UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare
Ph.D. in Agro-Food System

Cycle XXXVIII
S.S.D. AGRI-06/A AGRI-02/A

Genetic improvement of frost resistance in autumn-sown white lupin through high-throughput phenotyping and genomic selection

Coordinator:
Prof. Paolo Ajmone Marsan

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Matriculation n: 5215071

Academic Year 2024/2025

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Contents

Abstract

White lupin is a cool-season grain legume that is gaining importance for European agriculture due to its high seed protein content and other positive characteristics. Autumn sowing has a rising interest in sub-oceanic and sub-continental climatic regions to extend the crop cycle and anticipate harvesting, thereby reducing the growing risk of terminal drought and enhancing yield potential. Despite the trend towards milder winters, frost remains a major threat, as sudden events can be detrimental, especially for poorly-acclimated plants. The increasing year-to-year climatic variability hinders the field selection for frost survival.

The objective of this thesis is to support the genetic improvement of white lupin frost resistance by optimizing its evaluation under controlled conditions in a high-throughput phenotyping platform and exploring its prediction through genomic selection procedures. A methodological study was conducted to establish the experimental protocol and define the optimal screening temperature. Its results provided the foundation for a subsequent large-scale screening aimed at assessing the genetic variation for frost resistance in two reference populations, elucidating the trait genetic architecture, identifying putative candidate genes, assessing the potential of genomic selection through intra-population and inter-population predictive abilities of genome-enabled models, and quantifying the relative effects of frost resistance and other traits on field-based winter survival of a germplasm collection.

The methodological study included 11 white lupin genotypes covering a broad range of winter survival, along with 11 pea genotypes. This material underwent four freezing treatments at −7, −9, −11, and −13 °C, arranged in a group-balanced block design with species in the main plots and different genotypes in subplots. In this and following work, frost resistance was assessed as plant mortality as well as by a 10-level visual score of biomass injury. The methodological study also enabled the estimation of genotype lethal temperature 50 (LT_{50}). The large-scale screening involved two reference populations of white lupin, one comprising 144 landrace and cultivar genotypes and the other including 144 inbred lines, which were genotyped by 40,914 and 32,951 SNP markers, respectively, issued by genotyping-by-sequencing. This material was evaluated at −11 °C using an alpha-lattice design. A subset of 115 landraces and cultivars had also previously been evaluated for field-based winter mortality, onset of flowering, and seed dry weight.

A freezing temperature of −11 °C was identified as the optimal treatment to maximise genotype frost resistance variation based on plant mortality in white lupin. Plant mortality and biomass injury at this temperature were highly correlated with LT_{50} values (0.94 and 0.91, respectively). On average, white lupin exhibited lower resistance than pea (LT_{50} = −11.0 °C vs. −12.8 °C). White lupin selection for frost resistance is favoured by high broad-sense heritability for plant mortality and biomass injury (H^2 = 0.76–0.82), substantial genetic variation across genotypes, and the identification of promising genetic resources. GWAS detected 11 significant markers linked to 21 candidate genes; however, multiple non-significant peaks, likely associated with minor genes, pointed out a polygenic control of frost resistance. Genomic selection achieved high predictive ability in the intra-population scenario when applied to the inbred lines (0.67–0.68), encouraging its adoption to reduce phenotyping efforts. In the inter-population scenario, model transferability was feasible only for the genetically broader model constructed from landrace and cultivar data for prediction of the genetically narrower inbred line material, but the moderate predictive ability (0.39) suggested that this strategy should be used only when the development of a population-specific model was prevented by lack of time or resources. In the field, winter survival of landraces and cultivars was largely achieved through frost escape via delayed onset of flowering, while the contribution of frost resistance became substantial when the comparison was restricted to intermediate-flowering material. A lower but significant contribution was found for higher seed dry weight (being reportedly associated with a thicker root parenchyma in young plants and enhanced root protection).

Overall, this thesis (a) optimized the experimental protocol for large-scale frost resistance evaluation of white lupin in a phenotyping platform, (b) assessed the extent of genetic variation for white lupin frost resistance and identified elite genetic resources, (c) confirmed the polygenic control of frost resistance, (d) highlighted opportunities for genomic prediction in breeding line selection and in the identification of elite genetic resources, and (f) suggested that the simultaneous selection for intermediate phenology, frost and drought resistances, and seed size could be a viable strategy to develop autumn-sown varieties with high yield stability across years in Northern Italy and other regions with similar climatic characteristics.

List of figures

List of tables

Abbreviations

ANOVA	Analysis of variance
BL	Bayesian lasso
BLUE	Best linear unbiased estimate
BLUP	Best linear unbiased predictor
FDR	False discovery rate
GBS	Genotyping-by-sequencing
GEBVs	Genomic estimated breeding values
GLM	Generalized linear model
LD	Linkage disequilibrium
LT_{50}	Lethal temperature 50
QA	Quinolizidine alkaloids
REML	Restricted maximum likelihood
rrBLUP	Ridge regression best linear unbiased predictor
SNP	Single-nucleotide polymorphism

1. Background

1.1. White lupin

1.1.1. Protein crops for sustainable European agriculture

Legumes are high-protein crops that can address several major challenges in European agriculture. They help mitigate soil fertility loss through symbiosis with nitrogen-fixing rhizobia (Andrews and Andrews, 2017), improve soil structure (McCallum et al., 2004; Rochester et al., 2001), and enhance agricultural biodiversity, thereby contributing to weed control and disrupting pest and disease cycles (Księżak et al., 2023; Kocira et al., 2020). When included in crop rotations or intercropping systems, legumes can increase the yield of the main crop by up to 20%, providing an alternative to synthetic fertilisers (Kebede, 2021; Mudare et al., 2022; Zhao et al., 2022). As a consequence, increasing their cultivation has the potential to reduce greenhouse gas emissions associated with the synthesis, transport, and application of nitrogen fertilisers and agrochemicals (Nemecek et al., 2008; Jensen et al., 2012). Despite these environmental benefits and the growing demand for high-protein feed over recent decades (Sepngang et al., 2020), legumes still play a marginal role in European agriculture (European Commission, 2019).

Their marginalisation can be traced back to the Green Revolution, when biological nitrogen fixation was replaced by synthetic nitrogen fertilisers and agricultural funding started to be predominantly directed towards intensive cereal-based systems (Watson et al., 2017). Insufficient investment in research and breeding has led to a decline in the proportion of arable land allocated to legumes in the European Union, which fell from 4.7% in 1961 to just 1.8% in 2021 (Balázs et al., 2021). This has resulted in significant disparities in productivity, economic returns, and cultivated area between legumes and cereals (Annicchiarico, 2017): in the period 2015-2020, cereals accounted for 46% of European cropland (FAO, no date), whereas the three most widely grown grain legumes, namely faba bean (*Vicia faba* L.), pea (*Pisum sativum* L.), and soybean (*Glycine max* (L.) Merr.), jointly represented only around 3% (FAO, no date). To compensate for low internal production, the European Union imports up to 70% of its protein feed from international markets, 87% of which consists of soybean and derivatives (Clément et al., 2018). In 2023 alone, the European Union and the United Kingdom imported approximately 14 million tonnes of soybean and 18 million tonnes of soymeal (Eurostat, n.d.). Imported soybean mainly originates from the Americas (Sepngang et al., 2020), where its cultivation is a major driver of Amazon deforestation (Boerema et al., 2016), and its transport contributes significantly to global greenhouse gas emissions

(Poore and Nemecek, 2018). Furthermore, European livestock farming systems, which rely heavily on imported soybean, are highly vulnerable to the risks associated with global trade.

In this context, cool-season grain legumes such as pea, faba bean, chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* L.), and lupins (*Lupinus* spp.) are becoming increasingly important. They are well suited to rainfed cultivation and to inclusion in crop rotations with summer crops. Their use is not limited to animal feed; they can also contribute to the production of novel, plant-based, high-protein foods within the food industry (Lucas et al., 2015; van Loon et al., 2023). Nevertheless, there is an urgent need for enhanced plant breeding efforts targeting grain legumes to improve yield potential, close the yield gap with major cereals, and strengthen their role in European agriculture (Rubiales et al., 2021).

1.1.2. Taxonomy, domestication, and cultivation trends of lupins

Lupinus is a large genus within the Fabaceae family, comprising more than 170 species (Gresta et al., 2017), four of which have been domesticated and gained agricultural importance: white lupin (*L. albus* L.), yellow lupin (*L. luteus* L.), narrow-leafed lupin (*L. angustifolius* L.), and Andean lupin (*L. mutabilis* Sweet). White, yellow, and narrow-leafed lupins originate from Europe, where they are currently cultivated for both food and feed production. The domestication of white lupin dates back to 2000 BC in the northeastern Mediterranean region (including Greece, western Turkey, and the southern Balkans), where wild forms (var. graecus) are still present (Gladstones et al., 1998). Due to its ability to enhance soil fertility, white lupin spread across the Mediterranean basin and along the Nile Valley, ultimately becoming a major crop in various regions of the Roman Empire (Wolko et al., 2011; Kurlovich, 2002). Conversely, yellow and narrow-leafed lupins were domesticated much more recently. Yellow lupin was first cultivated as an ornamental plant in southern Europe as early as the 16th century but became a popular crop in central Europe by the mid-19th century (Święcicki et al., 2000). Narrow-leafed lupin was domesticated only in the early twentieth century in central Europe, from wild populations of Mediterranean origin (Berger et al., 2012; Mousavi-Derazmahalleh et al., 2018). Finally, Andean lupin is the only *Lupinus* species of agricultural importance not native to Europe. It was domesticated in South America and is not yet commercially cultivated in Europe (Lucas et al., 2015).

In line with the general trend for legume crops, the European cultivation of *Lupinus* spp. declined sharply during the second half of the 20th century. While covering 970,628 hectares in 1961, their cultivated area reached its lowest level of 55,608 hectares by 1998 (Figure 1.1). This decline was particularly severe during the 1980s, when epidemics of anthracnose, a fungal disease caused by *Colletotrichum lupini*, led to significant yield losses (Talhinhas et al., 2016) and widespread crop abandonment. However, a slight reversal of this trend was observed in the early 2000s.

Although *Lupinus* spp. cultivation has not yet returned to 1961 levels, the cultivated area increased to 288,843 hectares in 2023 (Figure 1.1). Furthermore, despite minimal breeding efforts, the average yield rose from 0.611 t/ha in 1961 to 1.781 t/ha in 2023 (Figure 1.1), likely due to improvements in agronomic practices.

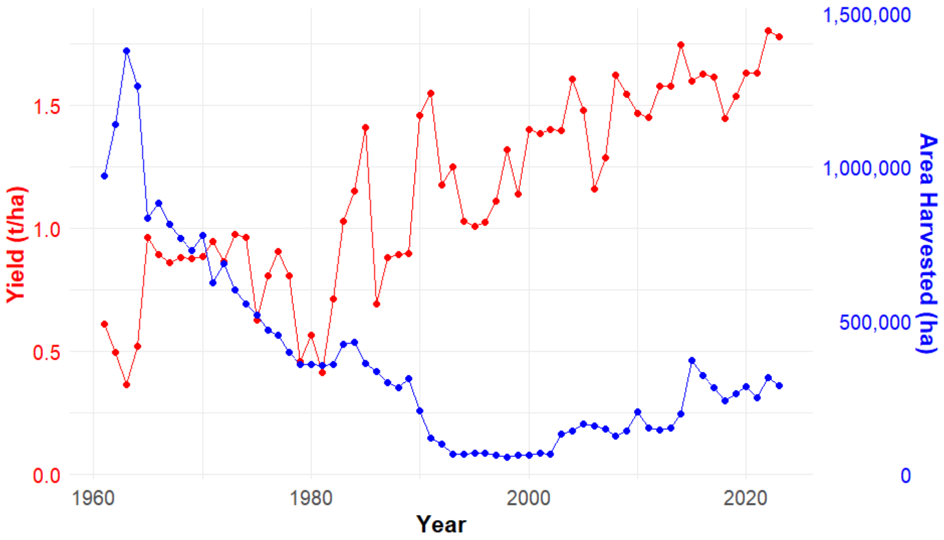


Figure 1.1. Trends in harvested area and average yield of lupins in Europe between 1961 and 2023. Data from FAO (n.d.).

Europe is the second-largest producer of lupins, accounting for 39.5% of global production in 2024, following Australia, which produced 51.6% (FAO, no date). In Australia, production is based on narrow-leafed lupin, and a significant market has been established. Most European production is consumed domestically, while Australia is effectively the sole exporter. Currently, lupins are mainly used for animal feeding, primarily for ruminants and aquaculture species, although there is increasing demand for human consumption (Lucas et al., 2015). Less than 4% of global production is consumed as human food, but lupin-based products are gaining attention as alternatives to animal proteins for the development of plant-based food as bakery products (Schrenk et al., 2019).

1.1.3. Botanical and agronomic characteristics of white lupin

Among *Lupinus* species, white lupin (Figure 1.2) is currently the most widely cultivated in southern Europe (Gresta et al., 2017). For this reason, and due to the increasing interest in this crop (as detailed in Section 1.1.4), it is the target crop for this thesis.

Figure 1.2. White lupin plants

White lupin is a diploid species (2n = 50, 451 Mb; Hufnagel et al., 2020), with epigeal germination (i.e., cotyledons are pushed above the ground; Figure 1.3b) and an indeterminate growth habit. It has a single, erect main stem reaching up to 1.2 m in height, with several branches. Each branch bears a terminal inflorescence with white or bluish flowers (Figure 1.3d; Huyghe, 1997), which develop into oblong pods containing three to six seeds each (Figure 1.3e). The seeds are flat, rectangular or square-shaped with rounded corners and are typically white (Figure 1.3a), sometimes exhibiting a salmon or pink tint or dark brown speckles (Foti and Abbate, 2000). Leaves are alternate, with five to seven leaflets (Figure 1.3c), smooth above and hairy beneath. The root system consists of a deep taproot (Figure 1.3f) nodulated by rhizobia, mainly belonging to the genus *Bradyrhizobium*, which establish an efficient nitrogen-fixing symbiosis (Msaddak, 2023; Lucas et al., 2015). Flowering time is determined by vernalisation requirements and the accumulation of growing degree days (Huyghe and Papineau, 1990; Rychel-Bielska et al., 2024). Self-pollination predominates, although outcrossing rates ranging from 5% to 10% may occur due to bumblebee activity, depending on genotype-specific attractiveness and environmental conditions (Faluyi and Williams, 1981; Williams, 1987).

White lupin can be sown either in autumn or early spring, depending on the location and genotype. Similar to other cool-season legumes, three germplasm classes can be distinguished: (i) spring types, adapted to early spring sowing in cold-prone areas of northern or continental Europe; (ii) Mediterranean types, adapted to autumn sowing in Mediterranean regions, where temperatures rarely fall below −8 °C, thus benefiting from water availability during the cool season while avoiding terminal drought; and (iii) winter types, which are also adapted to autumn sowing but exhibit greater frost tolerance than Mediterranean types, and are typically cultivated in areas with oceanic or mild continental winters (Stoddard et al., 2006; Annicchiarico and Iannucci, 2008).

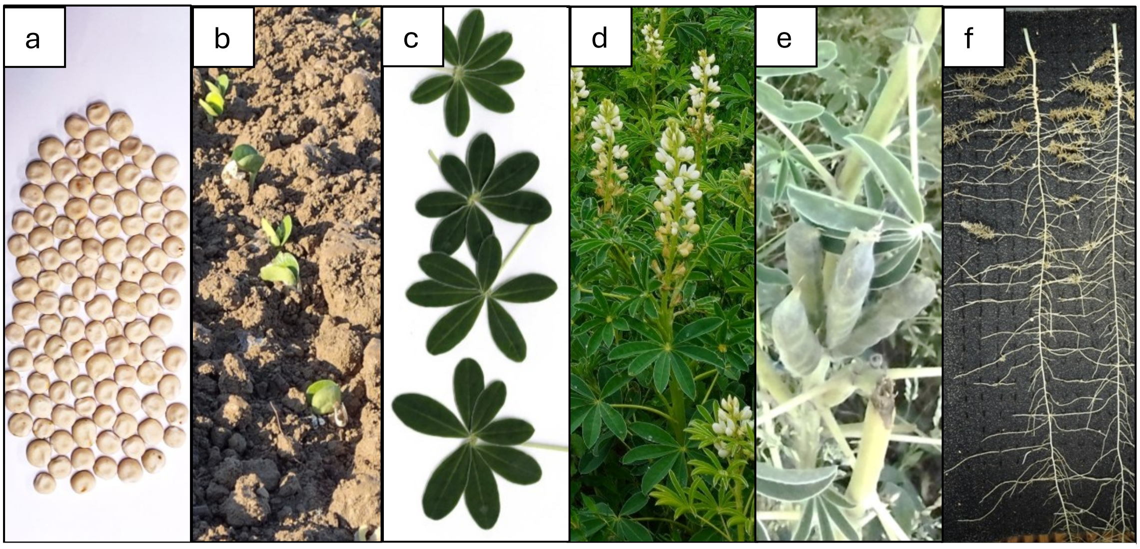


Figure 1.3. Morphological features of white lupin: (a) seeds, (b) seedling, (c) leaves, (d) inflorescences, (e) pods, and (f) root system.

Site selection is crucial because the species is sensitive to active lime (i.e., the soluble fraction of calcium carbonate, according to Drouineau (1942)) when it exceeds 1% (Papineau and Huyghe, 2004), and to alkaline soil reactions (Tang and Thomson, 1996). These factors often co-occur in calcareous soils, leading to impaired nutrient uptake, iron chlorosis, defective nodulation, and reduced growth and yield (Tang and Thomson, 1996; Kerley, 2000). Conversely, white lupin is well adapted to poor and acidic soils, owing to its characteristic cluster roots, which exude organic acids that mobilise phosphorus and other otherwise unavailable nutrients (Hufnagel et al., 2020; Müller et al., 2015). It achieves optimal performance at soil pH values between 4.5 and 7.5 (Huyghe, 1997). Thanks to its nitrogen-fixing symbiosis, nitrogen fertilisation is unnecessary, but inoculation with *Bradyrhizobium lupini* is a prerequisite, particularly in sites where lupins have not been cultivated recently (Clark, 2014). Potassium and sulphur fertilisation are recommended on deficient soils (Slesareva and Zaitseva, 2023; Cazzato et al., 2012).

White lupin is highly sensitive to waterlogging and weed competition (Foti and Abbate, 2000; Damalas and Koutroubas, 2022); therefore, heavy soils should be avoided, and tillage practices should aim to improve drainage and suppress weed germination. Recommended sowing practices include a sowing depth of 3–5 cm, row spacing of 40–60 cm, and a plant density of 60–80 seeds per square metre (Riethmuller, 1990; Foti and Abbate, 2000). Chemical weed control can be applied both pre-emergence and post-emergence; however, pre-emergence treatments are preferable, as cases of phytotoxicity have been reported with post-emergence applications (Juhász et al., 2025;

Kousta et al., 2024). Mechanical weeding can provide results comparable to those obtained through chemical treatments (Folgart et al., 2011).

Optimal harvesting occurs when seeds are fully formed, with a moisture content of approximately 15% (Levchenko et al., 2025). In southern Europe, under a Mediterranean climate and autumn sowing, harvesting takes place between late spring and early summer. Conversely, in northern regions with continental or subcontinental climates and spring sowing, harvesting occurs between mid-July and mid-September (López-Bellido et al., 1994; Terres Inovia, 2019). White lupin yield is highly variable, being affected by several factors such as geographical location, sowing time, soil type, genotype, and management. Typical yields range from 0.7 to 3.0 t/ha (Foti and Abbate, 2000), but under particularly favourable conditions they can exceed 6 t/ha (Annicchiarico et al., 2016; Prusiński, 2021).

The major diseases affecting white lupin are caused by fungal pathogens. *Pleiochaeta setosa* causes brown leaf spots and may pose a significant threat to autumn-sowing; however genetic tolerance has been progressively improved (Huyghe, 1997). *Uromyces lupinicolus* (rust) primarily develops during warm, dry summer periods, causing defoliation and biomass reduction, but it can be effectively controlled through chemical treatments (Huyghe, 1997). *Fusarium avenaceum* is the most aggressive *Fusarium* species affecting lupins; it attacks plant roots and, under excessively moist conditions, can lead to plant death. However, it is considered a serious threat only in fields where lupins are grown regularly, leading to inoculum build-up (Bateman, 1997). Anthracnose, caused by *Colletotrichum lupini*, is regarded as the most destructive fungal disease affecting white lupin. It is seed-borne (primary infection) and can rapidly spread within the crop via rain splash (secondary infections), causing initial stem twisting, necrotic lesions on stems and pods, and potentially resulting in total yield loss under favourable conditions (Thomas and Sweetingham, 2004). Current disease management strategies focus on the use of certified pathogen-free seed and the application of chemical protection (Alkemade et al., 2021).

1.1.4. White lupin as a crop of renewed interest

The increasing interest in white lupin is primarily due to the high protein content of its seeds (38–42% on a dry matter basis; Papineau and Huyghe, 2004). In southern Europe, where it is the most cultivated *Lupinus* species, it has demonstrated the highest crude protein yield per unit area (1.38 t/ha) compared with similar crops such as pea (1.01 t/ha), faba bean (1.01 t/ha), and narrow-leafed lupin (0.95 t/ha; Annicchiarico, 2008). Although soybean has an even higher seed protein content (48–52% on a dry matter basis; Boukid and Pasqualone, 2022), white lupin is likely to be a competitive alternative due to additional benefits provided by its cool-season growth cycle, such as reduced water use and the potential for rotation with summer crops.

Lupinus spp. proteins primarily consist of globulins and albumins in a 9:1 ratio and exhibit valuable techno-functional properties such as solubility, water and oil absorption, emulsifying capacity, foaming ability, and gelation capacity. These characteristics make them suitable ingredients for food and beverage products (Bader et al., 2011; Piornos et al., 2015), as well as for nutraceutical and cosmetic applications. Compared to narrow-leafed and yellow lupins, white lupin seeds have a higher essential amino acid index and protein efficiency ratio (Duranti et al., 2008). Additionally, they contain 8–12% oil on a dry matter basis, with excellent nutritional qualities, including a highly favorable $\omega-3/\omega-6$ fatty acid ratio (Boschin et al., 2008b), low starch content (2.8–3.27% on a dry matter basis), and high levels of polyphenols, carotenoids, and phytosterols (Boukid and Pasqualone, 2022). Consumption of white lupin has been associated with health benefits related to diabetes, hypertension, cardiovascular diseases, and obesity (Prusinski, 2017).

Nevertheless, the use of white lupin has long been limited by the presence of quinolizidine alkaloids (QAs) in the seeds. QAs are bitter, anti-nutritional compounds, namely a group of secondary metabolites synthesized by lupin species as a defence against pathogens and herbivores (Aniszewski, 2007; Mancinotti, 2022; Wink, 1993), and as seed nitrogen storage (Wink, 2019). Although the actual level of toxicity to humans remains debated (Schrenk et al., 2019), health authorities in various countries have established a threshold of 200 mg/kg of total QA content in lupin-based food products (ACNFP, 1996; ANZFA, 2001), while a threshold of 500 mg/kg is recommended for animal feed (FIRAG, 2017). A white lupin genotype is considered sweet-seeded when the QA content of its seeds is below 0.02%, making them palatable and safe for consumption. Various sweet-seed cultivars of white lupin have been developed through modern breeding and commercialized (Cowling et al., 1998; Gresta et al., 2017). If QA content exceeds the regulatory thresholds, debittering treatments such as soaking, cooking, fermentation, or ultrasound are required (Pelgrom et al., 2015; Erbaş et al., 2005).

1.1.5. Breeding challenges and priorities in white lupin

The long history of white lupin cultivation has led to the selection of key domestication traits, such as seed permeability and non-shattering pods (Kurlovich, 2002; Cowling et al., 1998). Formal genetic improvement began in the 1930s with the selection of the first sweet-seed cultivars (Cowling et al., 1998). Nearly a century later, low QA content remains the most important and challenging quality trait for breeders. Despite the established presence of sweet-seed cultivars on the market, the recommended QA threshold has been exceeded multiple times in food products (Schrenk et al., 2019), elite breeding lines (Jacob et al., 2017), and occasionally in commercial cultivars (Boschin et al., 2008a; Brand and Brandt, 2000; Muzquiz et al., 1994). To date, selection for low QA content has primarily relied on exploiting the *pauper* locus (Papineau and Huyghe, 2004). This recessive gene strongly reduces total QA levels by interfering with the biosynthetic pathway (Frick et al., 2017; Mancinotti et al., 2023). However, it does not guarantee reduction below threshold levels due to the presence of different allelic forms (Harrison and Williams, 1982), non-allelic gene interactions (Święcicki et al., 2019), and minor gene effects (Frick et al., 2017; Osorio and Till, 2022). Although QA content is also influenced by several environmental factors (Frick et al., 2017; Rodés-Bachs and Van der Fels-Klerx, 2023), it is unlikely to be significantly affected by genotype-by-environment (G×E) interactions (Schwertfirm et al., 2024; Cowling et al., 1998; Annicchiarico et al., 2014). The primary challenge for white lupin breeders is to further reduce QA content within germplasm carrying the *pauper* locus (Annicchiarico et al., 2025). Other seed quality traits on which breeders should focus include improved protein and oil contents, seed size (Annicchiarico et al., 2025), and reduced seed coat thickness, which accounts for approximately 18% of seed dry weight compared with about 9% in soybean (Cowling et al., 1998).

To fully realise the crop's potential and enhance its economic return, genetic improvement should also target tolerance to both abiotic and biotic stresses. Among the abiotic stresses, affecting white lupin, frost, drought, and soil calcareousness have the most significant impact. Frost survival is crucial for autumn-sowing, and its mechanisms are detailed in Section 1.2. Drought stress primarily affects the crop cycle in late spring, damaging pod development and seed filling (Huyghe, 1997). Drought survival relies on two distinct strategies: drought escape and drought resistance (i.e., intrinsic drought tolerance). Drought escape is achieved through early phenology, allowing the plant to complete its growth cycle before severe water deficits occur (Annicchiarico et al., 2018; Huyghe, 1997). Drought resistance encompasses physiological mechanisms independent of phenology, such as stomatal closure, higher stomatal conductance, or less reduction in net photosynthesis (Annicchiarico et al., 2018; Rodrigues et al., 1995). It is defined as a positive deviation from the genotype's expected yield based on its onset of flowering when drought affects the crop cycle (Pecetti et al., 2023). Finally, adaptation to calcareous soils represents one of the main limitations to crop expansion (Lucas et al., 2015). However, tolerance levels vary considerably within the species, and some tolerant landraces from Egypt and Italy have been identified (Annicchiarico and Thami Alami, 2012; Christiansen et al., 1999; Raza et al., 2001), highlighting the potential for genetic improvement of this trait.

Among biotic stresses, breeding efforts should primarily focus on resistance to anthracnose, caused by the fungal pathogen *Colletotrichum lupini*, which represents the most severe constraint on *Lupinus* spp. cultivation (Nirenberg et al., 2002; Talhinhas et al., 2016). As anticipated, this disease spread in the 1980s due to highly aggressive strains originating from South America (Alkemade et al., 2021; Dubrulle et al., 2020). Current management strategies rely mainly on the use of pathogen-free seed and chemical control (Thomas et al., 2008). Although genetic resistance is considered the most sustainable solution, no complete resistance has yet been found as the trait is polygenic (Adhikari et al., 2009; Alkemade et al., 2022; Jacob et al., 2017). Consequently, further breeding efforts are required.

Although the currently limited seed market for this crop forces breeding programmes to target wide adaptation (Annicchiarico et al., 2011), the large number of factors influencing its growth cycle suggest that varietal selection should focus on specific adaptation, based on local climatic conditions, disease pressure, and soil type. A sustainable compromise is the selection of white lupin varieties for contrasting agroclimatic conditions, namely Mediterranean, oceanic, and continental climates, and for either autumn or spring sowing (Annicchiarico et al., 2010).

1.2. Frost resistance

1.2.1. Mechanisms of frost injury

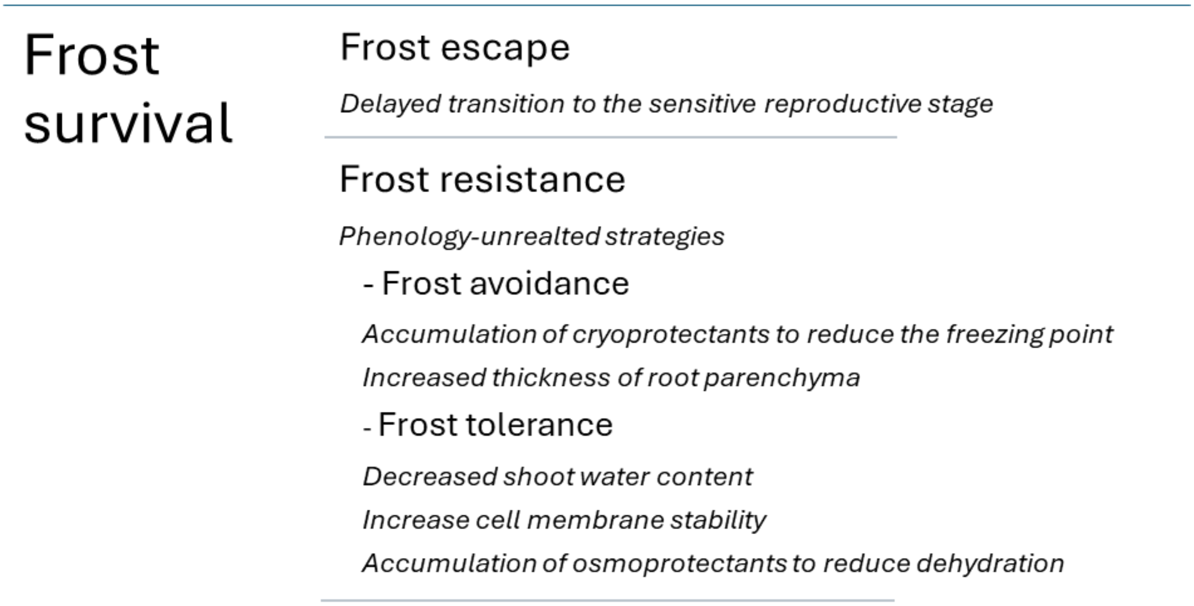
Low temperatures are among the most detrimental abiotic stresses for various crops. Low temperatures are classified as either chilling or freezing. Chilling temperatures range from 0 to 15 °C (Bhat et al., 2022) and can cause cold damage to susceptible crops such as soybean (Maqbool et al., 2010). Under such conditions, plants may experience reduced cell membrane fluidity, altered membrane protein function, and inhibition of several biochemical processes, including energy transduction, solute transport, and H^+-ATPase activity (Lucau-Danila et al., 2012; Muzi et al., 2016). Symptoms of cold stress in susceptible crops include reduced growth, leaf surface lesions, a water-soaked appearance, and withering (Morris, 1990; Smallwood and Bowles, 2002).

