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Winter hardiness in faba bean: Physiology and breeding

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ABSTRACT

Winter types of faba bean (*Vicia faba* L.) have existed for at least 200 years. Their superior use of the growing season confers strong yield advantages over spring beans. Nevertheless, yield increases have been slower than in many other crops. There are few sources of winter hardiness and efforts are in progress to combine favourable alleles from accessions such as Cote D'Or and BPL 4628 to increase the crop's tolerance to frost. Vernalization requirements are quantitative, as vernalization hastens flowering rather than allowing it. Hardening is associated with increases in fatty acid desaturation of membrane lipids and increases in content of soluble osmoprotectants such as proline. Other osmotically active factors such as glycinebetaine, trehalose and antifreeze proteins have not yet been reported for faba bean. Frost tolerance increases after hardening and shows good heritability ($h^2 = 0.89$). Three QTLs ($3.6 < \text{LOD} < 4.6$) have been identified for frost tolerance (explaining 8.6% of the phenotypic variation), and further QTLs for hardening response and cell membrane fatty acid composition. Information on responsive genes and the mode of their action is increasingly available from model plant species but most remains as yet untested in faba bean. There is clear potential for increasing the winter hardiness and yield of winter faba bean so it can be grown in a wider area than at present.

1. Introduction

Winter faba bean (*Vicia faba* L.) is sown in autumn, mainly in regions with mild winters north of the Pyrenees and Alps, and survives the winter as young plants with two to three leaves and a short shoot. In France, some 11 000 ha of winter faba bean was grown in 2006 (about 15% of the total area sown to faba bean) and in the UK, over 160 000 hectares of faba bean have been sown each year since 2001, of which about half is winter cultivars (data from DEFRA, UK). In Germany and further east in Europe, where harsher winters prevail, no winter faba bean is grown at present. Field trials in Germany in the 1970s and 1980s showed that the climate was not suitable for existing winter faba bean cultivars (von Kittlitz, 1974; Hauser and Böhm, 1984; Herzog, 1989b). Nevertheless, there is potential for expanding the range of winter faba bean through breeding for improved hardiness, particularly in the presence of climatic warming.

As a result of its head start, winter faba bean generally flowers and matures earlier than the spring type. An important feature is its capacity to develop two or more rather synchronous tillers whereas spring bean seldom tillers. Young winter faba bean plants show a considerable capacity for regrowth and healing of mechanical injury. Sowing rate is lower than in the spring type (20–30 rather than 35–60 seeds per m^2). The yield superiority of winter over spring faba bean was 14% in trials in Germany between 1981 and 1989 (Herzog and Geisler, 1991) and 47% in 2004 across three locations in UK (Table 1).

There are not many winter faba bean cultivars on the market. Some are well-tried, like Clipper, Hiverna, Irena, Karl, Olan, Punch and Target; others were recently released like Arthur, Diver and Gladice. Gladice is the only recent tannin-free winter faba bean cultivar and there is no low-vicine winter cultivar yet on the market. Only three companies are active in this field, Wherry and Sons (UK), Agri-Obtentions (France) and NPZ Lembke (Germany).

Bond and Crofton (1999) summarized the history of European winter faba bean. Small-seeded winter types named "Russian" and "Little Winter" were introduced to the UK in 1825 from unknown sources. All winter beans in the 1800s were small-seeded *minor* types and the medium-seeded *equina* types replaced them between 1925 and 1945. The only *minor* winter bean presently known is the old French population Côte d'Or. Faba bean was the second most important crop in the UK at the end of the 19th century, with more than 220 000 ha grown in 1873, about half

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Table 1
Grain yield (t ha^{-1}) in 2004 in England from winter and spring faba beans

	Locations		
	Bramham	Poringland	Thornaugh
Cultivar (winter bean)			
Target	5.05	3.91	3.72
Clipper	4.72	3.83	3.52
Wizard	4.39	4.11	4.25
Mean		4.17	
Cultivar (spring bean)			
Victor	1.88	2.85	2.67
Méli	2.43	3.20	3.76
Compass	2.38	2.65	3.27
Syncro	2.38	2.94	3.53
Mean		2.83	

Data from NIAB, UK.

being winter type. The “Russian” type was even recommended for Scotland, hence it may have been truly winter-hardy. Picard et al. (1985) reported that winter faba bean types like Côte d’Or were grown already in 1812 in remarkable amounts in the continental climate and at high altitude in the Côte d’Or region of Burgundy. In Munich, Kreutz (1930) conducted a long-lasting selection program on winter faba bean, apparently starting with spring types that survived winters. The resulting types were described as small-seeded and short, showing a restricted height as young plants, with a strong tendency to tiller and small leaflets.

Three germplasm pools are currently used for breeding winter faba bean: (1) that from PBI Cambridge (Bond et al., 1986) and now handled by Wherry & Sons in the UK; (2) that at Agri-Obtentions, France, mainly resulting from prior programs at Rennes (Berthelem, 1970) and Dijon; and (3) the material developed by Littmann at Timmdorf, studied by Herzog at Berlin and now handled by NPZ Lembke and the University of Göttingen. The Littmann material was said to contain genotypes originating from the Pyrenees (Bond and Crofton, 1999). Little further winter-hardy material is available from gene banks. At Göttingen, breeding research on winter faba bean has continued since 1988.

Autumn sowing of faba bean is traditional in the Mediterranean basin and similar climates. These cultivars endure mild frosts (about −6 °C) in southern Europe (e.g., cvs. Aquadulce, Enantia, Baraca and Alameda) or grow in very mild winters in North Africa (e.g., cvs. Chahbi, Giza 843 and Hudeiba 93). A main feature is their very early flowering and maturity, escaping the usual terminal drought of these semi-arid climates. These types of faba bean are grown also in Australia (e.g., cvs. Icarus, Farah and Mannafest) where breeding programs are in progress (Paull et al., 2006).

