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Conception d'un idéotype de luzerne pour un usage de mulch vivant au service des cultures de rente et étude génétique des caractères impliqués

Designing a lucerne ideotype for use as a living mulch for cash crop production and genetic analysis of key underlying traits

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Contexte général

Depuis les années 1960, les systèmes agricoles modernes, dits conventionnels, se sont largement appuyés sur l'utilisation intensive d'intrants chimiques, engrais azotés et phosphatés, herbicides, fongicides et insecticides, associés à une mécanisation croissante et à l'amélioration génétique des cultures (Jacques and Jacques 2012). Ces innovations ont permis d'atteindre des rendements élevés et de sécuriser la production alimentaire, réduisant considérablement le risque de famines. Ainsi, la production mondiale de denrées de base (céréales, cultures sarclées et tubercules) a presque doublé entre 1961 et 1996 (Tilman 1999).

Bien que ces systèmes aient significativement augmenté la productivité, ils sont aujourd'hui confrontés à des défis majeurs, liés à la fois à des facteurs environnementaux et socioéconomiques. L'utilisation accrue des intrants agricoles a entraîné une pollution diffuse des sols et des eaux, notamment par le lessivage des nitrates et les résidus de pesticides, contribuant à l'eutrophisation et à la contamination des écosystèmes. Parallèlement, les pratiques intensives ont dégradé la qualité des sols en réduisant la matière organique, en accélérant l'érosion et en appauvrissant la biodiversité microbienne, ce qui affaiblit leur fertilité. L'usage massif d'insecticides a fait décliner très fortement les populations d'insectes, tandis que les herbicides ont provoqué la disparition progressive de nombreuses adventices, qui constituaient une ressource alimentaire et un habitat pour la faune auxiliaire. Le déclin de ces organismes utiles, essentiels au contrôle naturel des ravageurs et à la pollinisation, traduit une véritable érosion fonctionnelle (Matson et al. 1997; Stoate et al. 2009; Kopittke et al. 2019; Zabel et al. 2019). Ces perturbations, en plus des changements climatiques, compromettent la stabilité des rendements agricoles, accélèrent l'érosion des sols et favorisent la prolifération de ravageurs et de maladies, tout en réduisant l'efficacité des intrants chimiques (Agrimonti et al. 2021; Pratap et al. 2024).

À ces enjeux environnementaux s'ajoutent des contraintes socioéconomiques importantes. La forte dépendance du secteur aux énergies fossiles, notamment pour la production d'engrais azotés, le fonctionnement des machines agricoles et le transport, le rend particulièrement vulnérable à la volatilité des prix du pétrole. Plusieurs travaux ont montré que les chocs pétroliers influent fortement sur le coût des intrants agricoles et, par conséquent, sur les prix

des denrées alimentaires (Zmami and Ben-Salha 2019; Kocaaslan 2023). Sur le plan social, ce modèle contribue aussi à accentuer les inégalités, en marginalisant les petites exploitations et en renforçant la dépendance vis-à-vis des grands acteurs de l'agro-industrie (Rose et al. 2021).

Face à ces défis, l'agroécologie s'impose comme une réponse systémique visant à repenser en profondeur les modes de production agricole. Elle applique les concepts écologiques aux systèmes agricoles, en tenant compte des interactions entre plantes, animaux, humains et environnement. L'agroécologie se définit à la fois comme une science, un ensemble de pratiques et un mouvement social œuvrant vers des systèmes alimentaires durables. Ses principes reposent sur la valorisation des cycles naturels, la réduction des intrants chimiques, la préservation de la santé des sols et de la biodiversité, ainsi que sur la recherche de synergies entre les différentes composantes du système. Ces principes se traduisent par une diversité de pratiques intégrées, allant de l'association cultures-élevage au recyclage des nutriments, en passant par l'agriculture de conservation (Wezel et al. 2014; Gliessman 2021).

La diversification constitue un pilier central de l'agroécologie. Elle prend des formes multiples, telles que les rotations culturales, l'agroforesterie ou encore les associations de cultures. Ces pratiques imitent la complexité des écosystèmes naturels et permettent d'améliorer la fertilité, de limiter l'érosion, de réguler les bioagresseurs et de renforcer la résilience des exploitations (Wezel et al. 2014; Paracchini et al. 2020). L'adoption à grande échelle se heurte toutefois à des obstacles économiques, institutionnels et sociaux liés aux coûts initiaux élevés, au manque de politiques incitatives, ou encore à la complexité de gestion des différentes composantes du système : sols, biodiversité, cultures, eau, climat (Bhujel and Joshi 2024).

Parmi les différentes formes de diversification, l'association culturale ou interculture constitue une modalité particulièrement étudiée. Elle repose sur la culture simultanée de deux espèces ou plus sur une même parcelle, de façon à ce qu'elles coexistent pendant une partie de leur cycle de croissance. Elles peuvent prendre différentes formes, en lignes, en bandes ou en mélange, et visent principalement à optimiser l'utilisation de la surface et des ressources disponibles (Malézieux et al. 2009). Cette pratique présente plusieurs avantages. Elle permet souvent d'augmenter la production totale par unité de surface, ou sur-production, ce que traduit un ratio d'équivalence en surface fréquemment supérieur à 1 (Brooker et al. 2014). La complémentarité entre espèces, morphologique ou physiologique, favorise une utilisation plus efficace de la lumière, de l'eau et des nutriments (Wezel et al. 2014). Cette diversification contribue

également à limiter la pression des adventices, des maladies et des ravageurs, réduisant ainsi le recours aux pesticides (Brooker et al. 2014; Gaba et al. 2015). Ces bénéfices s'accompagnent toutefois de contraintes. L'interculture demande une gestion plus fine, un choix d'espèces adapté et une organisation précise du semis à la récolte. Elle peut augmenter les besoins en main-d'œuvre et rendre la mécanisation plus difficile, notamment dans les systèmes à grande échelle. Lorsque les espèces sont mal associées, la compétition peut réduire fortement la production de certaines cultures (Brooker et al. 2014). Les associations entre légumineuses et céréales sont reconnues comme particulièrement efficaces, en raison de la fixation symbiotique de l'azote par les légumineuses et de la mise à disposition de cet azote à la culture non-légumineuse, combinée à la forte compétitivité des céréales pour l'absorption de l'azote. Ce principe a été démontré aussi bien pour les cultures annuelles (Ghaley et al. 2005; Mamine and Farès 2020), que pour les systèmes pérennes (Nyfeler et al. 2011).Parmi les différentes formes d'association entre légumineuses et céréales, les systèmes en mulch vivant constituent un cas particulier, où une céréale est implantée sur une légumineuse pérenne préétablie et maintenue comme couverture après la récolte des céréales (Hartwig and Ammon 2002). Parmi ces systèmes, l'association du blé avec la luzerne est l'un des exemples les plus étudiés (Cougnon et al. 2022).

La luzerne émerge comme une candidate particulièrement prometteuse pour ce rôle, grâce à sa pérennité, sa capacité à fixer l'azote, sa contribution à l'amélioration de la structure du sol et son fort potentiel de couverture, qui limite le développement des adventices (Cougnon et al. 2022). Ces atouts en font une plante de service attractive pour sécuriser durablement les agroécosystèmes céréaliers. Cependant, les variétés actuelles de luzerne ont été sélectionnées principalement pour la production fourragère dans des systèmes intensifs de monoculture. Elles se caractérisent par une forte vigueur végétative, une reprise précoce au printemps, des tiges hautes et droites et une production de biomasse élevée. Si ces traits sont avantageux pour maximiser le rendement en fourrage (Julier et al. 2017; Hawkins and Yu 2018), ils se traduisent, dans le mulch vivant, par une forte compétitivité vis-à-vis de la culture de rente, en particulier le blé. Dans les co-cultures blé–luzerne, cette compétition peut entraîner des pertes de rendement du blé pouvant atteindre 45 à 50 % (Humphries et al. 2004; Shili-Touzi 2009).

Ce paradoxe met en évidence un enjeu scientifique majeur : la luzerne possède de nombreux atouts pour son utilisation comme mulch vivant en association avec les céréales, mais les

variétés actuellement sélectionnées pour la production fourragère en monoculture présentent des traits qui les rendent trop compétitives vis-à-vis du blé. Trois verrous principaux se dégagent de ce constat :

1. Au niveau phénotypique, les traits morphologiques et phénologiques qui conditionnent l'équilibre entre services écosystémiques (fertilité et couverture du sol, régulation des adventices) et compétition avec la culture de rente restent encore mal identifiés et peu quantifiés.
2. Pour optimiser les programmes de sélection, il faudrait savoir si les évaluations conduites en monoculture dense ou en plantes espacées reflètent fidèlement les performances des variétés en conditions de mulch vivant.
3. Au niveau génétique, la base génétique de ces traits et le potentiel des outils moléculaires pour leur sélection restent encore peu explorés.

L'objectif général de cette thèse est de définir et caractériser un idéotype de luzerne adapté à l'usage en mulch vivant avec le blé, conciliant la fourniture de services écosystémiques et la limitation de la compétition avec la culture de rente, et d'acquérir des connaissances pour la sélection de cet idéotype. Pour répondre à cet objectif, la thèse s'articule autour de trois axes complémentaires, présentés sous la forme d'articles scientifiques.

Chapitre 1 : identifier les traits morphologiques et phénologiques de la luzerne qui conditionnent la compétition et la complémentarité avec le blé, à partir d'un large panel diversifié de ressources génétiques.

Chapitre 2 : comparer l'efficacité de la sélection en conditions pures (plants espacés et peuplements denses) à celle réalisée directement en association blé–luzerne, afin d'identifier le contexte le plus pertinent pour sélectionner des luzernes adaptées au mulch vivant.

Chapitre 3 : explorer la base génétique des traits clés de la luzerne par des approches de génétique quantitative et de sélection génomique.

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Literature review and synthesis

Towards lucerne ideotypes for living mulch systems

"This chapter could form the basis of a literature review paper to be submitted to a genetics journal, such as Euphytica"

1. Intercropping: concepts, benefits, and the breeding gap

1.1. Definition and typology

Defining intercropping is challenging due to variations in terminology influenced by different authors, crop types, and cropping techniques. Some definitions even differentiate between interplanting and intercropping, adding to the complexity. In this text, intercropping is broadly defined as the cultivation of two or more species in the same field for at least part of their life cycle, with potential benefits for productivity, resource use, and sustainability (Mousavi and Eskandari 2011; Neamatollahi et al. 2013). This definition excludes simple rotations or agroforestry systems, where temporal or spatial separation is greater, and emphasises the simultaneous coexistence of species.

Intercropping encompasses a wide diversity of practices, combining annual and perennial and herbaceous species in various configurations (Malézieux et al. 2009). It is usually classified according to spatial or temporal arrangement. Spatially, mixed intercropping refers to the random distribution of species in the same area without a fixed structure. Row intercropping involves growing species in alternating rows, while strip intercropping extends this principle to wider strips of several rows (*Fig.* 1). Temporally, species may be sown at the same time and share most of their growth period (synchronised intercropping), or they may overlap only partially, as in relay intercropping, where a second crop is introduced into a standing crop (Lithourgidis et al. 2011).

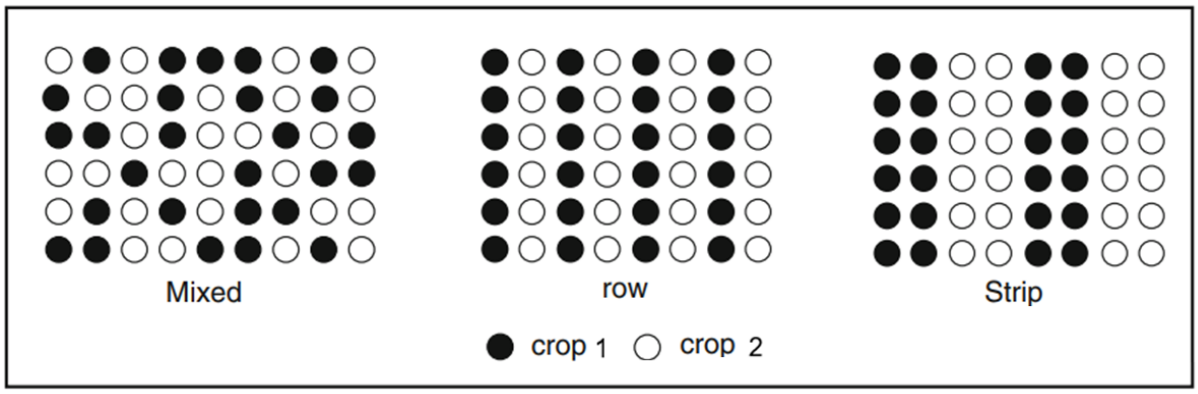

Figure 1. *Intercropping types* (Neamatollahi et al. 2013).

Intercropping effectiveness relies on the balance between negative interactions (competition for resources such as light, water, and nitrogen) and positive interactions (complementarity, facilitation), which together determine productivity and stability (Brooker et al. 2014). Interest in intercropping emerged in the 1970s as a strategy to improve agricultural sustainability and resilience (Glaze-Corcoran et al. 2020). Today, it is recognised as one of the most prominent examples of ecological intensification (Brooker et al. 2014), with a growing research interest over the past three decades (Lv et al. 2021). However, intercropping remains underutilised in modern large-scale agricultural systems (Renjan 2023). Cereal–legume associations represent the majority of intercropping uses, which explains why most examples in the literature focus on this type (Raseduzzaman and Jensen 2017).

1.2. Resource use efficiency and yield advantage

A key advantage of intercropping lies in its ability to use available resources more efficiently than monocultures. Species with contrasting functional traits can capture light, water, and nutrients in complementary ways, reducing competition and increasing overall productivity (Bedoussac et al. 2015). Differences in canopy architecture enhance light interception, while variations in rooting depth and morphology enable species to exploit soil resources from different layers (Duchene et al. 2017; Herve et al. 2017). Cereal–legume associations illustrate this complementarity particularly well. Legumes fix atmospheric nitrogen through symbiosis, while cereals are strong competitors for mineral soil nitrogen. When grown together, legumes are forced to rely more on biological fixation, thereby contributing more nitrogen to the system than in pure stands (Ghaley et al. 2005; Nyfeler et al. 2011; Mamine and Farès 2020).

The yield advantage of intercropping is usually expressed through the Land Equivalent Ratio (LER), which measures the relative land area required in sole crops to match the yield obtained in intercrops. LER values greater than 1 consistently indicate higher land-use efficiency. Classic studies have reported average LER values around 1.2–1.3 for cereal–legume combinations such as maize–bean or wheat–pea (Willey 1985; Lithourgidis et al. 2011). In practical terms, this means that 20–30 % more land would be required in monoculture to achieve the same yield produced by intercrops. Such advantages are not restricted to total biomass, intercropping also tends to stabilise yield performance across years and environments, making it a promising pathway for sustainable intensification (Raseduzzaman and Jensen 2017).

1.3. Ecological advantages

Beyond yield performance, intercropping contributes to soil conservation and fertility. The coexistence of species with contrasting rooting systems improves soil structure, porosity, and aggregate stability, enhancing water infiltration and reducing erosion. These diverse root systems also limit nutrient leaching and help stabilise the soil surface (Lithourgidis et al. 2011; Brooker et al. 2014). On the chemical side, legumes enrich soil fertility by fixing atmospheric nitrogen and releasing it through rhizodeposition and residue decomposition. In the rotations, this additional nitrogen, together with the return of organic matter from intercrop residues, increases soil organic carbon and nutrient availability for subsequent crops, while reducing dependence on synthetic fertilisers (Nyfeler et al. 2011; Bedoussac et al. 2015).

Intercropping also contributes to the reduction of weeds, pests, and diseases. Weed suppression results from improved resource use, which leaves less light, space, water, and nutrients available for weeds and limits their germination and growth (Gaba et al. 2015). High levels of diversity reduce pest and disease pressure through multiple mechanisms. The structural complexity of intercrops alters pest dynamics by making host plants less apparent and by providing habitats for natural enemies, thereby improving biological control (Iverson et al. 2014; Mir et al. 2022). Intercropping also tends to reduce disease pressure, as lower host density and physical barriers slow pathogen spread (Brooker et al. 2014; Cougnon et al. 2022).

1.4. Constraints and breeding mismatch

However, despite its multiple benefits, intercropping faces several important limitations that restrict its large-scale adoption. The first challenge lies in management complexity. Designing efficient species combinations requires careful adjustment of sowing dates, plant densities, and spatial arrangements, as well as fertilisation and harvest. These operations increase decision-making and labour costs. Mechanisation adds an additional layer of constraint. Intercrops are often composed of species with different heights, phenologies, or harvest times, making them poorly compatible with existing machinery optimised for uniform monocultures. These constraints are particularly critical in large-scale, mechanised systems, where uniformity is essential for efficient operations (Brooker et al. 2014; Van Oort et al. 2020).

A second limitation comes from the choice of species and their combinations. While legume–cereal associations for grain production and forage legume and grass associations are widely recognised as efficient due to their complementarity in nitrogen use, not all combinations result in positive outcomes. When species or varieties are poorly matched, competition for light, water, or nutrients becomes asymmetric, with one crop dominating at the expense of the other. These competitive effects are well documented, yet their intensity and direction remain highly context-dependent, varying with species traits, environment and management (Bedoussac et al. 2015; Duchene et al. 2017; Surault et al. 2024). This uncertainty makes it difficult to design robust species combinations. This problem is reinforced by plant breeding. Most varieties currently available have been bred exclusively under monoculture conditions, with traits optimised for sole cropping, such as high vigour, strong competitive ability, tall canopy architecture and responsiveness to high input levels. While desirable in monoculture, these traits can be maladaptive in intercropping, where reduced competitiveness or stronger niche complementarity would be more advantageous (Brooker et al. 2014). However, there remains a limited understanding of which traits consistently promote positive or negative interactions. Several studies have shown that performance in monoculture does not reliably predict performance under intercropping, underscoring the need to evaluate and select genotypes directly in these conditions (Maamouri et al. 2017; Annicchiarico et al. 2019; Sampoux et al. 2020). Very few breeding programmes include such evaluations. This lack of adapted material and knowledge leads to frequent yield losses and instability. This risk of yield instability is one of the main reasons why intercropping remains less attractive in commercial contexts, where maximising the main product is often prioritised (Lithourgidis et al. 2011; Raseduzzaman and Jensen 2017).

2. Living mulch systems: opportunities and constraints

2.1. Definition and particularities

Among the different forms of intercropping, living mulch systems stand out for their vegetative cover and multifunctional role. They involve service species sown before the cash crop and persisting as a living ground cover throughout the growing season (Hartwig and Ammon 2002). Living mulches can include either annual or perennial species. Annual species generally complete their life cycle during spring and may be incorporated into the soil to release nutrients, functioning as green manure, or left on the soil surface as mulch after the cash crop harvest (Hill et al. 2021). In contrast, perennial living mulches persist across several growing seasons without reseeding.

The temporal configuration of living mulch systems is unique among intercropping arrangements (Fig. 2). In synchronised intercropping, both species are sown simultaneously; in relay intercropping, the service crop is introduced later into a standing crop. By contrast, in living mulch systems, the service crop is already established before the cash crop and remains alive throughout its entire cycle, sometimes across multiple seasons when perennials are used (Gardarin et al. 2022; Dzvene et al. 2023). Unlike cover crops, which are typically sown between two cash crops and destroyed before the next sowing, living mulches maintain a permanent vegetative cover and a continuous interaction with the cash crop. This persistence distinguishes them from dead or surface mulches, which consist of plant residues left on the soil after destruction (Westbrook et al. 2022).