Cool-season legumes from temperate and continental regions are generally tolerant to cold. Although various factors, such as waterlogging and fungal diseases, influence their winter hardiness (i.e. winter plant survival), frost is regarded as one of the primary threats to many cool-season crops, including white lupin (Shield et al., 2000; Tantau et al., 2004). Consequently, this thesis focuses on frost stress caused by freezing temperatures. Frost can damage plant tissues through the formation of ice crystals. When frost occurs, intercellular fluids freeze more rapidly than intracellular fluids due to their lower solute concentration, resulting in a reduction of water potential of the intercellular space (Steponkus, 1979). This process draws water out of the cells, causing cellular dehydration, decreased cell volume, membrane deformation, reduced plasma membrane fluidity, increased leakage of ions, proteins, and lipids, and chlorophyll degradation (Steponkus, 1979; Bhat et al., 2022). Another negative effect of frost on cell metabolism is the overproduction of reactive oxygen species (ROS), which damage molecular and cellular components due to biomolecules' oxidation (Sachdev et al., 2021). Although cell dehydration is considered the most harmful effect of intercellular ice formation (Mazur, 1970), its consequences are generally reversible (Gabbrielli et al., 2022).

A more severe scenario arises during rapid or prolonged freezing events, or when very low minimum temperatures are reached, as the movement of liquid water into the intercellular spaces becomes insufficient to compensate for the water potential difference. This difference is then equilibrated solely through ice formation within the cells (Steponkus, 1979). Intracellular ice crystals are more damaging than intercellular ones, as they can destroy both cell membranes and organelles, ultimately leading to cell death (Beck et al., 2004; Levitt, 1980). Frost injury manifests as darkened, water-soaked, and flaccid tissue (Steponkus, 1979). In cool-season legumes, frost injury becomes lethal when it occurs in the epicotyl, where it disrupts the transport of water and nutrients between the roots and shoots, potentially resulting in plant death. If the epicotyl is affected, the plant may die within three weeks (Murray et al., 1988). Understanding the physiological responses of plants to freezing temperatures, and their mechanisms to avoid or resist ice formation, is essential for improving frost survival and overwintering.

1.2.2. Conceptual framework of frost survival strategies

The ability of plants to survive is the sum of several physiological and phenological strategies. Maqbool et al. (2010) highlighted the considerable inconsistency in the terminology used throughout the literature to define survival strategies; however, more than a decade later, this inconsistency persists. Therefore, the aim of this section is to clarify the terminology employed throughout this thesis, as summarised in Figure 1.4. This terminology is inspired by the definitions of drought survival components proposed by Turner (1986) and is consistent with that used in various reviews of frost survival, including those by Jahed et al. (2023), Janská et al. (2010), and Thapa et al. (2025).



Frost survival

Frost escape

Delayed transition to the sensitive reproductive stage

Frost resistance

Phenology-unrealted strategies

- Frost avoidance
  - *Accumulation of cryoprotectants to reduce the freezing point*
  - *Increased thickness of root parenchyma*
- Frost tolerance
  - *Decreased shoot water content*
  - *Increase cell membrane stability*
  - *Accumulation of osmoprotectants to reduce dehydration*

Figure 1.4. Overview of the main frost survival strategies

Two primary strategies enable frost survival: frost escape and frost resistance. Frost escape occurs through delayed flowering, which prevents the premature transition from the vegetative to the reproductive stage, the latter being highly sensitive to frost (Maqbool et al., 2010; Siddique et al., 1999). In various cool-season grain legumes such as chickpea, faba bean, and pea, this transition is primarily dependent on photoperiod, as flowering is induced only under long days in spring (Flores et al., 2013; Lejeune-Hénaut et al., 1999; Soltani et al., 2004). Conversely, in white lupin, the main factor regulating flowering time is vernalisation requirement, followed by the accumulation of growing degree days (Huyghe and Papineau, 1990; Rychel-Bielska et al., 2024). A high vernalisation requirement maintains the plant in the juvenile rosette stage, characterised by a low height-to-leaf-number ratio, but when it is met, the transition to the reproductive stage begins, leading to the development of highly sensitive reproductive organs and inducing hormonal changes. For instance, gibberellin levels increase, resulting in a reduced abscisic acid to gibberellin ratio (Huyghe and Papineau, 1990), whereas an elevated proportion of abscisic acid is known to induce growth retardation, which is required to maintain cold adaptation (Junttila et al., 2002).

Conversely, frost resistance represents the combination of frost survival components that are independent of phenology. It can be synthetically expressed as a positive deviation from the genotype observed frost survival relative to the expected according to its onset of flowering. Frost resistance, in turn, depends on two factors: frost avoidance, namely the plant's ability to prevent ice formation within its tissues, and frost tolerance, namely the plant's ability to withstand freezing (Levitt, 1980). Most resistance mechanisms underlying both frost avoidance and tolerance are induced by photoperiod shortening (Beck et al., 2004; Junttila, 1996; Rapacz et al., 2014) and by exposure to low, non-freezing temperatures in a process known as cold acclimation or hardening (Arbaoui and Link, 2008; Bourion et al., 2003; Link et al., 2010). This complex physiological process, detailed in Section 1.2.3, activates adaptive responses that enable survival under freezing conditions.

Among the components of frost resistance, one appears to be independent of hardening: the thickness of the root parenchyma, which depends on genotype and sowing time. According to Huyghe & Papineau (1990), a thicker parenchyma layer reduces roots frost damage: it is characterized by wide intercellular spaces and the capacity of cells to shrink significantly, and during freezing, water moves out of the cells, allowing for the formation of large ice crystals in the intercellular spaces, which reduces the risk of intracellular freezing (McCully et al., 2004).

1.2.3. The role of cold acclimation

Frost resistance is highly dependent on the degree of cold acclimation that plants acquire during the hardening period, which is influenced by both genotype and environmental conditions.

Hardening begins in autumn, when plants perceive a decrease in temperature and a reduction in photoperiod, transmitting this information to downstream components that induce the appropriate molecular, biochemical, and physiological adaptations (Theocharis et al., 2012). The reduction in photoperiod is sensed by chloroplasts and results in growth cessation, a prerequisite for cold acclimation (Rapacz et al., 2014). The temperature required to initiate the process as well as the minimum duration of the hardening period necessary to maximise frost resistance, are species-specific and, to date, have not been investigated in white lupin. However, some information can be extrapolated from other cool-season grain legumes. In faba bean, acclimation mechanisms are activated when temperatures fall below 7 °C, and frost resistance is maximised after two or three weeks of exposure to temperatures between 0 and 7 °C (Link et al., 2010). Conversely, in pea, a minimum of four weeks of cold acclimation is required to achieve maximum frost resistance (Swensen and Murray, 1983).

During hardening, chilling temperatures cause the rigidification of cellular membranes (Vaultier et al., 2006), which is detected by various receptors, including $Ca2^+$ influx channels, two-component histidine kinases, and G-proteins associated receptors (Xiong et al., 2002). These membrane receptors release secondary messengers, such as Ca^{2+}, into the cytosol. Specific sensors detect these secondary messengers and coordinate the transmission of the cold stress signal, thereby regulating the expression of transcription factors and cold-regulated genes (Jahed et al., 2023). The products of cold-regulated genes can be divided into two distinct groups: regulatory proteins that control the transduction of the cold stress signal, and proteins that are directly involved in the adaptive response (Janská et al., 2010). The latter group is responsible for the biochemical and physiological modifications that characterise the hardening process.

One of the most significant adaptive responses is the accumulation of cryoprotective compounds, also known as osmoprotectants, which contribute to both frost avoidance and tolerance. Common cryoprotectants synthesised by plants include potassium ions (K^+); low-molecular-weight nitrogenous compounds such as proline and glycine betaine; sugars such as saccharose, raffinose, stachyose and trehalose; sugar alcohols such as sorbitol, ribitol and inositol; and glycerol (Jahed et al., 2023; Janská et al., 2010). These compounds lower the freezing point by increasing the concentration of intercellular and intracellular solutes, enabling water to remain in a liquid state even at sub-zero temperatures (Jahed et al., 2023). Some plants also synthesise antifreeze proteins, which bind to the surface of newly formed ice crystals, thereby influencing their subsequent shape and reducing their growth (Moffatt et al., 2006). However, if environmental pressure becomes too severe and these mechanisms are insufficient to prevent ice formation, cryoprotectants can also act in conjunction with dehydrin proteins (DHNs), cold-regulated proteins (CORs) and heat-shock proteins (HSPs) to maintain the osmotic balance of cells, stabilise cell membranes and cytoplasmic proteins, preserve hydrophobic interactions, and scavenge reactive oxygen species (ROS; Janská et al., 2010).

Since cell membranes are highly sensitive to ice formation, their protection from mechanical damage is crucial. Membrane reinforcement occurs not only through the production of protective compounds but also via changes in membrane composition, such as an increase in the proportion of unsaturated fatty acids in phospholipids (Rajashekar, 2000; Arbaoui and Link, 2008). Other mechanisms contributing to frost tolerance include a reduction in shoot water content (Sallam et al., 2015), a decrease in photosynthetic activity, and alterations in pigment synthesis (Jahed et al., 2023).

1.3. Frost resistance genetic improvement in white lupin

1.3.1. Enhancing crop yield potential through autumn sowing

Sowing time significantly influences the exposure of white lupin, as with other cool-season grain legumes, to the most detrimental climatic stresses affecting the crop, namely frost and drought. Spring sowing is currently adopted in northern Italy and other European sub-oceanic or sub-continental climatic regions to avoid winters and minimise exposure to frost events. In Mediterranean climates, autumn sowing is more common; a five-year study conducted under the Mediterranean conditions of southern Spain reported around 60% increase in grain yield when shifting from spring to autumn sowing (López-Bellido et al., 1994). However, as climate change is increasing the likelihood of drought stress in late spring not only across the Mediterranean basin, but also northwards and eastwards (Alessandri et al., 2014), autumn sowing is becoming common also in European sub-oceanic and sub-continental climatic regions. This strategy aims to anticipate harvesting, reduce the risk of terminal drought, and extend the crop cycle, thereby enhancing yield potential (Huyghe and Papineau, 1990; Stoddard et al., 2006).

Nevertheless, frost stress is expected to remain a major constraint to white lupin autumn sowing, as milder winters are likely to reduce plants' cold acclimation (Annicchiarico and Iannucci, 2007; Herzog, 1989) and to accelerate cold de-acclimation (i.e., loss of frost resistance: Rapacz et al., 2014), while the risk of early and late frost events is expected to increase (Francis and Skific, 2015). The use of late-flowering genotypes allows the highly sensitive reproductive organs to escape frost and enhances frost survival, but their consequent delayed ripening is associated with an increased risk of terminal drought (Pecetti et al., 2023). In this scenario, a white lupin ideotype for autumn-sowing should combine intermediate phenology with phenology-independent frost and drought resistances, as defined in Sections 1.2.2 and 1.1.5, respectively. Together, these traits can ensure higher yield potential and stability across both cold, moisture-favourable years and relatively mild, drought-prone years (Annicchiarico et al., 2011).

The focus of this thesis is therefore on improving the genetic frost resistance of white lupin, a crucial adaptive trait to enhance its suitability for autumn sowing in European sub-oceanic and sub-continental climatic regions, within the current context of climate change.

1.3.2. Phenotypic selection under controlled conditions

Although frost survival is a key target for white lupin breeders, genetic improvement is challenged by a scarcity of studies on its genetic variation and limited knowledge of its genetic control. Germplasm pools of landraces from the Azores, Italy, Greece, Madeira, and the Canary Islands have been identified as valuable genetic resources for winter hardiness based on field experiments (Annicchiarico et al., 2010; Huyghe et al., 1997; Papineau and Huyghe, 2004). However, field evaluation is influenced by site- and year-specific factors such as the duration, intensity, and frequency of frost events, average winter temperature, and potential dehardening of plants due to increased temperatures preceding stress, as well as by other factors including diseases and waterlogging. As a consequence, the wide and increasing climatic variation across years adversely affects the applicability, efficiency, and replicability of field selection (Avia et al., 2013). Frost resistance, a key component of frost survival, can be assessed and selected under controlled conditions, where plants are evaluated at the vegetative stage to eliminate any phenological effects, and where hardening, frost intensity, and duration are standardised to overcome field limitations. Moreover, assessments under controlled conditions enable off-season selection (Stoddard et al., 2006).

A large-scale evaluation of white lupin under controlled conditions is still lacking, but it is essential for identifying elite genetic resources and studying the trait genetic control, as well as in breeding programmes for the selection of inbred lines. However, it requires an ad-hoc phenotyping platform, consisting of a growth chamber capable of reaching freezing temperatures (Figure 1.5). Numerous experimental factors influencing the final survival/mortality must be standardised to ensure experimental reproducibility:

- Soil type and drainage (Russell et al., 1978), along with the amount and frequency of irrigation, since free water in the soil can increase ice formation and cause mechanical damage to the roots.
- The stage of plant growth and development, which can influence cold acclimation.
- The level and duration of hardening (Swensen and Murray, 1983), namely the period of cold acclimation. The literature reports a variety of hardening conditions for experiments on cool-season legumes: Hoffmann-Bahnsen and Herzog (2001) proposed a prolonged hardening period for white lupin of 42 days at 8/2 °C day/night temperatures, whereas

most studies on pea have used 4 °C over 2 to 4 weeks (Auld et al., 1983; Homer et al., 2016; Meyer and Badaruddin, 2001; Murray et al., 1988).

- The cooling rate towards the freezing temperature, which should not exceed 2 °C per hour to allow sufficient time for water redistribution (Murray et al., 1988).
- The intensity and duration of frost treatments.
- The length of the recovery period after which mortality can be evaluated, as an accurate assessment of damage can only be made after a minimum recovery period of three weeks under favourable temperatures (Murray et al., 1988; Figure 1.6).

Figure 1.5. Phenotyping platform for frost resistance assessment, established at CREA-ZA, Lodi (Italy).

Figure 1.6. Mortality assessment of white lupin genotypes following a three-week recovery period: two frost-susceptible genotypes (on the left and right) are compared with a frost-tolerant genotype (in the centre).

The genotype lethal temperature 50 (LT_{50}) is a common indicator of frost resistance under controlled conditions. It is defined as the freezing temperature at which 50% mortality occurs. Its computation requires testing genotype mortality across a range of freezing temperatures, which are then used to fit a logit or probit regression model (Lei and Sun, 2018), with plant mortality as a function of the freezing temperature. Consequently, this method may be less practical than evaluating plant mortality at a single optimal freezing temperature when assessing frost resistance in large numbers of genotypes, such as in selection trials (Waalen et al., 2011). However, such an optimal temperature should maximise the variation in genotype mortality, and since no information is currently available for the target crop, its identification is a priority. In addition, genotype frost resistance at one optimal temperature can also be expressed using a visual score based on the quantification of necrotic biomass areas, as already proposed for other cool-season grain legumes by Ali et al. (2016), Arbaoui and Link (2008), and Beji et al. (2020). This biomass injury score is particularly useful when only a small number of plants per genotype are evaluated, a situation in which estimating plant mortality is less reliable.

Finally, although an outstanding consistency between platform-based frost resistance and field-based winter hardiness cannot be expected, as winter survival results from many factors, its quantification holds practical importance for the exploitation of artificial screening results. Correlations among plant mortality under field and growth chamber assessments in pea were close to 0.7 in Homer et al. (2016) and ranged from 0.5 to 0.6 in Auld et al. (1983). Correlations close to 0.5 have been reported for other legume species, such as faba bean (Arbaoui et al., 2008) and red clover (Zanotto et al., 2021).

1.3.3. Genomic selection

The need for an ad-hoc phenotyping platform for frost resistance assessment is a major limiting factor in improving this trait through breeding programs. Therefore, genomic techniques based on molecular markers can complement phenotypic selection under controlled conditions and have become particularly feasible with the advent of next-generation sequencing, especially genotyping-by-sequencing (GBS; Elshire et al., 2011). White lupin is a diploid species with genome size of approximately 451 Mb, organized into 25 chromosomes (2n = 50; Hufnagel et al., 2020). One study demonstrated that GBS can generate thousands of single-nucleotide polymorphism (SNP) markers for its genetic analysis (Książkiewicz, et al. 2017). Genome-wide association studies (GWAS) can identify associations between molecular markers and phenotypic variability within a population, providing insights into the genetic architecture of traits and enabling the detection of candidate genes in linkage disequilibrium with significant SNPs. This approach has been further facilitated by the recent publication of a high-quality white lupin genome (Hufnagel et al., 2020). Markers associated with frost survival and/or resistance have already been identified in various legumes, such as faba bean (Sallam et al., 2016; Windhorst et al., 2024), pea (Lejeune-Hénaut et al., 2008; Beji et al., 2020), and red clover (*Trifolium pratense* L.; Zanotto et al., 2023). In white lupin, several markers have been found to be associated with other agronomically important traits, including flowering time (Rychel-Bielska et al., 2024), adaptation to calcareous soils (Annicchiarico et al., 2023), drought tolerance (Pecetti et al., 2023), and anthracnose resistance (Alkemade et al., 2022).

For a quantitative trait under polygenic control, the most effective marker-based approach is genomic selection (GS; Meuwissen et al., 2001; Heffner et al., 2009). The fundamental principles underlying GS are:

- The development of a prediction model through the combination of phenotypic data and all available genome-wide markers. The simultaneous use of all markers captures the effects of numerous genes, including minor ones, rather than only those in linkage with significant SNPs identified in the GWAS. Marker effects can be estimated even if their number exceeds the number of observations by treating them as random effects with a common variance.
- The computation of genomic estimated breeding values (GEBVs), which represent predicted phenotypes attributable solely to the genetic component, as sum of the products of marker genotypes and their corresponding effects. Once the model is fitted, GEBVs are computed for new individuals using genotyping data alone, significantly reducing the need for phenotyping.

In the classical genomic selection scheme, prediction models are trained on a sample of genotypes (the training population) that is representative of the target genetic base (the reference population). These models are initially evaluated based on their prediction accuracy (i.e., correlation between observed and predicted breeding values), or their predictive ability (i.e., correlation between observed and predicted phenotypes), through cross-validation (Lorenz et al., 2011). Prediction accuracy can be calculated by dividing the predictive ability by its potential maximum (which corresponds to the square root of heritability when a single phenotype is associated with each genotype).

Pioneer GS studies have shown promising results for various white lupin traits. Predictive ability values ranged from 0.47 to 0.78 for grain yield in landrace genotypes and breeding lines under both favorable and drought stress conditions (Annicchiarico et al., 2019a; Pecetti et al., 2023), from 0.58 to 0.81 for tolerance to anthracnose (Schwertfirm et al., 2024; Rychel-Bielska et al., 2020), and from 0.40 to 0.87 for various morphophysiological and seed quality traits (Annicchiarico et al., 2020a; Annicchiarico et al., 2023; Annicchiarico et al., 2025). However, the applicability of GS for frost resistance in white lupin has not yet been explored. Limited data are available for cereals: a prediction accuracy of 0.59 was reported for frost injury in wheat (*Triticum aestivum* L.; Michel et al., 2019), and a predictive ability of 0.87 was reported for rye (*Secale cereale* L.; Erath et al., 2017).

GS can be applied to the selection of inbred lines as well as to the identification of promising genetic resources within germplasm collections (Jarquin et al., 2016). A key practical concern for genomic prediction models is their transferability across different populations. Previous studies on cool-season grain legumes suggest that genomic prediction models developed using a world germplasm collection (a genetically broad population) can be used to predict traits in breeding line populations (genetically narrower). For example, such models have been used to predict seed weight and oil content in white lupin (Annicchiarico et al., 2025) and seed protein content and seed weight in pea (Crosta et al., 2023). However, this transferability comes at the cost of a 35–50% reduction in predictive ability compared to predictions made within the breeding line populations themselves. Conversely, these studies also report a lack of model transferability for other traits, such as seed protein content in white lupin (Annicchiarico et al., 2025) and grain yield in pea (Crosta et al., 2023). The transferability of prediction models is primarily relevant when models are trained on genetically broad populations and applied to genetically narrower populations, as the inference space is expected to be broader in this direction compared to the reverse scenario (Annicchiarico et al., 2021).

1.4. Research objectives and structure of the thesis

Based on the background presented in Chapter 1, the present thesis aims to provide insights into white lupin frost resistance and develop practical tools for its genetic improvement. Enhancing frost resistance is expected to facilitate the northward expansion of autumn sowing, increase crop yields, improve economic returns for farmers, and thereby promote cultivation along with its associated environmental benefits. This study utilises a recently established high-throughput phenotyping platform (Figure 1.5) located at CREA-ZA in Lodi, Italy, which is described in detail in Section 2.3.2.

The work presented here began with a methodological study (Chapter 2), which established a robust experimental protocol for assessing frost resistance under controlled conditions. This protocol was developed following an extensive literature search on cool-season grain legume screenings. Frost resistance was evaluated in 11 genotypes of the target crop, and 11 genotypes of pea, based on plant mortality and biomass injury visually scored following four freezing treatments at −7, −9, −11, and −13 °C, as well as on LT_{50} values. The genetic material was selected to represent a wide range of winter mortality observed in previous field trials conducted in northern Italy. The methodological study was performed with the following objectives:

- Comparing the two species in terms of frost resistance.
- Optimising the phenotyping protocol by determining the optimal freezing temperature for each species.
- Verifying the consistency of genotypic plant mortality responses across both platform and field conditions.

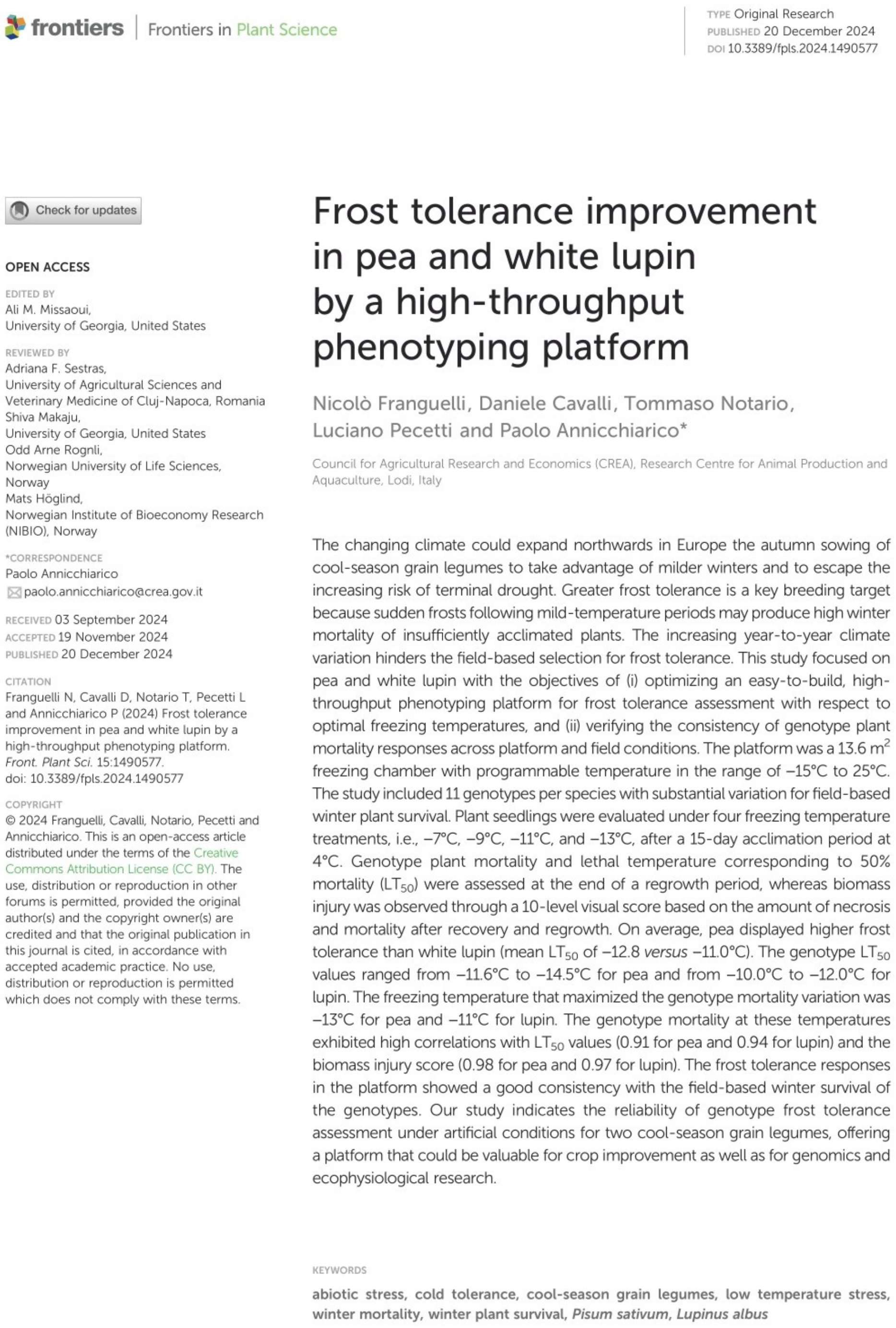
Building on the results of the methodological study, the thesis proceeds with the first large-scale assessment of white lupin frost resistance (Chapter 3). Two populations, comprising 144 landrace and cultivar genotypes and 144 breeding lines, were evaluated for plant mortality and biomass injury visually scored at the optimal freezing temperature. These populations had also previously been genotyped using GBS, yielding 40,914 and 32,951 SNP markers, respectively. Furthermore, 115 landraces and cultivars had been assessed in the field for winter mortality and phenology. The study aimed to:

- Detect genetic variation in frost resistance within each population and identify promising genetic resources.
- Assess the relationship between mortality under controlled and field conditions.
- Provide insights into the applicability of marker-based techniques through analysing trait genetic architecture, identifying candidate genes based on GWAS results, and evaluating

the applicability of genomic selection within each reference population and in an inter-population scenario.

Finally, the last part of the thesis (Chapter 4) investigates the relationship between three factors related to frost survival and winter hardiness of white lupin. These factors are frost escape, frost resistance, and greater seed weight (which is assumed to be associated with faster root parenchyma elongation). The aim of this chapter is to quantify the extent to which frost resistance measured under controlled conditions can predict winter mortality in the field, compared with the other factors. Although this investigation is limited by the availability of only one year of field data, it is justified by a high practical relevance. It provides a breeding strategy for selecting traits that can effectively improve the overwintering of autumn-sown white lupin and evaluates the effective exploitation of controlled-condition screenings.

2. Frost resistance improvement in pea and white lupin by a high-throughput phenotyping platform



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Frost tolerance improvement in pea and white lupin by a high-throughput phenotyping platform

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The changing climate could expand northwards in Europe the autumn sowing of cool-season grain legumes to take advantage of milder winters and to escape the increasing risk of terminal drought. Greater frost tolerance is a key breeding target because sudden frosts following mild-temperature periods may produce high winter mortality of insufficiently acclimated plants. The increasing year-to-year climate variation hinders the field-based selection for frost tolerance. This study focused on pea and white lupin with the objectives of (i) optimizing an easy-to-build, high-throughput phenotyping platform for frost tolerance assessment with respect to optimal freezing temperatures, and (ii) verifying the consistency of genotype plant mortality responses across platform and field conditions. The platform was a 13.6 m^2 freezing chamber with programmable temperature in the range of –15°C to 25°C. The study included 11 genotypes per species with substantial variation for field-based winter plant survival. Plant seedlings were evaluated under four freezing temperature treatments, i.e., –7°C, –9°C, –11°C, and –13°C, after a 15-day acclimation period at 4°C. Genotype plant mortality and lethal temperature corresponding to 50% mortality (LT_{50}) were assessed at the end of a regrowth period, whereas biomass injury was observed through a 10-level visual score based on the amount of necrosis and mortality after recovery and regrowth. On average, pea displayed higher frost tolerance than white lupin (mean LT_{50} of –12.8 *versus* –11.0°C). The genotype LT_{50} values ranged from –11.6°C to –14.5°C for pea and from –10.0°C to –12.0°C for lupin. The freezing temperature that maximized the genotype mortality variation was –13°C for pea and –11°C for lupin. The genotype mortality at these temperatures exhibited high correlations with LT_{50} values (0.91 for pea and 0.94 for lupin) and the biomass injury score (0.98 for pea and 0.97 for lupin). The frost tolerance responses in the platform showed a good consistency with the field-based winter survival of the genotypes. Our study indicates the reliability of genotype frost tolerance assessment under artificial conditions for two cool-season grain legumes, offering a platform that could be valuable for crop improvement as well as for genomics and ecophysiological research.

KEYWORDS

abiotic stress, cold tolerance, cool-season grain legumes, low temperature stress, winter mortality, winter plant survival, *Pisum sativum*, *Lupinus albus*

2. Frost resistance improvement in pea and white lupin by a high-throughput phenotyping platform

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The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Note:

To ensure consistency throughout the thesis in the terminology used to describe components of plant frost survival, the terms employed in the published article have been modified in this version to align with the definitions provided in Section 1.2.2. Specifically, the term *frost tolerance*, when referring to plant frost survival in the field, has been replaced with *frost survival*; the terms *intrinsic frost tolerance* and *frost tolerance*, when referring to the phenology-independent components of frost survival, have been replaced with *frost resistance*; and the term *frost avoidance*, that sometimes was used to describe delayed flowering as a mechanism to avoid frost stress at the reproductive stage, has been replaced with *frost escape*.

2. Frost resistance improvement in pea and white lupin by a high-throughput phenotyping platform

2.1. Abstract

The changing climate could expand northwards in Europe the autumn sowing of cool-season grain legumes to take advantage of milder winters and to escape the increasing risk of terminal drought. Greater frost survival is a key breeding target because sudden frosts following mild-temperature periods may produce high winter mortality of insufficiently acclimated plants. The increasing year-to-year climate variation hinders the field-based selection for frost survival. This study focused on pea and white lupin with the objectives of (i) optimizing an easy-to-build, high-throughput phenotyping platform for frost resistance assessment with respect to optimal freezing temperatures, and (ii) verifying the consistency of genotype plant mortality responses across platform and field conditions. The platform was a 13.6 m^2 freezing chamber with programmable temperature in the range of −15 °C to 25 °C. The study included 11 genotypes per species with substantial variation for field-based winter plant survival. Plant seedlings were evaluated under four freezing temperature treatments, i.e., −7 °C, −9 °C, −11 °C, and −13 °C, after a 15-day acclimation period at 4 °C. Genotype plant mortality and lethal temperature corresponding to 50% mortality (LT_{50}) were assessed at the end of a regrowth period, whereas biomass injury was observed through a 10-level visual score based on the amount of necrosis and mortality after recovery and regrowth. On average, pea displayed higher frost resistance than white lupin (mean LT_{50} of −12.8 versus −11.0 °C). The genotype LT_{50} values ranged from −11.6 °C to −14.5 °C for pea and from −10.0 °C to −12.0 °C for lupin. The freezing temperature that maximized the genotype mortality variation was −13 °C for pea and −11°C for lupin. The genotype mortality at these temperatures exhibited high correlations with LT_{50} values (0.91 for pea and 0.94 for lupin) and the biomass injury score (0.98 for pea and 0.97 for lupin). The frost resistance responses in the platform showed a good consistency with the field-based winter survival of the genotypes. Our study indicates the reliability of genotype frost resistance assessment under artificial conditions for two cool-season grain legumes, offering a platform that could be valuable for crop improvement as well as for genomics and ecophysiological research.

2.2. Introduction

Legume cultivation has a highly positive impact on agri-food systems by increasing the availability of biologically fixed nitrogen, enhancing soil quality, promoting biodiversity, and mitigating the impact of weeds and pests (Karkanis et al., 2016; Nemecek et al., 2008). For European agriculture, greater legume cultivation would help reduce its significant deficit and reliance on imported high-protein feedstuff (which contributes to Amazon deforestation: Boerema et al., 2016) and meet the increasing industry demand for novel protein-rich foods (Lucas et al., 2015; van Loon et al., 2023). Pea (*Pisum sativum* L.) and white lupin (*Lupinus albus* L.) are promising cool-season grain legume crops for southern Europe. Compared to other pulses, pea has a higher yield and energy production, while lupin maximizes protein yield per unit area due to its outstanding seed protein content (Karkanis et al., 2016; Annicchiarico, 2008; Cernay et al., 2016). However, greater plant breeding effort is indispensable to reduce the yield gap with cereals and increase the economic sustainability of these crops (Rubiales et al., 2021).