Winter faba bean offers several advantages over the spring type. It makes better use of moisture available in winter and especially in early spring, it partly escapes summer drought, and it tends to mature in August when conditions are favourably warmer and dryer. It partly escapes *Sitona* weevil and aphid attacks because it is already further developed and less sensitive than spring bean when the insects are most numerous and active (young plant and flowering, respectively). Autumn sowing avoids problems of damp spring soils impeding sowing, and in turn spring sowing allows a bean crop to be produced when autumn planting conditions have been poor. The main disadvantage of winter faba bean is the risk of winter-kill. A further drawback is the more traditional growth habit of this material: rather tall, low in harvest index, and prone to lodging. Although the material is vigorous, less breeding progress on earliness of flowering and maturity has been made than in spring types. In a dry warm year, spring bean may even mature before winter bean, but then the yield margin is even more in favour of the winter type. Since very few breeders work with this material, the general breeding progress is slow. As with other winter crops, it is hard to see how the growing season can effectively be brought under 6 months to allow two cycles per year. Winter faba bean sown at Göttingen in March and April matured on time in August or September, but with low yield and no opportunity for selection for winter traits, while glasshouse-grown plants matured too late in spite of seed and plant vernalization.

2. Breeding of winter bean

Faba bean cultivars are either lines or populations, and in the latter case these are often synthetic cultivars (Link and Ederer, 1993). The instability of the available systems of cytoplasmic-genic pollen sterility precludes commercial-scale production of true hybrid cultivars. Breeding of faba bean is hampered by its partial allogamy (about 50% with large variation). Pollinators are bumblebees, honeybees and other solitary bees (see companion paper in this issue). Heterosis for grain yield is strong, mostly >30%. There is even heterosis for a very specific trait: autofertility, the ability to self-pollinate spontaneously, i.e., without the need for visits from pollinators (Drayner, 1959; Link, 2006). Winter bean flowers rather early in the season, and autofertility might be of especial importance for yield when pollinator activity is limited (Stoddard, 1986). Since there is heterosis for general vigour and winter hardiness (see below), there seem to be more arguments in favour of maximizing heterosis in winter than in spring faba bean.

A crucial step in bean breeding is testing of inbred lines, yet production of selfed seed is a bottle-neck. In an open field, seed production is economical but may suffer from contamination with cross-pollen. In pollinator-excluding cages, controlled selfing can be enforced, but seed setting is poor without pollinator visits so technical staff are needed to trip flowers manually at a relatively high cost.

A consequence for faba bean breeding is that with the minimum possible number of seeds per inbred line, the maximum amount of information on its genetic value has to be gathered. One step (but not two consecutive steps) of cheap open field propagation seems to be adequate and tolerable (Link, 1995). Here, winter bean has a clear advantage. A single winter bean plant in low plant density may yield more than 75 seeds, enough to sow 3 m^2 of a field test. Thus, as few as 10 plants of an inbred line, sown to propagate in an open field, e.g. as topcross or polycross, will produce enough seed for a 30 m^2 field test of its offspring; this allows a reasonable test in two locations. In spring bean, 10 such plants may yield seed for only about 10 m^2, scarcely enough for a single location test.

The most important objectives of winter faba bean breeding that differ from those of spring bean breeding are resistance and tolerance to frost stress and to winter-specific pathogens. In detail these are:

- appropriate vernalization requirement;
- adequate hardening and dehardening behaviour;
- frost tolerance without hardening and after hardening;
- frost tolerance after dehardening;
- tolerance of continuous snow-cover;
- tolerance of frost-drought (desiccation from wind and solar radiation under frost);
- tolerance of high moisture levels in soil in winter;
- endurance of mechanical soil movement due to cycles of frost and thawing, referring especially to root morphology and tensile strength;

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- resistance against diseases that attack specifically in winter and early spring.

With the exceptions of frost tolerance and disease resistance, these are seldom handled individually, but they are implicitly tested in field trials.

3. Vernalization

Two important, specific features of winter crops are vernalization and hardening. These two processes have partial but not complete independence, as shown by Lee et al. (2001) who described the gene HOS1 (high expression in response to stress) that had a large impact on both in *Arabidopsis thaliana* L. They occur in parallel at low positive temperatures but the outcomes are different. There appears to be no correlation between vernalization requirement and frost hardening ability of faba bean (Soja and Steineck, 1986).

The major purpose of a vernalization requirement is to prevent flowering in late autumn and postpone it until spring. A short cold period after emergence in autumn followed by temporary warm weather should not induce flower bud formation. Thus, a winter crop should achieve a vernalized status only after exposure to a sufficiently extended period of cold. An additional effect of long days is a very valuable insurance against onset of flowers in autumn. Winter bean indeed responds to vernalization (at about 4 °C) but unvernalized plants eventually flower on a higher node. The vernalization requirement of winter bean is about 30 days. Temperatures above 23 °C inhibit flowering, especially if at night. Seeds can also be vernalized by cool temperatures. After vernalization, more genotypes showed a significant positive response (enhancement of flowering) to long day photoperiod than without (Evans, 1959; Soja and Steineck, 1986; Ellis et al., 1988; Fujime et al., 2000). Among Mediterranean, North African and European spring faba bean types the response to an exposure to low temperatures varied from nil to a marked acceleration of flowering and quantitative genotypic differences were shown (Soja and Steineck, 1986). In winter types of pea (*Pisum sativum* L.), the most important major locus for winter frost resistance co-segregated with the allele for delayed flowering (Hr; Lejeune-Henaut et al., 2004), showing the importance of appropriate (i.e., late) flowering time for winter pea. Referring to winter faba bean, only one comment on this topic was found in the literature. The vernalization requirements of most winter beans are probably adequate. Japanese cultivars with susceptibility to prolonged snow cover developed flower buds before winter, whereas snow-tolerant cultivars (Rinrei, Mairudo Green) differentiated their flowers much later (Fukuta and Yukawa, 1998). The molecular analysis of FLC, a major vernalization gene in Arabidopsis, provided clues about metabolic details of vernalization (Sung and Amasino, 2005) that may be confirmed in legumes.