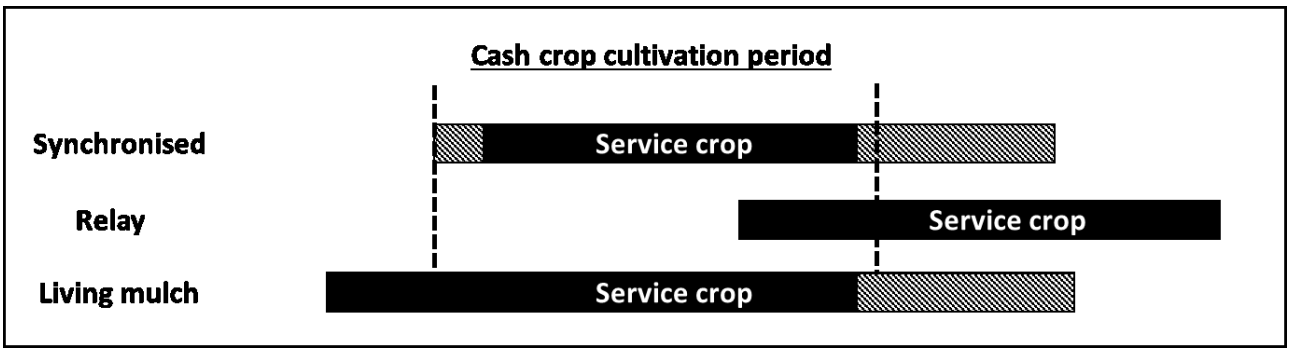

Figure 2. *intercropping with service crop (modified from Gardarin et al., 2022). The dashed lines indicate the variability in sowing and harvest dates of the service crops.*

2.2. Ecological and agronomic advantages

Living mulch systems are primarily designed for ecosystem service delivery. Their main objectives are weed suppression, soil protection, and biodiversity enhancement rather than direct yield increase (Hartwig and Ammon 2002). Weed control is often considered the central mechanism through which living mulches influence crop performance. The permanent ground cover acts as a physical and biological barrier, competing with weeds for light, space, and nutrients, thereby reducing their establishment and offering a non-chemical strategy for weed management (Gerhards 2018; Bhaskar et al. 2021; Westbrook et al. 2022; Gardarin et al. 2022). Beyond weed suppression, living mulches maintain continuous biological activity above and below ground. Persistent roots enhance soil aggregation, infiltration, and microbial activity, while the canopy protects against erosion and moderates temperature and moisture fluctuations. These functions create major ecological benefits by improving soil quality, nutrient cycling, and biological regulation. However, this same continuous presence can also intensify competition (Hill et al. 2021; Devi et al. 2023). Because the living mulch is already established when the cash crop emerges, it often has a competitive advantage, capturing early light, water, and nitrogen that may penalise the crop. Managing this trade-off between service provision (ecological advantages) and competition (affecting agronomic performance) remains one of the key challenges limiting the adoption of living mulch systems.

In this context, selecting appropriate species combinations is essential to balance these ecological and agronomic effects. It depends on the cropping system, local climate, and plant growth dynamics. Ideally, the service crop and the cash crop should belong to different botanical families to maximise functional complementarity and reduce direct competition (Bhaskar et al. 2021; Devi et al. 2023). Leguminous species (*Fabaceae*) are the most widely used living mulches, followed by grasses (*Poaceae*) and brassicas (*Brassicaceae*). Cereal crops, particularly wheat and maize, are the most frequent cash crops, reflecting their dominance in arable contexts (Gardarin et al. 2022). Among these associations, cereals grown on perennial legumes stand out as the most common and promising configuration. In addition to the services listed above, they add the key advantage of an efficient nitrogen cycling (Neamatollahi et al. 2013; Raza et al. 2019; Dzvene et al. 2023; Guerchi et al. 2023). In this context, the lucerne–winter wheat association constitutes the core focus of the present study.

3. Lucerne as a living mulch candidate

3.1. Lucerne among perennial legumes

Perennial legumes have long been explored as living mulch species in cereal-based systems, including white clover (*Trifolium repens*), birdsfoot trefoil (*Lotus corniculatus*), sainfoin (*Onobrychis viciifolia*), and lucerne (*Medicago sativa*). When maintained beneath annual cash crops such as wheat or maize, these species can potentially provide continuous ground cover, fix atmospheric nitrogen through symbiosis with Rhizobium bacteria, suppress weeds, limit erosion, and buffer temperature and moisture fluctuations at the soil surface (Gardarin et al. 2022; Cougnon et al. 2022; Dzvene et al. 2023). However, despite similar ecological functions, they differ markedly in persistence, adaptability, and interaction with cash crops, leading to contrasting agronomic performances.

White clover is the most widely studied living mulch species in temperate and humid regions. Its creeping stolons and prostrate growth habit enable rapid soil coverage and effective weed suppression once established, explaining its success in organic and reduced-input contexts (Westbrook et al. 2022; Leoni et al. 2024). It regrows well after mowing and steadily supplies nitrogen, partly through fine, easily mineralized roots and high exudate release that enhance nitrogen transfer to companion crops (Louarn et al. 2015). However, its shallow root system limits benefits to deep-rooted cereals, and poor drought tolerance reduces persistence under heat or water stress, leading to gaps in soil cover (Cougnon et al. 2022; Dzvene et al. 2023).

Birdsfoot trefoil and sainfoin exhibit slower establishment than clover but show greater tolerance to low fertility, calcareous soils, and periodic drought (Cougnon et al. 2022). Their moderate, upright vigour limits direct competition with the cash crop, resulting in smaller yield penalties for the cereal, though at the expense of weaker weed suppression and slower nitrogen cycling. In many field trials, these species contribute to longer-term soil fertility, but require reseeding after two or three seasons, reducing their potential as stable living covers (Bhaskar et al. 2021; Dzvene et al. 2023). Sainfoin, though more persistent, is sensitive to waterlogging and winter frost (Leoni et al. 2022).

Cultivated lucerne represents a distinctive functional type among perennial legumes, particularly due to the traits selected in improved varieties. It combines deep rooting, strong regrowth, and an erect growth habit. Its taproot system, capable of reaching several metres, confers exceptional drought resilience and allows resource capture from deeper soil horizons where other species fail (Annicchiarico et al. 2015; Julier et al. 2017; Cougnon et al. 2022). Through this architecture, lucerne improves soil porosity, aggregate stability, and deep carbon

sequestration, contributing to long-term structural restoration of degraded soils (Bhaskar et al. 2021; Leoni et al. 2022). In addition, when associated with grasses, its strong regrowth and dense canopy contribute to effective suppression of both annual and perennial weeds, offering a valuable non-chemical weed management strategy in low-input systems (Tahir et al. 2022; Leoni et al. 2022). Because of its longevity, often persisting for four to five years without reseeding, lucerne can serve as a true perennial base in crop rotations, maintaining permanent soil protection, while providing ecosystem services, including nitrogen enrichment, soil-carbon accumulation, and biodiversity enhancement (Cougnon et al. 2022). These attributes make it a good candidate for living mulch in organic or conservation agriculture, where sustained ground cover and reduced reliance on fertilisers and herbicides are major objectives.

However, the characteristics that make lucerne persistent and service-efficient also make it a dominant competitor in intercropping with cereals (Humphries et al. 2004; Carof et al. 2007a, b; Shili-Touzi 2009; Leoni et al. 2024). In winter wheat and lucerne living mulch, grain yield has been shown to decrease by 60–80 % when sown into established lucerne stands (without suppression), mainly due to reductions in ear density and grain number per ear, resulting from intense competition for light and nitrogen (Carof et al. 2007a). Comparable losses of around 50 % were reported under similar conditions, with severe nitrogen limitation and reduced wheat tillering identified as the primary causes (Shili-Touzi 2009).

This trade-off between ecological and agronomic advantages is a central challenge in living mulch systems. Lucerne, which delivers the strongest ecological advantages, also exerts the strongest competitive pressure on the cash crop. Understanding and optimising this balance is therefore essential for making lucerne a viable perennial living mulch species within sustainable cereal systems.

3.2. Agronomic strategies to mitigate competition

To mitigate the competitive effects of lucerne as a living mulch, two main agronomic management strategies have been explored. The first involves mechanical control, primarily through repeated mowing of lucerne during the cereal growing season to maintain crop dominance. An initial mowing in early spring helps limit both lucerne and weed pressure, while the resulting surface mulch reduces soil evaporation and restricts subsequent weed emergence. However, this method requires specialised equipment and precise GPS-guided machinery to

avoid damaging cereal rows, and it incurs significant costs in terms of time, labour, and energy inputs (Arvalis 2022). Although mowing reduces lucerne competition compared with no control, it still leads to yield penalties, with wheat yield declining by about 45% when lucerne was mown every 4–8 weeks from January to April (Humphries et al. 2004).

In parallel, chemical control of lucerne has been explored as a strategy to reduce its competitive effect on wheat during the growing season. In Carof et al (2007a), a reduced herbicide dose (data not provided) was applied mainly for weed management but also contributed to slowing lucerne regrowth. As a result, wheat yields under these conditions were comparable to the no-mulch control. In Shili-Touzi (2009), the sulfonylurea herbicide *Aminugec 600* was applied at one-quarter of the recommended dose, either early or late in the wheat cycle. This treatment reduced yield losses to 33 % in the early treatment and 42 % in the late treatment compared to pure wheat.

3.3. Breeding perspectives

Lucerne genetic diversity has not been explored as a possible option to reduce competition between lucerne and the cash crop in living mulch contexts. However, lucerne displays remarkable phenotypic and genetic diversity within the *Medicago sativa* complex, which includes subspecies *sativa* and *falcata*, their diploid and autotetraploid forms, wild and cultivated populations, and a wide range of morpho-physiological types from prostrate, dormant forms to tall, highly productive ones (Julier et al. 1995). This diversity results from multiple biological and ecological factors, including its autotetraploid and outcrossing reproductive system, high heterozygosity, and long history of adaptation to contrasting environments, from cold continental to dry Mediterranean climates across the world (Wang and Şakiroğlu 2021). Such variability provides a valuable genetic reservoir for breeding programmes targeting sustainable agriculture, particularly for the design of ideotypes specifically adapted to living mulch. These ideotypes should maintain persistence and ecological advantages while expressing reduced competitiveness with the cash crop, avoiding the need for external regulation (Cougnon et al. 2022).

Despite this potential, current breeding programmes have not explicitly targeted these objectives. Moreover, no study has yet characterised how lucerne genetic diversity affects its competitive and facilitative interactions with cash crops in living mulch contexts. The key traits

and their influence on the balance between ecological and agronomic advantages, therefore, remain largely unknown.

4. Key traits for lucerne ideotypes in living mulch systems

4.1. Lucerne development and morphological diversity

The identification of key traits for lucerne ideotypes in living mulch contexts mainly concerns the ability of plants to coexist with the cash crop by limiting early competition for light and nutrients. The intensity of this competition determines both the agronomic and ecological outcomes of the association. Consequently, the most relevant traits are related to morphological and phenological aspects, because they directly affect canopy development, resource capture, and growth dynamics during the critical phases of cash crop establishment.

Lucerne development follows a sequence of vegetative and reproductive stages characterised by morphological and physiological changes that determine its growth dynamics, persistence, and competitive ability. During spring and early summer, lucerne exhibits its most vigorous growth. Rising temperatures and increasing day length stimulate rapid shoot elongation, leaf expansion, and active nitrogen fixation. Root biomass also increases as the plant accumulates reserves to support subsequent regrowth (Mueller and Teuber 2007). In autumn, as temperatures and day length decline, lucerne enters an acclimation phase that initiates autumn dormancy. Growth slows as assimilates are redirected toward the crown and roots, allowing the plant to store reserves and improve winter survival (Undersander et al. 1997). This physiological process underlies both persistence and spring regrowth capacity. The depth of dormancy varies among cultivars and is classified into 11 autumn-dormancy ratings, ranging from highly dormant (1–2) to very non-dormant (10–11) (Munjal et al. 2018). In winter, lucerne cultivars with high dormancy ratings exhibit little or no top growth, but non dormant cultivars, grown under mild winters, continue producing biomass. Morphologically, lucerne shows a wide spectrum of growth habits, from erect to prostrate forms. Erect types that facilitate mechanical management, generally produce high biomass but tend to compete more strongly for light with companion crops, whereas prostrate types develop close to the ground, favouring soil cover (Favero et al. 2009; Pecetti et al. 2021). Growth habit is closely linked to dormancy, with prostrate forms generally associated with high dormancy (Liu et al. 2015). The timing and intensity of these growth activity phases, together with growth habit, determine how quickly

lucerne occupies space and uses resources, influencing its competitive effect when used as a living mulch.

4.2. Interspecific interaction traits

Competition between lucerne and wheat begins at an early stage after wheat establishment. Early competition determines the final number of wheat shoots and ears, fixed during the initial growth phases. Humphries et al (2004) showed that a winter-dormant lucerne variety reduced yield losses compared with a winter-active one. Light competition is the main limiting factor during this initial stage (Amossé et al. 2013). The peak of light competition occurs during wheat stem elongation (BBCH 30), when lucerne resumes growth. Its rapid shoot elongation and canopy expansion shade young wheat plants, reducing light availability and photosynthetic activity. Reduced light limits leaf expansion and tiller survival, leading to reduced tiller number, grain number per spike, and spike density (Carof et al. 2007a; Hiltbrunner and Liedgens 2008). These results highlight the importance of lucerne dormancy level and early spring growth in determining the intensity of light competition and its consequences for wheat yield formation.

After this first phase, light competition continues, but resource limitation progressively shifts to nitrogen as both species reach full vegetative activity, especially during the first year of lucerne establishment. A second critical peak of competition appears at the start of flowering (BBCH 61), when wheat nitrogen demand is highest. Experimental findings have shown that nitrogen transfer from lucerne remains limited during the first season, thereby constraining wheat grain formation (Shili-Touzi 2009). It is important to note that nitrogen fixation in lucerne is proportional to its biomass; therefore, during subsequent seasons, more vigorous lucerne plants with higher biomass accumulation fix larger amounts of nitrogen than in the first year (Louarn et al. 2015). Furthermore, when wheat is sown into an already established lucerne, the well-developed lucerne root system gives it a strong competitive advantage, reducing wheat access to nutrients and water (Cougnon et al. 2022). This illustrates that the intensity of competition evolves not only with phenology but also with lucerne growth during spring and related morphological traits such as height and leaflet size.

Another source of yield loss, particularly pronounced at harvest, is lodging of the association, which reduces harvest efficiency and grain yield (Hiltbrunner 2007). Lodging is strongly influenced by lucerne growth habit, height, and stem composition. Erect lucerne types, which

are generally tall and vigorous, are prone to lodging because of their high centre of gravity and great biomass concentration in the upper canopy. In contrast, prostrate or intermediate types maintain a lower profile and greater structural stability, making them less susceptible (Tang et al. 2022).

Altogether, these phenological and morphological traits can be considered the main factors defining the intensity of competition between lucerne and wheat. Yet, they remain poorly documented in living mulch. Identifying the optimal combination of these traits is essential for designing an ideotype capable of simultaneously suppressing weeds, fixing nitrogen, and limiting competition with the wheat. Once these key traits are defined, the next step is to determine the most suitable selection procedures in which they can be effectively expressed and evaluated. Finally, understanding their genetic determinism will enable their integration into breeding strategies and the development of varieties specifically adapted to living mulch.

5. Selection strategies for lucerne as a living mulch

5.1. Current breeding focus and limitations

Over the past 50 years, lucerne breeding has largely been shaped by the requirements of intensive forage systems. Early programmes focused on total biomass yield, persistence, and stand longevity under frequent cutting and satisfactory P and K fertilisation. With time, additional goals such as forage quality (high protein, low fibre contents) and resistance to major diseases and pests were progressively incorporated (Li and Brummer 2012; Annicchiarico et al. 2014; Hawkins and Yu 2018). More recently, tolerance to abiotic stresses such as drought, salinity, and soil acidity has gained importance in response to climatic variability and declining water availability (Annicchiarico et al. 2014, 2022). These efforts produced vigorous, widely adapted cultivars with strong early growth, erect canopy architecture, and rapid regrowth, traits that ensure high forage yield in monoculture (Maamouri et al. 2015; Grieder et al. 2021).

However, because selection was almost exclusively conducted for pure stand uses, and under uniform, high-input conditions, cultivars have been implicitly selected for competitive dominance rather than coexistence (Grieder et al. 2021). This conventional breeding therefore fails to predict performance in mixtures, where growth depends on interspecific interactions such as competition for resources, complementarity, or facilitation with companion species (Maamouri et al. 2017; Annicchiarico et al. 2019; Louarn et al. 2020; Bourke et al. 2021).

Standard breeding trials also fail to capture the plasticity of functional traits and the emergent effects of interspecific interactions (Moore et al. 2022). As a result, they do not measure traits that explain how plants interact, such as competitive strength, ability to share resources, or capacity to support the cash crop (Hill 1990; Bourke et al. 2021). By overlooking these aspects, traditional breeding trials ignore the functional diversity and environmental responses that ultimately determine performance in mixtures (Dubey et al. 2024).

5.2. Toward new breeding strategies

Breeding for intercropping, therefore, requires a reorientation of breeding objectives from maximising individual performance to optimising intercrop-level outcomes. This reorientation implies identifying the traits that favour coexistence and defining the most appropriate selection designs in which they should be evaluated. Two complementary strategies can be considered: indirect selection, based on monoculture performance, and direct selection, carried out in intercrop conditions. The relative efficiency of these approaches depends on the genetic correlation between genotypic values expressed in the two environments. This parameter measures the extent to which genotypic rankings are conserved between monoculture and mixture. When the correlation is high and positive, the same trait effects govern performance in both contexts, and selection in monoculture can effectively predict intercrop performance. Conversely, a low or non-significant correlation indicates that genotypic expression differs between environments because of changes in competition intensity or facilitation, making indirect selection unreliable. In such cases, direct evaluation under intercropping conditions is necessary to capture the interactions that determine compatibility and combined yield (Sampoux et al. 2020; Bourke et al. 2021; Dubey et al. 2024).

Direct selection within intercrops is more complex to implement and requires dedicated breeding designs (Sampoux et al. 2020). To support this process, the concepts of General Mixing Ability (GMA) and Specific Mixing Ability (SMA) have been developed. Originally adapted within quantitative genetics from diallel crossing methods, these approaches assess how well a variety performs when grown with others (Dawson and Goldringer 2012). GMA reflects the average performance of a variety across multiple combinations, highlighting its general compatibility and adaptability. In contrast, SMA captures the deviations observed in specific pairings, revealing synergistic or antagonistic interactions (Forst et al. 2019; Haug et al. 2021).

However, in lucerne, these concepts have received very limited attention. Most breeding programmes have continued to evaluate genotypes in monoculture, and only a few studies have explored their behaviour in mixtures. Research conducted in forage legume–grass associations has shown that lucerne traits such as height, shoot number, or regrowth capacity can express differently in mixtures compared with pure stands, leading to changes in genotype ranking and significant genotype × mixture interactions (Maamouri et al. 2015, 2017). These results confirm that lucerne performance in monoculture is not always predictive of its performance when associated with another species (Grieder et al. 2021; Surault et al. 2024). Nevertheless, such work has so far been limited to forage mixtures, where both components contribute to total biomass production. In contrast, the use of lucerne as a living mulch beneath cereal crops raises distinct challenges, since the objective is not joint forage yield but maintaining cereal performance alongside lucerne ecological services. To date, no study has explicitly addressed the key question of the optimal evaluation and selection environment for lucerne, whether in pure stands or directly under living-mulch conditions, to identify ideotypes adapted to this use. This represents a major gap for future breeding research and highlights the need to integrate trait-based selection with modern genetic and modelling approaches, including QTL analysis, genomic selection, and ecophysiological modelling, to better characterise and predict the performance of lucerne as a living mulch.

6. Genetic and genomic approaches for lucerne improvement

6.1. Genetic diversity

The *Medicago sativa* complex encompasses a broad genetic base shaped by multiple centres of origin, polyploidisation events, and extensive natural and human-mediated gene flow. The two main subspecies, *M. sativa* subsp. *sativa* (diploid or tetraploid, purple-flowered, coiled pods) and *M. sativa* subsp. *falcata* (diploid or tetraploid, yellow-flowered, sickle-shaped pods), represent the cultivated and wild ends of the diversity spectrum (Brummer et al. 1994; Şakiroğlu and Brummer 2011). Numerous other forms, including subsp. *caerulea* (diploid, purple-flowered), subsp. *hemicycla* (diploid hybrid with variegated flowers and intermediate pod coiling), subsp. *varia* (tetraploid hybrid with intermediate traits), and subsp. *glomerata* (diploid with yellow flowers and tightly coiled pods), have arisen through hybridisation and introgression across ploidy levels, producing a taxonomic continuum that complicates classification yet enriches the genetic reservoir (Small 1986). The species outcrossing and

autotetraploid reproductive system, coupled with open pollination, maintains high heterozygosity and continuous allelic recombination (Julier et al. 2000; Flajoulot et al. 2005; Herrmann et al. 2018). This genetic structure underlies the remarkable morphological and physiological diversity observed across natural and cultivated populations, encompassing variation in growth habit, dormancy level, stress tolerance, and biomass productivity. Despite strong domestication for forage production, modern varieties still retain a large proportion of this diversity, although cultivated gene pools display approximately 30% less genetic variation than wild relatives (Muller et al. 2006)

Because of lucerne allogamous reproductive mode and its low multiplication rate, breeding programmes rely on the development of synthetic varieties, created through crosses of multiple parents during 3 to 4 generations. This variety format preserves genetic diversity and accumulate favourable traits (Parajuli et al. 2021), and maximises heterosis expression as the species suffers from severe inbreeding depression caused by the expression of deleterious recessive alleles in the homozygous state (Milic et al. 2010; Parajuli et al. 2021).

6.2. Advances in genomic resources and breeding applications

The identification of an ideotype typically follows a structured, multi-step process integrating genetics, physiology, and environmental adaptability. The process begins with defining the target environment and breeding objectives, followed by the identification of key morphological and physiological traits that optimise performance under those conditions (Gauffreteau 2018). This process includes the identification of quantitative trait loci (QTL) associated with these traits, enabling their genetic dissection and supporting the development of breeding strategies through marker-assisted or genomic selection.