Cool-season grain legumes are typically sown in autumn in mild-winter regions and in late winter or early spring in cold-prone regions of Europe. The changing climate is expected to expand the autumn sowing northwards, allowing crops to benefit from milder winters and escape the increasing risk of terminal drought through earlier crop maturity (Alessandri et al., 2014). Crop frost survival is a key breeding target in this context, not only to withstand low temperature stress in cold regions but also because sudden frost events following mild-temperature periods may produce high winter plant mortality due to insufficient cold acclimation (Annicchiarico and Iannucci, 2007). Various stresses may concurrently affect winter survival, including frost, waterlogging, and fungal pathogens (Bélanger et al., 2006). However, frost has prominent importance and can be faced by plants through frost escape and frost resistance mechanisms (Janská et al., 2010). Frost escape is based on delayed flowering (aimed to protect the very sensitive reproductive organs) (Maqbool et al., 2010; Siddique et al., 1999), which is primarily achieved through greater vernalization requirements in white lupin (Huyghe and Papineau, 1990) and by photoperiodic control and/or high growing degree days requirements in pea (Summerfield and Roberts, 1988; Lejeune-Hénaut et al., 1999; Weller et al., 1997). In target regions possibly subjected to both low winter temperatures and terminal drought, late flowering and crop maturity may ensure frost escape and a higher yield in cold, relatively moisture-favorable years while being associated with greater drought susceptibility and lower yield in relatively mild, drought-prone years (Annicchiarico et al., 2011, 2019b). For plant breeders, this dilemma can be coped with by selecting materials with intermediate flowering times but frost and drought resistances. Drought resistance could be expressed by a positive deviation from the genotype yield expected according to its onset of flowering (Annicchiarico et al., 2017; Pecetti et al., 2023). Frost resistance of cold-acclimated plants could likewise be expressed by a positive deviation from the genotype winter plant survival expected according to its onset of flowering. The frost resistance mechanisms of cool-season grain legumes are based on physiological modifications to prevent or resist intracellular ice formation (Bourion et al., 2003), such as decreased shoot water content during cold acclimation (Sallam et al., 2015), increased cell membrane stability through changes in the lipid-to-protein ratio and the membrane lipid unsaturation level (Arbaoui and Link, 2008), and accumulation of osmoprotectant compounds such as proline, glycine betaine, mannitol, sucrose, raffinose, stachyose, and specific proteins that protect against dehydration (Link et al., 2010).

Breeding for improved frost survival under field conditions is complicated by the wide and increasing climate variation across years, which reduces the applicability, efficiency, and replicability of the selection (Avia et al., 2013). A reliable assessment of frost resistance in controlled environments could overcome these limitations and allow, in addition, for off-season selections (Stoddard et al., 2006). Its assessment on seedlings rules out any effect of flowering time and focuses, therefore, on frost resistance. The assessment requires a period of cold acclimation (hardening) above 0 °C temperature, the duration of which increases the frost resistance of relatively winter-hardy material (Swensen and Murray, 1983). Hardening was performed at 4 °C over 2 to 4 weeks in most freezing resistance studies on pea (Auld et al., 1983; Murray et al., 1988; Meyer and Badaruddin, 2001; Homer et al., 2016). Subsequently, slow cooling toward the stress temperature is essential to ensure sufficient time for water redistribution, with a cooling rate not exceeding 2 °C/h (Murray et al., 1988). An accurate assessment of mortality can only be made after a minimum recovery period of 3 weeks under favorable temperatures (Murray et al., 1988). Besides plant mortality, genotype frost resistance could also be expressed by a visual score based on the amount of necrotic areas and other traits (Ali et al., 2016; Beji et al., 2020). The assessment of the genotype lethal temperature 50 (LT_{50}), i.e., the freezing temperature corresponding to 50% of mortality, requires the evaluation of plant mortality across a set of freezing temperatures and may, therefore, be operationally less adequate than the evaluation of plant mortality at just one optimal freezing temperature when assessing frost resistance in large numbers of genotypes as in selection trials (Waalen et al., 2011). Such optimal temperature should ensure the maximization of genotype variation for plant mortality, and may approach the genotype mean value of LT_{50} in studies including a sample of genotypes representative of the crop frost resistance variation. Various studies suggested that this temperature may fall in the range of −7 °C to −9 °C (Auld et al., 1983; Swensen and Murray, 1983; Murray et al., 1988; Cousin et al., 1993; Homer et al., 2016) for pea based on small sets of genotypes mostly selected several decades ago, whereas no information is available for white lupin. For pea, an official frost resistance evaluation test prescribes the assessment of candidate varieties at −8 °C freezing temperature (Wery et al., 1994).

We recently established an easy-to-build, high-throughput phenotyping platform for frost resistance assessment represented by a 13.6 m^2 growth freezing chamber with programmable temperature to be used for the selection and genomic prediction of frost resistance in cool-season grain legumes. This study assessed plant mortality, LT_{50} values, and the biomass injury visual score of 11 genotypes of pea and 11 of white lupin encompassing a wide range of winter mortality in earlier field trials in northern Italy, with the objectives of (i) optimizing the frost resistance platform with respect to optimal freezing temperatures for each species and (ii) verifying the consistency of genotype plant mortality responses across platform and field conditions.

2.3. Materials and methods

2.3.1. Plant material

The experiment included 11 genotypes of pea and 11 of white lupin comprising commercial cultivars, landraces, and breeding lines, which were selected within each species to represent a wide variation in winter survival based on the results of previous field trials in northern Italy (Table 2.1). Based on winter plant mortality observed under field conditions in separate earlier experiments, we classified the genotypes into three broad classes of winter hardiness: high, intermediate, and low. One pea genotype, namely, the French landrace Champagne, was selected as a standard of extreme field-based winter hardiness according to Prieur and Cousin (1978) and Dumont et al. (2011).

2.3.2. Frost resistance evaluation experiment

The phenotyping platform consisted of a freezing chamber 4.80 m long × 2.84 m wide × 2.46 m high, with programmable temperature in the range of −15 °C to 25 °C. The chamber was equipped with eight Combo 300-W (C-LED, Bologna) lamps arranged in two rows, placed at a height of 1.6 m from the floor and about 0.9 m above the plant material. Individual test plants were sown at a depth of 2.5 cm into polystyrene plug trays composed of cells measuring 5 cm × 5 cm and 15 cm in depth filled with a commercial growing substrate that included peat corrected for acidity (pH = 6.0) and mineral compound fertilizer NPK (substrate SER CA-V7, Vigorplant, Piacenza, Italy). The plants were placed side by side on four large trolleys fitting into the chamber. Each experimental unit included a set of 10 adjacent plants.

The frost resistance of the 22 genotypes was tested under four freezing treatments: −7 °C, −9 °C, −11 °C, and −13 °C. Plant acclimation took place at 4 °C over 15 days, a shorter duration than in most of the earlier pea freezing resistance studies but consistent with the trend toward milder winters and reduced hardening periods in agricultural environments caused by the changing climate.

The experiment included four experimental units (organized in blocks) per genotype and treatment. Within each treatment, the genotypes were arranged according to a group balanced block layout (Gomez and Gomez, 1984) holding species on main plots and the different genotypes of the two species on subplots. Operationally, the four blocks were subdivided into two growth cycles of two blocks each, which were performed sequentially using exactly the same protocol.

Table 2.1. Name, origin (for cultivars or landraces), plant winter mortality in autumn-sown field experiments in northern Italy, and suggested winter hardiness based on these experiments for 11 pea and 11 white lupin genotypes.

Genotype name [alias] (Origin)	**Mortality (%)**[a]	**Winter hardiness**
Pea breeding lines and parent lines[b]		
KI_L38	3.3	High
KA_37	21.3	High
KI_118	67.0	Low
KA_19	86.0	Low
Isard	10.1	High
Kaspa	51.0	Intermediate
LSD ($P < 0.05$)	16.0	
Pea commercial cultivars[c]		
Dolmen (France)	2.6	High
Dove (France)	6.8	Intermediate
Kaspa (Australia)	7.8	Intermediate
Guifilo (Spain)	9.5	Intermediate
Catania (France)	24.7	Low
LSD ($P < 0.05$)	7.4	
Pea landraces		
Champagne (France)[d]	–	High
Lupin breeding lines[e]		
PLI4-3	16.1	High
PLI7-50 [Arsenio]	37.3	Intermediate
PLI-P3	88.8	Low
LSD ($P < 0.05$)	19.3	
Lupin commercial cultivars and landraces[f]		
Ludet (France)	0.0	High
Adam (France)	5.9	High
Amiga (France)	81.6	Low
Calabria [LAP 0108] (Italy)	2.2	High
GR56 [LAP 0019] (Greece)	8.6	High
E80 [LAP 0041] (Portugal)	33.6	Intermediate
Egypte11 [LAP 0086] (Egypt)	97.9	Low
LA 559 [LAP 0079] (Ethiopia)	93.0	Low
LSD ($P < 0.05$)	12.8	

[a] LSD relative to the values of subsets of genotypes as identified by the sub-headings in the first column.
[b] One environment with an absolute minimum temperature of −11.6 °C [see Annicchiarico et al. (2019)].
[c] Average of two autumn sowing dates in one environment with an absolute minimum temperature of −7.8 °C [see Annicchiarico and Iannucci (2007)].
[d] Described as highly winter-hardy by Prieur and Cousin (1978) and by Dumont et al. (2011).
[e] One environment with an absolute minimum temperature of −13.5 °C (Lodi, cropping season 2005–2006) (Annicchiarico, unpublished data).
[f] One environment with an absolute minimum temperature of −9.0 °C [see Annicchiarico et al. (2010)].

The seeds were pre-germinated on filter paper in Petri dishes for approximately 48 h at 19 °C before being transplanted into the plug trays. The evaluation protocol included (i) 10 days of growth at 22.5 °C with 12 h of daylight, (ii) 15 days of cold acclimation (hardening) at 4 °C with 10 h of daylight, (iii) 12 h of cooling at −3 °C in the dark, (iv) 4 h of freezing treatment, (v) 6 days of recovery at 4 °C with 10 h of daylight, and (vi) 15 days of regrowth at 15/20 °C (night/ day) with 12 h of daylight (Supplementary Table 2.1). The plants were irrigated every 2 days during growth, recovery, and regrowth, while irrigation was suspended from the beginning of hardening to the end of the freezing treatment. The decrease in temperature toward the freezing point and the subsequent increase in temperature occurred at a rate of 1 °C/h, according to a pattern described in Supplementary Figure 2.1 for one test temperature. Air and soil temperatures were monitored with two Tinytag Plus 2 TGP-4510 (Gemini, Chichester) dataloggers to ensure compliance with the protocol.

2.3.3. Data collection

Frost resistance was assessed using two criteria: plant mortality (i.e., the number of dead plants/total number of plants after hardening), and the level of injury to the aerial biomass measured through a visual score on individual plants and then averaged over plants of the experimental unit. The biomass injury visual score comprised the following 10 levels of increasing damage, which were based on observations at the end of the recovery period to evaluate mild injuries and at the end of regrowth to assess severe damage, such as mortality (Murray et al., 1988): (1) no visible damage, (2) loss of leaf turgidity for lupin and presence of dried tendrils for pea, (3) presence of dotted necrosis for lupin and leaf yellowing for pea, (4) presence of few necrotic spots, (5) up to 50% of leaf biomass necrotized, (6) between 50% and 90% of leaf biomass necrotized, (7) almost 100% of leaf biomass necrotized, (8) all of the biomass necrotized but a new shoot has started to grow, (9) the plant is severely damaged, with a very high expected probability of death, and (10) the plant is dead. For mortality assessment, plants that scored 9 and 10 were considered dead.

2.3.4. Statistical analysis

To compute the LT_{50} values, we fitted the following generalized linear model with the probit link function ϕ^{-1}:

$$\phi^{-1}\left[E\left(m_{g,b}\right)\right] = \beta_g T + \nu_g + \alpha_b$$

In the equation, the expectations of plant mortality ratios $E(m_{g,b})$ are binomially distributed and depend on the fixed effects of genotype g^{th} and block b^{th}, as well as on the frost treatment temperature T, expressed as a covariate, with the slope β_g depending on the genotype. The significance of each factor was assessed via a likelihood ratio test. Two standard model control techniques were applied to test the reliability of the model: a graphical assessment of raw residuals and Pearson's residuals against fitted values, and the test of homogeneity of the means. The LT$_{50}$ values were computed for each genotype within block according to the procedure described in Lei and Sun (2018), namely, as the opposite of the ratio between the intercept ($v_g + a_b$) and the angular coefficient (β_g) of the model.

An analysis of variance, including the factors genotype and block, was performed separately for each species to detect significant differences among genotypes for (i) LT$_{50}$, (ii) proportion of plant mortality following each freezing treatment, and (iii) biomass injury visual score following each freezing treatment. Mortality data were first transformed by using the arcsine square root transformation. We reported original data along with least significant difference (LSD) values back-transformed from LSD values obtained from the analysis of transformed data, and assessed the genotype differences by using Duncan's test. The mean values of species for plant mortality and LT$_{50}$ were compared according to the group balanced block lay-out, i.e., by testing the species factor on an error term represented by the species × block interaction. The consistency of genotype frost resistance assessments based on LT$_{50}$, plant mortality, and biomass injury score values was determined by using Pearson's correlation analysis.

Statistical models were fitted by the *glm()* and *lm()* functions from the R-package "stats". Duncan's test was performed by using the *duncan.test()* function, and LSD values were computed by using the *LSD.test()* function from the R-package "agricolae" (Steel et al., 1997).

2.4. Results

On average, pea exhibited greater frost resistance than white lupin as indicated by the lower LT$_{50}$ (−12.8 *versus* −11.0 °C; $P < 0.01$) and lower plant mortality at the lowest freezing temperature (0.50 *versus* 0.91; $P < 0.01$) in the analysis of variance-based species comparison. Within pea, the genotype values of LT$_{50}$ ranged from −14.5 °C for the breeding line KI_L38 to −11.6 °C for the cultivar Kaspa (Table 2.2). The pea genotype plant mortality values showed no mortality at −7 °C freezing temperature and did not differ significantly ($P > 0.05$) at −9 °C (Figure 2.1). They displayed significant differences ($P < 0.01$) at lower temperatures and achieved the largest variation, ranging from 0.11 to 0.83, at −13 °C (Figure 2.1, Table 2.2). The biomass injury visual score of the pea genotypes decreased with increasing freezing temperature but exhibited significant ($P < 0.01$) and similar extents of overall genotype variation across all freezing temperatures (Figure 2.1). The injury score values showed a high correlation ($r \geq 0.77$, $P < 0.01$) across the four freezing temperatures (Supplementary Table 2.2). The high correlation ($P < 0.001$) of genotype mortality at −13 °C with biomass injury score for the same temperature ($r = 0.98$) and LT$_{50}$ ($r = 0.91$), and that between genotype values of the last two traits ($r = 0.92$), indicated a strong consistency between the main indicators of pea genotype frost resistance.

Table 2.2. LT_{50} value, plant mortality proportion at the two lowest freezing temperatures, and biomass injury visual score (VS) after the highest and lowest freezing temperatures for 11 pea genotypes classified into three winter hardiness classes based on field-based winter mortality data.

Genotype	**Winter hardiness**	**LT_{50} (°C)**	**Mortality, −11 °C**	**Mortality, −13 °C**	**VS, −7 °C**	**VS, −13 °C**
KI_L38	High	−14.5 a	0.03 ab	0.21 abc	4.9 bcd	7.9 ab
Dove	Intermediate	−13.5 b	0.00 a	0.11 a	3.7 ab	8.1 ab
Isard	High	−13.4 b	0.00 a	0.15 ab	2.7 a	7.8 a
Champagne	High	−13.1 c	0.00 a	0.39 bcd	3.7 ab	8.4 bc
Dolmen	High	−13.0 cd	0.00 a	0.53 de	4.5 bc	8.8 cd
KA_37	High	−13.0 cd	0.08 abc	0.51 cd	4.8 bc	8.9 cd
Guifilo	Intermediate	−12.8 d	0.03 ab	0.58 de	4.0 b	8.9 cd
KI_118	Low	−12.4 e	0.22 cd	0.59 de	6.0 de	9.1 de
Catania	Low	−12.0 f	0.23 cd	0.79 e	5.7 cde	9.6 e
KA_19	Low	−11.9 f	0.18 bcd	0.81 e	5.5 cde	9.4 de
Kaspa	Intermediate	−11.6 g	0.30 d	0.83 e	6.5 e	9.6 e
LSD ($P < 0.05$)		0.3	0.07	0.12	1.1	0.6

Column means followed by different letter differs at P < 0.05 according to Duncan's test.

Table 2.2 reports the results of pea genotype comparisons relative to LT_{50}, plant mortality at the two lowest freezing temperatures (those displaying a significant genotype variation), and the biomass injury score for the highest and lowest temperatures (i.e., the extreme temperature range). In general, the pea genotype mean separation was more sensitive for LT_{50} (where KI_L38 outperformed any other genotype at $P < 0.05$) than for the other traits (Table 2.2). All genotypes assigned to the high winter hardiness class on the basis of field observation exhibited low to fairly low values of LT_{50}, plant mortality, and injury score, while all genotypes assigned to the low winter hardiness class showed fairly high to high values of these traits (Table 2.2). However, two genotypes in the intermediate winter hardiness class, namely, Dove and Kaspa, exhibited high and low frost resistance, respectively, according to all traits. The highly winter-hardy genotype Champagne displayed high, but not outstanding, frost resistance.

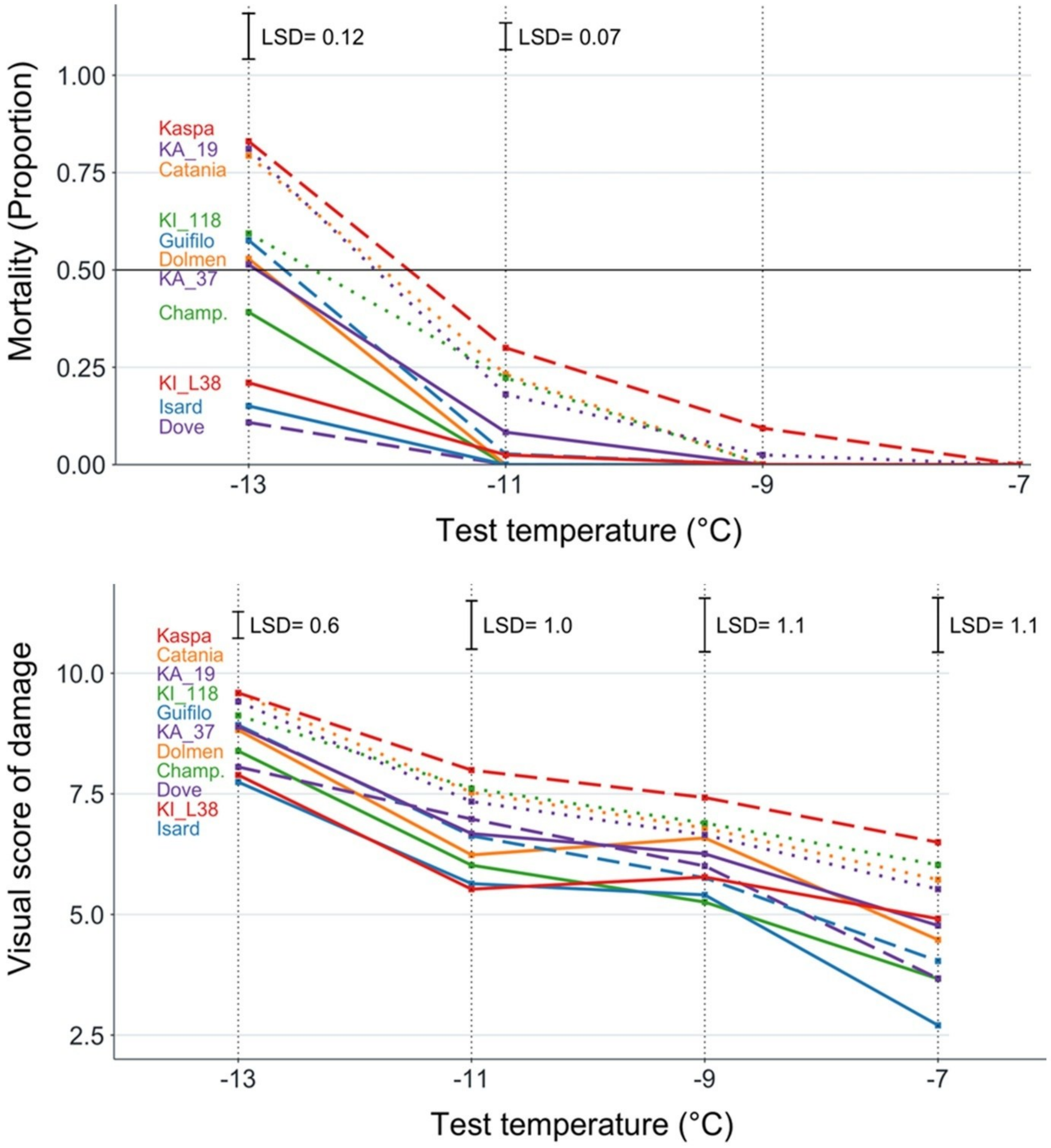


Figure 2.1. Plant mortality and biomass injury visual score for four freezing temperature treatments of 11 pea genotypes classified into three winter hardiness classes based on field-based winter mortality data (solid line, high winter hardiness; broken line, intermediate winter hardiness; dotted line, low winter hardiness). Least significant difference values at $P < 0.05$ reported only in the presence of overall genotype differences at $P < 0.05$.

The white lupin genotypes displayed LT_{50} values ranging from −12.0 °C for the Greek landrace GR56 to −10.0 °C for the Egyptian landrace Egypte11 and the Portuguese landrace E80. We observed no lupin genotype plant mortality at −7 °C, and significant ($P < 0.01$) plant mortality variation only at −11 °C and −13 °C freezing temperatures (Figure 2.2). However, the widest variation for genotype mortality, in the range of 0.26 to 0.88, took place at −11 °C in this species (Figure 2.2, Table 2.3). The variation for the biomass injury score achieved significance ($P < 0.01$)

only for the two intermediate temperatures while being flattened toward low values at −7 °C and high values at −13 °C (Figure 2.2). A high consistency among major indicators of genotype frost resistance was observed also in white lupin according to correlations ($P < 0.001$) of genotype mortality at −11 °C with the biomass injury score for the same temperature ($r = 0.97$) and LT_{50} ($r = 0.94$), or that between values of the last two traits ($r = 0.91$). High correlations were also observed for other indicators of frost resistance (Supplementary Table 2.3).

Table 2.3. LT_{50} value, plant mortality proportion, and biomass injury visual score (VS) for two freezing temperatures showing significant differences for 11 white lupin genotypes classified into three winter hardiness classes based on field-based winter mortality data.

Genotype	Winter hardiness	LT_{50} (°C)	Mortality, −11 °C	Mortality, −13 °C	VS, −9 °C	VS, −11 °C
GR56	High	−12.0 a	0.27 a	0.71 a	4.92 cd	6.73 abc
Calabria	High	−11.9 ab	0.41 ab	0.82 ab	3.88 abc	6.90 abc
PLI4-3	High	−11.8 b	0.26 a	0.82 ab	3.41 a	5.86 a
Adam	High	−11.5 c	0.26 a	0.93 bc	3.80 abc	6.29 ab
Ludet	High	−11.5 c	0.43 ab	0.85 abc	3.67 ab	7.07 abc
PLI-P3	Low	−11.1 d	0.46 ab	0.93 bc	3.97 abc	7.29 abc
Arsenio	Intermediate	−11 d	0.51 ab	0.94 bc	4.09 abc	7.59 bcd
Amiga	Low	−10.6 e	0.64 bc	1.00 c	4.75 bcd	8.86 de
LA559	Low	−10.1 f	0.68 bcd	1.00 c	6.24 e	8.30 cde
E80	Intermediate	−10.0 f	0.84 cd	1.00 c	5.61 de	9.36 e
Egypte11	Low	−10.0 f	0.88 d	1.00 c	5.50 de	9.25 e
LSD ($P < 0.05$)		0.2	0.10	0.07	1.00	1.40

Column means followed by different letter differs at P < 0.05 according to Duncan's test.

The results for major indicators of frost resistance reported for each white lupin genotype in Table 2.3 indicated also for this species a good consistency between frost resistance in the phenotyping platform and winter hardiness based on field observations. The five genotypes belonging to the high winter hardiness class were the top-ranking ones for frost resistance according to LT_{50} values, plant mortality, or the biomass injury score for −11 °C, whereas three genotypes in the low winter hardiness class out of four were bottom-ranking for all of these frost resistance indicators. The only inconsistencies were represented by the genotype E80, which was susceptible to frost while belonging to the intermediate winter hardiness class, and the breeding line PLI-P3, which displayed intermediate frost resistance while being assigned to the low winter hardiness class. In white lupin, too, LT_{50} exhibited more sensitive genotype mean separation than plant mortality or the injury score (Table 2.3).

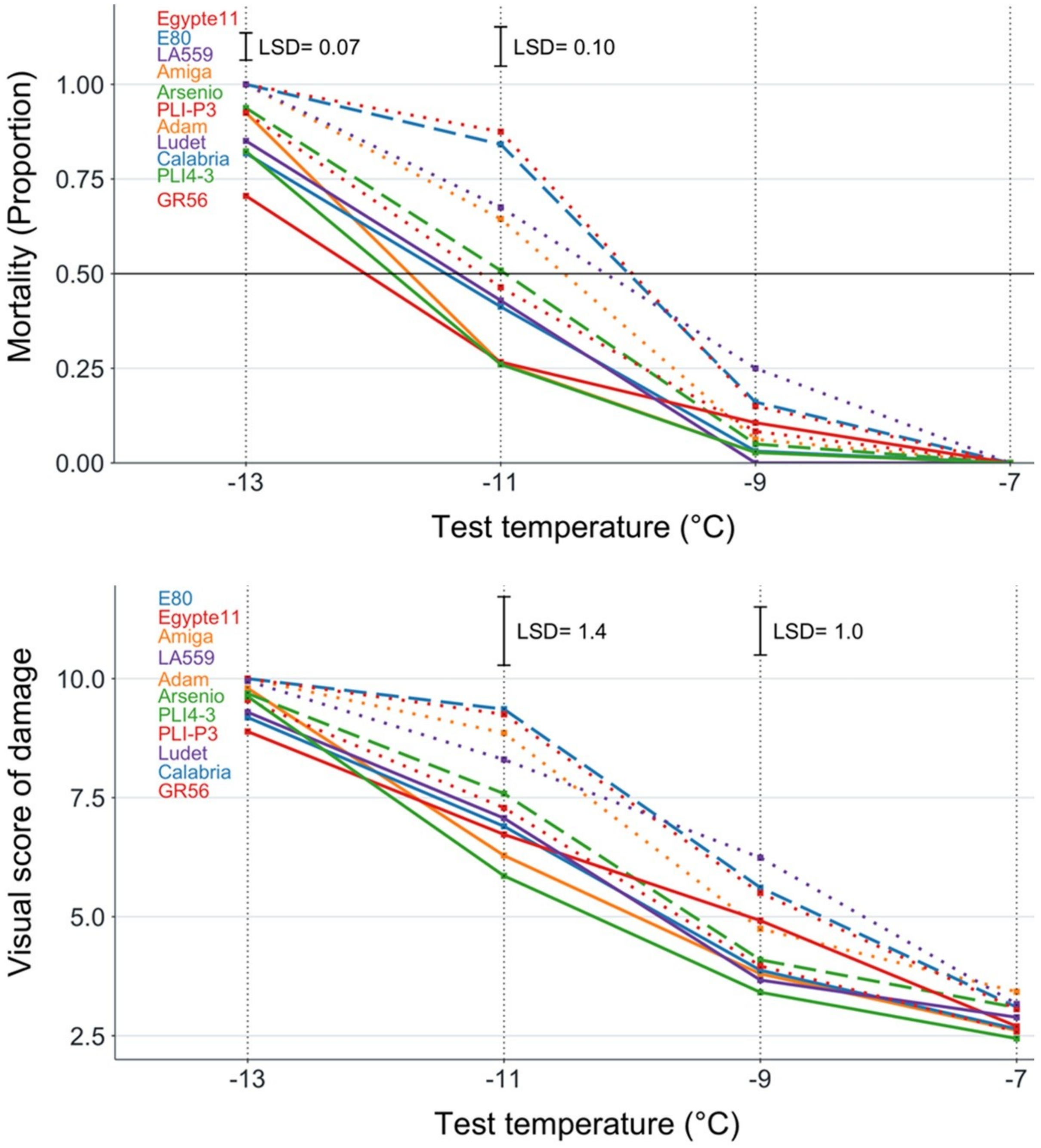


Figure 2.2. Plant mortality and biomass injury visual score for four freezing temperature treatments of 11 white lupin genotypes classified into three winter hardiness classes based on field-based winter mortality data (solid line, high winter hardiness; broken line, intermediate winter hardiness; dotted line, low winter hardiness). Least significant difference values at $P < 0.05$ reported only in the presence of overall genotype differences at $P < 0.05$.

2.5. Discussion

On average, pea exhibited greater frost resistance than white lupin in this study. This result agrees with the greater average winter plant survival of pea relative to white lupin in a field-based assessment of a large number of varieties across climatically contrasting Italian environments (Annicchiarico and Iannucci, 2007). In general, however, pea is credited an intermediate winter hardiness among the cool-season grain legumes, being considered less winter-hardy than faba bean or lentil and more winter-hardy than chick pea (Murray et al., 1988).

Our results indicated a good consistency between major indicators of genotype frost resistance observed in the phenotyping platform, namely, LT_{50}, plant mortality at the freezing temperature that maximized the genotype variation, and biomass injury score for the same temperature or a slightly higher one. LT_{50} exhibited a more sensitive genotype mean separation than the other indicators. This characteristic, however, requires multiple freezing temperatures (four in our study), making it less suitable for evaluating large genotype numbers than the frost resistance assessment based on one optimal freezing temperature (i.e., the one that maximizes the genotype variation for plant mortality). The optimal freezing temperature differed for the two species according to our results, being about −13 °C for pea and −11 °C for white lupin. When used for evaluation at an optimal freezing temperature, our platform could accommodate up to 216 genotypes in each of several evaluation cycles (each cycle acting as a replicate), using experimental units (replicates) of 10 plants each as in this study (or 144 genotypes, using experimental units of 15 plants). Our results suggest that the biomass injury score may concur to the frost resistance evaluation along with plant mortality or act as the only frost resistance indicator in case the platform included more genotypes per evaluation cycle with less plants per experimental unit (e.g., 432 genotypes with five plants per replicate), a situation that makes the estimation of plant mortality less reliable. A similar score was adopted by Beji et al. (2020) in a pea experiment including four plants per replicate, and is frequently adopted in other grain legumes under similar circumstances (Arbaoui and Link, 2008). Work by Humplík et al. (2015) suggests that the biomass injury assessment of the individual plants could be automated by image analysis, albeit hardly with large time savings and with a need for placing plants into individual pots.