4. Hardening

As for vernalization, the hardening responses of the winter and spring germplasm pools of faba bean are not clearly separate. Spring faba bean is able to acquire some winter hardiness, it survives Mediterranean winters (Schill et al., 1998) and has repeatedly survived mild winters in Germany (Herzog, 1989b). Faba bean shows increased frost hardiness already after a few days of exposure to low non-freezing temperature and a maximum status of hardening is reached in 2–3 weeks (Herzog, 1988). Hardening involves energy-demanding physiological processes, thus requiring appropriate light quality and intensity (200–400 μmol m^{-2} s^{-1}). Experiments with 5–8 °C day and 2 °C night temperatures resulted in similar rates of hardening and maximum frost tolerance. Since dehardening commences above 7 °C, and very strongly so above 10 °C, a temperature of 2–5 °C accompanied by short days (10 h; Herzog, 1988) is adequate for hardening. The genetic distinctness of the frost tolerance response of hardened plants and that of plants without hardening treatment has been demonstrated in *Arabidopsis* (Thomashow, 1990) and in *Solanum* (Stone et al., 1993). The correlation between hardened and non-hardened frost tolerance responses of winter faba bean was only moderate ($r = 0.59$; $r = 0.54$; Arbaoui, 2007) and this confirms at least partial independence of these traits.

Hardening results in modifications of the plant cell membrane, including changes in the lipid-to-protein ratio and membrane lipid unsaturation (Hughes and Dunn, 1996; Uemura et al., 2006). Arbaoui and Link (in press) found altered desaturation of faba bean leaf lipids in response to cold hardening, including a significant decrease in oleic acid content and a significant mean increase in linolenic acid (in leaves from 51% to 57% and in stem from 32% to 41%), resulting in weak to moderate correlations with frost tolerance. The association of increased desaturation with decreased temperature also exists at higher temperatures, as a decrease in cultivation temperature from 30 °C to 20 °C resulted in a desaturation of the major fatty acids in faba bean leaves, mainly of 18:2 to 18:3 (Lem et al., 1980).

Further hardening-induced changes in lipid composition of other species have yet to be tested in faba bean. In Arabidopsis, the lipid composition of the plasma membrane changed significantly due to hardening, as the proportion of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine rose and the proportion of phospholipids in total lipids increased (Park and Chen, 2006). Collins et al. (2002) found a significantly higher level of unsaturated fatty acids in white clover genotypes that survived a frost test than in the unselected population. Fatty acid desaturase (fad) mutants of *Arabidopsis*, deficient in the production of polyunsaturated fatty acids, partly within the chloroplasts, were killed by low temperatures, unlike wild types (Hughes and Dunn, 1996). Phospholipase D was involved in lipid hydrolysis and freezing tolerance of *Arabidopsis*, and phospholipase $\alpha 1$ and phospholipase δ were involved in post-freezing recovery (Li et al., 2008).

5. Frost stress

The winter 2002/2003 was the most recent one that was too severe at Göttingen for almost all winter beans (less than 1% survival). Four spells of harsh frost (below −10 °C) occurred, the lowest temperature of −16 °C coming during a period of five nights with frost below −10 °C and strong frost throughout the days. Snow cover was <1 cm and 6–7 h of sunshine per day together with a dry wind from the east caused high evaporation, i.e., severe frost-drought. The winter 2004/2005 with only one such spell of a single night at −17 °C and a snow cover of 7 cm barely caused winter-kill except among spring bean types.

The physiological changes resulting from hardening include an increase in the content of highly soluble compounds called compatible solutes or osmoprotectants that accumulate without disturbing metabolism and protect against dehydration. Examples are proline, glycinebetaine and related compounds, mannitol, sucrose, raffinose, stachyose, and specific proteins.

Proline accumulation during hardening is correlated with gain in frost tolerance in many species, e.g., wheat (Dörffling et al., 1990) and barley (Dobslaw and Bielka, 1988) and recently demonstrated in faba bean (Arbaoui et al., 2008). In wheat, the rate of change of the two traits with time was not equal (Windt and van Hasselt, 1999), and in faba bean maximum proline accumula-

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tion occurred before maximum frost tolerance (Balko, unpublished data) suggesting that proline accumulation was a precondition for hardening, but not the sole cause. Sánchez et al. (2004) doubted whether the main effect of proline accumulation in pea was a direct osmotic contribution, since it was not clear whether its accumulation was primarily in the cytosol or in the organelles. Xin and Browse (1998) demonstrated a positive role for proline in freezing tolerance with an *Arabidopsis* mutant that accumulated 30-fold more proline. Nanjo et al. (1999) corroborated this with an antisense construct in *Arabidopsis* that reduced proline hydrolysis, while Konstantinova et al. (2002) found increased freezing tolerance in transgenic tobacco that accumulated proline.