Since the early 2000s, genomic studies of lucerne have relied on its close relative, the diploid, autogamous model legume *Medicago truncatula*, which has a relatively small genome of approximately 860 Mb. Although *M. truncatula* has played a key role in legume research, notably advancing fundamental knowledge, its utility for lucerne genomics remains limited by differences in ploidy level, reproductive biology and evolutionary divergence.

Over the past five years, major advances have been achieved with the development of high-quality reference genomes across the Medicago sativa complex, encompassing both sativa and

falcata forms, and covering diploid (Li et al. 2020; Shi et al. 2024) and autotetraploid (Shen et al. 2020; Long et al. 2022) accessions, including both wild and cultivated materials. The cultivar 'XinJiangDaYe' provided the first chromosome-scale reference genome at the autotetraploid level, with a total assembled size of 2.74 Gb (N50 contig = 459 kb) (Chen et al. 2020). To centralise and facilitate access to these resources, the Multi-Omics Database of *Medicago sativa* (MODMS) was established, providing a comprehensive platform integrating reference genomes, transcriptomes, molecular markers, and multi-omics data across diverse genotypes and ploidy levels (Fang et al. 2024). Together with recent advances in high-throughput genotyping technologies such as genotyping-by-sequencing (GBS), which can be applied at both the individual and population levels, these developments have revolutionised lucerne breeding by enabling efficient genome-wide SNP identification and the fine mapping of complex quantitative traits (Li et al. 2014; Julier et al. 2018; Hawkins and Yu 2018).

The continuous improvement of lucerne genomic resources has greatly strengthened the capacity to conduct genome-wide association studies (GWAS) and genomic prediction (GP) in this species. These tools have already been successfully applied to numerous agronomic and adaptive traits, including plant height, biomass yield, fall dormancy, regrowth capacity, persistence, and resistance (McCord et al. 2014; Sakiroglu and Brummer 2017; Zhang et al. 2019; Pecetti et al. 2021; Jiang et al. 2022). Reported QTL generally explain between 10 % and 30 % of the phenotypic variance, consistent with the polygenic nature of most lucerne quantitative traits. In parallel**,** genomic prediction accuracies in lucerne typically range from 0.2 to 0.8, depending on population structure, heritability, and modelling approach. Traits with moderate to high heritability ($h^2 \approx 0.4–0.8$), such as dormancy, height, disease resistance and persistence, tend to yield the most stable predictions across environments (Annicchiarico et al. 2015, 2022; Pégard et al. 2023, 2025).

These findings provide a promising basis to extend these approaches to traits controlling lucerne performance as a living mulch. Identifying the genetic determinism and genomic predictability of these traits will be essential for their integration into breeding schemes aimed at developing lucerne ideotypes that sustain ecological functions while limiting competition with cash crops.

7. Synthesis: knowledge gaps and thesis objectives

Lucerne offers a unique opportunity to enhance the ecological performance of cereal-based systems when used as a living mulch. Its nitrogen fixation, persistence, and strong capacity for soil cover make it one of the most promising service crops for agroecological intensification. However, its practical use remains constrained by excessive competition with the companion crop, particularly during early spring growth, which can cause severe yield losses in cereals such as wheat.

To date, management strategies such as mowing or partial chemical suppression have been proposed to mitigate these effects, but they remain labour-intensive, costly, and inconsistent with the objectives of low-input and conservation agriculture. A more sustainable approach lies in breeding lucerne varieties specifically adapted to living mulch use, that can maintain ecological services while limiting competition with cash crops. Yet, achieving this requires a deep understanding of the phenotypic, ecological, and genetic mechanisms controlling interspecific interaction, which remain poorly explored.

From the synthesis of current knowledge, three main research gaps are identified:

1. **Trait-level knowledge gap:**

The morphological and phenological lucerne traits determining the balance between improved ecosystem service provision (weed suppression, nitrogen fixation) and limited competition with the cash crop remain insufficiently identified and quantified under living-mulch conditions.

2. **Selection-design gap:**

The extent to which traits assessed in spaced plant nursery and dense monoculture reflects wheat performance under living-mulch conditions remains unknown.

3. **Genetic and genomic gap:**

The genetic diversity, the genetic determinants and the genomic predictability of traits controlling lucerne-wheat interaction and service provision have not yet been characterised, limiting the application of marker-assisted and genomic selection for living-mulch breeding.

Addressing these gaps is essential to designing lucerne ideotypes adapted to living mulch use, combining ecological services with reduced competitiveness toward the cash crop.

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Chapter 1

Lucerne genetic diversity for living mulch: Identifying key traits and evaluating their impacts on wheat development

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Résumé

La luzerne (*Medicago sativa*) est une légumineuse fourragère tolérante à la sécheresse qui offre de nombreux services écosystémiques. Récemment, dans le cadre de la transition vers l'agroécologie, son utilisation en tant que plante de service a émergé. Elle est alors utilisée comme culture de couverture, au sein de laquelle des cultures annuelles de rente, comme le blé, sont implantées. Grâce à sa capacité à fixer l'azote atmosphérique, à limiter les adventices et à améliorer la fertilité du sol, la luzerne présente un fort potentiel pour accompagner la transition vers des systèmes agricoles plus durables. Cependant, les essais menés en association avec le blé montrent que les variétés actuelles, issues de la sélection pour le fourrage, sont trop compétitives vis-à-vis de la culture de rente, entraînant une réduction de ses rendements.

Cette étude visait à analyser la diversité au sein du complexe *M. sativa* afin d'identifier les caractères favorisant l'efficacité de la luzerne en tant que mulch vivant, en se concentrant sur la compétition pour la lumière et l'azote entre la luzerne, le blé et les adventices. Trente accessions représentatives de la diversité génétique de la luzerne ont été cultivées pendant deux ans en association avec un blé d'hiver. Les effets de la dormance et du port de la luzerne ont été évalués sur la dominance relative du blé aux stades précoces et sur l'abondance des adventices. Aux stades plus avancés, l'influence de la hauteur et de la verse de la luzerne sur la biomasse et l'état azoté du blé a également été analysée.

Les résultats ont montré que la dormance et le port de la luzerne influençaient fortement la croissance du blé aux stades précoces. Les accessions dormantes et prostrées favorisaient la dominance du blé en réduisant la compétition, tandis que les accessions non dormantes et dressées exerçaient une forte suppression des adventices mais entraient en compétition avec le blé. Les accessions hautes et dressées ont amélioré l'état azoté du blé uniquement la deuxième année. La verse de la luzerne a également affecté la croissance du blé, les accessions hautes réduisant sa biomasse la première année. Une combinaison de dormance modérée, de hauteur intermédiaire et de bonne résistance à la verse offrirait le meilleur compromis, soutenant les performances du blé tout en contribuant au contrôle des adventices. Aucune des accessions testées ne rassemblait l'ensemble de ces caractères favorables, soulignant la nécessité de programmes de sélection dédiés à la création de cette variété.

Mots-clés : luzerne, blé, compétition, couverture végétale, adventices.

Abstract

Context. Lucerne (M. sativa) can offer ecosystem services as a perennial living mulch, supporting annual cash crops through weed suppression and nitrogen fixation. However, trials with wheat reveal that current lucerne varieties are excessively competitive, leading to reduced wheat yields. **Aims.** This study aimed to analyse the diversity within the M. sativa complex to identify traits that enhance lucerne effectiveness as a living mulch, focusing on the competition for light and nitrogen among lucerne, wheat and weeds. **Methods.** Thirty diverse lucerne accessions were cultivated as living mulch with a winter wheat, over two years. Lucerne dormancy and growth habit effects were evaluated on wheat relative dominance during the early stages and on weed abundance. In later stages, the effects of lucerne height and lodging on wheat biomass and nitrogen status were also assessed. **Key results.** Results indicated that lucerne dormancy and growth habit influenced wheat growth during early stages, with dormant and prostrate lucerne accessions reducing competition and enhancing wheat dominance. However, non-dormant and erect lucerne accessions effectively suppressed weeds but competed intensely with wheat. Tall and erect lucerne accessions supported wheat nitrogen status in the second year only. Lucerne lodging affected wheat growth, with tall lucerne reducing wheat biomass in the first year. **Conclusions.** Lucerne should exhibit slow growth, moderate height, and low lodging to optimise its benefits. No variety in our panel exhibited all these desirable traits. **Implications.** These findings highlight the need for breeding programs to combine lucerne beneficial traits as a living mulch into new varieties

Keywords: Alfalfa, cereal, competition, cover, weed.

1. Introduction

Modern agricultural systems have moved towards high yielding systems, relying on mechanisation, synthetic inputs and genetic improvement (Jacques and Jacques 2012). However, these systems face several problems, mainly related to soil and water pollution with impacts on human health, and loss of ecosystem biodiversity (Steffen *et al.* 2015). The current challenge is to meet the growing food demand while maintaining ecological integrity (Wezel *et al.* 2014; Gaba *et al.* 2015). One solution is to diversify cropping systems by increasing the number of crops grown (Brummer 1998), either in long rotations (Martin *et al.* 2020) or in intercropping (Yin *et al.* 2020).

A particular form of intercropping involves growing a main crop alongside a cover crop, known as living mulch. In this approach, the cover crop is maintained as a living ground cover throughout both the growing season and the off-season (Carof *et al.* 2007a; Mohammadi 2012). The living mulch provides beneficial ecosystem services when competition is managed (Carof *et al.* 2007a), such as reducing soil surface drainage (Leary and DeFrank 2000), decreasing soil nutrient loss, especially nitrogen and phosphorus, improving soil health (Martin *et al.* 2020), and providing weed suppression (Bhaskar *et al.* 2021). Throughout the main crop growing season, living mulch helps reduce soil waterlogging during crop establishment and growth (Humphries *et al.* 2004; Ma *et al.* 2024).

The intercropping of a legume crop with a non-legume crop, often a grass or a cereal, has been proven to be an efficient mixture due to nitrogen fixation and transfer from the legume to the non-legume, and the high competitiveness of the non-legume for nitrogen uptake, for annual crops (Ghaley *et al.* 2005; Mamine and Farès 2020), as well as for perennial crops (Nyfeler *et al.* 2011). Growing cereals on perennial legumes is the most common living mulch system, such as sowing winter wheat (*Triticum aestivum L.*) on lucerne (*Medicago sativa*) living mulch (Cougnon *et al.* 2022). Competition for resources is also observed in this mixture. Indeed, lucerne living mulch and wheat compete for various resources, especially light and nutrients (Carof *et al.* 2007b). It is established that nitrogen transfer from lucerne takes at least a year to occur (Louarn *et al.* 2015). Additionally, in this system where wheat is sown into an already established lucerne cover, lucerne has the advantage of a well-developed root system, making it more difficult for wheat to efficiently capture resources. Shili-Touzi (2009) verified this, measuring no nitrogen transfer from lucerne to wheat in the first year; instead, there was a clear

competition between the two crops for nitrogen. This competition becomes more pronounced at the beginning of wheat heading (Carof *et al.* 2007b). The same study highlights another major form of competition, which is the competition for light. A second study supports this finding, leading to wheat yield losses of up to 50%, even with high nitrogen inputs (Shili-Touzi 2009). In this system, light interception sharing depends on the temporal and spatial development of the species, including factors such as plant growth habit, height, and leaf area (Barillot *et al.* 2014; Bedoussac *et al.* 2015; Paul *et al.* 2024). The significant yield reduction can be explained by several factors, including shading of the cash crop by the cover crop during the early stages of wheat growth (Amossé *et al.* 2013), a decrease in the number of wheat tillers (Hiltbrunner and Liedgens 2008), a decrease in grain number per spike and spike density (Carof *et al.* 2007b), and plant lodging by harvest time (Hiltbrunner *et al.* 2007). Wheat yield loss represents a significant risk that can be reduced by limiting lucerne competitiveness. Various techniques can be used to manage competition from living mulch. Mechanical regulation strategies, while capable of limiting lucerne growth, come with considerable costs, including time and energy. Chemical regulation approaches, involving the use of reduced doses of herbicides, also present significant environmental concerns (Cougnon *et al.* 2022). Another possibility is to choose less competitive lucerne varieties. Humphries *et al.* (2004) demonstrated that, with mechanical regulation, a winter-dormant lucerne variety reduced the yield loss compared to a winter-active one.

Across all winter dormancy classes, current lucerne varieties were bred for monospecific stands, with programs mainly focusing on traits that optimise forage production, such as high biomass, erect growth habit, increased canopy height, as well as lodging and disease or pest resistance (Annicchiarico *et al.* 2015). The interest in developing lucerne varieties with modified architecture has been highlighted to increase the performance of lucerne-perennial grass mixtures (Maamouri *et al.* 2017). So far, lucerne genetic diversity has not been considered as a possible option to reduce competition between the two species in the living mulch system. However, the *M. sativa* complex, with the diploid and tetraploid subspecies *sativa* and *falcata*, contains a wide genetic diversity, ranging from the erect cultivated to the prostrate wild forms, and from the winter-dormant to the winter-active ones (Julier *et al.* 1995).

The aim of this study was to analyse the effects of the lucerne morphological and phenological traits in the outcome of competition between lucerne and wheat by studying a wide range of

genetic diversity within the *M. sativa* complex. We specifically focussed on the vegetative phases of wheat development. Ultimately, these results will contribute to identify the most relevant morphological and phenological traits that enhance the effectiveness of lucerne as a living mulch. The underlying hypotheses regarding these key traits are: *i)* strong lucerne winter dormancy allows good initial growth of wheat, *ii)* a prostrate growth habit ensures good soil cover against weeds, *iii)* low plant height reduces light competition with wheat, and *iv)* low lodging prevents wheat grain losses and facilitates harvest in this system. We used thirty diverse lucerne accessions as living mulch with one winter wheat variety, under low nitrogen input and without living mulch regulation during the wheat growing season. The traits related to the growth of lucerne and wheat, describing competition for light and nitrogen, were observed in a trial from November 2021 to June 2023. We focussed on critical periods, specifically the early stages of wheat growth (seedling and tillering) during the winter and the heading stage in the spring when competition is the most severe. The impact of lucerne traits with temporal and physiological relevance on wheat and weed responses was analysed by examining correlation among traits. Lucerne accessions were grouped based on their phenotypic similarity, and these groups were compared to determine which combinations of lucerne traits are the most beneficial for wheat growth and weed control.

2. Material and methods

2.1. Plant materials

Thirty lucerne accessions with various characteristics and origins were collected. These accessions were selected based on their subspecies classification (*sativa* or *falcata*), ploidy level (diploid or tetraploid), winter dormancy, and growth habit, which ranged from wild creeping to cultivated erect types. The dormancy values for the cultivated lucerne accessions registered in France were obtained from the French catalogue of varieties. For other cultivated accessions, the dormancy values were obtained from a previous study (Pégard *et al.* 2023). For wild *sativa* and *falcata* accessions, known to regrow slowly in the autumn and the spring, a dormancy score of 1 was given (Table 1). One winter bread wheat variety, Geny, developed by AgriObtentions and registered in France for organic farming, was chosen. This variety is characterised by its early maturity, intermediate soil coverage capacity, and relatively short stems.

Table 1. *List of lucerne accessions, including subspecies, ploidy level, types (cultivated or wild), winter dormancy (ranging from 1 (very dormant) to 10 (non-dormant or winter-active)), geographic origin. Accessions are either registered varieties, described in the UPOV plant variety database, or genetic resources ([1]) from the Centre of Genetic Resources (INRAE, Lusignan, France) or breeding materials ([2])*

Accession name	Subspecies	Ploidy	Type	Dormancy	Origin
Camporegio	falcata	4x	Cultivated	2	Italy
Coussouls	sativa	4x	Cultivated	6	France
Gabès[1]	sativa	4x	Cultivated	10	Tunisia
Glomerata[1]	sativa	2x	Wild	1	France
Greenmed	sativa	4x	Cultivated	2	Spain
Koalf 2-96[1]	sativa	4x	Cultivated	3	Hungary
Krasnokutskaya[1]	falcata	4x	Cultivated	2	Ukraine
L4332[2]	sativa	4x	Cultivated	3	France
L5323[2]	sativa	4x	Cultivated	3	France
L8988[2]	falcata	4x	Cultivated	1	France
Limory	sativa	4x	Cultivated	2	France
Ludelis	sativa	4x	Cultivated	3.5	France
Luzelle	sativa	4x	Cultivated	3	France
Malzeville[1]	falcata	4x	Wild	1	France
Marais de Luçon[1]	sativa	4x	Cultivated	5	France
Maron[1]	falcata	4x	Wild	1	France
Mezzo	sativa	4x	Cultivated	3.6	France
Miechowska	sativa	4x	Cultivated	3.4	Poland
Milfeuil	sativa	4x	Cultivated	5	France
Milky Max	sativa	4x	Cultivated	4.6	France
Monte Oscuro[1]	sativa	4x	Wild	1	Spain
Occitane	sativa	4x	Cultivated	7	France
Pancrudo[1]	sativa	4x	Wild	1	Spain
Quasifalcata[1]	falcata	2x	Wild	1	Russia
Romanica[1]	falcata	2x	Wild	1	Russia
Speeda	sativa	4x	Cultivated	8	France
Timbale	sativa	4x	Cultivated	4.5	France
Verdor	sativa	4x	Cultivated	7.4	France
Villanueva de Jara[1]	sativa	4x	Wild	1	Spain
Wugong[2]	sativa	4x	Cultivated	2	China

2.2. Experimental design

The experiment was carried out over successive growing seasons from April 2021 to June 2023 at the INRAE research station in Lusignan, France (46°26′08″N, 0°07′25″E). The station is characterised by an oceanic climate, with an average temperature of 12°C and an annual rainfall of approximately 805 ± 152 mm. The experimental climate data, including temperature and rainfall, are detailed in Table S1. The soil is classified as a clay loam, with a pH of 6.5. The entire biomass of the preceding crop (buckwheat) was incorporated into the soil. On 21 April 2021, lucerne seeds were planted at a rate of 10 kg·ha^{-1}, with a row spacing of 28 cm. Wheat was sown on 26 October 2021 and 28 October 2022 at a seeding rate of 250 seeds per m^2 between lucerne rows, giving a row spacing of 14 cm between lucerne and wheat. The experimental design was a randomised complete block design with three repetitions, with each plot measuring 6.7 m^2 (6 m × 1.12 m). Nitrogen fertilisation was applied at 40 kg·ha^{-1} using ammonium nitrate (33.5%N), spread at the end of the winter (early March of each year). The experiment was cut a few days before wheat sowing and the biomass, mainly composed of lucerne, was exported.

2.3. Plant Measurements and processing

In mixtures, it was not possible to discern the lucerne growth habit, even the prostrate-growing plants straightened up when they reached the wheat row. The growth habit was noted in March 2022 in a nursery established in April 2021, where the same lucerne accessions were cultivated with a spacing of 70 cm, and four repetitions comprising 10 plants each. The growth habit was rated from 1 (upright) to 9 (prostrate). In the trial, lucerne and wheat height (i.e., stem height) was measured manually at 15-day intervals from January to June in both 2022 and 2023, with three random measurements per plot. The average of these measurements was calculated to determine the mean value for each plot. The plant height dynamics was described by fitting a logistic equation to plant measurements as a function of thermal time (Eq.1) (France and Thornley 1984):

$$Y_i = \frac{L_i}{1+e^{-r_i(t-t0_i)}} \qquad (1)$$

Where, 'Y_i' is the plant height (cm) of the variety i, 'L_i' is the theoretical maximum plant height of the variety *i*, 'r_i' is the plant growth rate of the variety *i*, '$t0_i$' is the time in growing degree days (GDD) when the variety *i* reaches half of its maximum height, and 't' is the cumulative thermal growth time with a basis of 0°C. To characterise the competition for light between the two species, the average height difference was analysed by calculating the difference of the integrals between the two species during five-time intervals, representing the main development stages of wheat: seedling (120-300 GDD), tillering (300-600 GDD), stem elongation (600-900 GDD), heading (900-1200 GDD), and anthesis (1200-1511 GDD) (Fig. 1). The purpose of this analysis was to determine the wheat relative dominance (WRD) (cm) over lucerne at each stage (Eq.2).

$$WRD = \frac{1}{t_e - t_s}\left(\int_{t_s}^{t_e} Y_w(t)dt - \int_{t_s}^{t_e} Y_l(t)dt\right) \qquad (2)$$

Where, Y_w is the height of wheat over each stage interval from t_s (the start time) to t_e (the end time), and Y_l is the height of lucerne over the same stage interval.