The optimal freezing temperature for pea plant mortality at −13 °C contrasts with earlier results by Auld et al. (1983), Swensen and Murray (1983), Murray et al. (1988), Cousin et al. (1993), and Homer et al. (2016), which suggested an optimal temperature in the range of −7 °C to −9 °C. The contrast is even greater when considering that most of these studies adopted a longer hardening period than our study. The improved frost resistance of the current, recently bred germplasm sample (breeding lines or commercial cultivars), along with possible differences in the evaluation protocols, may partly account for the currently lower optimal freezing temperature. For example, differences in substrate type and drainage may affect plant mortality (Russell et al., 1978). Irrigation during the hardening period (as contemplated in some earlier studies) could increase ice formation and cause mechanical damage to the roots. Our slower thawing (1 °C/h) relative to some early studies could be less damaging to plants (Gusta and Fowler, 1977). Other possibly different factors may include the plant growth and development stage before hardening, the duration of the frost treatment, and the length of the regrowth period. Although the frost resistance evaluation of pea germplasm collections at −8 °C is quite frequent (Wery et al., 1994; Dumont et al., 2009; Beji et al., 2020), Prieur and Cousin (1978) suggested the selection of frost-resistant pea germplasm by a set of freezing cycles ultimately achieving −12 °C.

No prior assessment of genotype frost resistance variation and optimal temperature for frost resistance evaluation based on LT_{50} for plant mortality was available for white lupin. A study based on frost-induced leaf damage of cultivars and accessions estimated by chlorophyll fluorescence indicated an average value of −9.5 °C for LT_{50}, estimated as 50% of damaged leaves after a long hardening period (42 days at 8 °C/2 °C day/night temperature) (Hoffmann-Bahnsen and Herzog, 2001). In contrast, Papineau and Huyghe (1992) proposed to assess white lupin frost resistance at −16 °C freezing temperature after a 3-week hardening period at −4 °C.

The observed good consistency between platform-based frost resistance and field-based winter hardiness of pea and white lupin genotypes has practical importance for the exploitation of artificial screening results for these species. Correlations for pea plant mortality across field and growth chamber assessments were close to 0.7 in Homer et al. (2016) and in the range of 0.5–0.6 in Auld et al. (1983). Correlations close to 0.5 have been reported for other legume species such as faba bean (Arbaoui et al., 2008) and red clover (Zanotto et al., 2021). As anticipated, one cannot expect a very high consistency between platform-based and field-based plant mortality in grain legumes because the latter depends not only on frost resistance but also on frost escape through a delayed onset of flowering. Other factors may influence the genotype variation for field plant survival, such as greater tolerance to diseases whose attack is favored by frost damage, such as *Ascochyta* spp. for pea (Maufras, 1997), and a different susceptibility to imbibitional chilling of the germinating seed due to seed coat variation for rapidity of imbibition (Wery et al., 1994). Onset of flowering may actually explain the response of the lupin breeding line PLI-P3, which featured moderate frost resistance according to the freezing test while belonging to the low winter hardiness class according to field observations. The high winter mortality under field conditions of this line was associated with extreme earliness of flowering in Annicchiarico et al. (2011) (where this line is coded as P3), a feature that would definitely increase its sensitivity to frost because of the early differentiation of the floral apex (Huyghe and Papineau, 1990). The currently good but not outstanding frost resistance of the pea landrace Champagne (Table 2.2) in spite of its reportedly extreme field-based winter survival (Prieur and Cousin, 1978; Dumont et al., 2011) may be accounted for by frost escape under field conditions via delayed flowering caused by possession of the *Hr* (high response to photoperiod) gene (Dumont et al., 2009). Indeed the *Hr* gene reportedly co-segregated with the most important quantitative trait loci (QTL) for frost survival (Lejeune-Hénaut et al., 2008). Anyway, the reliability of our genotype classifications for winter hardiness suffered from the limited field-based evaluation it was based upon. For example, the pea variety Dove, here classified as intermediate for winter hardiness while showing high frost resistance according to freezing test results, exhibited moderately high frost survival across various cold-prone agricultural environments of France (UNIP-ITCF, 2001).

In conclusion, our results encourage the use of high-throughput phenotyping platforms such as the current one for the assessment of pea or white lupin frost resistance aimed at plant breeding, molecular studies for detection of QTL (e.g., Beji et al., 2020) and/or definition of genome-enabled prediction models, or for investigation of physiological mechanisms regulating frost resistance (e.g., Dumont et al., 2009).

The assessment under artificial conditions could overcome the increasing unpredictability of field-based evaluations. In addition, its focus on frost resistance (as implied by the evaluation of young plants that lack any differentiation of reproductive organs) facilitates the combination of frost resistance and drought resistance characteristics unrelated to flowering time in novel varieties featuring greater yield stability and adaptation to the increasingly variable climate conditions. Indeed our results for PLI-P3 and Champagne confirm that frost resistance is not necessarily related to plant mortality under field conditions for pea or white lupin genotypes, and a similar response was observed for a few faba bean genotypes (Arbaoui et al., 2008). For pea, a genomic selection model for intrinsic drought tolerance proved capable of producing material with a similar flowering time but with increased yielding ability under severe drought relative to its genetic base (Annicchiarico et al., 2020b), while a similar model is awaiting exploitation for white lupin (Pecetti et al., 2023).

2.6. Supplementary material

Supplementary Table 2.1. Experimental protocol summarizing temperature, length, light hours/day, and irrigation management of each phase.

	Length	Temperature	Light hours/day	Irrigation
Germination	48 hours	19 °C	Dark	
Growth	10 days	22.5 °C	12 h	Yes
Hardening	15 days	4 °C	10 h	No
Cooling	12 hours	−3 °C	Dark	No
Stress	4 hours	−7 °C; −9 °C; −11 °C; −13 °C	Dark	No
Recovery	6 days	4 °C	10 h	Yes
Regrowth	15 days	15/20 °C (night/day)	12 h	Yes

Supplementary Table 2.2. Correlations for LT_{50} value, plant mortality proportion at two freezing temperatures and biomass injury visual score (VS) after four freezing temperatures of 11 pea genotypes.

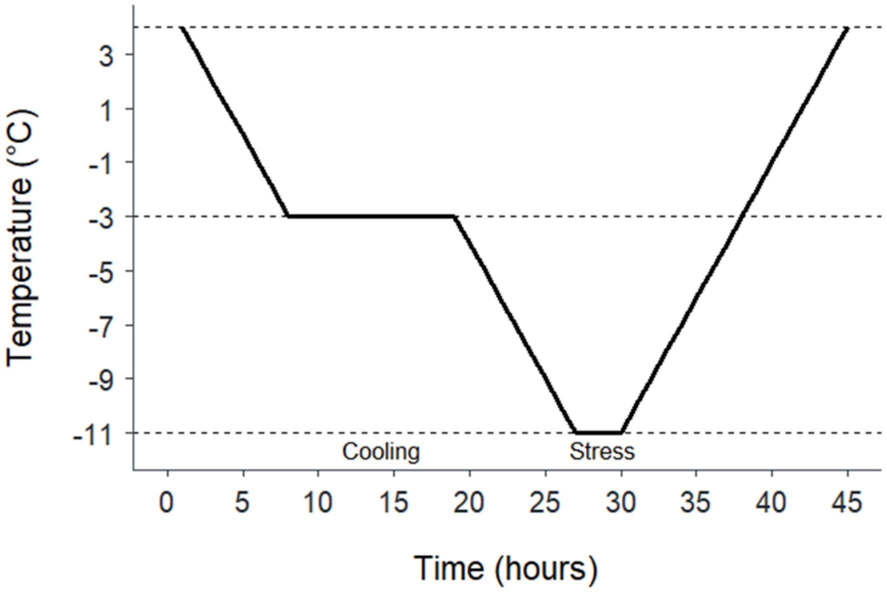
Trait	Mortality post −11 °C	Mortality post −13 °C	VS post −7 °C	VS post −9 °C	VS post −11 °C	VS post −13 °C
LT_{50}	0.81**	0.91***	0.64*	0.75**	0.85**	0.92***
Mortality post −11 °C	–	0.81**	0.90***	0.86***	0.87***	0.83**
Mortality post −13 °C	–	–	0.79**	0.76**	0.75**	0.98***
VS post −7 °C	–	–	–	0.89***	0.77**	0.81**
VS post −9 °C	–	–	–	–	0.85***	0.82**
VS post −11 °C	–	–	–	–	–	0.84**

*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; NS not significant ($P > 0.05$)

Supplementary Table 2.3. Correlations for LT_{50} value, plant mortality proportion at two freezing temperatures and biomass injury visual score (VS) after two freezing temperatures of 11 white lupin genotypes.

Trait	Mortality post −11 °C	Mortality post −13 °C	VS post −9 °C	VS post −11 °C
LT_{50}	0.94***	0.90***	0.78**	0.91***
Mortality post −11 °C	–	0.78**	0.76**	0.97***
Mortality post −13 °C	–	–	0.50 NS	0.76**
VS post −9 °C	–	–	–	0.78**

*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; NS not significant ($P > 0.05$)



Supplementary Figure 2.1. Temperature pattern during the cooling and freezing treatment at −11 °C.

3. Genetic variation and genome-enabled prediction of white lupin frost resistance in different reference populations

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Genetic Variation and Genome-Enabled Prediction of White Lupin Frost Resistance in Different Reference Populations

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Abstract

In various European regions, white lupin (*Lupinus albus* L.) production could increase by autumn sowing of winter-hardy varieties. This study aimed to explore the genetic variation, the genetic architecture, and the genomic prediction of frost resistance in two reference populations, one including 144 landrace and cultivar genotypes, and the other comprising 144 breeding lines. These populations were genotyped by 40,914 and 32,951 SNP markers, respectively, issued by genotyping-by-sequencing. The genotypes were phenotyped for mortality and a biomass injury score at freezing temperature of −11 °C under controlled conditions. Both traits, highly correlated, exhibited large genetic variation and high broad-sense heritability ($H^2 = 0.76$–0.82). A genome-wide association study highlighted their polygenic architecture and detected markers linked to candidate genes. The intra-population predictive ability of plant mortality achieved 0.41 for landrace and cultivar germplasm, and 0.67 for breeding lines. The cross-population predictive ability was higher when using the model constructed for landrace and cultivar germplasm to predict breeding lines (0.39) than the reverse (0.26). Landrace field survival was largely influenced by late phenology in addition to frost resistance. Our results revealed frost-resistant germplasm, confirmed the polygenic control of frost resistance, and highlighted genomic prediction opportunities for line selection and the identification of elite genetic resources.

Keywords: abiotic stress; cold acclimation; frost tolerance; genetic architecture; genetic resources; genomic selection; GWAS; *Lupinus albus*; winter hardiness; winter mortality

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3. Genetic variation and genome-enabled prediction of white lupin frost resistance in different reference populations

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NF: Methodology, Phenotypic data generation, Statistical analysis, Writing-original draft preparation.

DC: Methodology.

NN: Methodology, Molecular data generation.

LP: Methodology, Writing-review and editing.

TN: Methodology.

PA: Conceptualization and supervision, Methodology, Writing-original draft preparation, Funding acquisition.

Data Availability Statement:

The genotypic data used for this study are available in the Figshare repository at https://doi.org/10.6084/m9.figshare.30146242.v1, whereas phenotyping data are reported as Supplementary Materials.

3.1. Abstract

In various European regions, white lupin (*Lupinus albus* L) production could increase by autumn sowing of winter-hardy varieties. This study aimed to explore the genetic variation, the genetic architecture, and the genomic prediction of frost resistance in two reference populations, one including 144 landrace and cultivar genotypes, and the other comprising 144 breeding lines. These populations were genotyped by 40,914 and 32,951 SNP markers, respectively, issued by genotyping-by-sequencing. The genotypes were phenotyped for mortality and a biomass injury score at freezing temperature of −11 °C under controlled conditions. Both traits, highly correlated, exhibited large genetic variation and high broad-sense heritability (H^2 = 0.76–0.82). A genome-wide association study highlighted their polygenic architecture and detected markers linked to candidate genes. The intra-population predictive ability of plant mortality achieved 0.41 for landrace and cultivar germplasm, and 0.67 for breeding lines. The cross-population predictive ability was higher when using the model constructed for landrace and cultivar germplasm to predict breeding lines (0.39) than the reverse (0.26). Landrace field survival was largely influenced by late phenology in addition to frost resistance. Our results revealed frost-resistant germplasm, confirmed the polygenic control of frost resistance, and highlighted genomic prediction opportunities for line selection and the identification of elite genetic resources.

3.2. Introduction

White lupin (*Lupinus albus* L.) is a minor crop of increasing interest for European agriculture. As a rainfed cool-season grain legume, it can counteract soil fertility loss, enhance farming sustainability, and reduce water use (Nemecek et al., 2008). In addition, wider grain legume cultivation can reduce the European dependence on international markets for high-protein feedstuffs and their associated environmental costs (Watson et al., 2017; Boerema et al., 2016). Because of its outstanding seed protein content (nearly 40% on dry matter: Tadele (2015)), white lupin showed higher crude protein yield per unit area than other grain legumes such as pea (*Pisum sativum* L.), faba bean (*Vicia faba* L.) and narrow-leafed lupin (*Lupinus angustifolius* L.) in southern Europe (Annicchiarico, 2008). Such a high protein content is complemented by other favorable characteristics, namely, a high level of essential amino acids, valuable techno-functional properties, positive effects on human health with respect to diabetes, hypertension, cardiovascular diseases, and obesity (Prusinski, 2017), and a content of 8–12% of oil with excellent nutritional characteristics (Boschin et al., 2008b). Therefore, white lupin could meet the growing demand of pulses for novel food products (Boukid and Pasqualone, 2022; Quintieri et al., 2023). Nevertheless, higher crop yields are needed for this and other grain legumes to increase the economic sustainability of their cultivation in Europe (Gresta et al., 2017).

Frost and terminal drought are major factors limiting the yield of autumn-sown cool-season grain legumes in Europe. Winter hardiness (as estimated by winter plant survival) is essentially determined by the effect of frost (Shield et al., 2000), although other factors (such as waterlogging or fungal diseases) may affect it. Spring sowing is currently adopted in cold-prone regions of Europe to avoid frost events, but the changing climate is expected to promote the expansion of autumn sowing northward, allowing for potentially higher crop yields via a longer crop cycle and terminal drought escape due to earlier harvesting (López-Bellido et al., 1994). Even in Mediterranean environments, frost remains a major constraint because its damaging effects may be enhanced by poor plant acclimatation caused by mild winters (Annicchiarico and Iannucci, 2007). The changing climate is expected to increase the occurrence of mild winters on the one hand, and the risk of sudden, early or late frost events on the other (Francis and Skific, 2015). Late flowering by high vernalization requirement, which allows the sensitive reproductive organs under development to escape frost (Annicchiarico et al., 2010; Huyghe, 1997) , and frost resistance are the key components of winter hardiness. A late phenology, however, involves greater susceptibility to terminal drought (Annicchiarico et al., 2018; Pecetti et al., 2023). Therefore, white lupin breeding for autumn sowing in drought-prone regions should aim to combine the phenology-unrelated resistances to frost on the one hand and to terminal drought on the other in material with an intermediate phenology. Frost resistance, which could be synthetically expressed as a positive deviation from the genotype winter mortality value expected according to its onset of flowering (Franguelli et al., 2024), relies on physiological modifications that prevent or resist intracellular ice formation (Levitt, 1980; Sallam et al., 2015). Such modifications may be activated by daylength reduction (Beck et al., 2004; Junttila et al., 1996), and by exposure to low, non-freezing temperatures in a process known as cold acclimation or hardening (Bourion et al., 2003; Arbaoui and Link, 2008; Link et al., 2010).

There is limited knowledge on the extent of genetic variation for frost resistance in white lupin. Germplasm pools of landraces from the Azores, Italy, Greece, and Madeira and the Canary Islands have been reported as valuable genetic resources for winter hardiness observed in field experiments (Annicchiarico et al., 2010; Huyghe et al., 1997; Papineau and Huyghe, 2004). However, field-based winter survival depends on various factors, and its assessment is complicated by the wide and increasing climatic variation across years (which reduces the applicability and replicability of the results; Avia et al., 2013). Controlled environments, where hardening conditions and frost intensity and duration can be standardized, can overcome the limitations of field trials and allow for a rapid and possibly off-season evaluation of genotype responses (Stoddard et al., 2006). Plant evaluation at an early growth stage can rule out any effect of flowering time and focus, therefore, on frost resistance. A previous study developed a protocol for large-scale evaluation of white lupin frost resistance in a walk-in growth chamber, identifying −11 °C as the optimal freezing temperature (Franguelli et al., 2024). That study showed good consistency between genotype responses in this phenotyping platform (relative to plant mortality and a visual score of plant injury) and field-based winter mortality of the genotypes.

Large-scale germplasm evaluations under controlled conditions can be used for identifying elite genetic resources, selecting breeding material, and/or defining marker-assisted or genome-enabled procedures which could be used for future selection or identification of useful genetic resources in germplasm collections. The definition of such procedures achieved remarkable advances through the advent of next-generation sequencing techniques, such as genotyping-by-sequencing (GBS; Elshire et al., 2011). GBS proved to be capable of generating thousands of polymorphic single-nucleotide polymorphism (SNP) markers for white lupin genetic analyses (Ksiazkiewicz et al., 2017), and was used to define genomic prediction models for white lupin yielding ability in contrasting environments (Annicchiarico et al., 2019a), resistance to drought (Pecetti et al., 2023), adaptation to calcareous soils (Annicchiarico et al., 2023), and resistance to anthracnose (Alkemade et al., 2022; Schwertfirm et al., 2024). An issue of remarkable practical interest for genomic prediction models is the predictive ability of a model developed for a genetically broad reference population, such as an international germplasm collection, when applied onto a genetically narrower reference population, such as a collection of breeding lines (Crosta et al., 2023). For oligogenic traits, the possibility of identifying candidate genes through a genome-wide association study (GWAS) has been enhanced by the publication of a high-quality white lupin genome (Hufnagel et al., 2020) on which GBS-generated markers can be aligned. Markers associated with frost resistance have already been identified in various legumes, such as faba bean (Sallam et al., 2016), pea (Lejeune-Hénaut et al., 2008; Beji et al., 2020), and red clover (*Trifolium pratense* L.) (Zanotto et al., 2023).

This study focused on two white lupin reference populations, one including a world collection of landrace and cultivar genotypes (reference population 1) and the other comprising breeding lines issued from crosses of elite modern (sweet-seed) germplasm with landrace material (reference population 2). These populations were evaluated for frost resistance by the method described in (Franguelli et al., 2024). The study aimed at assessing (a) the genetic variation for frost resistance in each population; (b) the relationship between controlled and field conditions for plant mortality; (c) the genetic architecture of the frost resistance trait, and putative candidate genes, based on GWAS analyses; (d) the predictive ability of genome-enabled models developed for each reference population; and (e) the cross-population predictive ability of the genomic prediction models, particularly with respect to the model developed for landrace and cultivar material when used for the prediction of breeding lines.

3.3. Materials and methods

3.3.1. Plant material

Our study included two white lupin populations, one comprising landrace and cultivar genotypes (reference population 1), and the other including sweet-seed inbred lines from a breeding program (reference population 2). Reference population 1 consisted of 127 genotypes belonging to 107 landraces, along with 17 commercial cultivars, for a total number of 144 evaluated genotypes whose origins are given in Supplementary Table 3.1. The landraces were selected from a world collection described in Annicchiarico et al. (2010) and belonged to 11 regional germplasm pools reflecting the main historical white lupin cropping regions. Each accession was represented by at least one randomly chosen individual genotype, but two genotypes per accession were included in some cases. Genotypes selected from the same landrace were genetically distinct, as confirmed by the observed molecular marker diversity and consistent with the expected intra-landrace variation. The 17 commercial cultivars were classified into three phenological classes, namely (a) spring types, featuring nil or minimal vernalization requirement and adapted to early-spring sowing in cold-prone areas of northern or continental Europe; (b) Mediterranean types, with an intermediate vernalization requirement and adapted to autumn sowing in Mediterranean regions; and (c) winter types, with a high vernalization requirement and adapted to autumn sowing in regions with a subcontinental or suboceanic climate (Stoddard et al., 2006; Rychel-Bielska et al., 2024). All landraces, along with eight cultivars, had previously been evaluated for field-based winter plant survival and onset of flowering in Lodi (northern Italy), an environment characterized by a subcontinental climate, where the plants were exposed to an absolute minimum temperature of −9 °C (Annicchiarico et al., 2010).

Reference population 2 consisted of sweet-seed breeding lines originating from 16 crosses produced by a 4 × 4 factorial mating design. Each of four elite, sweet-seed cultivars or breeding lines, namely, the French cultivar Lucky, the French breeding line MB-38, the Italian variety Arsenio, and the Moroccan breeding line L27PS3, was crossed with each of four elite, bitter-seed landrace accessions, namely, the Italian landraces LAP123 and La246, the landrace La646 from the Canary Islands, and the Greek landrace Gr56. The selection of parent germplasm, which was based on desirable agronomical traits, and the development of inbred lines until the F_6 seed under insect-proof cages (to prevent cross-pollinations), were described earlier (Pecetti et al., 2023). The landrace Gr56 showed high winter hardiness in previous work (Annicchiarico et al., 2010). The lines underwent selection for low alkaloid content in F_3 and F_4 generations (Annicchiarico et al., 2025). Between four and eleven lines per cross contributed to the current population of 144 test lines. The number of lines issued from each of the eight parents was moderately balanced, ranging from

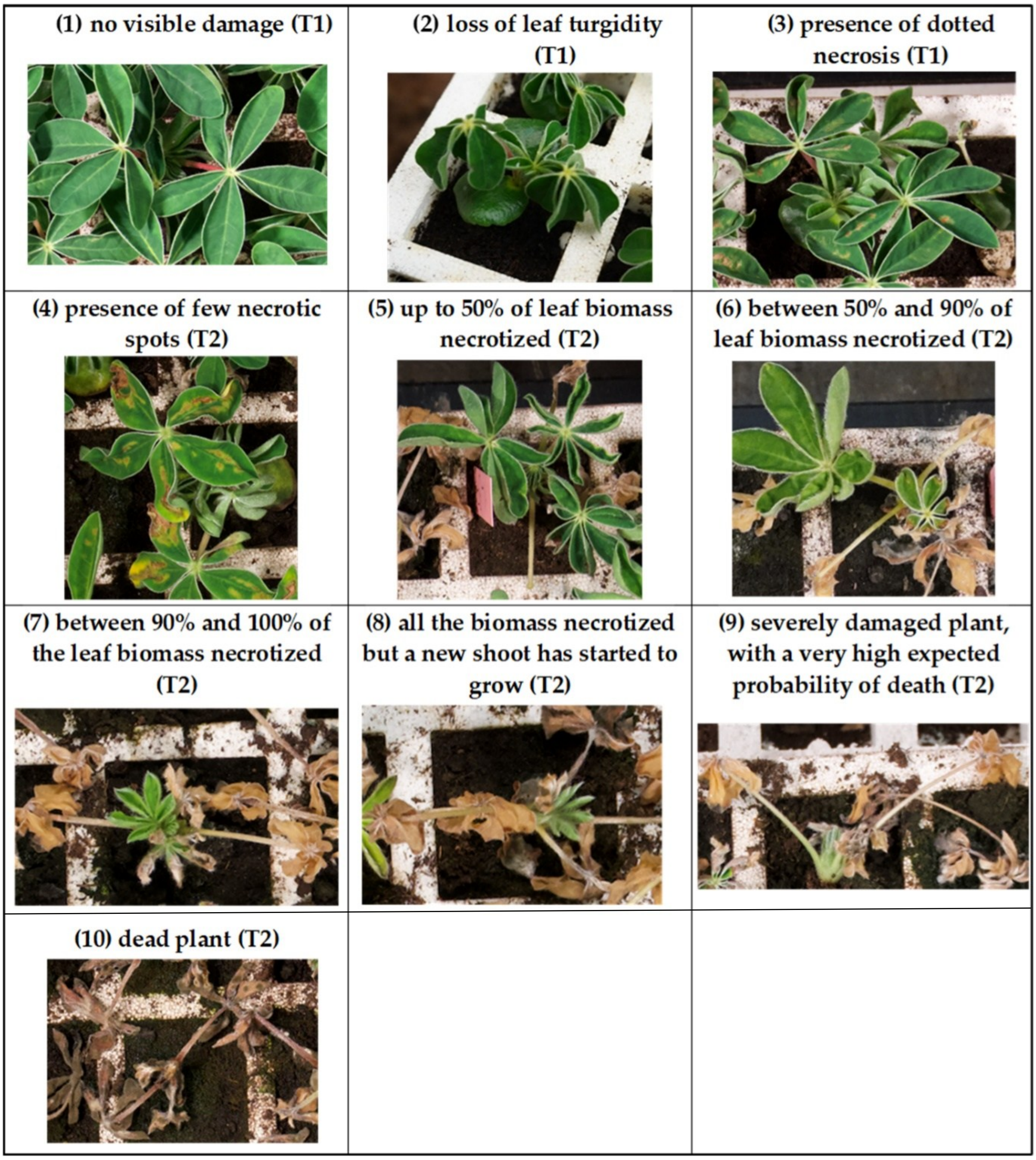
30 for MB-38 to 39 for L27PS3. The full list of breeding lines is provided in Supplementary Table 3.2 along with the indication of the parent germplasm. All landraces used as parental germplasm were also tested as single genotypes in reference population 1.

3.3.2. Phenotyping

The two populations were evaluated under two separate experiments performed in a high-throughput phenotyping platform according to a protocol for frost resistance assessments that was defined in a prior methodological study (in which frost resistance was termed as intrinsic frost tolerance; Franguelli et al., 2024). In brief, the platform consisted of a freezing growth chamber equipped with LED lamps. With a surface area of 13.63 m^2, it could accommodate up to 2160 plants placed in polystyrene plug trays with cells measuring 5 cm × 5 cm × 15 cm in depth. The trays were filled with a commercial growing substrate composed of pH-adjusted peat (pH = 6.0) and NPK mineral fertilizer (substrate SER CA-V7, Vigorplant, Piacenza, Italy), and were arranged side by side on four large trolleys. Each experiment was set up as an alpha lattice with 12 incomplete blocks of 12 experimental units each, and 4 replications. Each growing cycle comprised one replication. Each experiment unit included 15 plants of the relevant genotype.

The seeds were pre-germinated on filter paper in Petri dishes for approximately 48 h at 19 °C before being transplanted into the plug trays at a depth of 2.5 cm. The protocol included (i) 10 days of growth at 22.5 °C with 12 h of daylight, (ii) 15 days of cold acclimation (hardening) at 4 °C with 10 h of daylight, (iii) 12 h of cooling at −3 °C in the dark, (iv) 4 h of freezing treatment at −11°C, (v) 2 days of recovery at 4 °C with 10 h of daylight, and (vi) 19 days of regrowth at 15/20 °C (night/day) with 12 h of daylight (Supplementary Figure 2.1). The freezing temperature of −11 °C was identified as the one with the highest screening value for the species (Franguelli et al., 2024). The temperature decreased toward the freezing point and then increased at a rate of 1 °C/h. Plants were irrigated every two days during growth, recovery, and regrowth, while suspending the irrigation during hardening and the freezing treatment.

The frost resistance of the genotypes was evaluated by two traits: (a) the plant mortality proportion; (b) a visual score of aerial biomass injury. The latter trait was assessed on individual plants, and comprised the 10 levels of increasing damage described in Figure 3.1. This assessment was based on two observation timepoints: one six days after frost, when mild injuries are more evident; the other at the end of regrowth for severe damage and mortality observations, which can be accurately assessed after a minimum period of three weeks (Murray et al., 1988). Scores on individual plants were averaged across the experimental unit. Plants scoring 9 and 10 were classified as dead for mortality assessment.



(1) no visible damage (T1)

(2) loss of leaf turgidity (T1)

(3) presence of dotted necrosis (T1)

(4) presence of few necrotic spots (T2)

(5) up to 50% of leaf biomass necrotized (T2)

(6) between 50% and 90% of leaf biomass necrotized (T2)

(7) between 90% and 100% of the leaf biomass necrotized (T2)

(8) all the biomass necrotized but a new shoot has started to grow (T2)

(9) severely damaged plant, with a very high expected probability of death (T2)

(10) dead plant (T2)

Figure 3.1. Ten-level visual score of aerial biomass frost injury based on observations on each individual plant carried out after six days (T1) or three weeks (T2) after frost exposure.

3.3.3. Phenotypic data analysis

Plant mortality and visually scored biomass injury were analyzed separately for each reference population according to the following linear mixed model:

$$Y_{irb} = \mu + G_i + P_r + B_{rb} + \mathcal{E}_{irb}; \qquad \mathcal{E}_{irb} \sim N(0, \sigma_e^2)$$

where the response variable Y_{irb} is a function of the trait mean μ, genotype (G_i) and replicate (P_r) effects treated as fixed factors, a block within replicate random effect (B_{rb}), and with $\mathcal{E}_{irb}$ representing the model residual. The significance of each source of variation was assessed by analysis of variance (ANOVA). Lattice-adjusted genotype mean values of materials belonging to the reference population 1 underwent a second ANOVA including germplasm pool as a fixed factor and genotype within pool as random factor (hence, acting as error term for germplasm pools). We assessed the consistency of frost resistance response between landrace parent material (belonging to the reference population 1) and their progenies (averaged across relevant breeding lines of the reference population 2). Genotype mean values of landraces and cultivars that were previously evaluated for field-based winter plant mortality (Annicchiarico et al., 2010) underwent a linear regression analysis of field mortality as a function of mortality under controlled conditions. Values in the latter condition of genotypes belonging to the same landrace were averaged before the analysis. Landraces and cultivars were graphically grouped into three classes of onset of flowering, namely, early-flowering (18 entries), intermediate-flowering (76 entries), and late-flowering (21 entries), based on their flowering date (measured in days from January 1 to the point when 50% of the plants had at least one flower open). The accessions flowering earlier than the mean minus 0.5 × standard deviation were classified as early-flowering; those later than mean plus 0.5 × standard deviation as late-flowering; and the remaining ones as intermediate.

The model above, but holding genotype (G_i) as a random factor, was used for estimating genotype (σ_g^2), and experiment error (σ_e^2) variance components by the restricted maximum likelihood (REML) method. This analysis aimed to compute genetic coefficient of variation (CV_g) and broad-sense heritability on an entry mean basis (H^2) values as follows:

$$CV_g = (\sigma_g / \mu) \times 100$$

$$H^2 = \frac{\sigma_g^2}{\left(\sigma_g^2 + \frac{\sigma_e^2}{n}\right)}$$

where σ_g is the square root of the genotype variance component, and *n* is the number of experiment replications. We used H^2 values to compute best linear unbiased prediction (BLUP) values of genotypes according to DeLacy et al. (1996). These values were used to calculate Pearson correlations coefficients (*r*) between traits, and acted as phenotypic data for trait-marker analyses and genomic predictions.

All statistical analyses were performed with the statistical software R (version 4.4.1; R Core Team). Models were fitted by the *lm()* e *lmer()* functions from the R-packages “stats” and “lme4”, respectively.