The role of glycinebetaine in osmotic stress in cool-season legumes has not been demonstrated positively or negatively (reviewed by Stoddard et al., 2006). Glycinebetaine has been shown to occur in *Medicago truncatula* cv. Jemalong and not to occur in *Medicago sativa* cv. Hunter River, while *Lotus* sp. accumulated prolinebetaine instead of glycinebetaine (Naidu et al., 1992; Rhodes and Hanson, 1993). Transgenic tomato that produced glycinebetaine was more tolerant to cold than the wild type, showing the usefulness of glycinebetaine in improving cold-stress tolerance (Park et al., 2003). Proline and glycinebetaine application by spraying improved the tolerance and membrane stability of salt-stressed faba beans (Gadallah, 1999).

Hardening also affects carbohydrate metabolism. Bourion et al. (2003) found a close relationship between the soluble sugar concentration of leaves just before the frost event and the degree of freezing tolerance in spring and winter peas. In a range of legume species, including *Vicia* sp., the concentration of sugars in the root was negatively correlated to the level of frost damage (Ratinam et al., 1994). Antisense expression of a tomato late embryogenesis abundant galactosidase (LEA-GAL) gene resulted in a manifold increase in the level of raffinose along with a markedly increased tolerance towards frost of non-acclimated and cold acclimated petunia plants (Pennycooke et al., 2003). Trehalose is a carbohydrate that plays an important role as an abiotic stress protectant, stabilizing dehydrated enzymes and membranes. When *Lotus japonicus*, a legume model species, was treated with a trehalase inhibitor so that trehalose accumulation increased five-fold during salt stress, the plants produced 20% more biomass than without the inhibitor (López et al., 2006).

6. Other stresses: diseases, snow cover and waterlogging

The phenology of European winter bean enhances its exposure to certain diseases that are less common in spring bean. *Ascochyta fabae* is primarily a disease of winter bean and at Göttingen, winter bean is also frequently damaged early in spring by *Fusarium oxysporum*. It cannot be ruled out that further fungi are involved in early spring root rots of winter bean. Four major genetic loci for *Ascochyta* resistance have been marked with RAPD from two crosses, each locus explaining about 20% of the genotypic variation; one important source of resistance is the spring bean line 29H (Román et al., 2003; Avila et al., 2004). Genetic differences for *Ascochyta* resistance are as well known for winter bean cultivars; e.g., the UK cv. Target and the French cv. Irena are reportedly resistant. There is no evidence to link disease or pest resistance in winter bean with the two key antinutritional factors, tannin and vicine-convicine. In spring bean, however, zero tannin was correlated with susceptibility to *Fusarium* sp. at emergence (Bond et al., 1994).

Fukuta and Yukawa (1998) tested 41 bean cultivars for tolerance to long snow coverage (36 and 61 days) without severe frost. They identified Mairudo Green and Rinrei as outstandingly snowcover-tolerant genotypes; both were from the same germplasm pool and Rinrei is a dwarf mutation bred by radiation mutagenesis. Tolerant varieties were short in height, with prostrate growth habit, short internodes and petioles, small leaves that touched the soil and a high content and maintenance of non-structural carbohydrate in the plant tissue. This phenotype apparently reduced wounding risk from graupel fall and strong wind before snow coverage and increased resistance to snow mould (*Pythium iwayamai, Sclerotinia trifoliorum*; Fukuta and Yukawa, 1999; Fukuta et al., 2000; Fukuta et al., 2001). Similarly, Bond and Crofton (1999) reported on the development of the winter cv. Deneb from a plant that had survived the hard winter of 1947 in the UK. Deneb showed evidence of greater hardiness with smaller, darker green leaves and a more prostrate habit when tested in official trials. Essentially the same ideotype was described by Annicchiarico and Iannucci (2007) by calculating the ratio of plant height to number of leaves, with a favourable effect of a stunted, rosette-like habit. This relationship was identified only by calculating a partial correlation coefficient between this ratio and winter survival ($r = 0.47$; $P < 0.05$). The correlative impact of sown seed weight (large sown seed showed low winter survival and large young plants) was thus eliminated. Bond and Crofton (1999) also speculated about a general association between small-seededness and winter hardiness in grain legumes.

In South China, excess water in autumn-sown bean was reported to be common (Bond et al., 1994). Since waterlogging is one of the typical stresses of over-wintering crops in Northern latitudes, the material from South China should be investigated for possible resistance. Resistance against *Fusarium* sp. may also be connected to this aspect.

7. Field trials to assess winter hardiness

Series of field trials are the usual approach to investigate winter hardiness. They allow the ultimate validation of data since they show the agronomic reality. They are, however, very time-consuming, as often winters are either too mild or too hard instead of giving a good differentiation between genotypes. If the climatic diversity included is large, the probability of having a location with appropriate winter strength included increases. Nevertheless, genotype × environment interactions increase as well (Arbaoui, 2007; Arbaoui et al., 2008) probably because of the different relative importance of frost, waterlogging and the other components of winter survival. A good recommendation is to have two sowing dates with 2–4 weeks difference, to increase the probability of a good discrimination. A number of known checks with a spread of winter hardiness should be included. The locations should vary across years, to cover as many environments as possible.

8. Assessing frost tolerance

In addition to field trials and often even as an alternative, controlled frost tests are conducted. Several researchers have used plants in pots in a plant growth chamber, whereas others have conducted tests with detached leaves. A traditional method to assess frost tolerance is to grow winter beans in wooden boxes outdoors (Fig. 1) to expose them to winter weather. Any natural, protecting snow cover may be withheld. If necessary, the frost period may be shortened by moving the boxes inside. This approach has repeatedly allowed corroboration of known differences in winter hardiness (Balko, 2007, unpublished) and hence can be used to test novel material.