Weed abundance was noted on a scale from 1 (absence) to 9 (exceeding 70% coverage in the plot) in January, March, and April of each year. Around 20 May each year, at the wheat heading stage, lucerne lodging was evaluated on a scale of 1 (upright) to 9 (flat). Plants were collected by hand clipping within a subplot measuring 75 cm × 28 cm (two rows) situated at the centre of each plot. Wheat, lucerne, and weeds were separated and the number of wheat tillers (m^{-2}) was recorded. The samples were then oven-dried at 60°C for 72 h to determine the dry matter ($t \cdot ha^{-1}$). To assess wheat nitrogen nutrition, we measured the nitrogen concentration in the upper wheat leaves (N_{up}). According to Louarn et al. (2020), N_{up} is a valid proxy of plant nitrogen nutrition in mixed crops, proportional to the Nitrogen Nutrition Index (NNI). We randomly selected 15 upper wheat leaves (the flag leaves) from each plot, then dried and ground for analysis using the Dumas method (Hansen 1989).

Weed dry matter was not analysed because the sampling zones for wheat and lucerne biomass did not allow capturing the average weed abundance across the entire plot. Only visual notes of weed abundance at the entire plot level were retained. Lucerne height at the wheat anthesis stage was used to designate its aggressiveness. In addition to the wheat dry biomass, the single tiller dry matter (g) was calculated by dividing the wheat dry biomass by the tiller number. With

this approach, we evaluated wheat growth and potential productivity, as well as the distribution of nutrient supply and demand among individual tillers. Wheat aboveground dry biomass and N_{up} at heading stage were used as proxies for the potential grain biomass and its quality (Kichey *et al.* 2007; Zhang *et al.* 2016; Alpuerto *et al.* 2021).

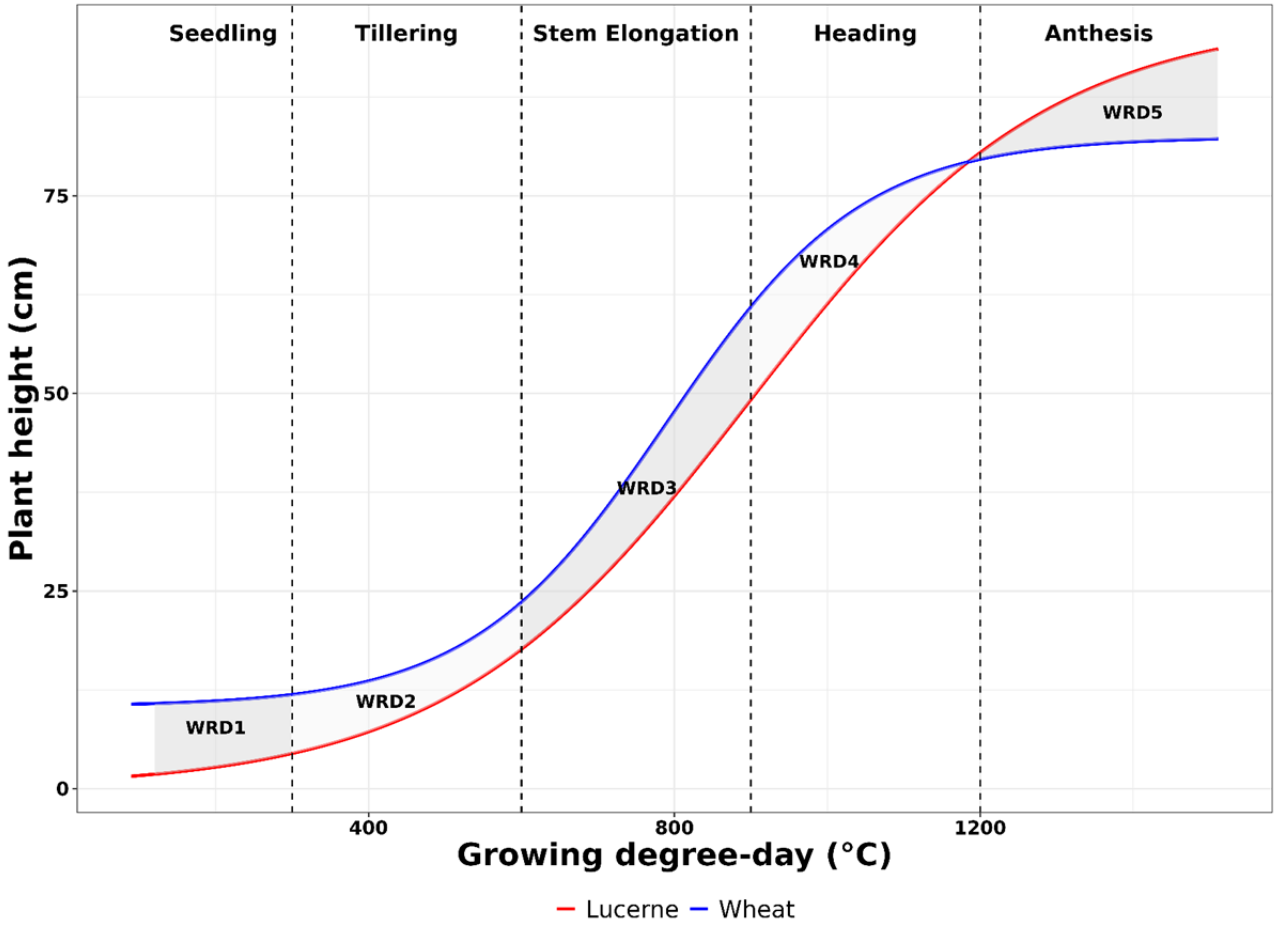

Figure 1. *Illustration of plant height dynamic for lucerne (var. L8988, red) and wheat (blue), through the five main growth stages of wheat in 2022. The shaded area between the two curves represents the difference in height integral between the two species, denoted by wheat relative dominance (WRD), followed by the number corresponding to each stage: 1: seedling, 2: tillering, 3: stem elongation, 4: heading, and 5: anthesis.*

2.4. Phenotypic spatial adjustment

To consider data heterogeneity due to microenvironmental conditions within the experimental trial, each trait was independently adjusted for spatial variability with breedR software (Munoz and Rodriguez 2020). The adjustment was performed using a linear mixed model (Eq. 3) for each year, incorporating the effects of lucerne accessions and spatial variability across rows, columns, and blocks within the plot.

$$Y_{ijk} = \mu + B_i + A_k + S_j + \epsilon_{ijk} \qquad (3)$$

Where, Y_{ijk} is the phenotype of the kth accession in the jth spatial location and the ith block, μ is the overall mean, B represents the fixed effect of the ith block, A represents the random effect of the kth lucerne accession, S is a matrix of the random effect of the jth spatial location (rows and columns) modelled using an autoregressive approach, ϵ_{ijk} is the residual effect. The adjusted data were used in the following analyses.

2.5. Statistical analysis

The statistical analysis was conducted using R Studio software, version 2023.12.1 (RStudio Team 2024). The effects of the experimental year, lucerne accession, and their interaction were assessed using a two-way ANOVA with the model (Eq. 4), to determine whether data from the two years could be studied together or separately and to evaluate the diversity among lucerne accessions.

$$Y_{ijk} = \mu + Z_j + A_k + (ZA)_{jk} + \epsilon_{ijk} \qquad (4)$$

Where, Y_{ijk} is the dependent trait, μ is the overall mean, Z represents the fixed effect of the jth experimental year, A represents the fixed effect of the kth lucerne accession, (ZA) is the interaction between the lucerne accession and the year, and ϵ_{ijk} is the residual effect.

Principal component analysis (PCA) and cluster analysis were used to group lucerne accessions based on the averages of the three repetitions for the following phenotypic traits: dormancy, growth habit, lodging, and lucerne height at wheat anthesis stage. PCA was performed on standardised data with the 'FactoMineR' package (Lê *et al.* 2008), and the optimum number of clusters was determined using the 'NbClust' package (Charrad *et al.* 2012). These groups were then used to identify whether phenotypically similar accessions had a similar effect on wheat

and weed responses. The effect of the groups was tested on all variables, including those related to lucerne, using a one-way analysis of variance (ANOVA). In order to verify the interdependence between the key traits of lucerne, i.e. dormancy, growth habit, height at wheat heading stage, and lodging, and the responses of wheat and weed abundance, a Pearson correlation analysis was performed using the average data from the three repetitions. The interdependence between traits was based on their temporal and physiological relevance. Specifically *(i)* lucerne dormancy and *(ii)* growth habit were tested for their effects on wheat relative dominance during the initial growth stages: seedling, tillering, stem elongation, and heading, as well as total tiller number and weed abundance in January, March, and April. The effect of *(iii)* lucerne height at wheat anthesis stage was tested on wheat yield components, represented in this study by single tiller dry matter, total wheat dry biomass, and wheat nitrogen nutrition. The effect of *(iv)* lucerne lodging was tested on wheat relative dominance at anthesis growth stage and total wheat dry biomass. In all analyses, the significance threshold was set at $p \leq 0.05$.

3. Results

3.1. Effect of year, accession and their interaction on phenotypic traits

Lucerne dormancy and growth habit are generally considered stable and little affected by environmental conditions. Therefore, we used a single value for each variable instead of two annual evaluations. Lucerne dormancy varied significantly among accessions, ranging from 1 (very dormant) to 10 (non-dormant) (Table 2). Lucerne growth habit ranged from 3 (prostrate) to 8 (erect).

The experimental year had a significant effect ($p \leq 0.05$) on all phenotypic traits. The effects of lucerne accessions and the interaction between year and accession were also significant. Since the interaction effect was significant, the data were analysed separately for each year (Table 2).

Lucerne height at the wheat anthesis stage showed notable differences between the two years. In 2022, the height ranged from 52 cm to 110 cm, while in 2023, the range of variation was slightly smaller, ranging from 63 cm to 103 cm. Lucerne lodging varied widely in 2022, ranging from 1 (high resistance) to 6 (relatively weak resistance), whereas in 2023, the variability decreased, with values ranging from 0.9 to 3.9 (Table 2).

During the seedling stage, wheat exhibited pronounced dominance over lucerne, with wheat relative dominance (WRD) values ranging from 1.9 to 10 cm in 2022 and increasing to 3.4 to 14 cm in 2023 (Table 2). At the tillering stage, WRD values for nearly all accessions increased, ranging from -3.8 to 15 cm in 2022 and from 0.5 to 13 cm in 2023. At the stem elongation stage, WRD ranged from 0.1 to 27 cm in 2022 and from 4 to 16 cm in 2023. At the heading stage, the WRD ranged from -0.9 to 32 cm in 2022 and from 1.6 to 20 cm in 2023. At the anthesis stage, the final growth stage for wheat in this experiment, wheat relative dominance was generally negative, with an average of -6.1 cm, with values ranging from -17 to 25 cm in 2022 and from -8.6 to 12 cm in 2023, with an average of -2.27 cm. The wheat tiller number also varied across the years, with values ranging from 196 to 290 tillers per m^2 in 2022, decreasing to 143 to 150 tillers per m^2 in 2023. Wheat dry biomass ranged from 2.2 to 3 t ha^{-1}in 2022 and from 1.2 to 3.2 t·ha^{-1} in 2023, while wheat single tiller dry matter ranged from 0.9 to 1.2 g in 2022 and from 0.8 to 2.1 g in 2023. For wheat N_{up}, the values ranged from 1.8 to 3.45 % in 2022 and from 1.8 to 2.8 % in 2023. Weed abundance notations varied significantly throughout the experiment, with a marked increase in 2023 compared to 2022. Across seasons, weed abundance ranged from 3.6 to 5.8 in January 2022 and from 6.1 to 8.4 in January 2023. In March, abundance increased from 3 to 7.5 in 2022 and from 5.2 to 7.9 in 2023, and in April, it ranged from 2.2 to 5 in 2022 and from 2.3 to 8 in 2023 (Table 2).

Table 2. *Summary of data, including minimum, maximum and mean values for 2022 and 2023, and significant p-value of the analysis of variance for year, accession, and their interaction effects on all phenotypic traits: lucerne dormancy, lucerne growth habit, lucerne lodging resistance, lucerne height at the wheat anthesis stage, wheat relative dominance (WRD) at different wheat growth stages (1: seedling, 2: tillering, 3: stem elongation, 4: heading and 5: anthesis), wheat dry matter biomass, wheat nitrogen nutrition (N_{up}), wheat tiller number, wheat single tiller dry matter, weed abundance in January, March, and April.*

		Data Description			p-value		
Trait	Year	Min	Max	Mean	Accession	Year	Year*Accession
Dormancy		1.00	10.00	3.30	< 0.001	< 0.001	< 0.001
Growth habit		3.08	8.31	5.39	< 0.001	< 0.001	< 0.001
Lucerne height (cm)	2022	52.43	110.11	93.63	< 0.001	< 0.001	< 0.001
	2023	63.51	103.27	91.55	< 0.001		
Lodging resistance	2022	1.04	6.07	3.32	< 0.001	< 0.001	< 0.001
	2023	0.90	3.90	2.36	< 0.001		
WRD1 (cm)	2022	1.95	10.35	6.47	< 0.001	< 0.001	< 0.001
	2023	3.42	14.35	8.83	< 0.001		
WRD2 (cm)	2022	-3.89	15.36	4.61	< 0.001	< 0.001	< 0.001
	2023	0.58	13.19	6.93	< 0.001		
WRD3 (cm)	2022	0.17	27.92	8.86	< 0.001	< 0.001	< 0.001
	2023	4.09	16.42	7.67	< 0.001		
WRD4 (cm)	2022	-0.90	32.50	7.60	< 0.001	< 0.001	< 0.001
	2023	1.65	20.88	6.85	< 0.001		
WRD5 (cm)	2022	17.52	25.36	-6.12	< 0.001	< 0.001	< 0.001
	2023	-8.64	12.89	-2.27	< 0.001		
Wheat dry matter yield (t ha^{-1})	2022	2.23	3.05	2.58	< 0.001	< 0.001	< 0.001
	2023	1.22	3.26	2.20	< 0.001		
Wheat nitrogen nutrition (%)	2022	1.89	3.45	2.93	< 0.001	< 0.001	< 0.001
	2023	1.85	2.81	2.47	< 0.001		
Wheat tiller number (m^2)	2022	196.92	290.36	232.56	0.002	< 0.001	0.016
	2023	143.94	150.48	147.03	< 0.001		
Single tiller dry matter (g)	2022	0.96	1.28	1.12	< 0.001	< 0.001	< 0.001
	2023	0.84	2.17	1.49	< 0.001		
Weed abundance-Jan	2022	3.62	5.88	4.92	< 0.001	< 0.001	< 0.001
	2023	6.16	8.48	7.35	< 0.001		
Weed abundance-Mar	2022	3.00	7.55	5.42	< 0.001	< 0.001	< 0.001
	2023	5.26	7.90	6.83	< 0.001		
Weed abundance-Apr	2022	2.24	5.08	3.38	< 0.001	< 0.001	< 0.001
	2023	2.31	8.01	4.37	< 0.001		

3.2. Lucerne diversity

The Principal Component Analysis (PCA) indicated that the first two principal components (PC1 and PC2) captured 56.2% and 27.9 % of the total variability, respectively (Fig. 2). PCA revealed a negative correlation between lucerne dormancy and growth habit (R = -0.76), as well as between growth habit, lucerne height at the wheat anthesis stage, and lodging. The positive correlation between lodging and height indicates that taller plants are more susceptible to lodging, which is largely due to the difficulties in characterising lodging in prostrate accessions. The optimal number of groups, determined by the Dunn Index (DI), was three (Fig. S1). The first group (G1) consisted of 12 accessions, mostly wild. Three cultivated accessions Krasnokutskaya, L8988, and Camporegio, which have some *falcata* origin, were also included. This group was characterised by its strong winter dormancy (1.2 ± 0.4), prostrate growth habit (7.5 ± 0.7), short height at the wheat heading stage (86 ± 12 cm in 2022 and 86 ± 8 cm in 2023), and the weakest lodging in our panel (3 ± 0.9 in 2022 and 1.8 ± 0.6 in 2023). The second group (G2) comprised 14 cultivated accessions characterised by a relatively high dormancy (3.6 ± 1.1), an erect growth habit (4 ± 0.5), a tall height at the wheat heading stage (100 ± 7 cm in 2022 and 95 ± 4 cm in 2023), and moderate lodging (3.4 ± 0.9 in 2022 and 3 ± 0.6 in 2023). The third group (G3) included four cultivated non-dormant accessions (8.1 ± 1.3), with an erect growth habit (3.4 ± 0.4), a tall height at the wheat heading stage (90 ± 14 cm in 2022 and 91 ± 10 cm in 2023), and moderate lodging (3.6 ± 2.1 in 2022 and 1.5 ± 0.4 in 2023). The PCA clearly shows the absence of prostrate, non-dormant accessions in our panel.

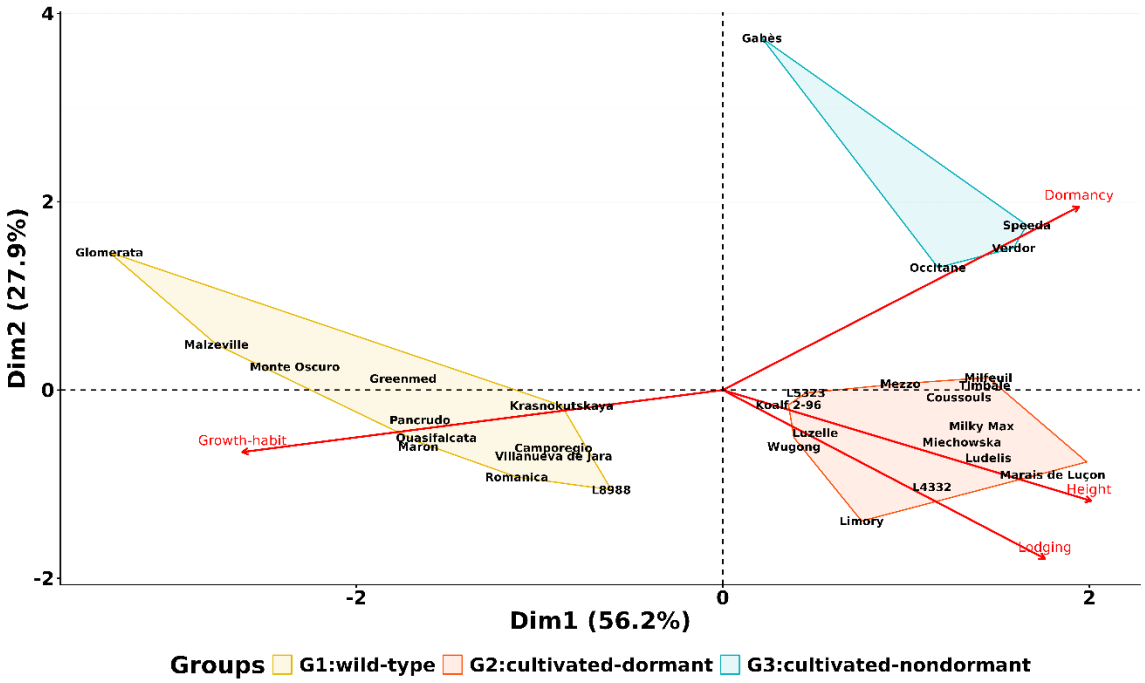

Figure 2. *Principal component analysis (PCA) on lucerne traits and accessions. The analysis was conducted with four phenotypic traits (dormancy, growth habit, lodging, and lucerne height at the wheat heading date) measured on thirty lucerne accessions. The forms show the three groups of accessions.*

3.3. Lucerne dormancy effect on wheat relative dominance, wheat tiller number and weed abundance

Wheat relative dominance

At the seedling stage, a significant negative correlation was observed between WRD and lucerne dormancy, with correlation coefficients of R = -0.8 in 2022 and R = -0.5 in 2023 (Fig. 3A). The effect of the accession group was significant ($p \leq 0.05$); wheat grown with lucerne accessions from Group 1 (wild-type) had the highest WRD values in both years: 9.3 ± 1.1 cm in 2022 and 11 ± 1.7 cm in 2023. The WRD values for Group 2 (cultivated-dormant) were lower, at 4.9 ± 1.1 cm in 2022 and 7.6 ± 1.8 cm in 2023, while Group 3 (cultivated non-dormant) recorded the lowest values, with 3.2 ± 1.6 cm in 2022 and 6.3 ± 2.0 cm in 2023 (Fig. 3A; Table S2).

From the tillering stage onward, one accession from Group 3, Gabès (the most winter-active accession in our panel, with a dormancy rating of 10), did not behave like the other accessions in its group; instead, it resembled those from Group 1. At the tillering stage, WRD remained negatively correlated with lucerne dormancy, with correlation coefficients of R = -0.7 in 2022 and R = -0.5 in 2023 (Fig. 3A). The group effect remained significant ($P \leq 0.05$), with Group 1 accessions showing the highest wheat dominance: 10.7 ± 2.9 cm in 2022 and 10.1 ± 2.1 cm in 2023. In contrast, wheat showed weak dominance or was even dominated by some accessions from Group 2 (0.9 ± 1.5 cm, ranging from -0.7 to 0.9 cm in 2022) and Group 3 (-0.7 ± 3.6 cm, ranging from -0.7 to 5.0 cm in 2022). In 2023, wheat showed improved dominance with only positive WRD values for both Group 3 (5.0 ± 2.5 cm) and Group 2 (4.7 ± 1.5 cm) (Fig. 3A; Table S2).