3.3.4. DNA isolation, GBS library construction, and sequencing

Genomic DNA was extracted from young leaves of landrace and cultivar genotypes (reference population 1) and from F_5 plants of each breeding line (reference population 2) using the DNeasy Plant Mini Kit (Qiagen, Milan, Italy). A Quant-iTTM PicoGreenTM dsDNA Assay Kit (P7589, Life Technologies, Trieste, Italy) was used to quantify the nucleic acid, and 1% agarose gel electrophoresis was used to verify its quality. A trial digestion was performed on 10% of the DNA samples using the Optizyme EcoRI restriction enzyme (25,000 U, Fisher BioReagents, Rodano, Italy) to compare bands of cut and uncut DNA. The reaction was performed at 37 °C for an hour and the enzyme was deactivated at 65 °C for 20 min. DNA samples were sent to The Elshire Group Ltd. laboratory (Palmerston North, New Zealand) for outsourced library preparation and sequencing. GBS data were generated according to Elshire et al. (2011), with the following modifications: 100 ng of genomic DNA, and 3.6 ng of total adapters were used, and the genomic DNA was restricted with ApeKI enzyme (NEB New England Biolabs, R0643L, Ipswich, MA, USA); the library was then amplified using Kapa Taq polymerase Alpha (KAPA Library Amplification Readymix, Kapa Biosystems KK2611, Cape Town, South Africa) by 14 PCR cycles. Sequencing was performed on a single Illumina HiSeq X lane, at 2 × 150 bp paired end. The adoption of ApeKI as the restriction enzyme, according to Elshire et al. (2011), was supported by the fact that about 60% of the white lupin genome includes repetitive DNA sequences (Hufnagel et al., 2020), which this enzyme tends to skip.

3.3.5. Genotype SNP calling procedures, data filtering and imputation

SNP calling was based on Legpipe2 pipeline default settings for diploid species (Nazzicari et al., 2024). For alignment, we used the *Lupinus albus* genome version 1.0 (Hufnagel et al., 2020), which was downloaded from https://www.whitelupin.fr/(accessed on 4 August 2025). The entire dataset, comprising landrace and cultivar genotypes (reference population 1) and breeding lines (reference population 2) was filtered for monomorphic markers, with a missing rate per marker < 1%, a missing rate per individual < 10%, and SNP heterozygosity < 30%. Due to relevant differences in allele frequency among reference populations 1 and 2, the two datasets were filtered separately for minor allele frequency > 5%. This process retained 40,914 SNPs for landrace and cultivar genotypes (reference population 1) and 32,951 SNPs for breeding lines (reference population 2). We used random forest imputation to estimate missing data (Breiman, 2001), using the R package MissForest (Stekhoven and Bühlmann, 2012), with the configuration ntree = 100, maxiter = 10, and encoding genotypes as categorical data (factors).

3.3.6. Analysis of linkage disequilibrium decay, population structure, and genome-wide association study

The analyses of LD decay and population structure, and the GWAS, were performed separately for the two reference populations. LD was estimated per chromosome as r^2 values for pairwise combinations of SNPs within a 100 kb window using the *LD.decay()* function from the R package "sommer" (Covarrubias-Pazaran et al., 2016). The r^2 values were plotted against physical distance and fitted by a polynomial curve, as described in Marroni et al. (2011). The critical value of r^2, representing the most meaningful LD decay threshold, was derived from the distribution of inter-chromosomal LD (distribution of r^2 estimates derived from SNPs located on different chromosomes), as described in Breseghello and Sorrells (2006). Inter-chromosomal r^2 estimates were square-root transformed to approximate a normally distributed random variable. The parametric 95th percentile of that distribution was assumed as a population-specific critical value of r^2 above which LD was likely attributable to physical linkage. For each chromosome, the LD extent was estimated as the physical distance at which the fitted polynomial curve of decay crossed the population-specific critical r^2 threshold.

The population structure was investigated by DPCA (Yendle and MacFie, 1989) performed on genotype data previously pruned for the excess of linkage disequilibrium, to avoid the strong influence of SNP clusters when estimating genetic relatedness (Laurie et al., 2010). Pruning was performed by the *snpgdsLDpruning()* function from the R package "SNPRelate" with a maximum r^2 threshold of 0.45. The k-means clustering algorithm was run iteratively for increasing values of K from 1 to 20 and repeated 300 times to identify the optimal numbers of genotype groups (K) based on the minimization of the Bayesian information criterion. The analysis was performed on the output of an ordinary principal component analysis (PCA) to benefit from dimensionality reduction, but all components were retained to avoid information loss. The final DPCA was carried out according to the optimal K value, which resulted equal to 2 for reference population 1 and 19 for reference population 2. The number of principal component (PC) axes retained for DPCA was determined by visual inspection of the plots of PC cumulative variance (100 PCs for reference population 1, and 80 PCs for reference population 2). The number of discriminant functions to be employed as covariates in the GWAS was determined by the a-score optimization criterion (which represents the propensity of DPCA toward overfitting). Based on this criterion, one discriminant function was retained for reference population 1, and 13 for reference population 2. The entire procedure was implemented using the functions *find.clusters()*, *dapc()*, and *oprim.a.score()* from R package "adegenet" (Jombart and Ahmed, 2011).

A GWAS was conducted for plant mortality and the visual injury score within each reference population according to the Blink model (Huang et al., 2019) using the R package "GAPIT" (Wang and Zhang, 2021). We accounted for the population structure by including the discriminant functions obtained by DPCA as model covariates. A visual examination of quantile–quantile (QQ) plots (Supplementary Figure 3.1) confirmed an appropriate compensation of population structure in the GWAS models. Significant SNPs were selected according to the FDR threshold at 5%. Based on the previously computed LD extent within each chromosome, candidate genes in association with the significant SNPs and corresponding putative functions were identified on the white lupin genome browser (www.whitelupin.fr) by scanning a genomic region corresponding to the chromosome-specific LD extent in both directions from each significant SNP.

3.3.7. Genomic selection

Genomic prediction models were assessed for plant mortality and the visual injury score using the R package "GROAN" (Nazzicari and Biscarini, 2022). We envisaged an intra-population prediction scenario for each population by comparing three statistical models (described below), which included all available SNPs (40,914 for reference population 1, and 32,951 for reference population 2). The predictive ability (i.e., the correlation between observed and predicted phenotypes) was estimated through a standard 10-fold cross validation repeated 10 times to ensure numerical stability (reporting the average results). The transferability of the predictive models in a cross-population scenario was investigated by fitting each of the three statistical models on reference population 1 and validating them on reference population 2, and vice versa. Only markers common to both populations were included in this analysis, namely a total of 30,412 SNPs.

The statistical models involved in this study were ridge regression BLUP (rrBLUP), Bayesian Lasso (BL), and BayesB. The rrBLUP model (Endelman, 2011) assumes a linear mixed additive model, where each marker is assigned an effect as a solution of the following equation:

$$y = 1\mu + Wq + \mathcal{E}$$

where y is the vector of observed phenotypes, μ is the mean of y, W is the genotype matrix (e.g., {0,1,2} for biallelic SNPs), $q \sim$ N (0, $I\sigma_q^2$) is the vector of marker effects, and ε ~ N (0, $I\sigma_e^2$) is the vector of residuals. This model, which is solved in a restricted maximum likelihood (REML) context, assumes that the effects of all loci have a common variance, making it suitable for traits influenced by a large number of minor genes.

BL and BayesB models fit the same general model as rrBLUP but within a Bayesian context, where different prior densities are assigned to marker effects, allowing them to have different variances (Meuwissen et al., 2001; Habier et al., 2011). BL assumes independent Laplace double-exponential priors, which impose a strong shrinkage for regression coefficients of marker effects with small values (Park and Casella, 2008). Bayes B assumes that marker effects follow a *t*-

distribution and that most loci have no effect on the trait and are, therefore, excluded from the prediction model (Meuwissen et al., 2001). Bayesian models use Markov chain Monte Carlo to estimate the model parameters (thereby introducing Monte Carlo sampling error as a source of variation). To ensure numerical stability of the results for models fit in one population and validation in the other, the number of iterations of the algorithm was set to 50,000.

3.4. Results

3.4.1. Frost resistance variation and relationship with field-based winter mortality

The frost resistance of the two reference populations was assessed in terms of plant mortality and a visual score of biomass injury following a freezing temperature of −11 °C applied to previously hardened plants. The genotype variation for these traits was wide (Figure 3.2) and statistically significant at $p < 0.001$ for both populations. The proportion of plant mortality ranged from 0.08 to 0.96 with a mean value of 0.53 for reference population 1 (landrace and cultivar genotypes), and from 0.05 to 1.00 with a mean value of 0.62 for reference population 2 (breeding lines). On average, population 2 tended to be more susceptible to frost than population 1 according to mean values of the biomass injury score (8.2 vs. 7.4). The values of plant mortality and biomass injury score of the individual genotypes are reported in Supplementary Table 3.1 for landrace and cultivar germplasm and Supplementary Table 3.2 for breeding lines. The top-ranking germplasm for plant survival was a genotype extracted from the Greek landrace Gr56 and the French winter-type cultivar Ludet (with mortality ≤ 0.09).

Broad-sense heritability values (H^2) were high for both traits, ranging from 0.76 to 0.82 (Figure 3.2). Plant mortality and the visual score of biomass injury provided highly consistent information on genotype frost resistance, as indicated by their strong correlations ($r = 0.97$ and 0.96 for reference populations 1 and 2, respectively; $p < 0.001$). Plant mortality tended to be more effective than the injury score in capturing the genetic variation for frost resistance according to its wider dispersal for genotype frequency data and its higher genetic coefficient of variation (CV_g) in Figure 3.2. Each of these traits exhibited somewhat higher CV_g values for reference population 1 relative to population 2, in agreement with the expectation of greater variation in a world collection of landrace and cultivar material than in a collection of inbred lines derived from a fairly restricted parent group.

Landrace and cultivar genotypes of reference population 1 were grouped into 14 germplasm pools. Landraces were classified into 11 pools according to their geographical origin, while cultivars were classified into winter, spring, and Mediterranean phenological types. An analysis of variance revealed significant differences ($p < 0.001$) between pools for mean values of plant mortality and biomass injury score. On average, winter-type cultivars were highly frost-resistant according to mortality and biomass injury score values, followed by Mediterranean-type cultivars (Table 3.1). The winter-type cultivar pool was more resistant than any landrace pool, whereas the pool of spring-type cultivars was highly susceptible (Table 3.1). However, few differences between germplasm pools were statistically significant, owing to the large variation within germplasm pools (as highlighted by the range of values in Table 3.1). The greatest difference emerging among landrace pools was the high average frost susceptibility of material from Maghreb, which contrasted with the moderately high resistance exhibited by landraces from Greece, Spain, and the Atlantic islands (Table 3.1).

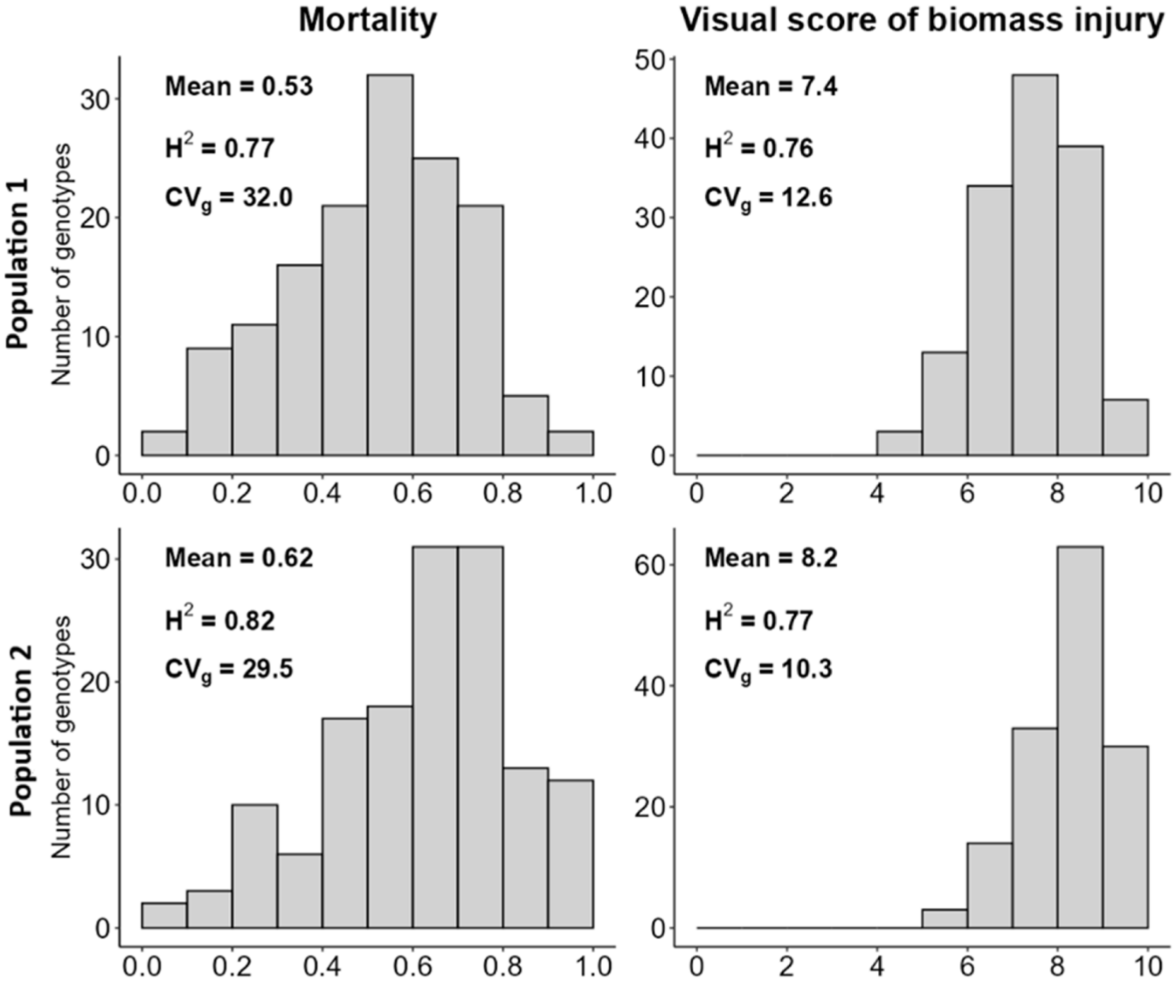


Figure 3.2. Distribution, mean, broad-sense heritability on an entry mean basis (H^2), and genetic coefficient of variation (CV_g) for proportion of plant mortality and visual score of biomass injury of landrace and cultivar genotypes (reference population 1) and breeding lines (reference population 2) of white lupin assessed at −11 °C freezing temperature in a phenotyping platform.

Table 3.1. Number of evaluated genotypes and mean and range values of proportion of plant mortality and visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform, for white lupin pools of landrace and cultivar genotypes.

Pool	Material	No. of genotypes	Mortality		Visual score	
			Mean[a]	Range	Mean[a]	Range
Winter-type	Cultivar	4	0.21 d	0.09-0.37	5.6 d	4.8-6.3
Mediterranean-type	Cultivar	5	0.33 cd	0.13-0.58	6.2 cd	4.4-7.5
Greece	Landrace	12	0.44 bc	0.08-0.70	7.0 bc	5.3-8.6
Spain	Landrace	11	0.48 bc	0.17-0.70	7.1 bc	5.7-8.3
Madeira & Canaries	Landrace	10	0.51 bc	0.27-0.73	7.3 b	6.2-8.5
Azores	Landrace	11	0.51 bc	0.18-0.80	7.2 b	5.1-8.7
West Asia	Landrace	12	0.52 ab	0.26-0.76	7.3 b	5.9-8.8
Turkey	Landrace	12	0.53 ab	0.16-0.73	7.5 b	6.1-8.9
Italy	Landrace	14	0.54 ab	0.26-0.75	7.5 ab	5.7-8.7
Portugal	Landrace	10	0.55 ab	0.25-0.76	7.7 ab	6.6-8.7
Egypt	Landrace	14	0.56 ab	0.16-0.82	7.7 ab	5.2-9.0
East Africa	Landrace	10	0.57 ab	0.21-0.95	7.6 ab	5.5-9.4
Spring-type	Cultivar	8	0.63 ab	0.12-0.90	7.9 ab	4.9-9.5
Maghreb	Landrace	11	0.71 a	0.45-0.96	8.4 a	7.4-9.7
LSD ($p < 0.05$)			0.16		0.9	

[a]Means followed by different letter differ at $p < 0.05$ according to Duncan's test.

Four landraces from reference population 1 had been used as parents for the development of the breeding lines (reference population 2). We found consistency between the landrace parent value and the mean value of their progenies for plant mortality and visual injury score. In particular, the parent landrace with the greatest resistance (the Greek accession Gr56) produced breeding lines with the highest resistance, whereas the parent landrace with the greatest frost susceptibility (LAP123) generated lines with the highest susceptibility (Table 3.2)

Table 3.2. Mean values of proportion of plant mortality and visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform for four white lupin parental landraces and the mean values of their progeny lines.

Landrace	Mortality		Visual score	
	Parent value	Progeny value	Parent value	Progeny value
Gr56	0.24	0.49	6.0	7.6
La646	0.39	0.57	6.6	8.0
La246	0.36	0.66	6.4	8.5
LAP123	0.72	0.77	8.6	8.9
LSD ($p < 0.05$)	0.34[b]	0.06	1.5[b]	0.6

A total of 115 landraces and cultivars from reference population 1 had previously been evaluated under field conditions for winter plant mortality and onset of flowering (Annicchiarico et al., 2010). The correlation between plant mortality under field conditions (as determined by stress resistance and stress escape by a late phenology) and plant mortality in the phenotyping platform (as determined essentially by frost resistance) was fairly low albeit significant ($r = 0.29$; $p < 0.01$). A linear regression of field-based plant mortality as a function of platform-based mortality with an indication of three phenological groups of accessions (early, intermediate, and late flowering) highlighted the role of phenology in determining the field mortality (Figure 3.3). Many accessions showing greater field mortality than the expected value based on platform mortality featured early flowering; whereas many accessions displaying lower field mortality than the platform-based expectation were characterized by late flowering (hence, greater phenology-based stress escape).

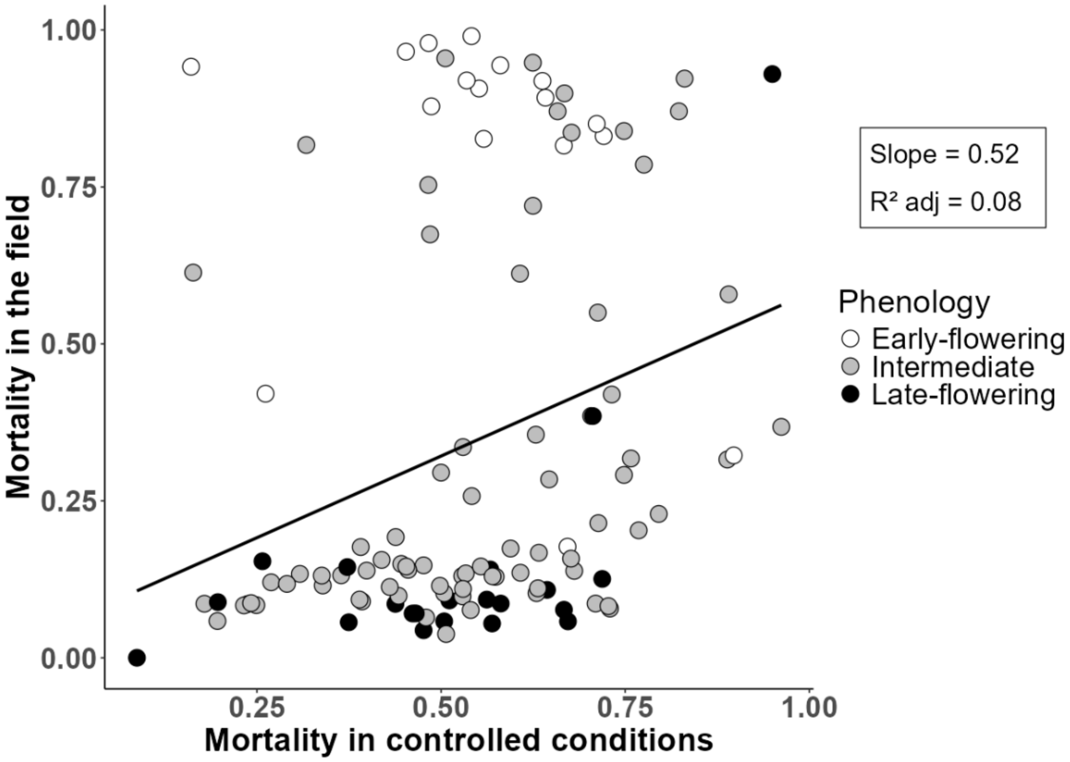


Figure 3.3. Linear regression of the proportion of plant mortality under field conditions as a function of the plant mortality in a phenotyping platform for 115 landraces and cultivars. Accessions are classified into three phenological classes based on onset of flowering (class range values of flowering in days from January 1 are: 94.0-115.0 for early-flowering, 115.1-120.6 for intermediate-flowering, and 120.7-130.7 for late-flowering).

3.4.2. Analysis of linkage disequilibrium decay and population structure, and genome-wide association study

The molecular characterization was based on 40,914 polymorphic SNPs for landrace and cultivar material (reference population 1), and 32,951 SNPs for breeding lines (reference population 2). The linkage disequilibrium (LD) decay plots for white lupin chromosomes based on Pearson's correlation (r^2) against physical distance are reported in Supplementary Figure 3.2 and Supplementary Figure 3.3, respectively, for these populations. The LD decay threshold (i.e., the critical r^2 value above which LD is likely attributable to physical linkage) was computed for each population as the 95th percentile of the distribution of pairwise inter-chromosomal r^2 estimates after a square root transformation. The threshold was 0.046 for reference population 1, and 0.067 for population 2. For each chromosome within each population, LD extent values are reported in Supplementary Table 3.3.

The population structure was investigated by discriminant principal components analysis (DPCA) on genotype data previously pruned for excess LD. For landrace and cultivar material, the DPCA identified one major discriminant function that classified the genotypes into two broad groups (Supplementary Figure 3.4). Group 1 included the entire germplasm pools from Azores and Greece, whereas group 2 comprised the pools from Italy, the Maghreb, and winter-type cultivars, along with most of the genotypes from Egypt, Madeira and the Canaries, West Asia, and spring-type cultivars. All the remaining pools were distributed almost equally between the two groups. The DPCA for reference population 2 grouped the genotypes mainly on the ground of the 16 crosses they derived from (Supplementary Figure 3.5). One discriminant function was retained for reference population 1 and 13 were retained for reference population 2 according to the a-score criterion, for use as covariates in the GWASs to compensate for population structure.

The results of the GWASs are summarized in the Manhattan plots reported in Figure 3.4 for landrace and cultivar material and in Figure 3.5 for breeding lines. Significant SNPs were selected according to the false discovery rate (FDR) threshold at 5%. For reference population 1, five SNPs (on chromosomes 5, 6, 8, 13, and 14) were associated with plant mortality, one SNP (on chromosome 16) was associated with the visual score of biomass injury, and one SNP (on chromosome 23) was associated with both traits. For reference population 2, three SNPs (on chromosomes 4, 19, and 21) were associated with plant mortality, and one SNP (on chromosome 2) was associated with both traits. The list of significant SNPs and their association scores, minor allele frequency, and estimated trait effects is provided in Supplementary Table 3.4. On the whole, the inconsistency for significant SNPs across populations, the presence of several significant SNPs in population 1, and the presence of several minor peaks of SNP association not reaching significance in both populations (Figures 3.4 and 3.5) indicated a polygenic genetic architecture of frost resistance-related traits.

Out of 11 significant SNPs in the GWASs, 10 were found to be associated with at least one candidate gene on the white lupin genome browser (www.whitelupin.fr), by searching within genomic regions corresponding to the chromosome-specific LD extent in both directions. Only the significant SNP on chromosome 23 (associated with both traits in population 1) was not associated with any candidate gene, suggesting either a false positive or, more probably, the presence of a regulatory region affecting the transcription of a relevant gene. The full list of candidate genes, with putative encoded proteins and their putative roles in frost resistance, is provided in Table 3.3.

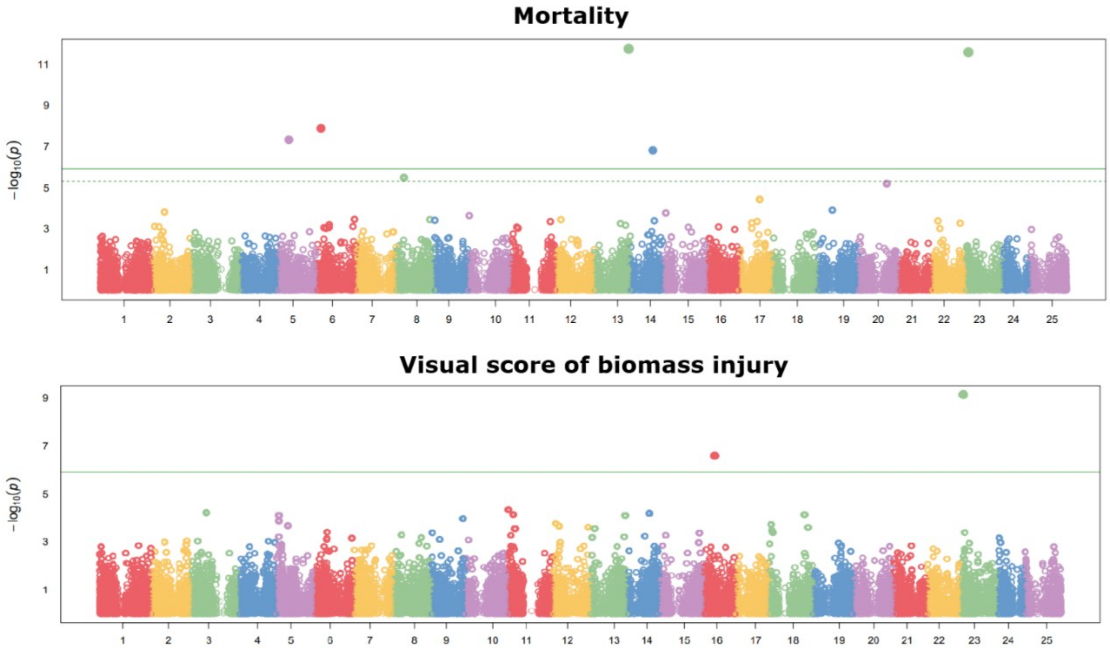


Figure 3.4. Manhattan plots showing the association scores of 40,914 SNPs (ordered along chromosomes) with the proportion of plant mortality and a visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform for 144 white lupin landrace and cultivar genotypes (reference population 1). The green continuous line is the Bonferroni threshold at 5%, whereas the green dashed line (where visible) is the false discovery rate threshold at 5%.

Figure 3.5. Manhattan plots showing the association scores of 32,951 SNPs (ordered along chromosomes) with the proportion of plant mortality and a visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform for 144 white lupin breeding lines (reference population 2). The green continuous line is the Bonferroni threshold at 5%, whereas the green dashed line (where visible) is the false discovery rate threshold at 5%.

Table 3.3. List of candidate genes associated with significant SNPs detected by a GWAS. The analysis was conducted on white lupin landrace and cultivar genotypes (reference population 1) and breeding lines (reference population 2), for proportion of plant mortality and a visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform. Candidate genes were identified using the white lupin genome browser (**www.whitelupin.fr**). Putative encoded proteins and their putative role in frost resistance are reported.

SNP	Population	Trait	Candidate Gene	Putative protein	Putative role
Chr02_14306413	2	Mortality, visual score	Chr02g0156401	Metallo-dependent phosphatase	Cold signal regulation
Chr02_14306413	2	Mortality, visual score	Chr02g0156391	Multi antimicrobial extrusion protein	
Chr02_14306413	2	Mortality, visual score	Chr02g0156411	UDP-N-acetylglucosamine--dolichyl-phosphate N-acetylglucosaminephosphotransferase	
Chr04_7632627	2	Mortality	Chr04g0256531	Hydrolase	Stabilization of cell wall
Chr04_7632627	2	Mortality	Chr04g0256541	Potassium transporter	Cryoprotection and osmoprotection
Chr05_4820341	1	Mortality	Chr05g0219341	CBS domain-containing protein (CDCPs)	
Chr05_4820341	1	Mortality	Chr05g0219331	Polyadenylate binding protein, human types 1, 2, 3, 4	
Chr06_1878682	1	Mortality	Chr06g0163651	Oxidoreductase	Enhancement of ROS scavenging
Chr06_1878682	1	Mortality	Chr06g0163661	mRNA splicing factor SYF2	
Chr08_3511620	1	Mortality	Chr08g0234251	Cellulose synthase (UDP-forming) chromatin regulator PHD family	Gene expression regulation
Chr08_3511620	1	Mortality	Chr08g0234241	QWRF family protein	
Chr13_15386976	1	Mortality	Chr13g0303311	Ribosomal protein S4/S9	Ribosomal biogenesis
Chr13_15386976	1	Mortality	Chr13g0303301	Transcription factor bHLH family	Cryoprotection and osmoprotection
Chr14_10127694	1	Mortality	Chr14g0368501	Leucine-rich repeat domain, L domain-containing protein	Primary cold sensor
Chr14_10127694	1	Mortality	Chr14g0368511	Plus-end-directed kinesin ATPase	
Chr16_5032297	1	Visual score	Chr16g0384801	Kinase RLK-Pelle-URK-1 family	Primary cold sensor
Chr19_17886074	2	Mortality	Chr19g0139891	Vacuolar protein sorting-associated protein	
Chr19_17886074	2	Mortality	Chr19g0139901	Methionine--tRNA ligase	
Chr19_17886074	2	Mortality	Chr19g0139881	Phosphatase 4 core regulatory subunit R2	
Chr21_1050253	2	Mortality	Chr21g0306351	Serine/threonine phosphatase, protein kinase CMGC-GSKL family	Cold signal regulation
Chr23_1146188	1	Mortality, visual score	-	-	

3.4.3. Genomic selection

The predictive ability of genomic selection was assessed with respect to plant mortality and the visual biomass injury score by three statistical models: rrBLUP, Bayesian Lasso, and BayesB. We envisaged an intra-population scenario by building and testing models on the same population (using 10-fold cross validations), as well as a cross-population scenario by constructing models on one population and testing their predictive ability on the other population. The three models usually performed similarly. However, Bayesian Lasso provided a slightly higher predictive ability in most cases, while rrBLUP outperformed the other models for both traits for intra-population predictions of reference population 1. Predictive ability values of the best-performing models for each trait, reference population, and prediction scenario are reported in Table 3.4. On average, the predictive abilities did not differ substantially between the two traits. For both traits, intra-population predictive abilities were high for the population of breeding lines (0.672–0.678) and moderately high for that of landrace and cultivar material (0.414–0.376). Remarkably, the model constructed from landrace and cultivar data exhibited a minimal or nil drop in predictive ability passing from intra-population prediction for the same population to cross-population prediction for the population of breeding lines (0.414 vs. 0.393, for plant mortality; 0.376 vs. 0.386, for the visual injury score; Table 3.4), indicating its broad applicability. In contrast, the model constructed for the reference population featuring smaller genetic diversity, i.e., the breeding line population, displayed a drastic decrease in predictive ability passing from intra-population prediction to cross-population prediction applied to the genetically broader reference population of landraces and cultivars (0.672 vs. 0.255, for plant mortality; 0.678 vs. 0.232, for the visual injury score; Table 3.4). However, the transferability to the population of breeding lines of the prediction model constructed for landrace and cultivar material involved a decrease in predictive ability in the range of 41–43% relative to the model specifically developed for the breeding lines (0.393 vs. 0.672, for plant mortality; 0.386 vs. 0.678, for the visual injury score; Table 3.4).