The first symptom caused by freezing temperatures, visible already while the frost holds, is a dry blackening of the tips and edges of leaflets. After thawing, leaflets and stems may become at

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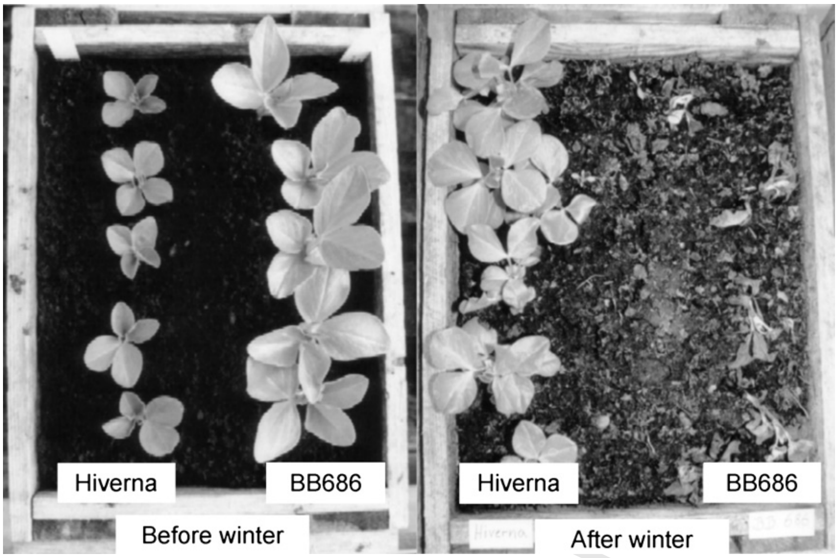

Fig. 1. An example of the difference in growth type and over-wintering behaviour of the winter bean Hiverna and the spring bean BB686 in provocation boxes in 2004 (Balko, unpublished).

first yellow to greyish and later black, they lose turgor (wilt), and especially the stems may develop a water-soaked, semi-translucent, crinkled appearance, later turning black as well. Even plants with no immediate symptoms may develop frost injury symptoms after several days of recovery. At first invisible since they are below the soil surface, roots and epicotyls may rot and become black, even though the plants at first show little wilting. The black zone extends upwards along the stem until the whole plant is dead.

In southern China, faba bean flowering in February regularly has to endure 3–4 weeks with frost, partly lower than −2 °C, and it was reported that flowers were tolerant to very mild frost, whereas young pods were not (Liu et al., 1987). In South Australia, however, a fraction of a degree of frost was enough to kill ovules within unfertilized flowers of cv. Fiord, leading to random growth of pollen tubes within the ovaries (Stoddard, unpublished observations, 1987).

Duc and Petitjean (1995) and Arbaoui et al. (2008); (Fig. 2) found that the results of artificial frost tests corroborated field data for overwintering and winter hardiness. Heritability in the data set of Arbaoui et al. (2008) was $h^2 = 0.83$ for grain yield and $h^2 = 0.90$ for overwintering. These two traits were correlated with $r = 0.83$ ($P < 0.01$), whereas $r = 0.41$ ($P < 0.05$) was the relationship between frost tolerance and overwintering (Fig. 2). Frost tolerance is apparently a major component of the winter hardiness of winter faba bean. Under artificial conditions, European winter bean cultivars like Côte d'Or and Hiverna are tolerant to temperatures down to −15 to −16 °C if adequately hardened (Herzog, 1987, 1989b). As reported by Picard et al. (1985), 61% of Côte d'Or plants survived even −25 °C in the field without snow-cover.

Gehriger and Vullioud (1982) used potted young plants for frost tests. Hardening of 34 days at 5 °C in the greenhouse was followed by testing. Their slow approach to the ultimate test temperature took 5 days from +5 °C to −3 °C, and thereafter frost increased by 3 °C per day until the test temperature was reached (between −6 °C and −15 °C). Duc and Petitjean (1995) allowed 3 weeks of hardening to young potted plants. Actual testing was a 3 weeks course of daily freeze–thaw cycles, with aggravating frost from 0 to −6 °C; thawing temperature was 3.5 °C, and day length was 9 h. They assessed leaf frost injury visually following Herzog's (1987) method. Arbaoui and Link (2007, in press) also assessed frost tolerance with young potted plants after 2–3 weeks of hardening. The test comprised six steps of aggravating frost, one step per night, with 4 h of frost, starting at −8 °C and culminating at −21 °C at which all plants were killed. After each step, injury, discoloration and loss of turgor were visually scored on leaves and stems and the scores were combined across the entire procedure. High heritabilities were achieved ($h^2 = 0.89$), although a large experiment was required (36 plants per genotype; Arbaoui et al., 2008). This work is continuing, in order to allow the detection of small, statistically significant differences in frost tolerance between highly frost-tolerant genotypes.