At the stem elongation stage, WRD maintained a negative correlation with lucerne dormancy score, with correlation coefficients of R = -0.6 in 2022 and R = -0.3 in 2023 (Fig. 3A). The group effect remained significant, with Group 1 accessions allowing wheat to reach the highest WRD values: 17 ± 5.4 cm in 2022 and 11 ± 3.1 cm in 2023. At this stage, wheat regained dominance with positive WRD values, and Group 3 accessions recorded higher WRD than Group 2, with values of 7.4 ± 4.0 cm in 2023 and 6.5 ± 1.4 cm in 2022 (Fig. 3A; Table S2).

At the heading stage, WRD showed a weak negative correlation with lucerne dormancy score (R = -0.34) in 2022 and a non-significant correlation (R = -0.08) in 2023 (Fig. 3A). Group 1 accessions continued to show the highest WRD values, with 14 ± 7.3 cm in 2022 and 8.5 ± 4.9 cm in 2023. In comparison, Group 2 had the lowest WRD values, with 2.7 ± 1.8 cm in 2022 and 5.2 ± 1.7 cm in 2023, while Group 3 recorded intermediate values, with 6.8 ± 6.9 cm in 2022 and 7.9 ± 6.5 cm in 2023 (Fig. 3A; Table S2).

Wheat tiller number

In 2022, the number of tillers showed a moderate negative correlation with lucerne dormancy, with a correlation coefficient of R = -0.29, and there was a significant difference between groups (Fig. 3B). Wheat cultivated with Group 1 accessions had the highest number of tillers, with an average of 245 ± 21 tillers m^{-2}, slightly more than those cultivated with Group 2 (222 ± 20 tillers m^{-2}), and Group 3 accessions (228 ± 16 tillers m^{-2}). In 2023, the correlation

between wheat tiller number and lucerne dormancy was non-significant (R = 0.13). Similarly, the effect of accession group on tiller number was also non-significant (Fig. 3B; Table S2).

Weed abundance

The correlation between weed abundance notations and lucerne dormancy was negative, with correlation coefficients of R = -0.64 in January, R = -0.37 in March, and R = -0.50 in April 2022. In 2023, the correlations were R = -0.64 in January, R = -0.41 in March, and R = -0.31 in April (Fig. 3C). Significant differences in weed abundance were observed among the three groups, showing a consistent pattern across both years. In January, plots cultivated with Group 1 accessions had the highest weed abundance, with 5.3 ± 0.3 in 2022, increasing to 7.7 ± 0.2 in 2023. Group 2 had intermediate values, with 4.8 ± 0.4 in 2022 and 7.1 ± 0.3 in 2023, while Group 3 had the lowest abundance, with 3.8 ± 0.2 in 2022 and 6.7 ± 0.7 in 2023. In March, Group 1 continued to have the highest weed abundance, with 6.4 ± 0.6 in 2022 and 7.3 ± 0.6 in 2023, compared to Group 2, which had averages of 4.6 ± 0.6 in 2022 and 6.4 ± 0.6 in 2023. Group 3 had the lowest weed abundance, with 5 ± 1.6 in 2022 and 6 ± 0.5 in 2023. In April, Group 1 continued to have the highest weed abundance, with 4 ± 0.6 in 2022 and 5.8 ± 1.2 in 2023. Group 3 followed, with 3 ± 0.7 in 2022 and 4.5 ± 2.1 in 2023. Notably, Group 2 had the lowest weed abundance, averaging 2.9 ± 0.2 in 2022 and 3 ± 0.3 in 2023 (Fig. 3C; Table S2). From March, we notice that, and similar to the wheat relative dominance (WRD) observations, the accession Gabès from Group 3 behaved similarly to the Group 1 accessions.

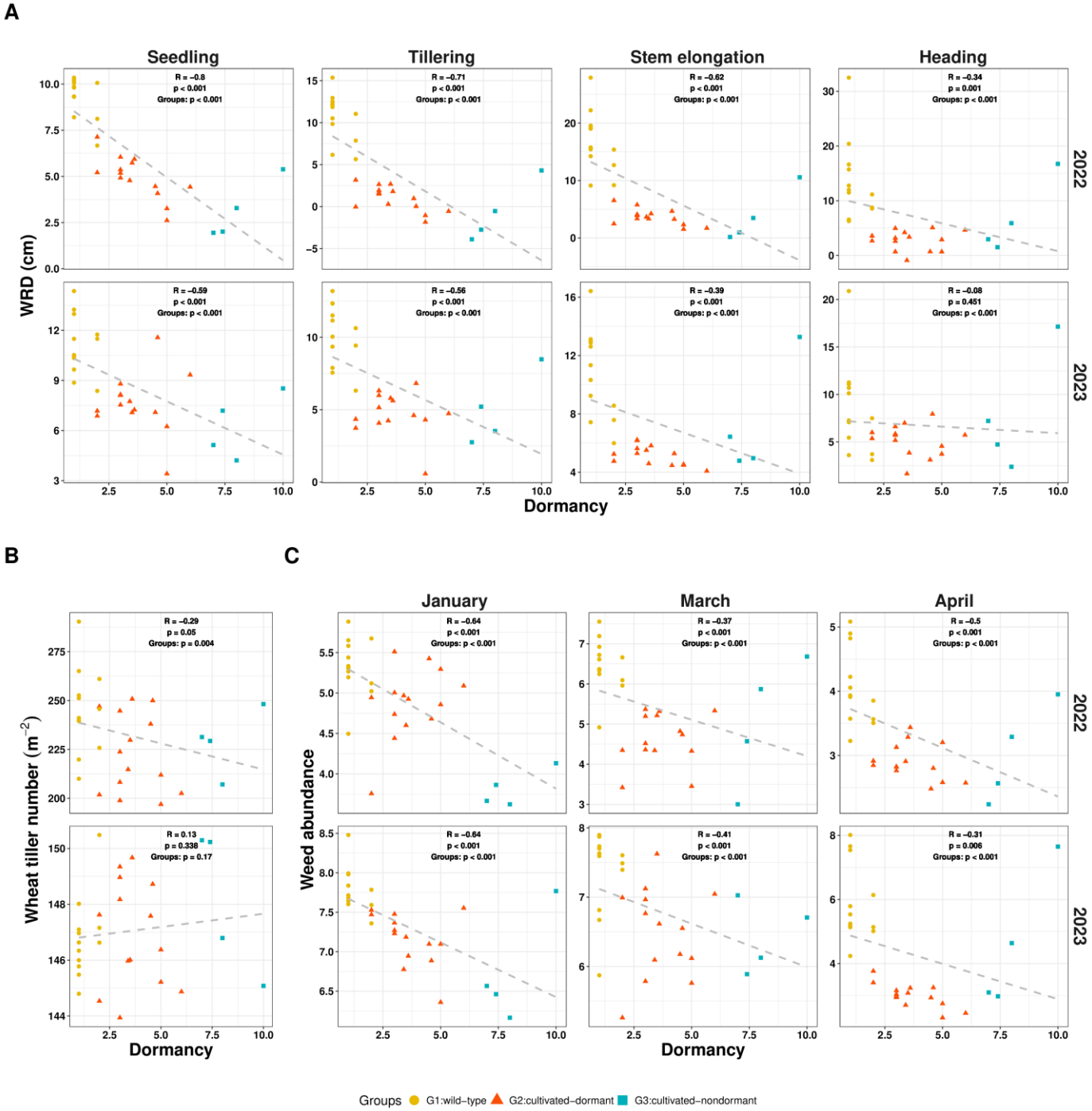

Figure 3. *Correlation between lucerne dormancy and wheat relative dominance during the wheat growth stages: seedling, tillering, stem elongation, and heading (A), wheat tiller number (B), and weed abundance in January, March, and April (C) for thirty lucerne accessions in 2022 and 2023.*

3.4. Lucerne growth habit effect on wheat relative dominance, wheat tiller number and weed abundance

Wheat relative dominance

At the seedling stage, a strong positive correlation was observed between wheat relative dominance (WRD) and lucerne growth habit (R = 0.92 in 2022 and R = 0.75 in 2023) (Fig. 4A). At the tillering stage, wheat dominance further increased, with WRD showing a strong correlation with lucerne growth habit (R = 0.93 in 2022 and R = 0.83 in 2023). The strong correlation continued during the stem elongation stage, with R = 0.88 in 2022 and R = 0.74 in 2023. During the heading stage, the positive correlation with lucerne growth habit persisted but weakened slightly in 2022 (R = 0.69) and decreased further to R = 0.34 in 2023. The effect of the accession group on this variable has been detailed previously (Fig. 4A).

Wheat tiller number

In the first year, the number of tillers exhibited a significant moderate positive correlation with lucerne growth habit, with a correlation coefficient of R = 0.44. However, in 2023, this correlation was non-significant, with a correlation coefficient of R = -0.13.

Weed abundance

Strong and significant positive correlations were observed between lucerne growth habit and weed abundance notations throughout both years. In 2022, the correlation coefficients ranged from R = 0.62 in January to R = 0.74 in March and R = 0.77 in April. Similarly, in 2023, the coefficients ranged from R = 0.77 in January to R = 0.60 in March and R = 0.73 in April (Fig. 4C).

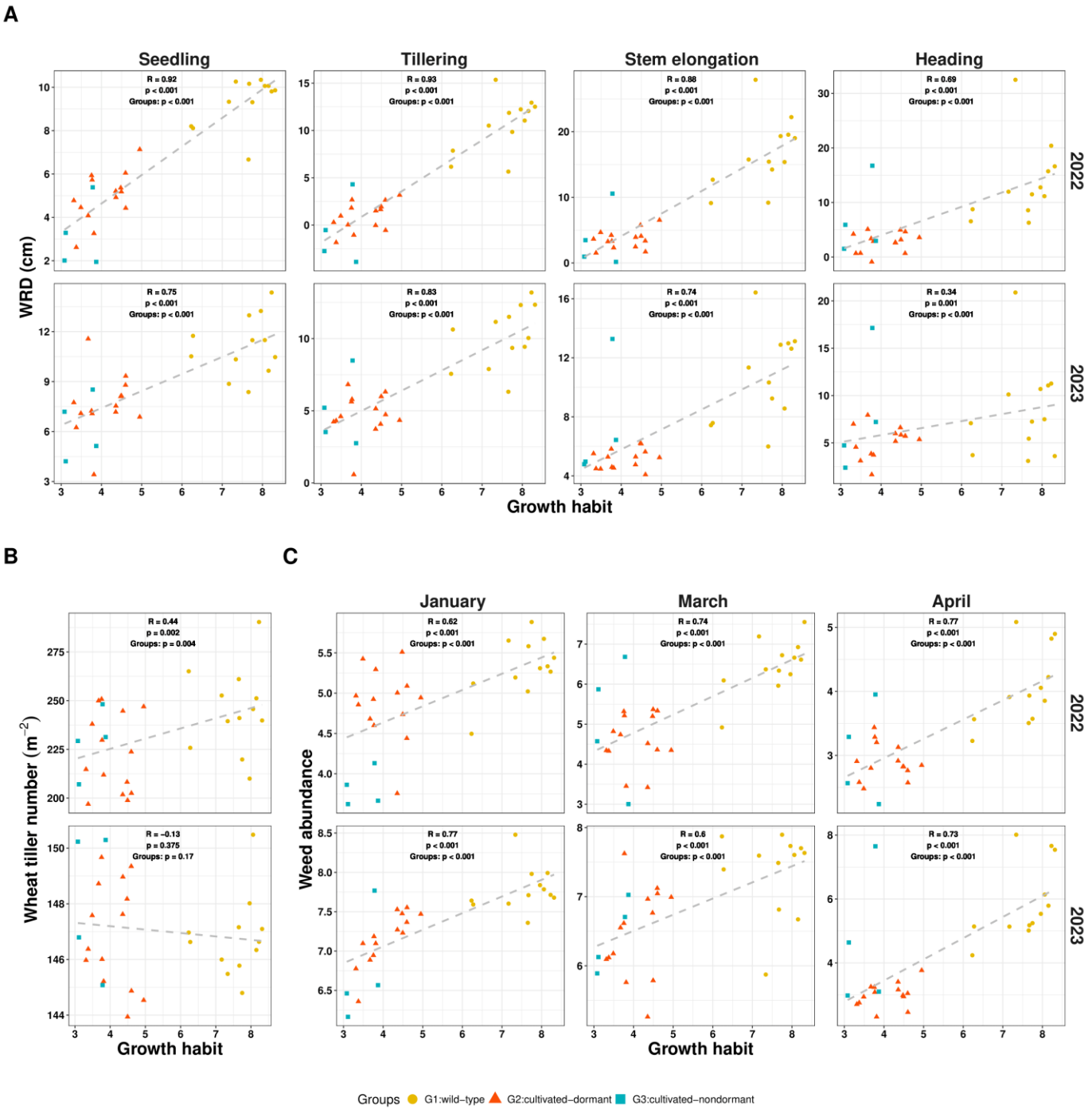

Figure 4. *Correlation between lucerne growth habit and wheat relative dominance during the wheat growth stages: seedling, tillering, stem elongation, and heading (A), wheat tiller number (B) and weed abundance in January, March, and April (C) for thirty lucerne accessions in 2022 and 2023.*

3.5. Effect of lucerne height on wheat dry biomass and wheat nitrogen nutrition at wheat heading stage

Wheat dry biomass

In 2022, wheat single tiller dry matter showed no correlation with lucerne height at the wheat anthesis stage (Fig. 5A). Similarly, there were no significant differences among the three groups, with average values of Group 1 (1.1 ± 0.09 g), Group 2 (1.1 ± 0.10 g), and Group 3 (1.1 ± 0.07 g) (Fig. 5A; Table S2). However, in 2023, a significant positive correlation was observed (R = 0.55) (Fig. 5A). Significant differences were noted among the three groups, with Group 3 showing the highest value (1.7 ± 0.3 g), followed by Group 2 (1.6 ± 0.2 g) and Group 1 (1.2 ± 0.2 g) (Fig. 5A; Table S2).

Wheat dry matter showed a negative correlation with lucerne height at the wheat anthesis stage in 2022 (R = -0.32) (Fig. 5B), and significant differences between the groups with Group 1 averaging 2.7 ± 0.1 $t \cdot ha^{-1}$, Group 2 at 2.5 ± 0.2 $t \cdot ha^{-1}$, and Group 3 at 2.5 ± 0.1 $t \cdot ha^{-1}$. The groups also displayed considerable variability among accessions (Fig. 5B; Table S2). In contrast, in 2023, wheat dry biomass showed a positive correlation with lucerne height at the wheat anthesis stage (R = 0.57) (Fig. 5B), indicating a reversal in trend from the previous year. The differences between the groups were significant, and the trend was also reversed. The highest wheat dry biomass in 2023 was recorded when wheat was cultivated with accessions from Group 3 (2.6 ± 0.5 $t \cdot ha^{-1}$), followed by Group 2 (2.4 ± 0.3 $t \cdot ha^{-1}$), while Group 1 showed the lowest biomass (1.8 ± 0.4 $t \cdot ha^{-1}$). In 2023, wheat dry biomass and single tiller dry matter followed a similar pattern, with Group 3 showing the best results for both variables (Fig. 5B; Table S2).

Wheat nitrogen nutrition

In 2022, wheat N_{up} showed a strong positive correlation with lucerne height at the wheat anthesis stage (R = 0.82) (Fig. 5C). Significant differences were observed among the three groups, with Group 2 demonstrating the highest N_{up} at 3.2 ± 0.1 %, followed by Group 3 (2.8 ± 0.5 %) and Group 1 (2.5 ± 0.3 %) (Fig. 5C; Table S2). In 2023, the correlation remained

strong, but slightly reduced compared to the previous year (R = 0.78) (Fig. 5C). The group order was maintained, with Group 2 showing the highest N_{up} at 2.6 ± 0.1 %, followed by Group 3 (2.3 ± 0.3 %) and Group 1 (2.2 ± 0.2 %) (Fig. 5C; Table S2).

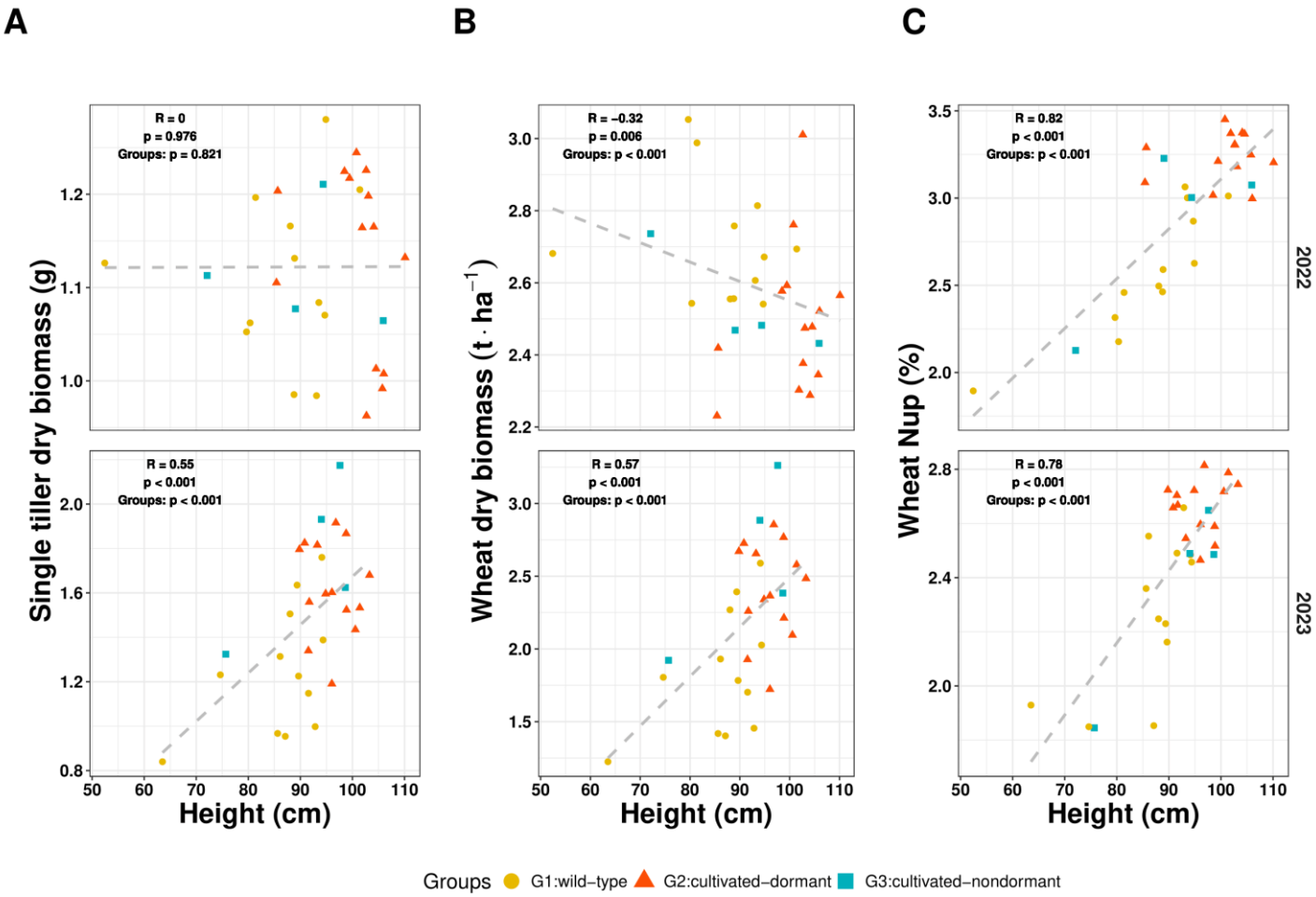

Figure 5. *Correlation between lucerne height at the wheat anthesis stage and wheat single tiller dry biomass (A), wheat dry biomass (B), and wheat nitrogen concentration, represented by Nup (C), for the thirty lucerne accessions in 2022 and 2023.*

3.6. Lucerne lodging effect on wheat relative dominance and wheat dry biomass

Wheat relative dominance

At the anthesis stage in 2022, a moderately negative correlation was observed between wheat relative dominance and lucerne lodging, with a correlation coefficient of R = -0.40 (Fig. 6A). Significant differences were noted among the groups, with Group 1 exhibiting a slight dominance of -0.1 ± 9.4 cm, while Group 3 recorded -4.8 ± 10 cm, and all accessions of Group

2 dominated wheat (WRD = -11 ± 3.9 cm) (Fig. 6A; Table S2). In 2023, the correlation between wheat relative dominance and lucerne lodging was non-significant, with a correlation coefficient of R = -0.02 (Fig. 6A). Similarly, no significant differences were found among the groups for WRD, with Group 1 recording -2.2 ± 5.7 cm, Group 2 at -2.6 ± 3.2 cm, and Group 3 at -1 ± 5.8 cm (Fig. 6A; Table S2).