Table 3.4. Predictive ability (as correlation between observed and predicted phenotypes) of the best-performing of three statistical models (rrBLUP, Bayesian Lasso, BayesB) for proportion of plant mortality and visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform in two white lupin reference populations (population 1: landrace and cultivar genotypes; population 2: breeding lines). Predictions for intra-population and cross-population scenarios.

Trait	Training set	Validation set	Model	Predictive abilities
Mortality	Population 1	Population 1	rrBLUP	0.414
Mortality	Population 2	Population 2	Bayesian Lasso	0.672
Mortality	Population 1	Population 2	Bayesian Lasso	0.393
Mortality	Population 2	Population 1	Bayesian Lasso	0.255
Visual score	Population 1	Population 1	rrBLUP	0.376
Visual score	Population 2	Population 2	Bayesian Lasso	0.678
Visual score	Population 1	Population 2	Bayesian Lasso	0.386
Visual score	Population 2	Population 1	Bayesian Lasso	0.232

3.5. Discussion

This study provided the first large-scale assessment of white lupin genotype frost resistance under controlled conditions, capitalizing on earlier work that optimized the evaluation procedure in these conditions (Franguelli et al., 2024). The assessment was based on two traits, namely, plant mortality and the visual injury score, which were highly correlated. The former trait tended to be more sensitive than the latter in detecting relevant genetic variation in the current experiment layout (which included 15 plants per experimental unit and 144 evaluated genotypes per cycle). However, the visual injury score may be preferable in the presence of fewer plants per experimental unit (as envisageable when evaluating more genotypes per growth cycle), owing to greater experiment error expected for plant mortality in that case. A similar visual injury score was adopted when testing four plants per experimental unit in pea (Beji et al., 2020) and faba bean (Arbaoui and Link, 2008).

As expected, the genetically broader reference population 1 (including an international set of landrace and cultivar genotypes) exhibited larger variation for frost resistance than the reference population of breeding lines. The average proportion of plant mortality around 0.5 displayed by the former population was close to the average mortality displayed at the same freezing temperature by a smaller set of genotypes used to identify −11 °C as the optimal temperature for frost resistance evaluation (Franguelli et al., 2024). Results for the individual genotypes (summarized in Supplementary Table 3.1 and Supplementary Table 3.2) can help identify genetic resources with outstanding frost resistance for use as parent germplasm in breeding programs. Actually, the highly frost-resistant landrace Gr56 was already used as one of the parents that generated the breeding lines of reference population 2. The substantial consistency between parent and progeny values that we observed for frost resistance traits is comforting for breeding and agrees with a similar result observed for key seed quality traits such as protein content, total content of quinolizidine alkaloids, and seed weight (Annicchiarico et al., 2025). In addition, the selection for higher frost resistance under controlled conditions could be favoured by high broad-sense heritability and substantial genetic variation according to the present results.

Winter-type and Mediterranean-type cultivars ranked in this order for frost resistance and tended to display greater resistance than any landrace germplasm pool. This result reflected the effort on cold tolerance improvement performed by a few breeding programs, especially in France, where autumn sowing was devised as a key strategy for crop yield improvement (Papineau and Huyghe, 2004). In addition, frost resistance may be important even in the Mediterranean climate (Annicchiarico and Iannucci, 2007; Stoddard et al., 2006). Landrace material from Greece, the Atlantic islands (Azores, Madeira and the Canary Islands) and Italy was previously reported as winter-hardy under field conditions (Annicchiarico et al., 2010; Huyghe, 1997; Papineau and Huyghe, 2004). While tending to confirm these findings in terms of frost resistance, our results indicated relatively modest differences between most germplasm pools for this characteristic, suggesting that frost escape by a late phenology has a key impact on field-based winter hardiness. Indeed, a multi-location evaluation of landrace germplasm pools highlighted the close relationship between a late phenology and the specific adaptation to a cold-prone environment of northern Italy of germplasm from the Azores and Greece (Annicchiarico et al., 2010). The modest correlation currently observed for plant mortality across phenotyping platform and field conditions ($r = 0.29$), and the importance of flowering time in accounting for plant mortality differences across these conditions (Figure 3.3), reinforced this finding, suggesting that phenology had a greater impact than frost resistance on the field-based winter survival of landrace accessions. Phenology emerged as a major determinant of the specific adaptation to cold-prone or mild-winter Italian environments also for other cultivar and breeding line materials (Annicchiarico et al., 2010). However, the modest correlation observed for plant mortality across platform and field conditions was emphasized by the wide variation for onset of flowering expressed by the evaluated material (Annicchiarico et al., 2010), and a higher correlation would occur for a material with a similar phenology. Delayed flowering is mediated by vernalization requirements and the accumulation of growing degree days (Adhikari et al., 2012; Huyghe and Papineau, 1990; Rychel-Bielska et al., 2024). Studies on other legumes reported higher correlations for plant mortality across artificial and field conditions, which approached 0.7 (Homer et al., 2016) and 0.5–0.6 for pea (Auld et al., 1983), and 0.5 for faba bean (Arbaoui et al., 2008) and red clover (Zanotto et al., 2021). As a matter of fact, a lack of correlation across these conditions may result not only because of the importance of phenology-based stress escape but also because of other factors such as waterlogging and fungal diseases, particularly *Pleiochaeta setosa*, *Fusarium* spp., and *Colletotrichum gloeosporioides* (anthracnose;

Huyghe, 1997; Bateman, 1997). As anticipated, our focus on selection for frost resistance (as allowed by the evaluation of young plants in our phenotyping platform) has particular interest for southern Europe and other regions that are prone to both frost and terminal drought, since selection for a late phenology would lead to material with high winter hardiness but high susceptibility to terminal drought.

The genetic improvement of white lupin frost resistance may rely not only on phenotypic selection under controlled conditions (which ideally requires a high-throughput phenotyping platform) but also on marker-assisted or genomic selection. The results of the DPCA that preceded the GWAS showed a weak population structure for reference population 1, with just a loose relationship with the geographic origin of landraces that confirmed earlier results (Alkemade et al., 2022; Hufnagel et al., 2021). The two major genetic groups identified in this population were not clearly related to frost resistance of the material, since frost-resistant, winter-type cultivars and frost-resistant landrace pools (such as those from Greece and the Azores) clustered in different groups. As expected, the population structure of reference population 2 reflected the crosses from which the lines originated. The slower average LD decay in reference population 2 compared to population 1 was consistent with the lower number of meiotic events that occurred in the breeding line population compared to the population of landrace and cultivar genotypes. Overall, LD decay values confirmed earlier observations (Hufnagel et al., 2021) of rapid LD decay in white lupin relative to other inbred crops, which complicates the identification of significant SNPs in this species.

The GWAS identified various population-specific SNPs associated with plant mortality and/or the biomass injury score. On the whole, it indicated a polygenic genetic control of frost resistance that agrees with the complexity of frost resistance mechanisms. These mechanisms are activated by exposure to low, non-freezing temperatures (during cold acclimation or hardening), and enhance plant survival during later freezing events by preventing intracellular ice formation or resisting ice damage. This occurs through a decrease in shoot water content (Sallam et al., 2015), an increase in cell membrane stability by changing the lipid-to-protein ratio and the membrane lipid unsaturation level (Arbaoui and Link, 2008), and an accumulation of cryoprotectant and osmoprotectant compounds that protect against dehydration (Link et al., 2010; Jahed et al., 2023). Various of the current candidate genes listed in Table 3.3 appear to be involved in the cold signaling pathway, acting as primary sensors, signal modulators, or regulators of gene expression. In particular, Chr16g0384801 and Chr14g0368501 encode, respectively, a putative receptor-like kinase (RLK) and a leucine-rich repeat (LRR) domain protein, both of which are membrane-localized and can act as primary sensors when low temperatures are perceived through changes in membrane fluidity (Chen et al., 2021). Chr21g0306351 and Chr02g0156401 encode, respectively, a putative serine/threonine phosphatase and a metallo-dependent phosphatase. Phosphatases are known to negatively modulate key signaling proteins involved in stress responses. In alfalfa (*Medicago sativa* L.), the activity of serine/threonine phosphatase type 2A (PP2A) is inhibited by cold, and its inactivation results in increased kinase activity and transmission of cold signals to the nucleus (Monroy er al., 1998). Chr08g0234251 encodes a chromatin regulator of the PHD family. In rye, ScPHD5, belonging to the plant homeodomain (PHD) family, regulates C-repeat binding factor (CBF) gene expression, one of the most important transcription factors in the response to low temperatures (Jung et al., 2024). Three other candidate genes are likely to be involved in protection from intra-cellular ice formation. Chr04g0256531 encodes a putative hydrolase and, in *Arabidopsis*, two hydrolase enzymes, namely XTH19 and XTH22, strengthen the cell wall during cold and sub-zero acclimation, making cells more resistant to freezing mechanical stress (Wu et al., 2024; Takahashi et al., 2020). Chr13g0303301 and Chr04g0256541 encode, respectively, a putative transcription factor of the bHLH family (which is associated with enhanced proline accumulation; Guo et al., 2021) and a putative potassium transporter (which regulates the uptake of potassium ions (K^+) in plant cell membranes; Mulet et al., 2023). Potassium ions and proline are among the major cryoprotectants and osmoprotectants, which lower the freezing point of cellular fluids, inhibit ice formation, and at the same time help maintain cell osmolarity and prevent severe dehydration caused by membrane damage due to ice formation (Jahed et al., 2023). Indeed, 11 genes encoding members of the bHLH protein family were detected as candidate genes for frost resistance in alfalfa (Li et al., 2022). Another negative effect of abiotic stress on cell metabolism is the overproduction of reactive oxygen species (ROS), which damage molecular and cellular components due to the oxidation of biomolecules such as lipids, carbohydrates, proteins, enzymes, and DNA (Sachdev et al., 2021). Chr06g0163651 encodes a putative oxidoreductase that can enhance ROS scavenging. Accumulation of oxidoreductase enzymes during cold acclimation was observed in *Solanum tuberosum*, *Festuca pratensis*, and *Arabidopsis* (Song et al., 2022; Kosmala et al., 2009; Kwon et al., 2007). Moreover, Chr13g0303311 encodes putative ribosomal protein S4/S9. Ribosomal proteins are crucial for ribosomal biogenesis, which allows the rapid synthesis of proteins involved in cold stress response, and various studies suggest the contribution of ribosome-related genes to frost resistance (Kosmala et al., 2009; Sandve et al., 2011). Finally, Chr19g0139891 and Chr05g0219341 encode, respectively, a putative vacuolar protein sorting-associated protein, and a putative CBS domain-containing protein (CDCP), which appear to be associated with cold response, although the underlying mechanisms are under investigation (Zhang et al., 2018; Kushwaha et al., 2009; Hao et al., 2021).

The polygenic genetic architecture of frost resistance traits encourages the use of genomic selection to account for the effect of many genes in linkage with SNPs. The presence of many relevant genes with a minor effect was also supported by the minimal or nil decrease in predictive ability observed for the model constructed from landrace and cultivar data when passing from intra-population prediction to cross-population prediction, in the presence of marked inconsistency between the two reference populations for significant SNPs in the GWAS. Our results confirmed the interest of genomic prediction models constructed for a genetically broad reference population, such as the world collection of landrace and cultivar materials, not only to identify promising genetic resources within large germplasm collections but also for a preliminary genomic selection of breeding lines. Within cool-season grain legumes, the transferability to a breeding line population of genomic prediction models constructed from data of a world germplasm collection have already emerged for seed weight and oil content in white lupin (Annicchiarico et al., 2025) and seed protein content and seed weight in pea (Crosta et al., 2023), albeit at the cost of a predictive ability drop in the range of 35–50% relative to intra-population prediction of the breeding lines. The current predictive ability drop of 41–43% agrees well with those findings. Hence, even in favorable cases, the model transferability is likely to be envisaged only when budget constraints impede the development of a specific prediction model for the targeted breeding line population. Of course, a lack of model transferability may emerge, as in the case of seed protein content of white lupin (Annicchiarico et al., 2025) and a highly complex trait such as pea grain yield (Crosta et al., 2023). The modest cross-population predictive ability exhibited by the model constructed using reference population 2 to predict reference population 1 confirmed earlier results (Annicchiarico et al., 2025) showing that a training reference population with relatively low genetic variation (such as that of the breeding lines) restricts the inference space for the prediction of breeding values in a genetically broad, independent reference population.

The intra-population scenario for the breeding lines showed high predictive abilities (in the range of 0.67–0.68). This result aligns with previous results obtained for frost biomass injury of cereal breeding populations, which showed a prediction accuracy of 0.59 for wheat (Michel et al., 2019) and a predictive ability of 0.87 for rye (Erath et al., 2017). In a marker-assisted selection prospect, a marker score was able to explain about 30% of the variation for faba bean resistance to winter and late-winter frosts (Windhorst et al., 2024). The currently high predictive ability values encourage the prediction of frost resistance aimed to restrict the number of breeding lines subjected to platform-based and/or field-based evaluation. The predictive ability value of 0.41 observed for intra-population prediction of plant mortality in the international collection of landraces and cultivars, while being expectedly lower than the value for breeding lines as a consequence of the wider genetic variation and faster LD of this material, is nevertheless useful for the identification of genetic resources with putative frost resistance to be verified by subsequent evaluation. The use of genomic prediction models for white lupin germplasm collections is justified by their large number of accessions, which exceeds 6300 just considering the main collections (Buirchell and Cowling, 1998) and prevents a thorough evaluation due to limited budgets.

In conclusion, this study can support the selection of winter-hardy white lupin cultivars targeted to autumn-sown environments by (a) highlighting the extent of genetic variation for frost resistance, (b) elucidating the impact of frost resistance and a late phenology on field-based winter plant survival, (c) identifying frost-resistant genetic resources, (d) showing the definite polygenic architecture of frost resistance, (e) revealing genomic regions hosting putative candidate genes, and (f) highlighting opportunities for genomic prediction models aimed at the selection of breeding lines or the identification of promising genetic resources. Our study indicated that the genomic selection of inbred lines can be valuable even via the cross-population prediction of models constructed from a genetically broad reference population such as a world germplasm collection, albeit less effective than intra-population prediction. The overall contribution of frost resistance on field-based winter survival was modest in the presence of large variation for the onset of flowering but increased substantially for a material with a similar phenology, emphasizing the need to combine selection for both frost resistance and a sufficient vernalization requirement. The highly positive impact of the late onset of flowering on winter plant survival, and the highly negative impact of the same trait on tolerance to terminal drought in earlier work (Annicchiarico et al., 2018; Pecetti et al., 2023), highlight the strategic importance to identify a convenient, intermediate vernalization requirement on the one hand and to exploit the genetic variation for resistance to frost and to terminal drought on the other, when breeding for autumn sowing in cold- and drought-prone regions. Genomic selection models for drought resistance, which have already been defined for breeding line (Pecetti et al., 2023) and landrace material (Annicchiarico et al., 2019a), could be used in combination with the current models to select simultaneously for frost and drought resistance. Evaluation under controlled conditions is promising for both germplasm selection and the refinement of genomic prediction models.

3.6. Supplementary Materials

Supplementary Table 3.1. List of 144 white lupin landrace and cultivar genotypes (reference population 1). For each genotype, the following information is reported: name, original accession name, type of material (landrace or cultivar), corresponding germplasm pool, origin, mean values of plant mortality proportion and visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform, and availability of field data (mortality and onset of flowering) associated with the accession.

Genotype	Accession	Material	Germplasm pool	Origin	Mortality	Visual Score	Field data
LAP0001b	Ac003	Landrace	Azores	Azores	0.18	5.1	Yes
LAP002acd	Ac045	Landrace	Azores	Azores	0.44	6.8	Yes
LAP0003c	Ac050	Landrace	Azores	Azores	0.67	8.0	Yes
LAP0005a	Ac079	Landrace	Azores	Azores	0.49	7.2	Yes
LAP0005d	Ac079	Landrace	Azores	Azores	0.54	7.1	Yes
LAP006bc	Ac085	Landrace	Azores	Azores	0.46	7.2	Yes
LAP0007c	Ac139	Landrace	Azores	Azores	0.33	6.9	Yes
LAP0007a	Ac139	Landrace	Azores	Azores	0.80	8.7	Yes
LAP0008abc	Ac140	Landrace	Azores	Azores	0.64	8.2	Yes
LAP0009ab	Ac155	Landrace	Azores	Azores	0.57	7.4	Yes
LAP0010bc	Ac156	Landrace	Azores	Azores	0.48	6.8	Yes
LAP0078ac	La020	Landrace	East Africa	Ethiopia	0.65	8.1	Yes
LAP0080a	La399	Landrace	East Africa	Ethiopia	0.67	8.2	Yes
LAP0081bc	La420	Landrace	East Africa	Sudan	0.48	6.9	Yes
LAP0082d	La422	Landrace	East Africa	Sudan	0.39	6.7	Yes
LAP0079d	La559	Landrace	East Africa	Ethiopia	0.95	9.4	Yes
LAP0083a	La629	Landrace	East Africa	Sudan	0.83	9.0	Yes
LAP0075b	La654	Landrace	East Africa	Kenya	0.21	5.5	Yes
LAP0075a	La654	Landrace	East Africa	Kenya	0.27	6.3	Yes
LAP0076b	La655	Landrace	East Africa	Kenya	0.80	9.0	Yes
LAP0077a	La656	Landrace	East Africa	Kenya	0.46	6.7	Yes
LAP0085d	Egypte003	Landrace	Egypt	Egypt	0.82	9.0	Yes
LAP0086d	Egypte011	Landrace	Egypt	Egypt	0.48	6.8	Yes
LAP0088a	Egypte016	Landrace	Egypt	Egypt	0.26	5.7	Yes
LAP0089b	Egypte022	Landrace	Egypt	Egypt	0.62	8.5	Yes
LAP0087d	Egypte026	Landrace	Egypt	Egypt	0.64	8.1	Yes
LAP0090bd	Egypte038	Landrace	Egypt	Egypt	0.68	8.2	Yes
LAP0091b	Egypte055	Landrace	Egypt	Egypt	0.57	7.7	Yes
LAP0091a	Egypte055	Landrace	Egypt	Egypt	0.59	8.2	Yes
LAP0092a	Egypte064	Landrace	Egypt	Egypt	0.16	5.2	Yes
LAP0093c	Egypte076	Landrace	Egypt	Egypt	0.75	8.6	Yes
LAP0094b	Egypte093	Landrace	Egypt	Egypt	0.33	6.3	Yes
LAP0094d	Egypte093	Landrace	Egypt	Egypt	0.68	8.4	Yes
LAP0095c	La356	Landrace	Egypt	Egypt	0.49	7.5	Yes
LAP0096a	La364	Landrace	Egypt	Egypt	0.78	9.0	Yes
LAP0011cd	Gr003	Landrace	Greece	Greece	0.48	7.4	Yes
LAP0012c	Gr005	Landrace	Greece	Greece	0.50	7.3	Yes
LAP0013c	Gr017	Landrace	Greece	Greece	0.47	6.8	Yes
LAP0014ad	Gr021	Landrace	Greece	Greece	0.58	7.9	Yes
LAP0015b	Gr025	Landrace	Greece	Greece	0.34	6.6	Yes
LAP0016a	Gr030	Landrace	Greece	Greece	0.57	7.7	Yes
LAP0017b	Gr033	Landrace	Greece	Greece	0.56	7.8	Yes
LAP0017a	Gr033	Landrace	Greece	Greece	0.70	8.6	Yes
LAP0018a	Gr049	Landrace	Greece	Greece	0.31	6.7	Yes
LAP0019d	Gr056	Landrace	Greece	Greece	0.08	5.3	Yes
LAP0019c	Gr056	Landrace	Greece	Greece	0.39	6.6	Yes
LAP0020a	Gr057	Landrace	Greece	Greece	0.23	5.4	Yes
LAP0097a	Ita001	Landrace	Italy	Italy	0.54	7.7	Yes
LAP0098ac	Ita002	Landrace	Italy	Italy	0.54	7.5	Yes
LAP0103c	Ita020	Landrace	Italy	Italy	0.26	5.7	Yes

LAP0099c	Ita037	Landrace	Italy	Italy	0.70	8.3	Yes
LAP0100a	Ita042	Landrace	Italy	Italy	0.71	8.2	Yes
LAP0104b	Ita049	Landrace	Italy	Italy	0.34	6.8	Yes
LAP0104d	Ita049	Landrace	Italy	Italy	0.55	7.7	Yes
LAP0101c	Ita051	Landrace	Italy	Italy	0.55	7.3	Yes
LAP0105abd	Ita057	Landrace	Italy	Italy	0.34	6.3	Yes
LAP0106c	Ita058	Landrace	Italy	Italy	0.56	7.2	Yes
LAP0107a	La246	Landrace	Italy	Italy	0.36	6.4	Yes
LAP0123b	LAP123	Landrace	Italy	Italy	0.69	8.4	Yes
LAP0123c	LAP123	Landrace	Italy	Italy	0.75	8.7	Yes
LAP0124c	LAP124	Landrace	Italy	Italy	0.67	8.6	Yes
LAP0057b	La197	Landrace	Madeira & Canaries	Madeira	0.63	8.0	Yes
LAP0058d	La198	Landrace	Madeira & Canaries	Madeira	0.73	8.5	Yes
LAP0059b	La641	Landrace	Madeira & Canaries	Canaries	0.27	6.2	Yes
LAP0059c	La641	Landrace	Madeira & Canaries	Canaries	0.48	6.9	Yes
LAP0060abc	La642	Landrace	Madeira & Canaries	Canaries	0.61	7.9	Yes
LAP0061d	La646	Landrace	Madeira & Canaries	Canaries	0.36	6.6	Yes
LAP0061c	La646	Landrace	Madeira & Canaries	Canaries	0.42	6.7	Yes
LAP0062c	La648	Landrace	Madeira & Canaries	Canaries	0.50	7.4	Yes
LAP0063bd	La652	Landrace	Madeira & Canaries	Canaries	0.57	7.4	Yes
LAP0064b	La653	Landrace	Madeira & Canaries	Canaries	0.51	7.2	Yes
LAP0054a	La060	Landrace	Maghreb	Morocco	0.89	9.3	Yes
LAP0049ad	La127	Landrace	Maghreb	Algeria	0.66	8.2	Yes
LAP0055ac	La150	Landrace	Maghreb	Morocco	0.89	9.2	Yes
LAP0050bcd	La568	Landrace	Maghreb	Algeria	0.62	7.9	Yes
LAP0051d	La572	Landrace	Maghreb	Algeria	0.58	7.4	Yes
LAP0051b	La572	Landrace	Maghreb	Algeria	0.64	8.3	Yes
LAP0056c	La680	Landrace	Maghreb	Morocco	0.63	7.6	Yes
LAP0052d	La686	Landrace	Maghreb	Algeria	0.96	9.7	Yes
LAP0053b	La688	Landrace	Maghreb	Algeria	0.71	8.6	Yes
LAP0109d	Maroc74	Landrace	Maghreb	Morocco	0.45	7.6	Yes
LAP0110ad	Maroc78	Landrace	Maghreb	Morocco	0.77	8.8	Yes
LAP0041abc	E080	Landrace	Portugal	Portugal	0.53	7.4	Yes
LAP0042c	E091	Landrace	Portugal	Portugal	0.67	8.3	Yes
LAP0042b	E091	Landrace	Portugal	Portugal	0.76	8.7	Yes
LAP0043d	E099	Landrace	Portugal	Portugal	0.68	8.5	Yes
LAP0044b	E104	Landrace	Portugal	Portugal	0.68	7.9	Yes
LAP0045a	E107	Landrace	Portugal	Portugal	0.25	7.2	Yes
LAP0046a	E112	Landrace	Portugal	Portugal	0.53	7.4	Yes
LAP0047a	E126	Landrace	Portugal	Portugal	0.40	6.6	Yes
LAP0047c	E126	Landrace	Portugal	Portugal	0.46	7.2	Yes
LAP0048bc	E132	Landrace	Portugal	Portugal	0.53	7.4	Yes
LAP0031acd	E002	Landrace	Spain	Spain	0.44	6.7	Yes
LAP0032bd	E003	Landrace	Spain	Spain	0.48	7.2	Yes
LAP0033d	E016	Landrace	Spain	Spain	0.27	5.9	Yes
LAP0034c	E036	Landrace	Spain	Spain	0.45	6.7	Yes
LAP0034a	E036	Landrace	Spain	Spain	0.61	7.9	Yes
LAP0035ab	E059	Landrace	Spain	Spain	0.67	8.2	Yes
LAP0036c	E064	Landrace	Spain	Spain	0.17	5.7	Yes
LAP0036a	E064	Landrace	Spain	Spain	0.70	8.3	Yes
LAP0037d	E068	Landrace	Spain	Spain	0.63	7.7	Yes
LAP0039c	E075	Landrace	Spain	Spain	0.50	7.3	Yes
LAP0040c	E191	Landrace	Spain	Spain	0.39	6.6	Yes
LAP0027a	La110	Landrace	Turkey	Turkey	0.71	8.4	Yes
LAP0028d	La120	Landrace	Turkey	Turkey	0.73	8.9	Yes
LAP0029a	La259	Landrace	Turkey	Turkey	0.56	7.7	Yes
LAP0030c	La431	Landrace	Turkey	Turkey	0.32	6.5	Yes
LAP0021ad	Tr001	Landrace	Turkey	Turkey	0.54	7.2	Yes
LAP0022e	Tr007	Landrace	Turkey	Turkey	0.43	6.7	Yes
LAP0022b	Tr007	Landrace	Turkey	Turkey	0.54	7.0	Yes
LAP0023b	Tr012	Landrace	Turkey	Turkey	0.16	6.1	Yes
LAP0024b	Tr016	Landrace	Turkey	Turkey	0.55	8.0	Yes

LAP0025a	Tr017	Landrace	Turkey	Turkey	0.56	7.6	Yes
LAP0025b	Tr017	Landrace	Turkey	Turkey	0.72	8.8	Yes
LAP0026d	Tr021	Landrace	Turkey	Turkey	0.53	7.4	Yes
LAP0071b	La406	Landrace	West Asia	Israel	0.50	7.4	Yes
LAP0072a	La409	Landrace	West Asia	Israel	0.72	8.3	Yes
LAP0073b	La415	Landrace	West Asia	Jordan	0.40	6.5	Yes
LAP0068d	La416	Landrace	West Asia	Lebanon	0.39	6.4	Yes
LAP0068c	La416	Landrace	West Asia	Lebanon	0.45	6.8	Yes
LAP0069bc	La418	Landrace	West Asia	Lebanon	0.59	8.0	Yes
LAP0065a	La425	Landrace	West Asia	Syria	0.76	8.8	Yes
LAP0066bcd	La427	Landrace	West Asia	Syria	0.53	7.1	Yes
LAP0074c	La432	Landrace	West Asia	Jordan	0.71	8.1	Yes
LAP0067d	La547	Landrace	West Asia	Syria	0.26	5.9	Yes
LAP0067b	La547	Landrace	West Asia	Syria	0.64	8.0	Yes
LAP0070abcd	La673	Landrace	West Asia	Lebanon	0.29	6.2	Yes
LAP0113ab	Amiga	Cultivar	Spring-type	France	0.67	8.0	Yes
LAP0111ab	Ares	Cultivar	Spring-type	France	0.73	8.5	Yes
Dieta	Dieta	Cultivar	Spring-type	UK	0.56	7.9	No
LAP0115ab	Energy	Cultivar	Spring-type	France	0.75	8.4	Yes
Feodora	Feodora	Cultivar	Spring-type	France	0.56	7.7	No
Frieda	Frieda	Cultivar	Spring-type	Germany	0.73	8.4	No
LAP0112ab	Lublanc	Cultivar	Spring-type	France	0.90	9.5	Yes
LAP0150abcd	Lucky	Cultivar	Spring-type	France	0.12	4.9	No
Arsenio	Arsenio	Cultivar	Mediterranean-type	Italy	0.29	6.4	No
LAP0156ab	Multitalia	Cultivar	Mediterranean-type	Italy	0.17	5.2	No
Murringo	Murringo	Cultivar	Mediterranean-type	Australia	0.58	7.5	No
Pecosa	Pecosa	Cultivar	Mediterranean-type	Chile	0.13	4.4	No
LAP0155a	Rumbo	Cultivar	Mediterranean-type	Chile	0.47	7.4	No
LAP0117ab	Adam	Cultivar	Winter-type	France	0.20	5.8	Yes
LAP0119ab	Aster	Cultivar	Winter-type	France	0.37	6.3	Yes
LAP0126ab	Ludet	Cultivar	Winter-type	France	0.09	4.8	Yes
LAP0118ab	Luxe	Cultivar	Winter-type	France	0.20	5.6	Yes

Supplementary Table 3.2. List of 144 white lupin breeding lines (reference population 2). For each line, the following information is reported: name, cross, female parent, male parent, and mean values of plant mortality proportion and visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform.

