012	**:**	Genome Research Working Group Conference of the GPZ. Martin Luther University Halle-Wittenberg. Germany.
19-21.09.2012	**:**	Resilience of agricultural systems against crises Conference. University of Gottingen. Germany.
09-11.05.2012	**:**	First Legume Society Conference. Novi Sad. Serbia.
17-19.09.2013	**:**	Agricultural development within the rural-urban continuum. University of Hohenheim. Germany.
24–26 .09.2013	**:**	Breeding for Nutrient Efficiency. University of Gottingen. Germany.
11-13.02.2014	**:**	Conference of the Genome Research Working Group. Max Plant Institute for Breeding Research. Germany.

Experiences

- *Methodology*: Plant breeding methods, DNA isolation, Gel documentation, Genetic engineering, Cytogenetic techniques, PCR, Electrophoresis, Molecular marker (RAPD, AFLP, SNPs), Association analysis, QTL mapping, Analysis of leaf fatty acid composition.

- *Softwares:* Plabstat, Plabplan, SAS (fair), R statistics, STATISTICA, TASSEL 3.0, QTL network, Mapmaker 3.0, Mapchart 2.0, STRTUCTURE 2.3.4 for population genetic structure

Field of study:

- Quantitative genetics, Population genetics, Molecular plant breeding and genetics, and Cytogenetics

Language:

- Arabic (mother language)
- English
- German