Wheat dry biomass

In 2022, a weak negative correlation was observed between wheat dry biomass and lucerne lodging (R = -0.30) (Fig. 6B). In contrast, in 2023, wheat dry biomass exhibited a weak positive correlation with lucerne lodging (R = 0.29), indicating a reversal in trend compared to the previous year. The differences among the groups regarding this variable have been previously detailed.

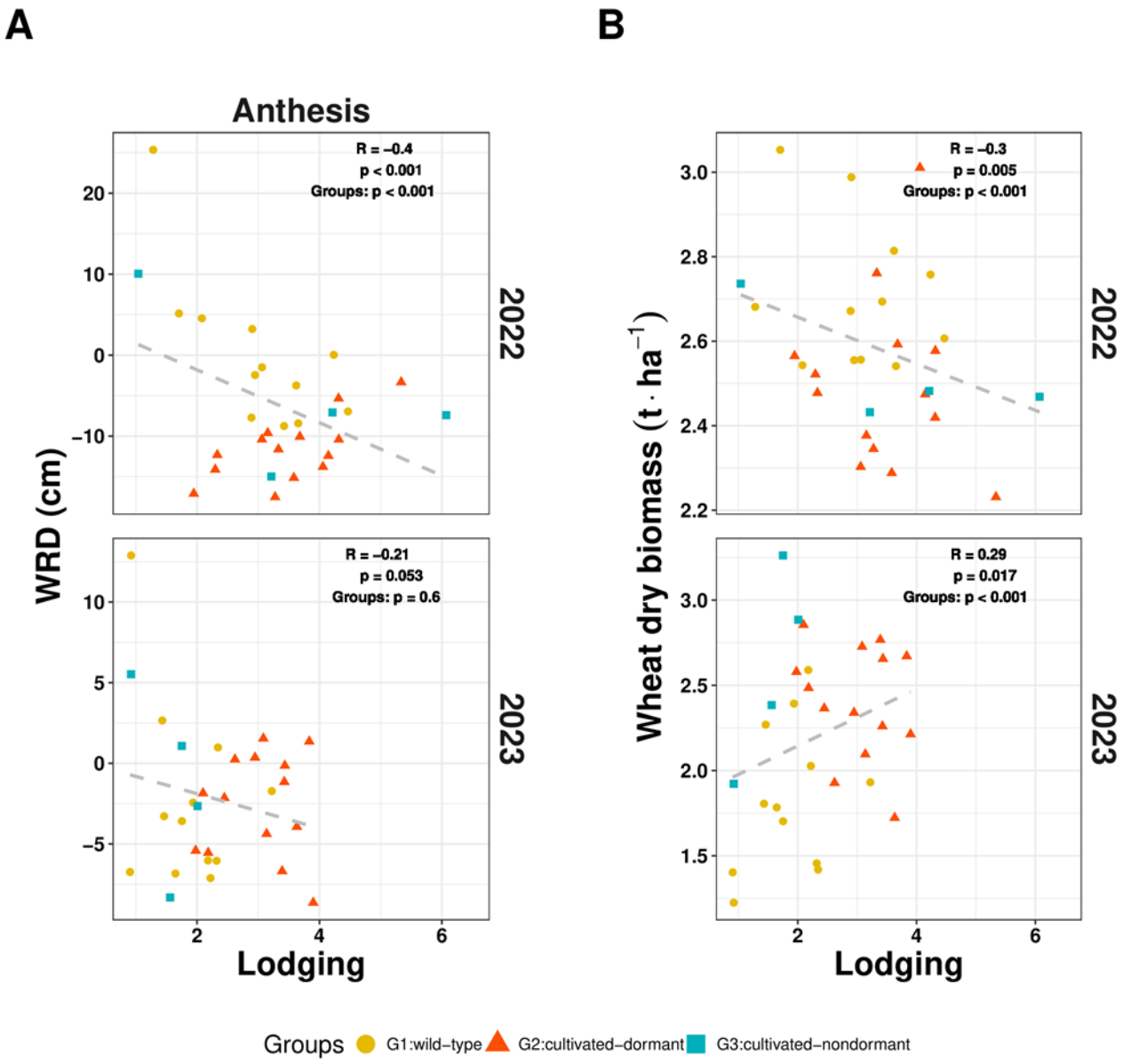

Figure 6. *Correlation between lucerne lodging and wheat relative dominance at the anthesis stage (A) and wheat dry biomass (B) for the thirty lucerne accessions in 2022 and 2023.*

4. Discussion

In this study, we based the analyses on the temporal and physiological relevance of lucerne traits and the responses of wheat and weeds. Lucerne dormancy and growth habit were influential during the early stages of wheat development and in determining weed abundance. In contrast, lucerne height at the wheat anthesis stage became critical during this late stage of wheat development, directly influencing wheat yield components such as total biomass, and

indirectly affecting nitrogen nutrition. Similarly, lucerne lodging played a significant role in determining wheat biomass.

4.1. Dormancy and growth habit effects

Lucerne dormancy significantly enhanced wheat relative dominance throughout the early development stages; seedling, tillering, stem elongation, and heading, over both experimental years. The higher the dormancy of lucerne (Group 1: wild-type), the more effectively wheat can dominate in these early development stages. These findings aligned with previous studies showing that wheat yield loss was lower on winter-dormant lucerne mulch than on winter-active lucerne (Bullied *et al.* 2002; Humphries *et al.* 2004). This reduced competition can be attributed to the temporal separation of growth periods: dormant lucerne halted growth in the winter and resumed in the late spring, while wheat thrived during the winter, resulting in weak competition between the two crops (Cougnon *et al.* 2022). However, our results indicated that winter-dormant lucerne was less competitive against weeds than non-dormant lucerne (Group 3: cultivated, non-dormant). The weed-suppressive role of non-dormant lucerne can be attributed to its continuous growth and biomass production during these periods (Barilli *et al.* 2017; Vincent-Caboud *et al.* 2019). Living mulch, such as non-dormant lucerne, suppresses weeds through several mechanisms, mainly by reducing weed germination through shading, which decreases light availability and quality (e.g., lowering the red to far-red ratio), and modifying soil temperature and moisture. Additionally, it competes with weeds for limited resources such as water and nutrients (Wiens *et al.* 2006; Radicetti *et al.* 2018; Bhaskar *et al.* 2021). Furthermore, the combination of lucerne and wheat enhances weed suppression by absorbing more photosynthetically active radiation, further limiting light availability to weeds (Shili-Touzi 2009). Lucerne dormancy should be selected carefully to exert sufficient negative effects on weed establishment while minimizing competition with wheat, even if the same competition effects are on play.

During these early stages of wheat growth, lucerne growth habit showed a positive correlation with wheat relative dominance. Specifically, prostrate lucerne (Group 1: wild type) promoted wheat relative dominance. This result was expected, as the prostrate growth of lucerne reduced overshadowing and minimised light competition with wheat. However, prostrate lucerne was less competitive against weeds, which contradicted our initial hypothesis and previous studies suggesting that prostrate or lateral growth forms of living mulches are more effective than erect

types in covering the soil and suppressing weeds (Biazzo and Masiunas 2000; Leoni *et al.* 2022; Cougnon *et al.* 2022). This may be explained by the nature of the accessions chosen in our study, which exhibited a strong negative correlation between dormancy and growth habit. The non- to moderately dormant accessions (Group 2 and Group 3) exhibited an erect growth habit, which induced strong competition with both weeds and wheat. In contrast, the dormant, prostrate accessions (Group 1) gave wheat and weeds the opportunity to dominate. However, we lacked data on how the prostrate non-dormant accessions might have influenced these results.

According to the literature, wheat yield loss in living mulches is primarily attributed to reduced tiller formation during the tillering stage (Hiltbrunner and Liedgens 2008), and a lower proportion of tillers developing into ears from the stem elongation to heading stages (Carof *et al.* 2007a). In our study, the number of wheat tillers in 2022 was similar to the sowing density, which indicates that there was no tillering. Additionally, the number of wheat tillers in 2022 was positively correlated with lucerne growth habit and negatively correlated with dormancy, likely due to the low competition between the dormant, prostrate accessions (Group 1) and wheat. However, in 2023, the number of tillers was low, and no correlation with either lucerne dormancy or growth habit was observed. This could be attributed to the early high weed abundance observed from January 2023, which increased competition. To achieve a balance between minimising competition with wheat and maximising competition with weeds, moderate dormancy in lucerne (i.e., significant growth in the winter) along with moderate height is likely to be an effective choice. However, due to the nature of our accessions, our conclusions are limited to the effect of dormancy, and we cannot determine whether growth should be prostrate or erect.

We observed that with the Gabès accession from Group 3, wheat and weeds exhibited similar traits values as with the dormant accessions from Group 1. This deviation can be attributed to frost damage that this accession experienced in mid-January 2022. The damage significantly impacted its survival and growth, reduced its competitiveness, and led to lasting effects that persisted into the subsequent growth stages and the following year. These findings are particularly noteworthy, as this accession exhibited early effectiveness against weeds. Subsequently, the depressed growth of this accession allowed wheat to maintain its dominance.

This combination of winter activity and frost susceptibility is also beneficial and may represent a favourable choice under similar climatic conditions.

4.2. Effect of lucerne height at the wheat anthesis stage

The anthesis stage represented the final phase of wheat development in our study. This stage marks the beginning of grain setting and filling. Before this stage, mature leaves act as sinks to store nitrogen. At anthesis, up to 95% of the nitrogen in grains is derived from remobilisation, primarily from the flag leaves, while a minor proportion of seed nitrogen is sourced from post-flowering nitrogen uptake (Barillot *et al.* 2016). Therefore, the nitrogen concentration in the upper wheat leaves (N_{up}) can serve as a representative proxy to reflect the whole-plant nitrogen status (NNI) and grain quality (Dreccer 2000; Kichey *et al.* 2007; Louarn *et al.* 2020; Alpuerto *et al.* 2021). Likewise, pre-anthesis biomass plays a crucial role in determining grain yield (Zhang *et al.* 2016). These findings suggest that early competition is inseparable from competition during the anthesis stage. Additionally, they highlight the necessity of incorporating dry matter biomass and N_{up} into our analysis. The correlation between wheat dry biomass and lucerne height at the wheat anthesis stage varied between the two years of the study. In 2022, the correlation was negative, showing that wheat cultivated with lucerne accessions from Group 1 exhibiting the highest biomass. However, in 2023, the correlation shifted to positive, so wheat cultivated with lucerne accessions from Groups 2 and 3 demonstrating the highest dry matter biomass and the largest single stem dry matter. We suppose that, in the first year, the lucerne accessions from Group 1, of low vigour, enabled wheat to dominate and consequently achieve high biomass. In contrast, during the second year, wheat benefited from the robust growth of lucerne accessions from Groups 2 and 3 during the previous year, which enriched the soil with nitrogen through root rhizodeposits and the mineralisation of root residues (Bullied *et al.* 2002; Cougnon *et al.* 2022). The positive correlation between lucerne height at the wheat anthesis stage and N_{up} could be explained by the proportional increase in nitrogen fixation with lucerne growth, which leads to greater nitrogen release through root rhizodeposits and root biomass mineralisation (Louarn *et al.* 2015).

The competition for nitrogen between wheat and lucerne is known to be intense at the onset of anthesis (Carof *et al.* 2007a; Shili-Touzi 2009). Wheat N_{up} ranged between 1.89% and 3.45% in 2022, and between 1.85% and 2.81% in 2023. This corresponded to NNI ranging from 0.23

(very severe stress) to 0.77 (moderate stress) in 2022. In 2023, nitrogen stress was even more severe, with the NNI ranging from 0.21 to 0.55, based on the formula by (Ziadi *et al.* 2010). These findings demonstrate that the wheat in our experiment was consistently subjected to nitrogen stress. Incorporating lucerne biomass into the soil during the wheat off-season, rather than exploiting it for biomass as done in this experiment, or applying appropriate nitrogen fertilisation during wheat growth, could potentially enhance soil nitrogen levels.

4.3. Lodging effect

At the anthesis stage, a negative correlation was observed between wheat relative dominance and lucerne lodging in 2022. The erect and vigorous lucerne accessions from Groups 2 and 3 dominated wheat, but they displayed high levels of lodging. This can be attributed to the traits of the selected accessions in this study, as the tall erect accessions were more susceptible to lodging than the shorter, prostrate ones, for which it was difficult to score lodging resistance. When lucerne plants lodged, they fell onto wheat plants, dragging wheat.

Wheat dry biomass also showed a contrasting correlation with lucerne lodging, being negative in 2022 and positive in 2023. In 2023, the variability in lodging among the accessions was minimal but the 2022 results aligned with our hypothesis. Several factors, beyond the nature of the selected accessions, may explain these findings. Firstly, under monoculture conditions for forage production, lucerne accessions from Groups 2 and 3 grow rapidly in the spring, but cuttings are done at early bud stage (April or May) to combine forage yield and quality while reducing lodging risks. However, in the living mulch system, cutting is delayed until after the wheat harvest, the risk of plant lodging is thus very high (Hiltbrunner *et al.* 2007). We hypothesise that the correlation between lodging and wheat dry biomass is indirect. The accessions with high lodging were from Groups 2 and 3. These accessions effectively suppressed weeds and fixed nitrogen due to their high growth rates, which allowed the wheat to develop strongly when grown alongside them in 2023.

The selection of lucerne accessions for living mulch systems should prioritise several key traits. During the early stage of wheat development, lucerne should display moderate dormancy and height. With this combination, lucerne could not overshadow wheat while still competing effectively against weeds. At the late stage of wheat growth, lucerne should grow slowly, have moderate height, and exhibit low lodging. These characteristics are also expected in systems

where lucerne growth is regulated just before to the wheat heading stage. Although no variety in our panel gathered all these traits due to genetic linkages, such varieties could potentially be developed through targeted breeding programmes. Interestingly, some accessions from Group 2, which included cultivated accessions, exhibited traits close to our objectives, despite being initially selected for forage production. The choice of wheat variety is also crucial in this system. Wheat varieties with strong ground cover in the winter could be preferable to reduce weed pressure before lucerne begins growing, along with tall stems and good resistance to lodging.

The results also suggest the necessity to focus on management practices that will be essential for establishing standards for this system. This includes incorporating lucerne biomass during the wheat off-season, nitrogen fertilisation strategies, row spacing, and the timing of wheat sowing in relation to the first or second year of lucerne growth. Additionally, climatic conditions in each region must be considered, as we observed that frost can be advantageous in reducing lucerne growth, allowing wheat to dominate.

5. Conclusion

This study reveals the complex dynamics between lucerne and wheat growth within a living mulch system, highlighting the importance of lucerne genetic diversity for some traits to optimise their interactions. This study is based on the vegetative phase of wheat and shows that early-stage competition is critical, as it significantly influences subsequent wheat development. Therefore, our results indicate that lucerne accessions that are moderately dormant and exhibit a moderate height are preferred, as this combination reduces competition for light while effectively suppressing weeds. The effects of lucerne height and lodging on wheat dry biomass varied over the two years of the study. Generally, lucerne should exhibit slow growth, moderate height, and low lodging to optimise its benefits (Fig. 7). Unfortunately, no variety in our panel exhibited all these desirable traits due to genetic linkages. Frost susceptibility could also be an option to have a natural regulation of lucerne during the winter.

Finally, there is a pressing need for breeding programmes focused on developing lucerne accessions that can effectively integrate weed suppression, optimal nitrogen nutrition, and reduced competition with wheat. Furthermore, similar experiments with various wheat varieties, or winter crops in general as cash crops, could yield beneficial results.

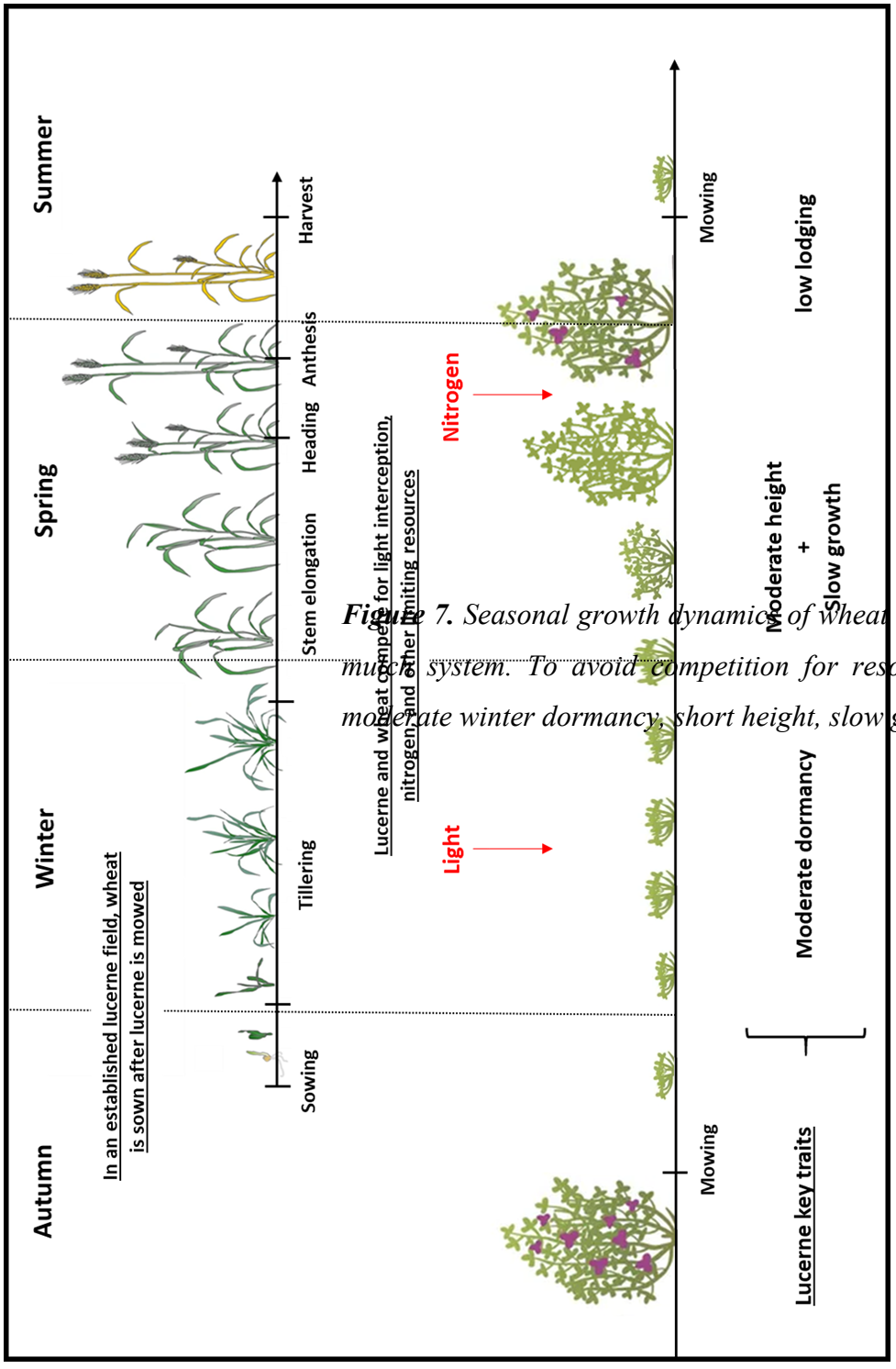

Figure 7. *Seasonal growth dynamics of wheat (Triticum aestivum L.) and lucern*
mulch system. To avoid competition for resources, particularly light and ni
moderate winter dormancy, short height, slow growth in spring, and low lodging