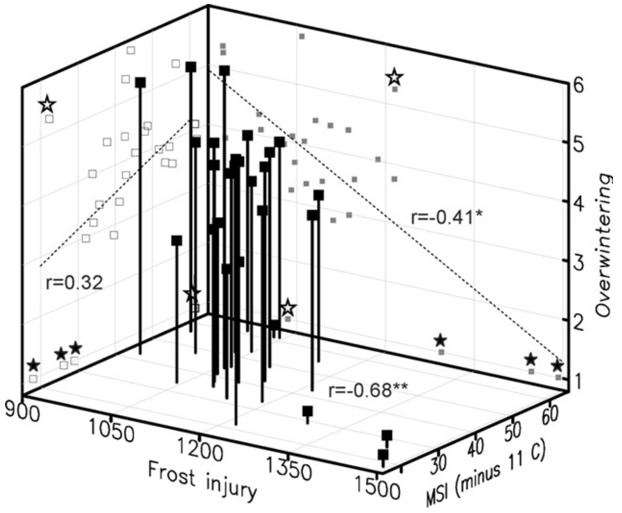

Fig. 2. Relationship between (1) frost injury (area under symptom progress curve); (2) electrolyte leakage (i.e., membrane stability index, MSI); and (3) over-wintering (visual score, 1 = total winter-kill; 12 European environments; Spearman rank correlation coefficients are displayed). The three spring bean accessions are identified by closed stars near the bases of the back walls. Two-dimensional relationships – between overwintering and MSI (open boxes) and between overwintering and frost injury (grey boxes) – are additionally displayed as projections of the data onto the two back walls. The winter bean Bulldog/1 (high over-wintering score, relatively high frost injury score and low MSI) is identified by open stars near the tops of the back walls, and the non-adapted, exotic line BPL4628 (lower frost injury score and higher MSI but very low over-wintering score) is shown also as open stars, near the bases of the back walls. The three entries with best over-wintering, visible as the top set of three points were Hiverna, Karl and the Winter Bean Population at Göttingen (Arbaoui, 2007; Arbaoui et al., 2008).

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Herzog (1987) developed an approach using detached leaves from hardened faba bean plants. The leaves were artificially frozen and injury was assessed by visual rating and by conductivity measurements (Flint et al., 1967). Freezing damages cell membranes; they may actually rupture and cell content may escape. Plant tissue, when placed in water, can be assessed for damage by measuring this leakage via electrical conductivity tests of the water–eluate mixture. A regression equation was established to estimate the test temperature T50 that induced a mean injury (score 4 on a scale from 0 to 8), provided that a test temperature in the vicinity of T50 was applied. This equation was based on the finding that a step of 1 °C in freezing temperature caused about 2.5 units increase in frost injury on this scale. Interestingly, unhardened leaves tended to be either barely or highly injured, showing a binomial or qualitative response, whereas hardened leaves exhibited all levels of intermediate injury, i.e., a quantitative response. Arbaoui et al. (2008) found the correlation of frost tolerance with electrolyte leakage to be somewhat higher ($r = 0.68$) than that with proline accumulation ($r = 0.58$) and that with changes in leaf fatty acid composition ($r = 0.48$), although all three coefficients were highly significant ($P < 0.05$; Fig. 2 and see below).

Photosynthesis is affected by freezing stress in general and specifically by membrane damage (Ensminger et al., 2006). Olszewski (1996) and Herzog and Olszewski (1998) applied the chlorophyll-fluorescence method to assess frost resistance of faba bean, barley, oats and oilseed rape. The crucial trait was the ratio Fv (before frost) / Fv (after frost). This technique was reliable only after a thorough phase of establishment and when all steps were very well standardized. For faba bean screening, this method when compared to a visual scoring of frost injury needed more labour hours, but was free of observer bias. Expenditure for apparatus and budget were higher, but the procedure could be partly automated. The two approaches did not differ in either reproducibility or sensitivity. Even though this approach was not more efficient and accurate than visual scoring in faba bean, in the other crops it was. Hoffmann-Bahnsen and Herzog (2001) used this chlorophyll-fluorescence method to identify frost-tolerant *Lupinus albus* types. They found Hiverna faba bean to be more frost-tolerant (by about 4 °C) than the most tolerant lupins, which were tolerant to a range from −9 to −11 °C.

Only very few reports present comparisons of these approaches. The available data do not yet allow a decision on which approach to recommend to practical breeding or to breeding research.

9. Physiological damage due to freezing

Generally, when plant shoots freeze, water moves from the cells to the intercellular space, and at −10 °C this amounts to more than 90% of the osmotically active water (Thomashow, 1998). The cause is the growth of ice crystals in the intercellular spaces of the tissues. The plant cells correspondingly suffer from dehydration. Furthermore, the ice crystals may disrupt the cell membranes. When the tissue thaws, the protoplast leaks cell contents and (further) loses turgor.

Plants have a number of strategies to survive freezing temperatures. The ability to avoid ice formation within the tissue (both intercellular spaces and intracellular) and hence to avoid the resulting dehydration of cells (Thomashow, 2001) is of major importance. One strategy is the depression of the freezing point by osmotic adaptation of cells (Nilsen and Orcutt, 1996) and thus the accumulation of a range of osmotically active (i.e. cryoprotective) substances is widespread. This can be observed during the hardening process. A further strategy to avoid ice formation is supercooling, the ice-nucleation-related ability of tissues to cool below the freezing point without actual ice formation (Sakai and Larcher, 1987; Reyes-Díaz et al., 2006), but this has not yet been reported in any *Vicia* species.

Frost tolerance includes processes that allow ice to form in plant tissues – mainly in the intercellular spaces or special compartments – and the resulting dehydration of cells without lethal consequences (Nilsen and Orcutt, 1996). In frost-tolerant genotypes, intercellular ice formation seems to be controlled and modified by antifreeze proteins. Intracellular ice formation, however, is generally lethal (for reviews see Uemura et al., 2006; Margesin et al., 2007).