Line	Cross	Female parent	Male parent	Mortality	Visual score
1.09	1	Lucky	Gr56	0.52	7.4
1.22	1	Lucky	Gr56	0.45	7.5
1.31	1	Lucky	Gr56	0.59	8.1
1.44	1	Lucky	Gr56	0.50	7.3
1.51	1	Lucky	Gr56	0.53	7.5
1.62	1	Lucky	Gr56	0.35	6.9
1.71	1	Lucky	Gr56	0.25	6.6
1.74	1	Lucky	Gr56	0.30	6.4
1.87	1	Lucky	Gr56	0.49	7.3
2.08	2	MB-38	Gr56	0.17	6.3
2.29	2	MB-38	Gr56	0.28	5.9
2.47	2	MB-38	Gr56	0.39	7.5
2.56	2	MB-38	Gr56	0.30	7.0
2.70	2	MB-38	Gr56	0.29	6.2
2.91	2	MB-38	Gr56	0.44	7.4
3.03	3	Arsenio	Gr56	0.77	8.9
3.07	3	Arsenio	Gr56	0.61	8.0
3.09	3	Arsenio	Gr56	0.74	8.9
3.17	3	Arsenio	Gr56	0.50	7.6
3.35	3	Arsenio	Gr56	0.68	8.2
3.39	3	Arsenio	Gr56	0.66	8.2
3.42	3	Arsenio	Gr56	0.41	6.8
3.75	3	Arsenio	Gr56	0.85	9.4
4.05	4	L27PS3	Gr56	0.16	5.9
4.07	4	L27PS3	Gr56	0.31	7.0
4.12	4	L27PS3	Gr56	0.67	8.4
4.23	4	L27PS3	Gr56	0.78	8.6
4.25	4	L27PS3	Gr56	0.29	6.8
4.53	4	L27PS3	Gr56	0.68	8.5
4.56	4	L27PS3	Gr56	0.49	8.3
4.57	4	L27PS3	Gr56	0.30	7.3
4.66	4	L27PS3	Gr56	0.86	9.6
4.67	4	L27PS3	Gr56	0.50	7.7
5.06	5	Lucky	La646	0.73	8.6
5.12	5	Lucky	La646	0.51	7.9
5.23	5	Lucky	La646	0.50	7.4
5.28	5	Lucky	La646	0.61	8.1
5.34	5	Lucky	La646	0.65	8.5
5.40	5	Lucky	La646	0.50	7.5
5.43	5	Lucky	La646	0.37	7.1
5.50	5	Lucky	La646	0.52	7.5
5.51	5	Lucky	La646	0.70	8.6
6.01	6	MB-38	La646	0.40	7.3
6.30	6	MB-38	La646	0.21	6.3
6.41	6	MB-38	La646	0.80	8.8
6.43	6	MB-38	La646	0.25	6.2
6.45	6	MB-38	La646	0.05	5.7
6.47	6	MB-38	La646	0.09	6.3
6.58	6	MB-38	La646	0.68	8.3
6.62	6	MB-38	La646	0.24	6.6
6.65	6	MB-38	La646	0.16	6.1
6.71	6	MB-38	La646	0.60	8.2
7.10	7	Arsenio	La646	0.46	7.5
7.25	7	Arsenio	La646	0.57	8.2
7.26	7	Arsenio	La646	0.38	7.2
7.41	7	Arsenio	La646	0.77	8.7

7.46	7	Arsenio	La646	0.56	8.3
7.47	7	Arsenio	La646	0.77	9.2
7.54	7	Arsenio	La646	0.72	8.7
7.56	7	Arsenio	La646	0.62	8.0
7.72	7	Arsenio	La646	0.58	7.9
8.02	8	L27PS3	La646	0.69	8.7
8.08	8	L27PS3	La646	0.71	8.6
8.14	8	L27PS3	La646	0.73	8.7
8.18	8	L27PS3	La646	0.87	9.7
8.34	8	L27PS3	La646	0.74	8.7
8.37	8	L27PS3	La646	0.91	9.7
8.49	8	L27PS3	La646	0.66	8.6
8.51	8	L27PS3	La646	0.61	8.1
8.55	8	L27PS3	La646	0.76	9.0
9.08	9	Lucky	La246	0.78	8.8
9.16	9	Lucky	La246	0.66	9.1
9.42	9	Lucky	La246	0.43	7.2
9.54	9	Lucky	La246	0.60	8.5
9.58	9	Lucky	La246	0.55	8.0
9.60	9	Lucky	La246	0.67	8.3
9.78	9	Lucky	La246	0.44	8.1
9.81	9	Lucky	La246	0.51	7.6
9.86	9	Lucky	La246	0.71	8.9
10.03	10	MB-38	La246	0.55	8.2
10.18	10	MB-38	La246	0.40	7.0
10.21	10	MB-38	La246	0.65	8.1
10.28	10	MB-38	La246	0.41	7.6
10.39	10	MB-38	La246	0.67	8.1
10.40	10	MB-38	La246	0.65	7.9
10.44	10	MB-38	La246	0.66	8.2
10.59	10	MB-38	La246	0.50	7.3
10.62	10	MB-38	La246	0.61	7.9
10.91	10	MB-38	La246	0.49	7.6
11.01	11	Arsenio	La246	0.55	8.1
11.02	11	Arsenio	La246	0.76	8.8
11.09	11	Arsenio	La246	0.77	9.2
11.10	11	Arsenio	La246	0.68	8.6
11.22	11	Arsenio	La246	0.69	8.7
11.23	11	Arsenio	La246	0.84	9.5
11.25	11	Arsenio	La246	0.73	8.4
11.37	11	Arsenio	La246	0.67	8.6
11.40	11	Arsenio	La246	0.44	7.1
11.57	11	Arsenio	La246	0.42	7.7
12.09	12	L27PS3	La246	0.87	9.5
12.12	12	L27PS3	La246	0.76	9.0
12.15	12	L27PS3	La246	0.66	8.4
12.25	12	L27PS3	La246	1.0	10.3
12.34	12	L27PS3	La246	0.82	9.3
12.36	12	L27PS3	La246	0.97	9.9
12.37	12	L27PS3	La246	0.75	8.8
12.44	12	L27PS3	La246	0.90	9.2
12.50	12	L27PS3	La246	0.92	9.8
13.08	13	Lucky	LAP123	0.91	9.2
13.19	13	Lucky	LAP123	0.65	8.1
13.24	13	Lucky	LAP123	0.94	9.7
13.27	13	Lucky	LAP123	0.65	8.4
13.31	13	Lucky	LAP123	0.75	8.5
13.32	13	Lucky	LAP123	0.89	9.2
13.45	13	Lucky	LAP123	0.91	9.1
13.50	13	Lucky	LAP123	0.91	9.3
13.55	13	Lucky	LAP123	0.60	7.7
13.60	13	Lucky	LAP123	0.68	8.3

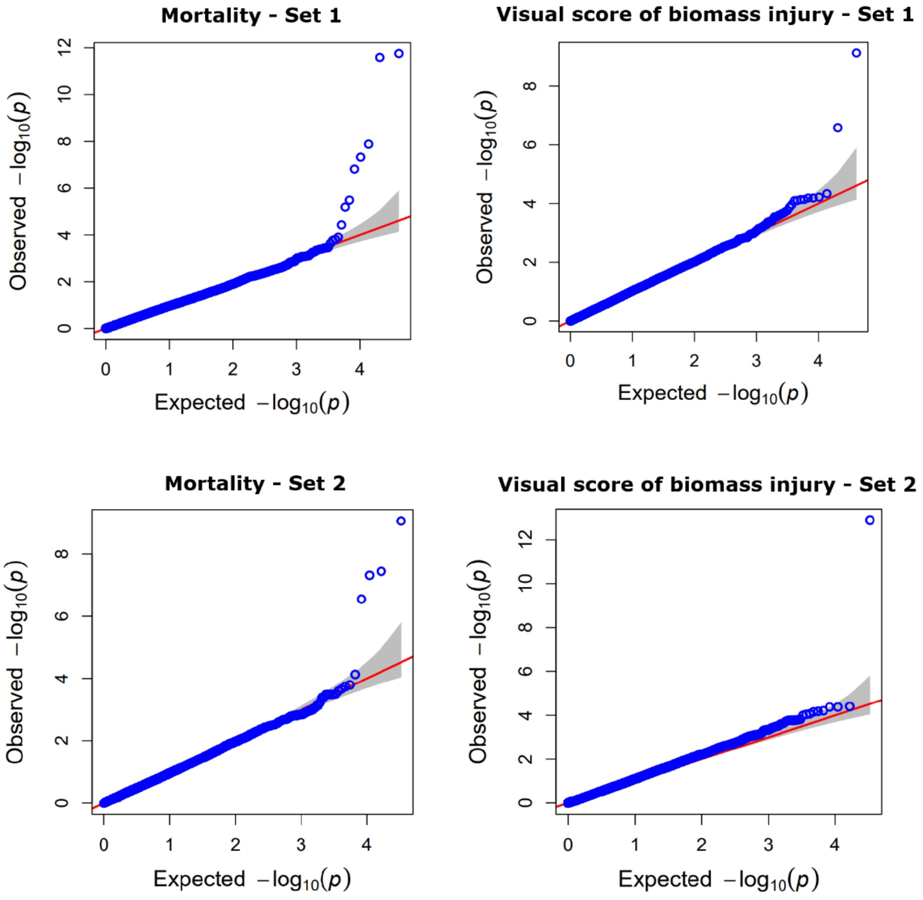
13.62	13	Lucky	LAP123	0.81	9.2
14.01	14	MB-38	LAP123	0.71	8.9
14.02	14	MB-38	LAP123	0.73	8.7
14.03	14	MB-38	LAP123	0.74	9.0
14.04	14	MB-38	LAP123	0.69	8.7
15.04	15	Arsenio	LAP123	0.65	8.0
15.05	15	Arsenio	LAP123	0.65	8.1
15.10	15	Arsenio	LAP123	0.59	8.1
15.17	15	Arsenio	LAP123	0.73	8.9
15.29	15	Arsenio	LAP123	0.76	8.7
15.32	15	Arsenio	LAP123	0.66	8.8
15.33	15	Arsenio	LAP123	0.67	8.9
15.36	15	Arsenio	LAP123	0.89	9.6
15.62	15	Arsenio	LAP123	0.89	9.4
15.91	15	Arsenio	LAP123	0.72	8.8
16.10	16	L27PS3	LAP123	0.85	9.3
16.20	16	L27PS3	LAP123	0.78	9.0
16.22	16	L27PS3	LAP123	0.83	9.3
16.29	16	L27PS3	LAP123	0.91	9.7
16.35	16	L27PS3	LAP123	0.94	9.4
16.37	16	L27PS3	LAP123	0.73	9.0
16.39	16	L27PS3	LAP123	0.96	9.4
16.52	16	L27PS3	LAP123	0.92	9.7
16.62	16	L27PS3	LAP123	0.74	8.8
16.77	16	L27PS3	LAP123	0.72	8.8
16.88	16	L27PS3	LAP123	0.81	9.3

Supplementary Table 3.3. Extent of linkage disequilibrium (LD) of white lupin landrace and cultivar genotypes (reference population 1) and breeding lines (reference population 2). LD extent values were estimated as the physical distances at which the LD decay curves crossed the population-specific critical r^2 threshold (0.046 for reference population 1; 0.067 for reference population 2), above which LD is likely due to physical linkage.

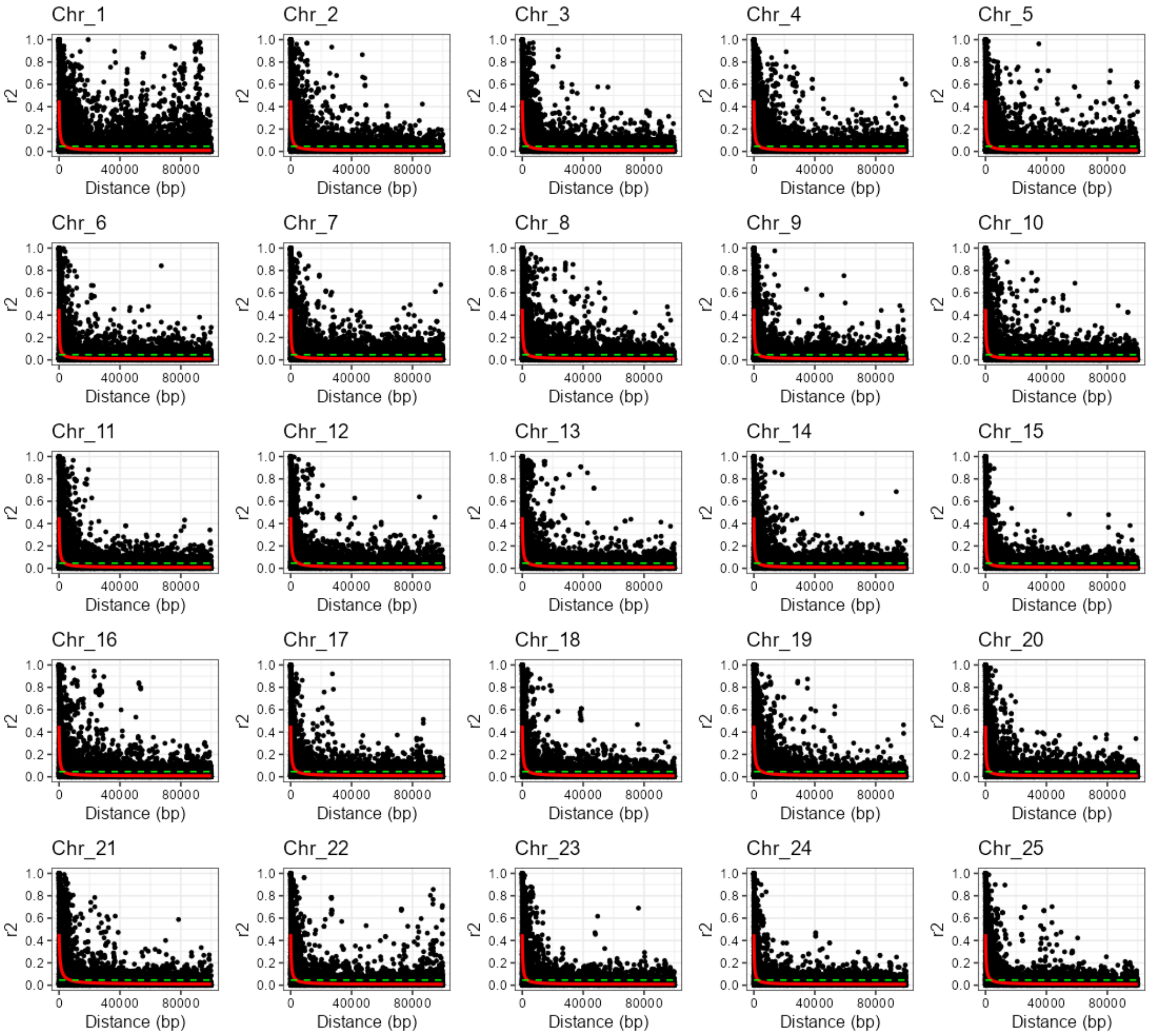
Chromosome	Reference population	LD extent (base pairs)
1	1	4134
1	2	10256
2	1	3649
2	2	9724
3	1	4295
3	2	9158
4	1	3611
4	2	11965
5	1	3368
5	2	7615
6	1	2862
6	2	5565
7	1	2577
7	2	5443
8	1	3794
8	2	5520
9	1	3152
9	2	5724
10	1	3117
10	2	5617
11	1	3751
11	2	6152
12	1	4970
12	2	10846
13	1	4044
13	2	7849
14	1	2940
14	2	6639
15	1	2513
15	2	3703
16	1	3151
16	2	6289
17	1	4464
17	2	8663
18	1	4213
18	2	12365
19	1	5291
19	2	8262
20	1	2837
20	2	5676
21	1	7248
21	2	7756
22	1	3822
22	2	6489
23	1	3475
23	2	6405
24	1	2791
24	2	4658
25	1	3925
25	2	5018

Supplementary Table 3.4. List of significant SNPs detected by a GWAS conducted on white lupin landrace and cultivar genotypes (reference population 1) and breeding lines (reference population 2) for plant mortality and a visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform, according to the false discovery rate threshold at 5%. The negative logarithm of *p*.value (-log10(*p*)), minor allele frequency (MAF), and estimated trait effect are reported for each SNP.

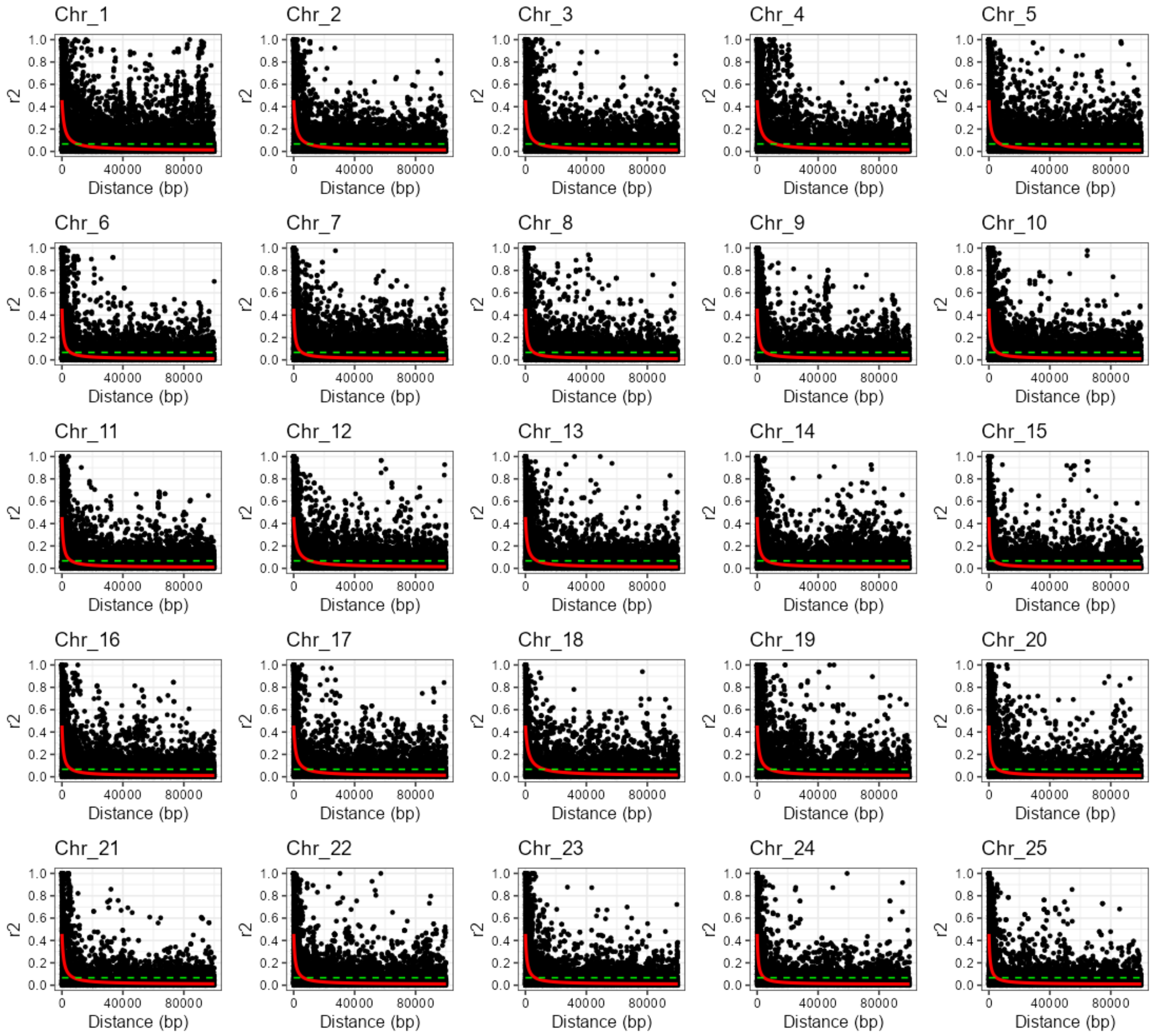
SNP	Population	Trait	$-\log_{10}(p)$	MAF	Effect
Chr05_4820341	1	Mortality	7.33	0.11	-0.07
Chr06_1878682	1	Mortality	7.88	0.19	0.06
Chr08_3511620	1	Mortality	5.49	0.31	0.04
Chr13_15386976	1	Mortality	11.75	0.33	-0.06
Chr14_10127694	1	Mortality	6.81	0.11	0.07
Chr16_5032297	1	Visual score of biomass injury	6.58	0.06	-0.61
Chr23_1146188	1	Mortality	11.59	0.15	-0.08
Chr23_1146188	1	Visual score of biomass injury	9.13	0.15	-0.48
Chr02_14306413	2	Mortality	9.05	0.06	-0.12
Chr02_14306413	2	Visual score of biomass injury	12.89	0.06	-0.73
Chr04_7632627	2	Mortality	7.31	0.29	0.06
Chr19_17886074	2	Mortality	6.55	0.10	-0.06
Chr21_1050253	2	Mortality	7.44	0.25	0.06



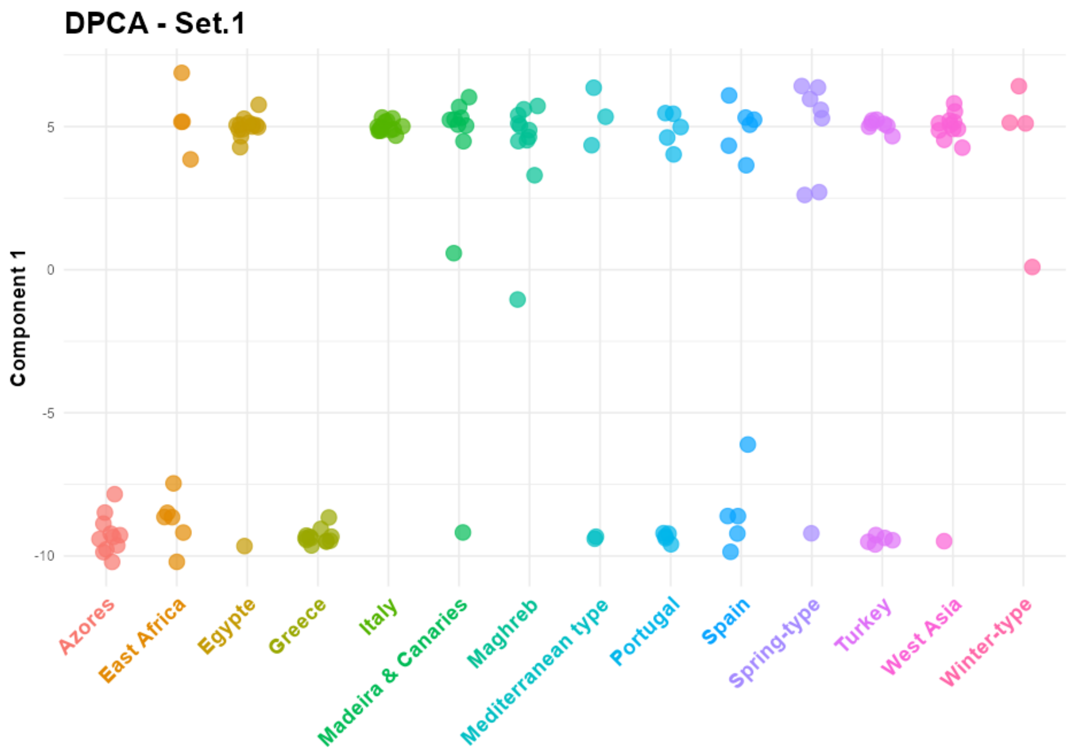
Supplementary Figure 3.1. Quantile-Quantile plots of expected vs. observed association scores for (a) 40,914 SNPs from 144 landrace and cultivar genotypes (reference population 1), and (b) 32,951 SNPs of 144 breeding lines (reference population 2), with plant mortality and visual score of biomass injury assessed at −11 °C freezing temperature in a phenotyping platform. The red line indicates equality between expected and observed quantiles; the grey area represents the associated 95% confidence interval.



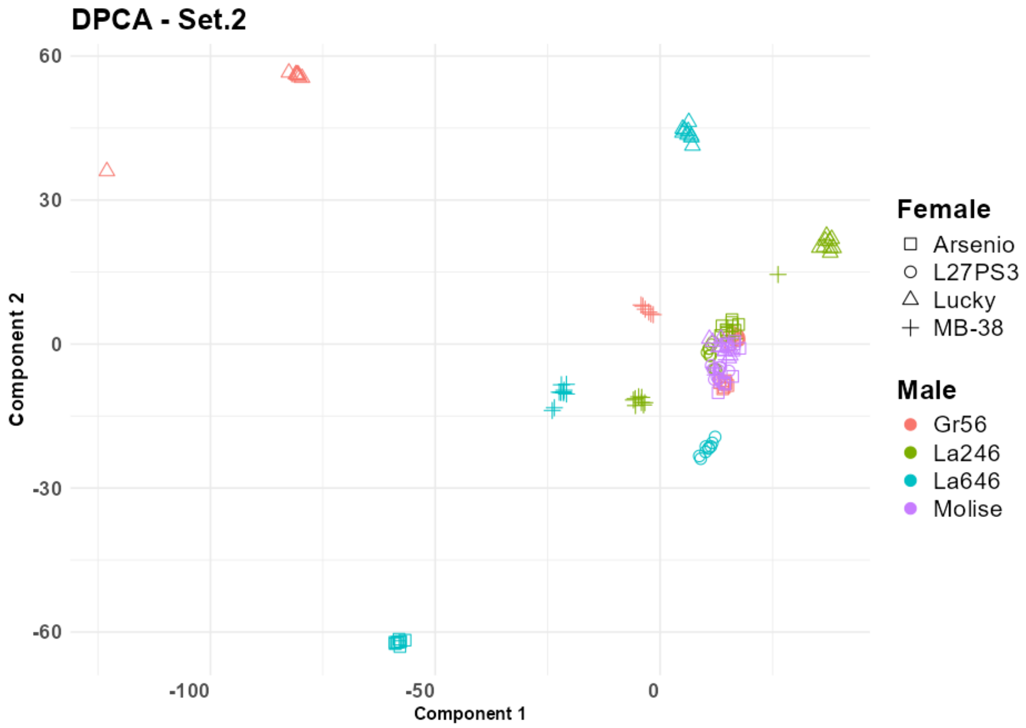
Supplementary Figure 3.2. LD decay plots for each chromosome for white lupin landrace and cultivar genotypes (reference population 1), based on r^2 values (Y axis) and physical distance in base pairs (X axis), estimated on pairwise combinations of 40,914 SNPs within a 100 kb window.



Supplementary Figure 3.3. LD decay plots for chromosome for white lupin breeding lines (reference population 2) based on r^2 values (Y axis) and physical distance in base pairs (X axis), estimated on pairwise combinations of 32,951 SNPs within a 100 kb window.



Supplementary Figure 3.4. Population structure of landrace and cultivar genotypes (reference population 1) based on the first discriminant function (component) of a discriminant principal component analysis. Genotypes are grouped into 14 gene pools based on origin (landraces) or phenological class (cultivars).



Supplementary Figure 3.5. Population structure of the breeding lines (reference population 2), based on the first two discriminant functions (components) of a discriminant principal component analysis. The symbol shapes represent the sweet-seed parent line (female), while the symbol colours represent the bitter-seed parent landrace.

4. Winter hardiness components in white lupin: frost escape, frost resistance, and seed weight

4.1. Abstract

Autumn sowing of white lupin is expanding northward to enable earlier harvesting and to avoid terminal drought. Enhancing winter survival is crucial for maximizing yield potential and economic returns; however, the actual contribution of frost survival-related traits remains debated. This study aims to assess the relative effects of frost escape, frost resistance, and seed weight on winter survival. A total of 115 landraces and cultivars evaluated for frost mortality under controlled conditions in Chapter 3 had also been assessed for field-based winter mortality, dry grain yield, and onset of flowering at Lodi (Northern Italy), and seed dry weight at Sanluri (Sardinia) during the 2004–2005 cropping season. A strong negative correlation between winter mortality and yield ($r = -0.88$; $P < 0.001$) was observed. Onset of flowering (as an indicator of frost escape), frost mortality under controlled conditions (as an inverse indicator of frost resistance), and seed dry weight (associated with root protection) together explained half of the phenotypic variability in field-based winter mortality in a multiple linear regression analysis (adjusted $R^2 = 0.49$). According to standardized β values, onset of flowering emerged as the main predictor, with an effect 1.76 times that of mortality under controlled conditions and 2.13 times that of seed dry weight. When the analysis was restricted to intermediate-flowering material, the effect of onset of flowering was 0.97 times that of mortality under controlled conditions, and the effect of frost resistance became prominent. The contribution of seed weight remained significant and moderate across both scenarios. Overall, simultaneous selection for these traits is expected to improve winter hardiness and, if combined with drought resistance and avoidance of late-flowering types, is likely to increase yield stability across years with cold-prone winters and/or drought-prone springs.

4.2. Introduction

The shift from spring to autumn sowing in suboceanic and subcontinental climate regions, such as northern Italy, aims to increase the yield potential of white lupin by taking advantage of a longer growing season and earlier harvesting, thus reducing the risk of terminal drought (Huyghe, 1997). However, improved winter hardiness (as estimated by winter plant survival) is a crucial breeding target. As detailed in Section 1.2.2, winter hardiness in white lupin is primarily determined by frost survival (Shield et al., 2000), although other factors, such as waterlogging or fungal diseases, may also have an impact. Frost survival, in turn, depends on frost escape and frost resistance mechanisms (Figure 1.4).

Frost escape occurs through delayed onset of flowering, which prevents the premature transition from the vegetative to the reproductive stage, the latter being highly sensitive to frost (Maqbool et al., 2010; Siddique et al., 1999). In white lupin, the primary factor regulating this transition is vernalisation requirement, followed by the accumulation of growing degree days (GDD), while photoperiod response appears less effective compared to other cool-season grain legumes (Huyghe and Papineau, 1990; Rychel-Bielska et al., 2024). Vernalisation requirement is genotype-specific and is satisfied by exposure to temperatures between 0 °C and 14 °C (Huyghe, 1991). For spring-type varieties 10 days at temperatures up to 12 °C are enough, whereas winter types require at least two weeks (optimally 3-4 weeks) at temperatures below 6 °C (Rychel-Bielska et al., 2024; Adhikari et al., 2012). White lupin maintains a juvenile rosette stage, characterised by a low height-to-leaf-number ratio, until vernalisation is fulfilled. Subsequently, the transition to the reproductive stage begins and is controlled by the total amount of accumulated temperature (expressed as GDD), leading to stem elongation and inflorescences development. This transition induces hormonal changes, such as an increase in gibberellin levels, thus lowering the abscisic acid to gibberellin ratio (Huyghe and Papineau, 1990), whereas an higher proportion of abscisic acid is recognized to delay growth and increase cold adaptation (Junttila et al., 2002). Studies on cereals indicate that the expression of genes involved in cold acclimation is downregulated once vernalisation is saturated, even before any generative development of the apex is visible under the microscope (Fowler et al., 1996; Laudencia-Chingcuanco et al., 2011).

Conversely, frost resistance, which is the main focus of this thesis, is the sum of phenology-independent components of frost survival. It is defined as a positive deviation from the genotype observed frost survival relative to the expected according to its flowering onset, and its mechanisms have been detailed in sections 1.2.2 and 1.2.3. In brief, it relies on frost avoidance and frost tolerance. Frost avoidance refers to the plant’s ability to prevent ice formation within its tissues, whereas frost tolerance is the plant’s ability to withstand the presence of ice crystals (Levitt, 1980). Both strategies primarily rely on a process of cold acclimation (hardening), induced by photoperiod shortening and exposure to low, non-freezing temperatures.

Most studies on frost resistance focus on aboveground organs, while root resistance mechanisms remain poorly understood (Ambroise et al., 2020). Huyghe and Papineau (1990) identified root parenchyma thickness as a key factor in root protection. Parenchyma tissue is characterized by wide intercellular spaces and the ability of cells to shrink significantly. During freezing, water moves out of the cells allowing for the formation of large ice crystals in the intercellular spaces, which reduces the risk of intracellular freezing (McCully et al., 2004). Root parenchyma thickness is unlikely to be influenced by cold acclimation; rather, it depends on genotype and sowing time. Seed weight is an easy-to-measure trait that significantly affects organogenesis and seedling growth: heavier seeds promote more vigorous seedlings with more extensive root systems and thicker parenchyma layers (Huyghe, 1993). Greater seed weight is expected to enhance crop establishment under severe conditions during the early growth of autumn-sown crops. However, late sowing compromise a sufficient elongation of the root parenchyma (Huyghe and Papineau, 1990).

In Chapter 3, the correlation between field-based winter survival and frost resistance (measured under controlled conditions) for 115 landraces and cultivars was relatively low, though statistically significant ($r = 0.29$; $p < 0.01$). A subsequent linear regression analysis of field-based plant mortality as a function of platform-based mortality, including an indication of three phenological groups of accessions (early-, intermediate-, and late-flowering), highlighted the role of phenology (via frost escape) on field mortality (Figure 3.3). Many accessions exhibiting higher field mortality than the expected value based on platform mortality were early-flowering, whereas those with lower field mortality than expected tended to be late-flowering.

The objectives of this chapter are (a) to further investigate the effect of frost resistance, as well as well as those of frost escape, and seed weight (higher seed weight is assumed to be associated with rapid root parenchyma elongation) on winter survival, and (b) to propose a viable strategy for developing autumn-sown varieties with high yield stability across years in Northern Italy and other regions with similar climatic conditions. Although the study is limited by the availability of only one year of field data, it is justified by the practical relevance of the research questions and by the need to assess the effectiveness of controlled-condition screenings.