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Supplementary Material

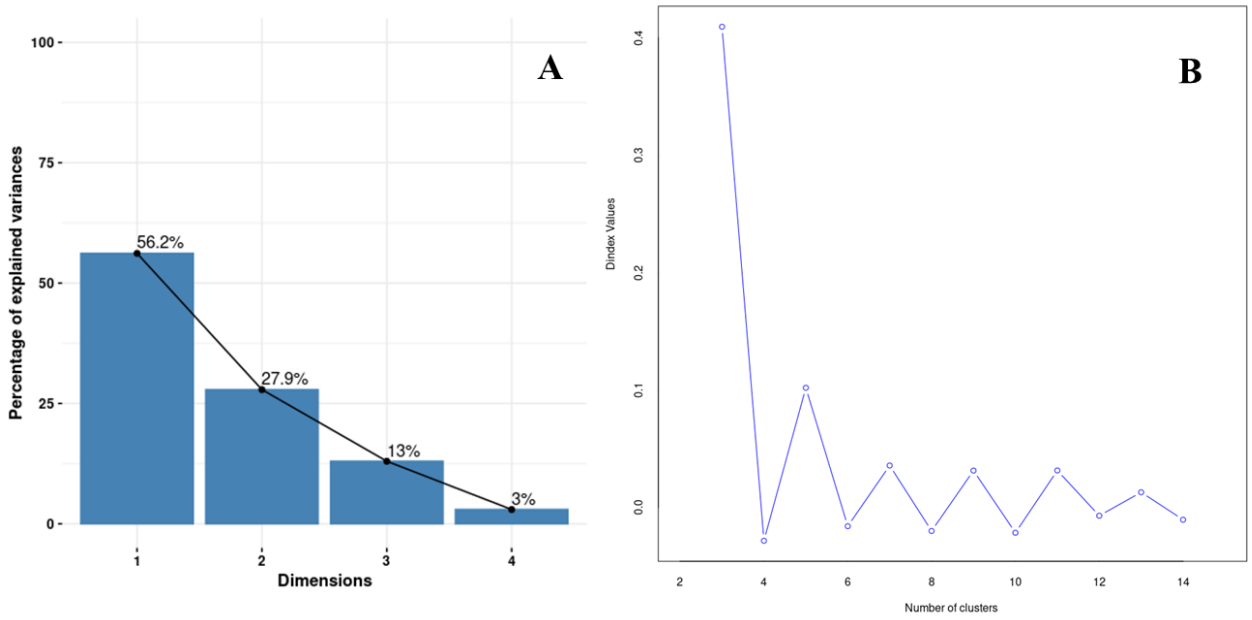

Figure 2S. *Principal component analysis (PCA) results. (A) Cumulative variance among principal components (PC) revealed the contributions of four PC. (B) Dunn Index values plotted as a function of the number of groups.*

Table S1. *Climate data in 2021-2023 in Lusignan (F)*

Month	Min / Max Temperatures (°C)			Rainfall (mm)		
	2021	2022	2023	2021	2022	2023
January	1.8/7.2	1.7/7.2	3.7/8	96	39,5	120
February	4.9/11.1	3.1/11.7	2/10.5	80	31	47,5
March	2.9/13.5	4.8/14.4	5.7/13.5	16	30,5	117,5
Avril	3.8/16.1	6.2/16.3	6.1/16	18,5	60,5	34
May	7.9/17.8	10.5/23.4	9.8/20.8	90,5	12	28,5
June	13.6/24.5	14.1/26.2	14.8/27.2	98	98,5	86
July	14.1/24.4	14.8/29.3	14.2/25.8	59,5	5,5	26,5
August	13.4/24.1	16.3/29.5	15.1/26.8	23,5	46,5	43,5
September	13.1/24.2	11.6/22.6	15.3/27.2	38	65,5	70
October	7.4/18	12.5/20.9	10.7/20.8	52,5	51	203
November	3.5/9.9	6.9/14.1	7.1/12.6	34	88,5	214,5
December	3.9/9.1	3.6/8.5	5.4/10	120	92	119,5

Table S2. *Descriptive statistics for all phenotypic traits in each group, in 2022 and 2023 (Group 1: wild type, Group 2: cultivated-dormant, Group 3: cultivated-non-dormant).*

Trait	*Group*	*Year*	*Mean*	*Standard deviation*	*Maximum*	*Minimum*
Dormancy	Group 1		1,25	0,45	2,00	1,00
	Group 2		3,69	1,18	6,00	2,00
	Group 3		8,10	1,33	10,00	7,00
Growth habit	Group 1		7,57	0,70	8,31	6,23
	Group 2		4,07	0,53	4,95	3,31
	Group 3		3,46	0,42	3,87	3,08
Lucerne height at the wheat heading stage (cm)	Group 1	2022	86,43	12,57	101,42	52,43
	Group 2		100,74	7,07	110,11	85,40
	Group 3		90,36	14,07	105,91	72,09
	Group 1	2023	86,41	8,93	94,36	63,51
	Group 2		95,98	4,22	103,27	89,81
	Group 3		91,49	10,72	98,63	75,68
Lodging	Group 1	2022	3,02	0,96	4,47	1,28
	Group 2		3,49	0,92	5,34	1,95
	Group 3		3,63	2,10	6,07	1,04
	Group 1	2023	1,86	0,66	3,22	0,90
	Group 2		3,01	0,65	3,90	1,98
	Group 3		1,56	0,46	2,01	0,92
Wheat relative dominance at seedling stage (cm)	Group 1	2022	9,35	1,13	10,35	6,67
	Group 2		4,94	1,16	7,13	2,62
	Group 3		3,16	1,61	5,38	1,95
	Group 1	2023	11,12	1,79	14,35	8,37
	Group 2		7,60	1,79	11,57	3,42
	Group 3		6,27	1,95	8,52	4,22
Wheat relative dominance at tillering stage (cm)	Group 1	2022	10,67	2,87	15,36	5,65
	Group 2		0,94	1,51	3,17	-1,84
	Group 3		-0,72	3,63	4,30	-3,89
	Group 1	2023	10,14	2,12	13,19	6,32
	Group 2		4,73	1,51	6,82	0,58
	Group 3		4,99	2,54	8,48	2,75
Wheat relative dominance at stem elongation stage (cm)	Group 1	2022	16,66	5,37	27,92	9,13
	Group 2		3,63	1,42	6,53	1,52
	Group 3		3,79	4,73	10,57	0,17
	Group 1	2023	10,71	3,05	16,42	5,99
	Group 2		5,15	0,68	6,22	4,09

	Group 3		7,37	4,01	13,27	4,79
Wheat relative dominance at heading stage (cm)	Group 1	2022	13,57	7,28	32,50	6,28
	Group 2		2,73	1,83	5,08	-0,90
	Group 3		6,78	6,89	16,74	1,51
	Group 1	2023	8,48	4,91	20,88	3,10
	Group 2		5,16	1,66	7,94	1,65
	Group 3		7,87	6,49	17,14	2,38
Wheat relative dominance at anthesis stage (cm)	Group 1	2022	-0,10	9,43	25,36	-8,75
	Group 2		-11,65	3,99	-3,34	-17,52
	Group 3		-4,85	10,59	10,05	-14,98
	Group 1	2023	-2,27	5,72	12,89	-7,12
	Group 2		-2,60	3,20	1,54	-8,64
	Group 3		-1,09	5,86	5,52	-8,32
Wheat dry biomass (t ·ha^{-1})	Group 1	2022	2,71	0,17	3,05	2,54
	Group 2		2,50	0,21	3,01	2,23
	Group 3		2,53	0,14	2,74	2,43
	Group 1	2023	1,83	0,43	2,59	1,22
	Group 2		2,40	0,34	2,85	1,72
	Group 3		2,61	0,58	3,26	1,92
Wheat nitrogen nutrition (Nup) (%)	Group 1	2022	2,58	0,36	3,06	1,89
	Group 2		3,24	0,14	3,45	3,00
	Group 3		2,86	0,50	3,23	2,13
	Group 1	2023	2,27	0,28	2,66	1,85
	Group 2		2,66	0,10	2,81	2,46
	Group 3		2,37	0,36	2,65	1,85
Wheat tiller number (m^{2})	Group 1	2022	245,17	21,57	290,36	210,00
	Group 2		222,78	20,30	250,79	196,92
	Group 3		228,98	16,86	248,17	207,10
	Group 1	2023	146,78	1,45	150,48	144,80
	Group 2		146,93	1,90	149,67	143,94
	Group 3		148,10	2,60	150,29	145,08
Single tiller dry biomass (g)	Group 1	2022	1,11	0,09	1,28	0,98
	Group 2		1,13	0,10	1,24	0,96
	Group 3		1,12	0,07	1,21	1,06
	Group 1	2023	1,25	0,29	1,76	0,84
	Group 2		1,62	0,21	1,92	1,19
	Group 3		1,76	0,37	2,17	1,32
Weed abundance-January	Group 1	2022	5,33	0,36	5,88	4,50
	Group 2		4,87	0,44	5,51	3,76
	Group 3		3,82	0,23	4,13	3,62
	Group 1	2023	7,78	0,28	8,48	7,36
	Group 2		7,16	0,34	7,55	6,36

	Group 3		6,74	0,71	7,77	6,16
Weed abundance-March	Group 1	2022	6,47	0,67	7,55	4,92
	Group 2		4,63	0,65	5,37	3,42
	Group 3		5,03	1,61	6,68	3,00
	Group 1	2023	7,36	0,60	7,90	5,87
	Group 2		6,49	0,65	7,62	5,26
	Group 3		6,44	0,52	7,03	5,89
Weed abundance-April	Group 1	2022	4,05	0,60	5,08	3,23
	Group 2		2,90	0,28	3,44	2,48
	Group 3		3,01	0,76	3,95	2,24
	Group 1	2023	5,89	1,21	8,01	4,24
	Group 2		3,00	0,38	3,76	2,31
	Group 3		4,59	2,17	7,65	2,98

Chapter 2

Evaluating selection design for breeding lucerne ideotypes adapted to living mulch systems

"This chapter was prepared as a research article for submission to Peer Community Journal"

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Résumé :

La luzerne présente un intérêt croissant comme mulch vivant dans les systèmes agroécologiques, en raison de sa capacité à fixer l'azote, à couvrir le sol et à limiter les adventices. Cependant, sa croissance vigoureuse cause une forte compétition avec les cultures de rente telles que le blé, rendant nécessaire la conception de nouveaux idéotypes adaptés à cet usage. L'amélioration variétale de la luzerne pour un usage en mulch vivant nécessite ainsi d'évaluer quels dispositifs de sélection prédisent le mieux la performance en mulch vivant.

Un panel diversifié de trente accessions a été testé pendant deux ans dans trois modalités de culture : plantes espacées, peuplements denses purs et mulch vivant luzerne–blé. Les principaux caractères de la luzerne liés à cet usage — hauteur de début de printemps, vitesse de croissance printanière, hauteur maximale et résistance à la verse — ont été évalués entre chaque modalité et le mulch vivant, ainsi qu'en relation avec le rendement et la qualité du blé mesurés en mulch. Les corrélations génétiques entre modalités pour la hauteur de début de printemps et la hauteur maximale se sont révélées fortes, faisant de ces caractères des cibles fiables pour une sélection sur plantes espacées. De plus, les associations de ces caractères avec les performances du blé ont montré des tendances similaires lorsqu'ils étaient mesurés en plantes espacées ou en mulch vivant, confirmant leur valeur prédictive. Le taux de croissance printanière a présenté des corrélations génétiques légèrement plus faibles que les hauteurs. À l'inverse, la verse a montré des corrélations faibles et instables entre modalités, nécessitant une évaluation directe en mulch vivant pour être sélectionnée de manière fiable.

Ces résultats soutiennent une stratégie de sélection en deux étapes : une sélection indirecte précoce pour les caractères héritables observés en peuplements espacés, suivie d'évaluations ciblées en conditions de mulch vivant pour intégrer les caractères liés à la compétition interspécifique.

Mots-clés : luzerne, sélection, compétition, densité, association, G×M

Abstract

Lucerne is gaining interest as a living mulch in agroecological systems due to its ability to fix nitrogen, provide soil cover, and suppress weeds. However, its vigorous growth can lead to competition with cash crops for light and nutrients, necessitating the development of new ideotypes. Breeding lucerne ideotypes adapted to living-mulch systems, requires evaluating which selection designs best predict performance under living mulch conditions. A diverse panel of thirty accessions was tested over two years in three cropping modalities: spaced plants, pure dense stands, and living mulch. Key lucerne traits relevant for this use, early spring height, spring growth rate, maximum height, and lodging resistance, were evaluated across modalities in comparison with the living mulch, and in relation to wheat yield and quality measured in the living mulch. Genetic correlations between modalities for early spring and maximum height were strong, making these traits reliable targets for early selection in spaced nurseries. Moreover, the associations of these traits with wheat performance displayed similar patterns when measured in spaced or living-mulch conditions, confirming their predictive value for intercrop outcomes. Spring growth rate showed slightly lower genetic correlations. In contrast, lodging exhibited weak and unstable correlations across modalities. These traits, therefore, require direct evaluation under intercropping conditions to be reliably selected. Altogether, these results support a breeding strategy that begins with trait-based indirect selection in spaced pure stands to exploit heritable variation, followed by targeted evaluations in living-mulch conditions to capture competition traits.

Keywords: Alfalfa, breeding, competition, density, intercropping, G×M

1. Introduction

Intercropping, the practice of cultivating two or more crop species simultaneously for at least part of their life cycle, is increasingly promoted as a key strategy for sustainable intensification (Maitra et al., 2021). By combining species with complementary characteristics, it can enhance overall productivity and land-use efficiency, improve soil fertility and structure, and provide natural regulation of weeds, pests, and diseases (Brooker et al. 2014; Gaba et al. 2015; Li et al. 2020). However, intercropping remains underutilised in large-scale agriculture, largely due to the difficulty of managing competition between crops and the associated yield losses. The intensity of this competition is determined not only by planting density and management practices but, critically, by the choice of species and varieties (Brooker et al. 2014; Maitra et al. 2021). Current varieties were developed almost exclusively under monoculture conditions, with breeding objectives centred on maximising total yield in dense stands. Traits beneficial in monoculture may not confer advantages in intercrops (Bourke et al. 2021; Dubey et al. 2024). Breeding designs usually include spaced-plant nurseries where traits that show a high heritability and a good correlation with dense conditions are scored, and dense pure stands where traits with a low heritability, such as yield, are measured. These classical breeding designs, conducted in spaced-plant conditions and pure dense stands, do not capture the interspecific interactions that drive intercrop performance, such as complementarity in resource use, facilitation, or niche differentiation (Li *et al.*, 2020; Bourke *et al.*, 2021; Moore *et al.*, 2022).

Breeding for intercropping, therefore, requires reorienting the focus from maximising individual species performance to optimising intercrop performance. This involves not only defining which traits are most relevant for intercrop adaptation, but also deciding in which selection designs they should be evaluated. The efficiency of indirect selection in monoculture

depends on the genetic correlation between monocrop and intercrop performances. When correlations are high, selection in monoculture can be effective, but when they are low, monoculture becomes a poor predictor of intercrop performance (Sampoux et al. 2020; Bourke et al. 2021; Dubey et al. 2024). The low correlation between traits measured in spaced-plant and dense designs can also be described as an interaction between genotypes and selection designs (G×M), leading to genotype re-ranking between monoculture and intercrop conditions. Such re-ranking reflects the plasticity of trait expression across contexts and further reduces the efficiency of monoculture selection (Moore et al. 2022). Under these conditions, direct evaluation in intercrops is required to capture favourable interactions between partners, even though such approaches are complex to implement and demand substantial resources and experimental efforts (Sampoux et al. 2020). However, spaced-plant selection remains essential, as it allows screening a very large number of individuals and therefore enables high selection intensity at early stages, while reducing the need for complex evaluations (Dubey et al., 2024).

Living mulches represent a specific practice of intercropping, in which a service plant is established before the cash crop and maintained as a permanent cover during and beyond the crop growing season (Hartwig and Ammon 2002). Legumes are especially valuable as living mulches because of their ability to fix atmospheric nitrogen and improve soil fertility. Lucerne has emerged as a particularly promising legume candidate for this role thanks to its perennial growth, nitrogen-fixing ability, and capacity to improve soil structure and suppress weeds (Cougnon et al. 2022). However, lucerne varieties were bred for forage production, with ideotypes favouring rapid spring regrowth, tall erect stems, and high biomass (Julier et al. 2017; Hawkins and Yu 2018). While these traits are advantageous in monoculture and in mixture with perennial forage grasses, they make lucerne highly competitive as a living mulch. In wheat–lucerne living mulch, wheat yield losses can reach 45–50% (Humphries et al. 2004; Shili-Touzi

2009). Designing lucerne ideotypes for living mulch requires balancing service provision with reduced competition against wheat. An effective living mulch lucerne variety should exhibit late and moderate spring regrowth to allow wheat establishment and tillering, maintain intermediate height during wheat stem elongation and grain filling to limit shading, and display strong lodging resistance to avoid physical smothering of wheat plants (El Ghazzal et al. 2025). This raises the breeding challenges of the most appropriate context for selecting these traits. Lucerne has already been studied for this in forage mixtures. These studies showed that key traits (height, shoot number, regrowth) express differently in mixtures compared with monoculture, with changes in genotype ranking, reflecting significant G × M interactions (Maamouri et al. 2015, 2017). Such interactions mean that lucerne performance in monoculture is not always predictive of its performance in mixtures (Grieder et al. 2021; Surault et al. 2024). However, no studies have yet examined these questions in the specific context of lucerne used as a living mulch with wheat, where the targeted services differ from those of forage mixtures and aim to combine the ecological services provided by lucerne with the maintenance of wheat yield and quality.

This study aimed to evaluate selection designs for breeding lucerne ideotypes adapted to wheat–lucerne living mulch systems. To this end, a diverse panel of thirty lucerne accessions was evaluated over two years in three distinct cropping modalities, (i) spaced plants (representing early-stage nursery selection), (ii) dense pure stands (representing selection under monoculture), and (iii) dense living mulch with wheat (representing direct selection under intercropping). Key lucerne traits relevant for this use, early spring height, spring growth rate, maximum height, and lodging resistance , were evaluated: (i) across the three modalities, to assess how trait expression and genotype ranking vary among selection environments, and thereby to infer the potential efficiency of indirect selection in pure stands compared with direct

selection in living mulch; and (ii) in relation to wheat yield performance in mulch, to test whether lucerne traits measured in pure stands are predictive of living mulch performance Fig.1).

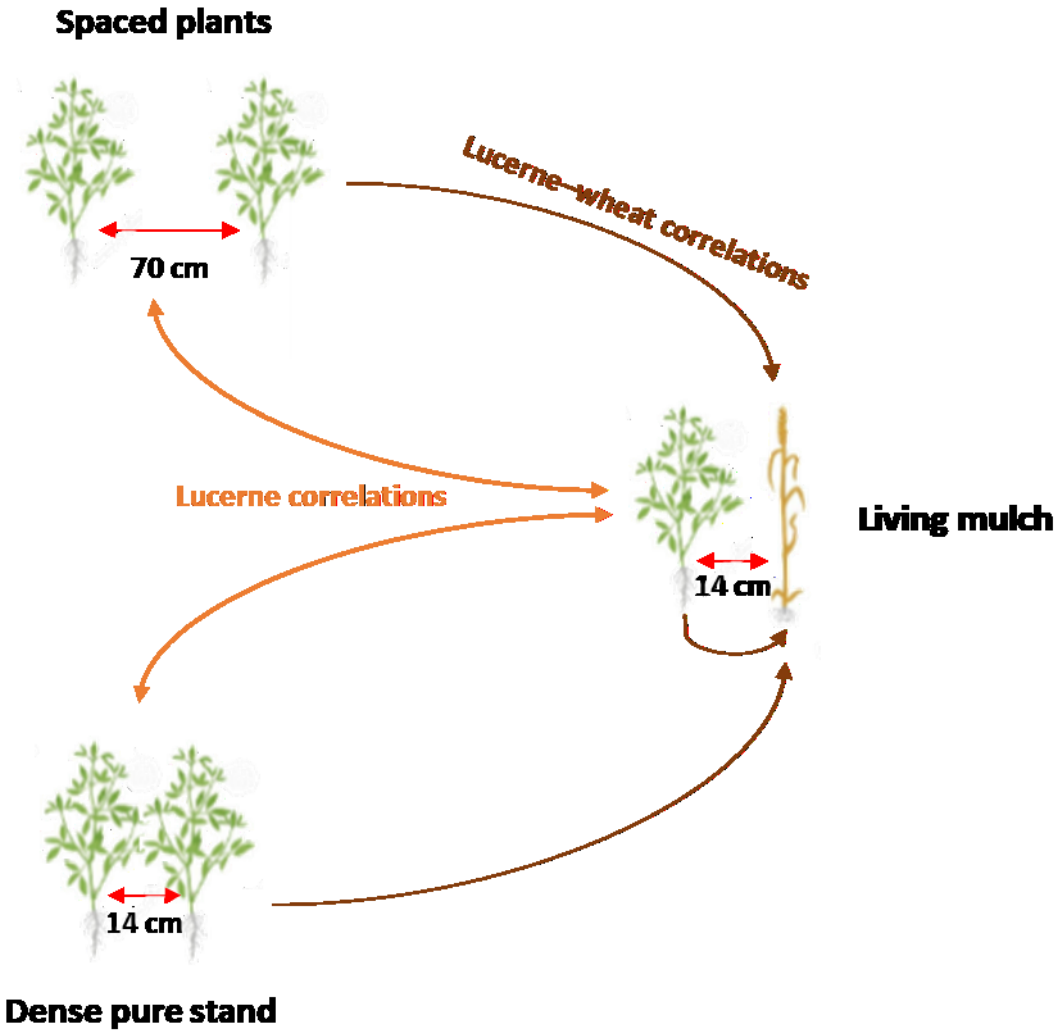

Figure 1. *Experimental modalities and analytical relationships for evaluating selection designs in lucerne.*

2. Materials and methods

2.1. Plant material

The plant material consisted of the same panel of thirty lucerne (*Medicago sativa* L.) accessions described in (El Ghazzal et al. 2025). These accessions represented a wide range of phenological and morphological characteristics, encompassing two subspecies (*sativa* and *falcata*) and two ploidy levels (diploid and tetraploid) (Table S1). In addition, the winter wheat (*Triticum aestivum L.*) variety Geny was used for the lucerne–wheat living mulch experiment.