Griffith and Yaish (2004) reviewed “antifreeze proteins” and their role in overwintering plants. Their physiological function is likely in inhibiting the intercellular recrystallization (growth) of ice rather than in altogether preventing this ice formation and they may additionally stabilize membranes, preventing damage by ice. They may further work in conjunction with volunteer protein ice nucleators to channel ice crystallization. Plant antifreeze proteins are homologous to pathogenesis-related proteins. In winter rye, antifreeze proteins exhibited antifungal activity in addition to their hydrolytic and ice-binding role. Antifreeze proteins have been identified in some forage legumes (Avice et al., 2003 and references in Griffith and Yaish, 2004) but not yet in any grain legumes. The existence and activity of osmoprotective substances in faba bean needs further investigation.

10. Availability of genetic variation

Winter faba bean breeders are faced with Côte d’Or being apparently a sole, outstanding source for frost tolerance and winter hardiness (although not for disease resistance). Thus, genotypes are sought with (1) higher frost tolerance than Côte d’Or; or (2) similar frost tolerance due to complementary genes to those found in Côte d’Or. Mutagenesis, interspecific crosses and genetic transformation are alternative ways by which to release additional variation, yet, none of these seems to attract the due attention of breeders and researchers (see below).

Several lines inbred from Côte d’Or are available, and there seems to be genetic variation among them. Further useful sources of hardiness are the cvs. Hiverna, Webo, Wibo, Karl and Diva, and the current Winter Bean Population at Göttingen. An exotic, rather frost-tolerant genotype is BPL4628, an ICARDA accession from China (Duc and Petitjean, 1995; Arbaoui et al., 2008). The inbred line (Côte d’Or/1 × BPL4328)-95 performed well (Arbaoui et al., in press).

The following genotypes out of 208 accessions showed superior frost resistance (values rounded off): Côte d’ Or (−16 °C), Hiverna (−15 °C), the lines ILB3187 (Cixi Dabaican), ILB2999 (PAK-40841), ILB14 (Aleppo), ILB345 (Egypt; all −14 °C; Olszewski, 1996). Further promising accessions for winter hardiness and frost tolerance should be sought in the mountainous regions of Western and Central Asia like the Hindu Kush; material from these regions frequently showed frost tolerance, probably due to being adapted to a high risk of frost early in the vegetative phase (Olszewski, 1996).

In spite of all known winter beans belonging to *minor* and *equina* types, recently Bond has developed two rather winter-hardy *major* type lines (Bond 64621, Bond 3353; D.A. Bond, personal communication, 2007).

Accessions of *Vicia johannis* and *Vicia narbonensis* were found to show superior tolerance to frost (Birch et al., 1985). Since interspecific crosses between *V. faba* and other species of the genus have not yet been successful (Wijaya, 2003), this genetic variation is unlikely to be transferable to *V. faba* in the near future.

Correlation studies are an important source of relevant data for breeding. Herzog (1978) reported that chlorophyll content and the

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chlorophyll A:B ratio did not seem to be promising criteria. Yet, high dry matter content per area of leaf seemed to be indicative for winter beans and for successful hardening. In a study with 10 genotypes of diverse origin, including Côte d'Or and Aquadulce, Herzog and Saxena (1988) found more than 83% of the variation in frost tolerance of detached leaves to be explained by four traits, expressed by hardened young plants: low relative water content, slow plant development, short plant height, and small leaf area. This combination essentially corroborates the one mentioned earlier for field-based over-wintering.

11. Heterosis

Heterosis for frost tolerance and winter hardiness is of interest because it can be exploited in synthetic cultivars (Gehriger and Vullioud, 1982; Bond and Crofton, 1999; Ghaouti et al., in press). In the *Arabidopsis* cross Columbia-0-G1 × C24-G1, the parental mean of the LT50 (50% of maximum electrolyte leakage) was −7.8 °C, whereas for the average of the reciprocal F_1 hybrids it was −10.3 °C, a highly significant difference. The leaf contents of soluble sugars (fructose, glucose, sucrose and raffinose) were dramatically higher in the F_1 plants after cold acclimation than in the parental lines, while the content of proline was only moderately higher. Correlation analyses showed that only raffinose content was consistently related to leaf-freezing tolerance. The expression of cold-related genes like COR78 (see below) was seemingly not involved in the expression of this heterosis (Rohde et al., 2004).

Two diallel tests showed that frost tolerance in faba bean under artificial conditions (potted plants) followed a quantitative genetic model (Duc and Petitjean, 1995). They authors found high favourable heterosis and high heritability, and concluded that the favourable alleles for frost in the line 285 derived from Côte d'Or were dominant over susceptibility. They recommended the cross between this line and the line BPL4628 from China (ICARDA catalogue). In this cross and others, Arbaoui and Link (2007) found between 0 and 11% higher frost tolerance in F_1 hybrids than in their parental means, the F_2 generation showing about half of this superiority. Line BPL4628 showed a high general combining ability, corroborating the data of Duc and Petitjean (1995).

Following this approach, screening to identify new, useful genetic diversity for frost tolerance could be conducted with F_1 hybrids from crosses between a most frost-tolerant line from Côte d'Or and the candidate accessions, instead of (or in addition to) testing the accession *per se*. Accessions whose F_1 hybrids outperform Côte d'Or promise to contain dominant favourable alleles that are not available in Côte d'Or. Transgressive segregants should be obtainable from these hybrids (Arbaoui and Link, 2007).