4.3. Material and methods

4.3.1. Data collection

This study included 115 white lupin accessions, previously evaluated for frost mortality under controlled conditions in Chapter 3, and for field-based winter mortality, onset of flowering, seed dry weight, and grain yield in Annicchiarico et al. (2010). The accessions include 107 landraces representing 11 regional germplasm pools corresponding to the main historical white lupin cropping regions, and 8 French cultivars, comprising four spring types and four winter types. The complete list is provided in Supplementary Table 3.1, where landraces and cultivars used in this study are marked with “Yes” in the column labelled “Field data”. Nine cultivars had been evaluated under controlled conditions but not in the field and could not be used for this study.

Frost mortality under controlled conditions was evaluated on at least one randomly chosen genotype per accession, although in 20 cases two genotypes per landrace were assessed. Genotypes selected from the same landrace were genetically distinct, as confirmed by the observed molecular marker diversity, consistent with the expected intra-landrace variation. The evaluation followed a −11 °C freezing treatment, as described in Section 3.3.2.

Field-based winter mortality, onset of flowering, and dry grain yield were evaluated during the 2004–2005 cropping season at Lodi, on these and other accessions. Lodi, in northern Italy, is representative of a subcontinental climate characterised by extended frosts in winter and moderate terminal drought. The sowing date was October 14th, while further details on field agronomic management are reported in Annicchiarico et al. (2010). Minimum, maximum, and mean air temperatures (°C), as well as cumulative precipitation (mm), were recorded on-site, aggregated by a ten-day period, and are reported in Figure 4.1. Onset of flowering was measured as the number of days from January 1st to the date on which 50% of the plants flowered. Winter plant mortality was assessed by counting plants at emergence and at the end of winter along one linear metre in each of two rows. Dry grain yield was measured on a plot basis and converted in t/ha. Due to some missing data for seed dry weight in the Lodi dataset, data collected in Sanluri (Sardinia), Italy, during the same cropping season (2004–2005), and belonging to the same study, were used. Sanluri is representative of the Mediterranean climate with fairly mild winters and terminal drought and heat stress. Despite the substantial climatic differences between Lodi and Sanluri, the use of data from the latter environment is justified by the high genetic correlation between these environments for seed dry weight ($r_g = 0.86$; $P < 0.01$; Annicchiarico et al., 2010). Both soils, in Lodi and Sanluri, were suitable for white lupin cultivation (pH ≤ 7.4; active lime ≤ 0.4%).

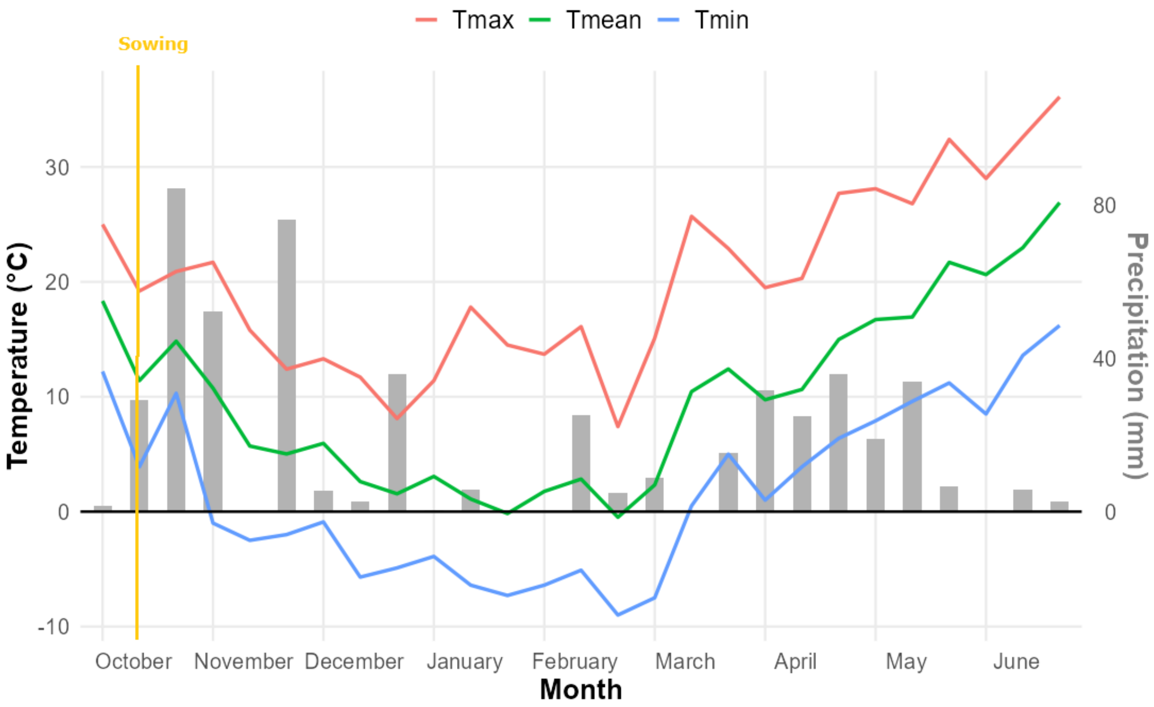


Figure 4.1. Maximum (Tmax), minimum (Tmin), and mean (Tmean) air temperatures (°C), and cumulative precipitation (mm) recorded during the 2004-2005 white lupin cropping season, at Lodi, Italy. Data are aggregated on a ten-day period.

4.3.1. Statistical analysis

Frost mortality data had already been analysed according to an alpha-lattice design, adjusted means (BLUE values) had been computed (Section 3.3.3), reported in Supplementary Table 3.1, and used in this study. When two genotypes from the same landrace were assessed, the average mortality value was calculated to obtain a single representative value for each accession. Conversely, data collected in Annicchiarico et al. (2010), namely field-based winter mortality, onset of flowering, dry grain yield, and seed dry weight were analysed ex novo, according to an alpha-lattice design with three replications and nine incomplete blocks within each replication. The following linear mixed model was employed to compute adjusted genotype means (BLUE values):

$$Y_{irb} = \mu + G_i + P_r + B_{rb} + \mathcal{E}_{irb}; \qquad \mathcal{E}_{irb} \sim N(0, \sigma_e^2)$$

where the response variable Y_{irb} is a function of the trait mean μ, the genotype (G_i) treated as a fixed factor, and replicate (P_r) and block within replicate (B_{rb}) treated as random factors, with $\mathcal{E}_{irb}$ representing the model residual. The same model but holding genotype (G_i) as a random factor, was used for estimating genotype (σ_g^2), and experiment error (σ_e^2) variance components by the restricted maximum likelihood (REML) method. This analysis aimed to compute genetic coefficient of variation (CV_g) and broad-sense heritability on an entry mean basis (H^2) values as follows:

$$CV_g = (\sigma_g / \mu) \times 100$$

$$H^2 = \frac{\sigma_g^2}{\left(\sigma_g^2 + \frac{\sigma_e^2}{n}\right)}$$

where σ_g is the square root of the genotype variance component, μ is the trait mean, and n is the number of experimental replications.

BLUE values of 115 landraces and cultivars evaluated under field and controlled conditions along with those restricted to 76 accessions with intermediate onset of flowering constituted the two datasets object of this study, which were analysed separately. Accessions with onset of flowering within the interval defined by the mean minus 0.5 × standard deviation and the mean plus 0.5 × standard deviation were classified as intermediate. The intermediate onset of flowering dataset was used to investigate a scenario in which extreme flowering types are avoided, which reflects an expected breeding generation. Pearson correlation coefficients between traits were computed and a multiple linear regression analyses with field-based winter mortality as function of frost mortality under controlled conditions, onset of flowering, and seed dry weight was performed. Based on Pearson correlation coefficients, onset of flowering was chosen as the initial variable in both scenarios (supplementary tables 4.1 and 4.2). Subsequently, variables were added one at a time from the remaining ones, based on the adjusted R^2 (R^2_{adj}) maximisation criteria, provided that their partial regression coefficient was significantly different from zero ($P \leq 0.05$) (Supplementary Table 4.3). Finally, to compare the contribution of each independent variable in determining field-based winter mortality, standardized β coefficients (β_{std}), which are independent of the units of measurement, were calculated according to the following formula:

$$\beta_{std} = \frac{\sigma_x}{\sigma_y} \times \beta$$

where the standardized β coefficient for each independent variable is equal to the ratio between the standard deviation of the independent variable and that of the dependent variable (field-based winter mortality), multiplied by its partial regression coefficient.

4.4. Results

4.4.1. Phenotypic and genetic variation

Climatic data (Figure 4.1) confirmed the occurrence of prolonged frost stress during the winter cropping season, with freezing temperatures up to –9 °C. A strong negative correlation ($r = -0.88$; $P < 0.001$; Supplementary Table 4.1) was observed between field-based winter mortality (%) and yield (t/ha).

Table 4.1 summarises the mean, range, broad-sense heritability on an entry mean basis (H^2), and genetic coefficient of variation (CV_g%) for yield (t/ha), field-based winter mortality (%), onset of flowering (days from January 1st), seed dry weight (mg), and frost mortality under controlled conditions (%). Yield exhibited considerable variability, ranging from 0 to 9.04 t/ha, with a mean of 4.72 t/ha and a genetic coefficient of variation of 47.9%. The lowest-yielding accession was the Turkish landrace Tr001 (0 t/ha), which exhibited 99.1% winter mortality, whereas the highest-yielding accession was the Italian landrace Ita057, with 13.1% winter mortality. These contrasting performances further highlight the primary role of winter survival in determining yield. Plant mortality ranged from 8.2% to 96.2% under controlled conditions, with a mean of 53.6%, and from 0.1% to 99.1% in the field, with a mean of 33.6%. Overall mortality was higher under controlled conditions, reflecting the lower frost temperature experienced by plants (−11 °C vs. −9 °C). High phenotypic variability was observed under both conditions, but the genetic coefficient of variation was particularly high for field-based winter mortality (CV_g = 96.3%), indicating substantial genetic variability for this trait. The onset of flowering had a mean of 117.7 days and relatively low genetic variability (CV_g = 4.2%). Seed dry weight averaged 0.33 mg, with intermediate genetic variability (CV_g = 23.2%). Broad-sense heritability values on an entry mean basis (H^2) were high for all traits, ranging from 0.77 to 0.98.

Table 4.1. Mean, range, broad-sense heritability on an entry mean basis (H^2), and genetic coefficient of variation (CV_g%) for yield (t/ha), field-based mortality (%), onset of flowering (days from January 1st), seed dry weight (mg), and frost mortality under controlled conditions (%), for 115 landraces and cultivars.

Data	Mean	Min	Max	H^2	*CVg%*
Yield (t/ha)[a]	4.72	0	9.04	0.92	47.9
Winter mortality (%)[a]	33.6	0.1	99.1	0.98	96.3
Onset of flowering (days)[a]	117.7	94.0	130.7	0.87	4.2
Seed dry weight (mg)[b]	0.33	0.19	0.69	0.93	23.2
Controlled conditions mortality (%)[c]	53.6	8.2	96.2	0.77	32.0

[a] Evaluation performed during the 2004-2005 cropping season at Lodi, in northern Italy, representative of the subcontinental climate with extended frosts in winter and moderate terminal drought.

[b] Data collected during the 2004-2005 cropping season, at Sanluri (Sardinia), in Italy, representative of the Mediterranean climate, with fairly mild winters and terminal drought and heat stress.

[c] Assessed at −11 °C freezing temperature in a phenotyping platform.

4.4.1. Assessment of winter mortality predictors

Multiple linear regression analysis revealed that the onset of flowering (as an indicator of frost escape), frost mortality under controlled conditions (as an inverse indicator of frost resistance), and seed dry weight (assumed to be associated with rapid root parenchyma elongation and thus root protection) are significant predictors of field-based winter mortality. This was true both when all accessions were included in the multiple linear regression analysis and when the analysis was restricted to accessions with intermediate onset of flowering (Supplementary Table 4.3). The regression model fitted on all 115 accessions achieved an adjusted R^2 (R^2_{adj}) of 0.49, indicating that these predictors jointly explain around half of the variability in the response variable. Although this value is moderate, it is substantially higher than the adjusted R^2 of 0.08 obtained using frost mortality under controlled conditions alone (Figure 3.3). When the analysis was restricted to accessions with intermediate flowering times, the adjusted R^2 decreased from 0.49 to 0.31. This reduction reflects the narrower variability in onset of flowering and mainly the exclusion of early-flowering accessions with high field-based mortality.

Table 4.2 presents the partial regression coefficients (β) and standardized partial regression coefficients (β_{std}) for each predictor across both scenarios, allowing for a direct comparison of their relative effects. Across scenarios, winter mortality decreases with the onset of flowering and seed dry weight but increases with frost mortality under controlled conditions. The effect of the onset of flowering was particularly relevant when analysed using the full dataset, emerging as the strongest predictor ($\beta_{std} = -0.51$), followed by frost mortality under controlled conditions ($\beta_{std} = 0.29$) and seed dry weight ($\beta_{std} = -0.24$). However, the effect of onset of flowering diminished in the intermediate-flowering subset ($\beta_{std} = -0.33$) reflecting the reduced variability in this trait, becoming slightly lower than the effect of frost mortality under controlled conditions ($\beta_{std} = 0.34$), while the effect of seed dry weight remained stable ($\beta_{std} = -0.24$).

Table 4.2. Coefficients (β) and standardized coefficients ($\beta_{standardized}$) from the multiple regression models assessing the effects of onset of flowering (days from January 1st), seed dry weight (mg), and frost mortality under controlled conditions (%) on field-based winter mortality (%). Estimates are reported for two datasets: one including all accessions, and the other restricted to accessions with intermediate flowering time.

	All accessions		**Intermediate-flowering accessions**	
	β^a	β_{std}	β^a	β_{std}
Intercept	4.07***	-	10.15***	-
Onset of flowering (days)	−0.03***	−0.51	−0.08**	−0.33
Controlled condition mortality (%)	0.52***	0.29	0.53***	0.34
Seed dry weight (mg)	−1.10**	−0.24	−1.28*	−0.24

[a] Statistical significance is indicated as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

4.5. Discussion

The main limitation of this study is the availability of field data from a single environment (one year and location), making the results site- and year-specific, considering that numerous factors affect winter survival (Bélanger et al., 2006). Although the following considerations primarily pertain to the experimental context, climatic data collected during the crop cycle and previous findings in the literature allow for broader interpretations. Winter temperatures during the cropping season in Lodi were cooler than average, and minimum temperatures, grouped on a 10-days basis, were below zero by the first decade of November, with negative peaks of −5.7 °C in December, −7.3 °C in January, −9.0 °C in late February, and −7.5 °C in early March, with extended frost exposure until late winter. During this period, mean temperatures over each 10-day period ranged from −0.5 °C to 5.9 °C, with a maximum of 17.8 °C recorded in mid-January (Figure 4.1).

The strong negative correlation between winter mortality and yield ($r = -0.88$; $P < 0.001$; Supplementary Table 4.1) confirms that winter survival is a major determinant of agronomic performance and highlights the need to improve winter hardiness in autumn sown white lupin to enhance its productivity, economic returns, cultivation and environmental benefits (Chapter 1). The high broad-sense heritability ($H^2 = 0.98$) and genetic coefficient of variation ($CV_g\% = 96.3$) for field-based winter mortality are encouraging. However, high heritability does not imply replicability across years and environments due to numerous environmental factors affecting winter mortality and the expected high genotype-by-environment (G×E) interactions. Annicchiarico and Iannucci (2007) assessed winter mortality on 11 white lupin cultivars across two locations and two sowing times per location, reporting a broad-sense heritability across environments of 0.73, which is lower than in the present study but still moderately high. Nevertheless, the increasing climatic variability across years is expected to negatively affect the replicability of mortality assessments (Avia et al., 2013). This study compares the relative effects of frost resistance (mortality under controlled conditions), frost escape (delayed onset of flowering), and seed weight (assumed to be associated with rapid root parenchyma elongation and thus root protection) on field-based winter survival. According to a multiple regression analysis, all these variables are statistically significant ($P < 0.05$).

Considering all landraces and cultivars, onset of flowering emerged as the main predictor, with an effect 1.76 times that of mortality under controlled conditions and 2.13 times that of seed dry weight. According to the regression model, an increase of one day on onset of flowering reduces the probability of death by 3%. However, when the analysis was restricted to intermediate-flowering material, the effect of onset of flowering was 0.97 times than that of mortality under controlled conditions. In this scenario, each additional day on onset of flowering can reduce the probability of death by 8%, highlighting the importance of accounting for onset of flowering even when extreme types are excluded from evaluation. The association between delayed flowering and winter survival is well documented in various cool-season legumes (Link et al., 2010; Maqbool et al., 2010). In pea, the flowering locus *Hr*, which controls photoperiod response (Murfet, 1973), co-localizes with a major QTL explaining winter survival, highlighting the key role of delayed flowering in enhancing winter hardiness (Lejeune-Hénaut et al., 2008). In white lupin Annicchiarico et al. (2010) had already identified late flowering as a specific adaptation trait to cold-prone autumn-sown environments. Although early-flowering accessions in thesis study began flowering at the beginning of April, the expression of genes involved in cold acclimation is likely downregulated earlier, as soon as once vernalisation is saturated, even before any generative development of the apex is microscopically visible, as observed in cereals (Fowler et al., 1996; Laudencia-Chingcuanco et al., 2011). Breeding for frost escape in white lupin is facilitated by the high genetic correlation of onset of flowering across environments (r_g = 0.76–0.96; Annicchiarico et al., 2010) and recent advances in marker-assisted selection for vernalization responsiveness (Rychel-Bielska et al., 2024; Surma et al., 2025).

Frost mortality under controlled conditions increased the adjusted R^2 by only 0.071 when considering all accessions and by 0.112 for intermediate-flowering accessions. This limited improvement reflects its low, albeit significant, correlation with field winter mortality ($r = 0.29$; $P < 0.01$). As anticipated in Chapter 3, studies on other legumes have reported higher correlations for plant mortality between artificial and field conditions, approaching 0.7 (Homer et al., 2016) and 0.5–0.6 for pea (Auld et al., 1983), and 0.5 for faba bean (Arbaoui et al., 2008) and red clover (Zanotto et al., 2021). A possible explanation to the low correlation obtained in this study is that accessions were evaluated under controlled conditions as one or two randomly chosen genotypes. While for cultivars the selected genotype is expected to be highly representative due to genetic uniformity within cultivars, this does not apply to landraces, which are genetically diverse populations. This genetic diversity, confirmed by a recent molecular study (data not yet published), reduced the representativeness of the tested genotypes, negatively affecting the correlation between controlled and field mortality. For example, among the landraces assessed with two genotypes, E064 showed the greatest difference between genotypes: one exhibited 70% mortality and the other 17% (Supplementary Table 3.1), while in the field its mortality was 19%, confirming that a single evaluated genotype may not represent the entire landrace. Considering these limitations, and the stronger correlations reported for similar crops, the results of this study support the genetic improvement of frost resistance under controlled condition.

Seed dry weight showed an inverse correlation with field winter mortality, confirming that larger seeds enhance tolerance to unfavourable winter conditions (Huyghe, 1993), likely through faster root parenchyma elongation, and thus root protection (Huyghe and Papineau, 1990). However, its additive effect in the model was modest, as the adjusted R^2 increased by only 0.043 in both cases. An explanation of the low effect of seed dry weight on winter mortality is that frost injury becomes lethal when affects epicotyl (Murray et al., 1988), while on the roots it negatively impacts water and mineral absorption and overall plant fitness (Ambroise et al., 2020), but additional stresses, likely present in the field, are required to kill plants. This is further supported by the lack of correlation between seed dry weight and frost mortality under controlled chamber conditions, where no additional stresses occur.

The full model fitted including all accessions, proved an adjusted R^2 of 0.49. This moderate value indicates that frost escape, frost resistance, and seed dry weight together explain approximately half of the phenotypic variability in winter mortality. Besides the reduced representativeness of genotypes tested under controlled conditions, other factors contribute to the unexplained variance. Factors other than frost may have affected winter survival, such as waterlogging and fungal diseases, particularly *Pleiochaeta setosa*, *Fusarium* spp., and *Colletotrichum gloeosporioides* (anthracnose; Huyghe, 1997; Bateman, 1997). De-acclimation, defined as the loss of frost resistance due to a mild weather spell, can increase frost injury when followed by a rapid drop to freezing temperatures and is a typical threat in late winter or spring (Gu et al., 2008; Rapacz et al., 2014). Specific resistance to de-acclimation and/or the ability to quickly reacclimate upon exposure to low temperatures are expected to be critical for winter survival. Although winter rainfall was not extreme (Figure 4.1), no waterlogging or relevant diseases were reported in Annicchiarico et al. (2010), and temperature trends do not indicate a high probability of de-acclimation during the trial, these factors might be secondary sources of unexplained variance (Figure 4.1) in winter mortality.

This study demonstrates that improving winter survival in autumn-sown white lupin can enhance crop yield and cultivation, providing practical guidance for its selection. A minimum vernalisation requirement is necessary to avoid early-flowering varieties, which rapidly lose frost resistance and are more susceptible to late frost events, which are expected to become more frequent in the future (Francis and Skific, 2025). Since a late onset of flowering increases the risk of damage from terminal drought, the selection of an intermediate flowering onset is recommended. Frost resistance assessed under controlled conditions was particularly effective when evaluated in intermediate-flowering accessions, although its predictive power was limited by the potential lack of representativeness of genotypes under controlled conditions. Finally, seed weight is an easy-to-measure trait with a modest contribution to winter survival and a positive association with yield. The simultaneous selection of these traits is expected to improve winter hardiness and, if associated with drought resistance, is likely to increase yield stability across years with cold-prone winters and/or drought-prone springs.

4.3. Supplementary material

Supplementary Table 4.1. Correlations among frost mortality under controlled conditions (%; following −11 °C), field-based winter mortality (%), onset of flowering (days from January 1st), seed dry weight (mg), and yield (t/ha) for 115 landraces and cultivars.

	Winter mortality (%)	**Onset of flowering (days)**	**Seed dry weight (mg)**	**Yield (mg)**
Controlled conditions mortality (%)	0.29**	−0.04	0.10	−0.25**
Winter mortality (%)	-	−0.62***	−0.43***	−0.88***
Onset of flowering (days)	-	-	0.42***	0.58***
Seed dry weight (mg)	-	-	-	0.54***

*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; NS not significant ($P > 0.05$)

Supplementary Table 4.2. Correlations among frost mortality under controlled conditions (%; following −11 °C), field-based winter mortality (%), onset of flowering (days from January 1st), seed dry weight (mg), and yield (t/ha) for 76 landraces and cultivars with intermediate flowering time

	Winter mortality (%)	**Onset of flowering (days)**	**Seed dry weight (mg)**	**Yield (mg)**
Controlled conditions mortality (%)	0.36**	−0.04	−0.02	−0.35**
Winter mortality (%)	-	−0.41***	−0.34**	−0.89***
Onset of flowering (days)	-	-	0.27*	0.42***
Seed dry weight (mg)	-	-	-	0.47***

*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$; NS not significant ($P > 0.05$)

Supplementary Table 4.3. Forward selection of independent variables in a multiple regression model assessing the contribution of onset of flowering (days from January 1st), seed dry weight (mg), and frost mortality under controlled conditions (%; following −11 °C frost treatment) to explain field-based winter mortality (%). Independent variables were retained based on their statistical significance and the maximization of adjusted R^2. The analysis was conducted on two datasets: one including all accessions, and the other restricted to accessions with intermediate onset of flowering.

Independent variable			**R^2_{adj}**	
Controlled condition mortality (%)	**Onset of flowering (days)**	**Seed dry weight (mg)**	**All accessions**	**Intermediate -flowering accessions**
-	$P < 0.001$	-	0.385	0.157
$P < 00.01$	$P < 0.001$	-	0.451	0.269
$P < 0.001$	$P < 0.01$[a]	$P < 0.05$[b]	0.494	0.312

[b] Onset of flowering (days) resulted significant at $P < 0.01$ in the analysis restricted to intermediate flowering accession, and at $P < 0.001$ when all accessions were included.

[b] Seed dry weight (mg) resulted significant at $P < 0.05$ in the analysis restricted to intermediate flowering accession, and at $P < 0.01$ when all accessions were included.

5. Conclusions

Autumn sowing has rising interest for cool-season grain legumes in various European sub-oceanic and sub-continental climatic regions, where the shift from spring sowing can extend the crop cycle and anticipate harvesting, thereby reducing the increasing risk of terminal drought. Despite the trend towards milder winters, frost is expected to remain a major threat, as sudden events can be detrimental to poorly-acclimated plants, making genetic improvement for frost resistance essential. Phenotypic selection under controlled conditions can be very important in this respect, allowing to focus on frost resistance, which is independent from the effect of phenology (based on the assessment of genotypes at the vegetative stage) and to overcome field limitations caused by climatic variability across years. The genome-enabled prediction of frost resistance could be the second element of a breeding strategy (by which phenotyping under controlled conditions and genome-enabled prediction reinforce each other).

This thesis aimed to contribute to the genetic improvement of frost resistance in white lupin, a grain legume with increasing interest as a high-protein food or feed crop, by investigating the trait genetic control and providing practical guidance for phenotypic and genomic selection. A methodological study involving 11 genotypes with substantial variation in field-based winter survival (Chapter 2) laid the foundation for a subsequent large-scale evaluation of two reference populations, comprising 144 landrace and cultivar genotypes and 144 inbred lines (Chapter 3). They were conducted it a high-throughput phenotyping platform located at CREA-ZA, Lodi, Italy (Section 2.3.2). Some 107 landraces and 8 cultivars that had previously been evaluated for winter survival under field conditions in Lodi were used to assess the effects of frost resistance and other traits that may contribute to field-based winter survival (Chapter 4).

According to the experimental protocol detailed in Section 2.3.2, a –11 °C freezing temperature was identified as optimal treatment capable of maximising the genotypic variability for frost mortality (Section 2.4). The high consistency observed among the results from plant mortality at –11 °C and the lethal temperature 50 (LT_{50}) based on four freezing treatments indicates the reliability of measuring frost resistance using a single optimal freezing treatment. This method is more suitable than LT_{50} computation for evaluating large numbers of genotypes, as expected when screening germplasm collections or breeding lines. Biomass injury, visually scored, provided indication highly consistent with those for plant mortality, although it was less sensitive in detecting genetic variation (Section 3.4.1). This indicator may play an important role in frost resistance evaluation when only a limited number of plants per genotype are available, a situation that reduces the reliability of mortality estimates.

Consistently with previous field-based results (Annicchiarico and Iannucci, 2007), white lupin was found to be less resistant than pea (LT_{50} = –11 °C vs. –12.8 °C; Section 2.4). The frost resistance improvement of white lupin is supported by high broad-sense heritability estimates for both plant mortality and biomass injury following exposure to –11 °C (H^2 = 0.76–0.82), substantial genetic variation (particularly for plant mortality), consistency between parental and progeny responses, and the identification of genetic resources exhibiting exceptional frost resistance that can be used as parental germplasm in breeding programmes (Section 3.4.1). However, winter-type and Mediterranean-type cultivars tended to show greater frost resistance than landrace germplasm pools, indicating that the limited breeding efforts previously carried out for this trait, especially in France (Papineau and Huyghe, 2004), was successful.

The studies of GWAS on plant mortality and visually scored biomass injury, conducted on the two reference populations, identified 11 significant SNPs in linkage disequilibrium with 21 candidate genes, 12 of which have a putative role in the frost stress response (Table 3.3). Nevertheless, a polygenic trait architecture is suggested by the presence of several peaks in the Manhattan plots that did not reach the significance threshold and are likely to be associated to minor-effect genes (Section 3.4.2). This interpretation is further supported by the minimal decrease in predictive ability of the genomic prediction model fitted on the genetically broad population (landraces and cultivars) when moving from intra-population to cross-population prediction of inbred lines (0.414 vs. 0.393 for plant mortality), despite the marked inconsistency between the two reference populations regarding significant SNPs identified in the GWAS.

Genomic selection, considered the most effective form of marker-based selection for polygenic traits (Section 3.4.3), demonstrated high predictive abilities (0.67–0.68) in the intra-population scenario for inbred lines, encouraging its adoption to restrict the number of lines from a genetic base that are subjected to platform-based evaluation (thereby reducing the phenotyping costs). Despite the lower predictive ability (0.41) observed in the intra-population scenario for landrace and cultivar genotypes, attributable to the greater genetic variation and faster LD decay in this material, genomic selection could still be employed to preliminarily identify genetic resources with putative frost resistance within extensive germplasm collections, although subsequent phenotypic validation is recommended. Prediction according to the inter-population scenario indicate that genomic prediction models fitted on a genetically broad population could also be used to predict plant mortality or biomass injury levels in a breeding population for which a genomic prediction model is not available, albeit with predictive ability 41–43% lower than that observed in the intra-population scenario for inbred lines. Conversely, models fitted on inbred lines are poorly suited to predict genetically broader material. Therefore, model transferability is likely feasible only from a genetically broader to a genetically narrower population and is recommended in the absence of a phenotyping platform or when budget constraints impede the development of a specific prediction model for the target breeding line population. In such a case, subsequent platform-based (if feasible) and/or field-based evaluation of the best-ranking lines would be needed.

Frost escape emerged as the predominant factor determining winter survival in the collection of landraces and cultivars evaluated under field conditions in Lodi. Almost all early-flowering accessions exhibited higher field-based winter mortality than expected based on frost resistance results obtained under controlled conditions (Figure 3.3). Multiple linear regression analysis confirmed that flowering onset was negatively correlated with field mortality, and its absolute effect, according to standardised β coefficients, was 176% greater than that of plant mortality under controlled conditions (i.e., frost resistance) (Chapter 4). The importance of frost resistance became more evident when the comparison was restricted to intermediate-flowering accessions, thereby excluding the overwhelming stress escape effect due to extreme flowering types. In this case, the relative effect of onset of flowering decreased, becoming 97% of the effect of plant mortality under controlled conditions. A lower but still significant contribution to field-based winter survival was also attributed to higher seed dry weight, being reportedly associated with a thicker root parenchyma in young plants (thereby enhancing root protection).

A white lupin ideotype for autumn sowing, in sub-oceanic and sub-continental climatic regions, should combine high yield potential with yield stability across years featuring cold-prone winters and drought-prone springs. The highly positive effect of a late onset of flowering on winter plant survival conflicts with the markedly negative impact of the same trait on tolerance to terminal drought (Annicchiarico et al., 2018; Pecetti et al., 2023). Selecting for intermediate phenology allows simultaneous escape from both frost and drought, and can be pursued by exploiting recent advances in marker-assisted selection for vernalisation responsiveness (Rychel-Bielska et al., 2024; Surma et al., 2025). Genomic selection models for drought resistance, which have already been developed for breeding lines (Pecetti et al., 2023) and landrace material (Annicchiarico et al., 2019a), could be used in combination with the current models to select simultaneously for frost and drought resistance, thereby complementing phenotypic selection. Finally, seed dry weight is an additional, easy-to-measure trait with a modest effect on winter survival and grain yield (Annicchiarico et al., 2010), which could be incorporated into the selection process.

Overall, this thesis contributes to the genetic improvement of frost resistance in white lupin by:

- Establishing a protocol for large-scale trait assessment under controlled conditions.
- Characterising trait variation and identifying promising genetic resources for breeding.

- Highlighting the polygenic architecture of frost resistance and detecting genomic regions hosting candidate genes.
- Assessing the value of genomic selection for frost resistance to support breeding line selection and the screening of germplasm collections.
- Contributing to define a breeding strategy for autumn-sown varieties in regions possibly subjected to both frost and drought stress.

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