12. QTL and marker-assisted selection

Arbaoui et al. (in press) analyzed 101 recombinant inbred lines (RIL) from the cross Côte d'Or/1 × BPL4628 for frost tolerance and for leaf fatty acid composition, with and without hardening. They identified several lines that showed significant and marked superiority compared to the better parent Côte d'Or/1 (e.g., the lines 33, 69, 95) and work on this material is continuing. The cross was used to identify putative QTL for frost tolerance and fatty acid composition. Three useful QTL for unhardened frost tolerance were detected, and for one of them the exotic parent carried the favourable allele. After cross-validation, the QTL jointly explained 8.6% of the genotypic variance. For oleic acid content, three QTL were detected in unhardened leaves that explained 40.6% of the genotypic variance after cross-validation. This fatty acid was significantly correlated with unhardened frost tolerance. More experiments, especially with further parental lines, are necessary before embarking on a marker-assisted selection for frost tolerance in winter faba bean. Nevertheless, once reliable QTL for frost tolerance are detected, it might be considerably more efficient to work with a marker-assisted approach than to rely on the phenotypic frost test approach, given the large number of replications, and thus time, labour and infrastructure needed to establish adequate heritability of the results (Arbaoui et al., in press).

Avia and Lejeune-Hénaut (2007) identified several QTL for frost tolerance in the model species *Medicago truncatula*, thus opening new options for the application of comparative genetics among different legume species in this area (I. Lejeune-Hénaut, personal communication, 2008).

13. Knowledge on frost tolerance from molecular-genetic research, mainly in *Arabidopsis*

Hundreds of genes are known to be up- or down-regulated due to cold (COR, cold-regulated genes; Thomashow, 2001; Park and Chen, 2006). Early studies in *Arabidopsis* resulted in the identification of four COR-genes, COR6.6, COR15a, COR47 and COR78 (Hajela et al., 1990; Thomashow, 1998). The gene COR78 is as also known as RD29A (responsive to drought). A COR-gene identified later in a legume, *Medicago sativa* ssp. *falcata*, was named MfCAS30 (*Medicago falcata* cold acclimation specific; Pennycooke et al., in press).

The gene COR78 on chromosome V of *Arabidopsis* at At5g52310 codes for a desiccation-responsive, temperature-induced 78 kDa protein, with sequence similarity to the late embryogenesis abundant proteins (Nakashima and Yamaguchi-Shinozaki, 2006). It is thought to have a direct protecting function in frost and drought stress. This gene contains a *cis* element in its promoter, called CRT(C-repeat) that shares the CCGAC sequence as a core element with many COR-genes (Yamaguchi-Shinozaki and Shinozaki, 1994). The cold-induced genes that are jointly activated by this common C-repeat motif are called the CBF regulon. As examples, COR78 in *Arabidopsis* and MfCAS30 in *Medicago falcata* are activated by the CRT element in response to low temperature and to dehydration stress via the transcription factor protein, CRT-binding factor (CBF). One of the corresponding genes coding for this transcription factor, on *Arabidopsis* chromosome IV, contains two specific regions in its promoter that are activated by a further protein named ICE (inducer of CBF-expression). ICE, in turn, is produced as a very specific and rapid response to cold stress, but its regulation is only partly understood (Zhu et al., 2007). Mitogen activated protein kinases (MAPKs), calcium and abscisic acid are all involved in both cold and drought signal transduction (Kaur and Gupta, 2005). The activation of stress-responsive genes via the CRT-binding factor seems to be very widespread (Novillo et al., 2007). This gene, when transferred from *Arabidopsis*, increased the stress tolerance of the grass *Festuca arundinacea* (Zhao et al., 2007). A combination of the *Arabidopsis* CBF-gene (transcription factor) and the stress-inducible promoter of the cold-responsive gene COR78 when used as transgene improved the tolerance of tobacco to both drought and cold (Kasuga et al., 2004).

Several different CBF genes exist, and apparently play different roles in an intricate and complex pattern of regulation (Novillo et al., 2007). Many further transcription factors are involved in a highly complex net of signalling and metabolic pathways. Frost stress, for instance, stimulates the accumulation of reactive oxygen species such as hydrogen peroxide. Being damaging agents they cause injury, but also they are signals that induce protective mechanisms (Suzuki and Mittler, 2006). It is unclear whether to choose as a breeding objective an upstream gene like CBF or ICE that shows an apparently specific response to the stress (in this case, frost), or a downstream gene like COR78 or MfCAS30 that

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regulates a specific aspect of the frost tolerance response. The number of candidate genes is, of course, smaller at the more upstream levels of metabolism.

Recently a step forward in the techniques for regeneration of transgenic *V. faba* plants has been reported, although transformation of faba bean remains very laborious, slow and with low efficiency. Few transgenes have been so far introduced (*bar*, *uidA*, *nptII*, *sfa8*, *lysC*). Molecular-genetic progress in *Arabidopsis*, *Medicago*, *Lotus* and *Pisum* will enlarge the list of genes with potential for use in improving abiotic stress tolerance (Hanafy et al., 2005; Link et al., in press).

14. Conclusions

As has been shown, very little molecular-genetic detail is yet available on the winter hardiness and frost tolerance of winter faba bean. It is a pressing task for faba bean breeders and researchers to follow up the *Arabidopsis*, *Medicago* and *Lotus* research on abiotic stress, to identify shortcuts in the path to apply any further accruing pertinent results, and to achieve results in this crop. Beyond this, the search for and identification of new, useful genetic diversity within *V. faba* should allow a next step forward. Gepts et al. (2005) called for a major advance in legumes within a few years in detecting new QTLs and new, useful alleles by genetic and association mapping. The identification and incorporation into the breeding germplasm of new, not yet used frost-tolerance alleles that retain superior growth, productivity and grain yield and the provision of pertinent knowledge to plant breeders is one of the major challenges in this field.

Q5 Uncited references

Guy and Carter (1984), Herzog (1989a), and Link and Arbaoui (2005).

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