2.2. Experimental design

The study was conducted at the INRAE Lusignan experimental station (46°25′N, 0°07′E, France) over two growing seasons (2021–2023). Three independent experiments were established, corresponding to different cropping modalities: spaced plants "*spaced*", dense pure stands "*dense*", and lucerne–wheat living mulch "*mulch*". Each modality followed a randomised complete block design, with four replicates in *spaced* and three in *dense* and *mulch*.

In *spaced*, plants were first sown in a greenhouse and transplanted to the field on 21 April 2021, with 70 cm between rows and 70 cm between individuals within rows. In *dense* and *mulch*, lucerne was sown on 21 April 2021 at a density of 10 kg·ha^{-1}. Row spacing was 14 cm in *dense*, and 28 cm in *mulch* to allow inter-row wheat sowing. In *mulch*, wheat was sown between lucerne rows at 250 seeds·m^{-2} on 26 October 2021 and 28 October 2022. Plots measured 6.7 m² (6 m × 1.12 m) in both *dense* and *mulch*. Nitrogen fertilisation was applied in these two modalities at 40 kg·ha^{-1} (ammonium nitrate, 33.5% N) in early March of each year. The field received an initial irrigation of approximately 25 mm following transplanting/sowing, and then relied solely on natural rainfall (Table S2).

2.3. Phenotyping

In the three modalities, lucerne growth during spring was assessed through a sequence of complementary measurements. The early spring height (cm), used as a proxy for dormancy, was measured as stretched plant height in late March, with greater values indicating lower dormancy. The maximum height (cm) was recorded at the wheat heading stage, when competition between species was the most critical. Between these two reference points, successive stretched height measurements were taken from March to May and used to estimate the spring regrowth rate ($cm \cdot GDD^{-1}$), calculated as the slope of a linear regression of stretched height against accumulated growing degree days (GDD, base 0 °C) (Table S3).

Lodging is a combined trait, reflecting both stem lodging and growth habit, as prostrate accessions naturally combine short standing height with great stretched height, which may mimic lodging. It was assessed differently according to modality. In spaced plants, it was calculated as the ratio of natural canopy height (from drone imagery) to stretched height, with values close to one indicating upright plants (Table S3). In *dense* stands, lodging was visually scored at the end of May on a 1–9 scale and rescaled to 0–1 (1: fully erect). Measurements were averaged per block from ten plants in *spaced*, and on three random places in *dense* and *mulch*.

In the living mulch experiment, wheat traits were also recorded to characterise yield components. Plant height (cm) was recorded on the same date as lucerne maximum height. In addition, traits related to crop nutrition and yield were assessed. Nitrogen uptake (%) was estimated at heading stage, from 15 flag leaves per plot analysed for N content using the Dumas method (Hansen 1989), providing a reliable proxy of crop nitrogen nutrition index (NNI) (Louarn et al., 2020). Dry biomass ($t \cdot ha^{-1}$) was determined at heading stage by harvesting two central rows over a 0.75 m × 0.28 m subplot, separating wheat from lucerne and weeds, and oven-drying at 60 °C for 72 h. Grain yield ($q \cdot ha^{-1}$) was measured at physiological maturity (late June) by harvesting two central rows over 2 m per plot, threshing, and adjusting values to 15 %

moisture content. Grain protein (%) was analysed from subsamples of the harvested grain by near-infrared reflectance spectroscopy (NIRS) following AFNOR (2020).

2.4. Genotyping

Genotyping was carried out to characterise genetic relationships among accessions in subsequent quantitative genetic analyses. Leaflet sampling, DNA extraction, and GBS library construction were performed at the plant level following Julier et al. (2018), using a double digestion with PstI and MseI restriction enzymes. SNP calling and allele frequency estimation followed the pipeline described by Pégard et al. (2023), with raw reads mapped to the *Medicago sativa* 'B' haploid reference genome (Chen et al. 2020).

Each accession was represented by 40 individual plants, a sample size shown to be sufficient for reliable estimation of allele frequencies (Herrmann et al. 2010). Only biallelic SNP with MAF > 5 % and no missing data were retained, resulting in 33,963 SNP. Allele frequencies of the accessions were obtained from the average of the individual genotypes.

2.5. Statistical analyses

All analyses were performed in R (RStudio Team 2024). To correct the field heterogeneity, all phenotypic data were first adjusted for block effects and spatial heterogeneity (row and column). A Genomic Best Linear Unbiased Prediction (GBLUP) model was fitted separately for each trait (Eq.1).

$$Y = \mu + W\gamma + Ss + Gg + \epsilon \quad (1)$$

where Y is the vector of phenotypic observations, and μ is the overall mean. where Wγ represents the fixed effect of block, Ss represented the random spatial effect across rows and

columns within the field, following $s \sim \mathbb{N}(0, S\sigma_s^2)$, where S_S was the spatial covariance matrix derived from a tensor product of row and column splines, and σ_s^2 was the spatial variance. Gg represents the random additive genetic effects, $g \sim \mathbb{N}(0, G\sigma_g^2)$, where G was the Genomic Relationship Matrix (GRM), computed from 33,963 SNP following VanRaden (2008) and Forni et al. (2011) (Eq.2), and σ_g^2 was the additive genetic variance. The last effect is the residual $\varepsilon \sim \mathbb{N}(0, I\sigma_\varepsilon^2)$, where I is the identity matrix and σ_ε^2 is the residual variance.

$$G = \frac{MM\prime}{K} \quad \text{with} \quad K = \frac{trace(MM\prime)}{n} \quad (2)$$

where M is the genotype matrix centred by allele frequency and n is the number of accessions. The adjusted phenotypic values were used for subsequent analyses. Genetic correlations of lucerne traits across modalities (*dense* vs. *mulch* and *spaced* vs. *mulch*) were estimated using the same mixed model described above, extended to a multi-trait form.

The phenotypic variation of each trait was first analysed across cropping modality, year, accession, and their interactions using a linear mixed model (LMM) (Eq. 3):

$$Y = \mu + X_1\mathfrak{y} + X_2\beta + X_3(\mathfrak{y} \times \beta) + Z_1a + Z_2a\mathfrak{y} + Z_3a\beta + Z_4a\mathfrak{y}\beta + \varepsilon \quad (3)$$

where Y is the vector of phenotypic observations, and μ is the overall mean. X_1, X_2, X_3 represented respectively the incidence matrices of the fixed effects cropping modality ($\mathfrak{y}$), year (β) , and their interaction ($\mathfrak{y}\times\beta$). Z_1, Z_2, Z_3, Z_4 represent the incidence matrices linking observations to the random effects, accession effect $a \sim \mathbb{N}(0, I\sigma_a^2)$, its interaction with modality $a\mathfrak{y} \sim \mathbb{N}(0, I\sigma_{a\mathfrak{y}}^2)$, its interaction with year $a\beta \sim \mathbb{N}(0, I\sigma_{a\beta}^2)$,and their joint interaction $a\mathfrak{y}\beta \sim \mathbb{N}(0, I\sigma_{a\mathfrak{y}\beta}^2)$. The last effect is the residual $\varepsilon \sim \mathbb{N}(0, I\sigma_\varepsilon^2)$, where I is the identity matrix and σ_ε^2 is the residual variance.

Fixed effects were tested using Type III ANOVA, while the random effects were tested by Likelihood Ratio Tests (LRT) comparing full and reduced models differing by a single random effect. Finally, differences between modalities were evaluated using estimated marginal means (EMM) with contrasts between *spaced* vs. *mulch* and *dense* vs. *mulch* within each year.

Finally, to evaluate the relevance of lucerne traits measured in pure stands for future selection under living mulch conditions, Pearson correlations were estimated between lucerne traits measured in each modality and wheat yield components measured in mulch, separately for each year.

Genetic correlations across modalities and phenotypic correlations with wheat performance were prioritised. Heritability estimates were omitted because spatial and residual variance structures led to unstable, model-dependent values.

3. Result

3.1. Phenotypic variation of lucerne traits across modalities and years

All lucerne traits were significantly affected by cropping modality, year, and their interaction, as well as by accession. The accession × modality interaction was also significant for all traits ($p < 0.001$; Table S4; Fig. S1).

In 2022, plants grown in *mulch* reached an average early spring height of 23.3 ± 9.7 cm, being taller than in *dense* plots (-3.23 ± 0.51 cm) but not significantly different from *spaced* ones (0.19 ± 0.51 cm). In 2023, plants in *mulch* averaged 21.8 ± 7.4 cm, and were taller than in *dense* (-3.57 ± 0.51 cm) and particularly than in *spaced* (-8.24 ± 0.51 cm) (Fig. 2).

The spring regrowth rate averaged 0.10 ± 0.02 cm·GDD^{-1} in *mulch* in 2022, with faster growth than in *dense* (-0.0183 ± 0.0018 cm·GDD^{-1}), while no difference was observed with *spaced*. In

2023, *mulch* plants showed an average regrowth rate of 0.11 ± 0.01 cm·GDD^{-1}, faster than in *dense* (-0.0043 ± 0.0018 cm·GDD^{-1}) and particularly faster than in *spaced* (-0.0311 ± 0.0018 cm·GDD^{-1}).

The maximum height in *mulch* reached 72.8 ± 10.4 cm in 2022, exceeding that of *dense* stands (-11.68 ± 1.04 cm), while *spaced* plants were slightly taller (+2.56 ± 1.02 cm). In 2023, mulch plants averaged 76.8 ± 9.4 cm, taller than in *dense* (-5.74 ± 1.04 cm) and markedly taller than in *spaced* (-22.63 ± 1.02 cm).

Lodging averaged 0.61 ± 0.09 in *mulch* in 2022, higher than in *spaced* (+0.06 ± 0.02) but not significantly different from *dense*. In 2023, lodging averaged 0.69 ± 0.10 in *mulch*, with no significant differences among modalities (Fig. 2).

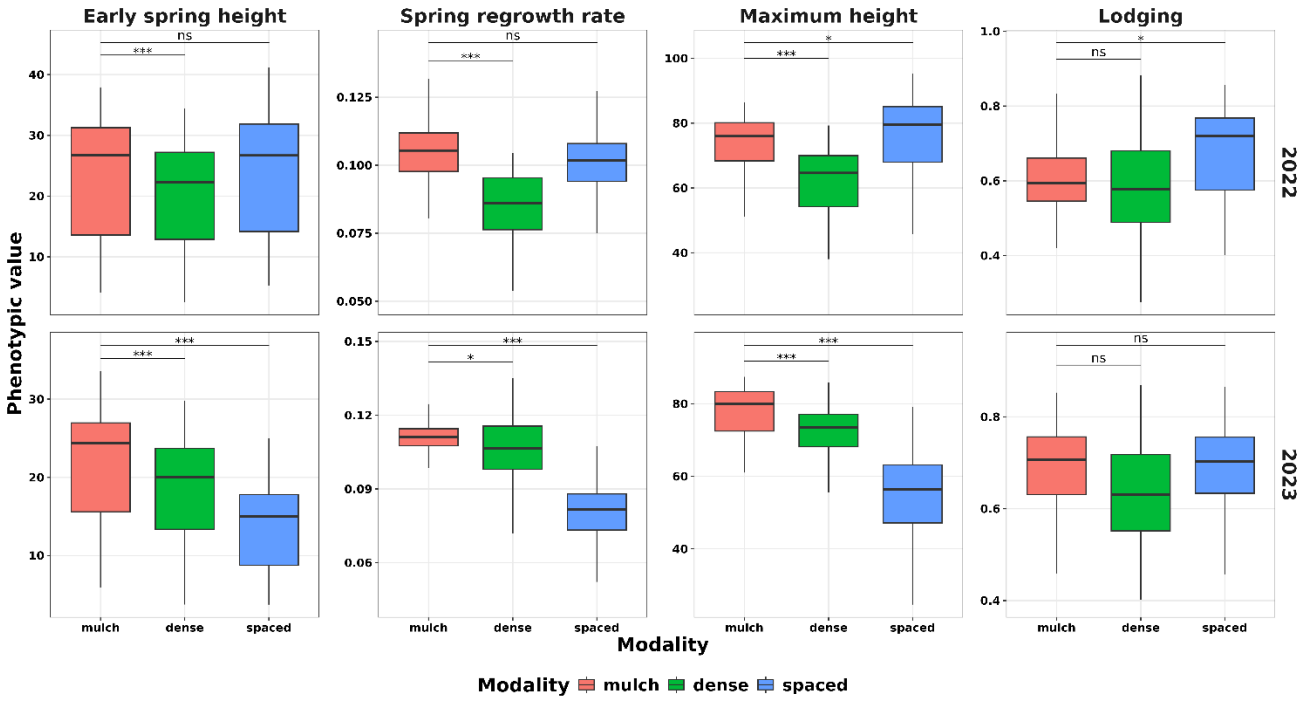

Figure 2. *Phenotypic variation of lucerne traits across cropping modalities (dense, mulch and*

*spaced) in 2022 and 2023. Stars indicate significance of pairwise contrasts between modalities. (*** P ≤ 0.001; ** P ≤ 0.01; * P ≤ 0.05; ns = not significant).*

3.2. Genetic correlations among lucerne traits across modalities

Genetic correlations between modalities were high for most traits, but their magnitude differed between comparisons (*dense* vs. *mulch* and *spaced* vs. *mulch*) and among traits (Fig. 3).

For early spring height, correlations were very high in both years (0.91 to 0.98) and almost identical between the two comparisons, *dense–mulch* and *spaced–mulch*. Similarly, maximum height showed strong correlations in both years (0.83 to 0.94) between *dense–mulch* and *spaced–mulch*, indicating highly stable accession ranking across modalities for these traits (Fig. 3). These early spring and maximum height traits were also correlated between the two comparisons in both years (0.7–0.8 in *dense vs. mulch* and 0.6–0.8 in *spaced vs. mulch*) (Fig. S1).

For spring regrowth rate, correlations were slightly higher between *dense* and *mulch* (0.75 in 2022; 0.85 in 2023) than between *spaced* and *mulch* (0.67 in 2022; 0.63 in 2023), showing that accession ranking was more consistent between dense and mulch (Fig. 3). Spring regrowth rate in *mulch* was also positively correlated with maximum height in the other modalities (Fig. S2), with correlations of 0.74 in 2022 and 0.54 in 2023 for *dense–mulch*, and slightly higher and more stable correlations of 0.67 in 2022 and 0.79 in 2023 for *spaced–mulch*.

For lodging, correlations were positive but moderate between *dense* and *mulch* (0.44 in 2022; 0.85 in 2023). In contrast, the pattern reversed between *spaced* and *mulch* (–0.29 in 2022; –0.64 in 2023), revealing greater instability of genetic ranking between these two modalities for this complex trait. Nevertheless, lodging in mulch remained positively associated with height traits

in spaced (Fig. S2), with early spring height (r = 0.80 in 2022; 0.70 in 2023) and maximum height (r = 0.64 in 2022 and 2023), highlighting that taller plants in *spaced* conditions tended to lodge more under *mulch* management.

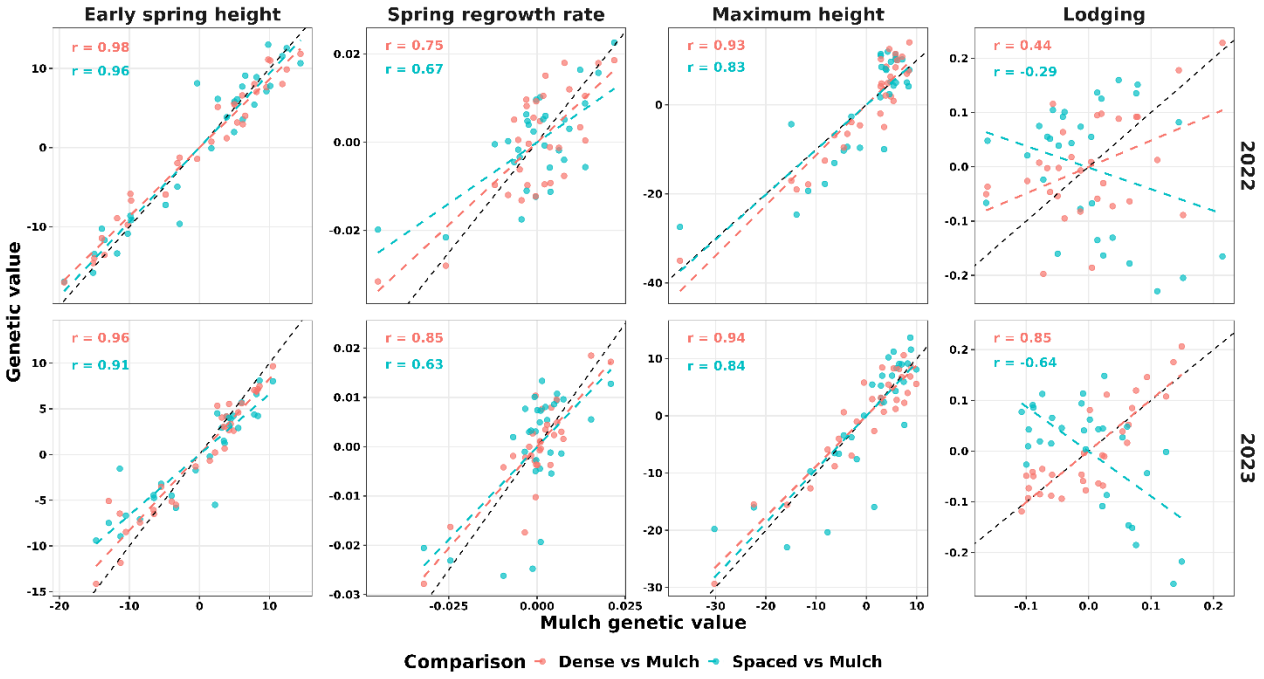

Figure 3. Genetic correlations of lucerne accession additive genetic values (BLUP) between modalities (*dense* vs. *mulch* and *spaced* vs. *mulch*) in 2022 and 2023. The black dashed line represents the 1:1 slope, and the coloured dashed lines show the fitted linear regressions for each comparison.

3.3. Phenotypic correlations between lucerne and wheat traits

Phenotypic correlations between lucerne and wheat traits showed more differences between years than between modalities (Fig. 4).

In the mulch modality, where lucerne and wheat grew together, lucerne early spring height was positively correlated with wheat maximum height (r = 0.67 in 2022; r = 0.91 in 2023) and with grain protein content in both years (r = 0.72 in 2022; r = 0.68 in 2023). It was negatively correlated with wheat biomass (r = –0.65) and grain yield (r = –0.49) in 2022, but these relationships became positive in 2023 (r = 0.76 and 0.76, respectively). A similar pattern was observed for lucerne maximum height, which was positively correlated with wheat maximum height (r = 0.86 in 2022; r = 0.91 in 2023) and with grain protein content (r = 0.87 in 2022; r = 0.53 in 2023), while being negatively correlated with biomass (r = –0.39) and grain yield (r = –0.65) in 2022, before becoming positive in 2023 (r = 0.61 and 0.56, respectively). Spring regrowth rate was positively correlated with wheat maximum height (r = 0.48 in 2022; r = 0.49 in 2023) and with grain protein content (r = 0.40 in 2022). Lodging was significantly and negatively correlated with wheat maximum height (r = –0.55 in 2022; r = –0.71 in 2023) and with grain protein content (r = –0.71 in 2022; r = –0.58 in 2023). Overall, tall lucerne was associated with greater wheat height and higher grain protein content in both years and showed contrasting correlations with wheat yield and biomass between the two years.

The same general pattern was observed when using lucerne trait values measured in pure *dense* conditions to correlate with wheat traits measured in *mulch*. With the traits measured in *spaced*, correlations between lucerne early spring height, spring regrowth rate, and maximum height with wheat traits showed a similar direction, though with different magnitudes. The main difference concerned lodging, which was positively correlated with wheat maximum height (r = 0.65 in 2022; r = 0.68 in 2023) and with grain protein content (r = 0.57 in 2022; r = 0.60 in 2023), negatively correlated with wheat biomass (r = –0.51) and grain yield (r = –0.51) in 2022, but positively in 2023 (r = 0.60 and 0.63, respectively).

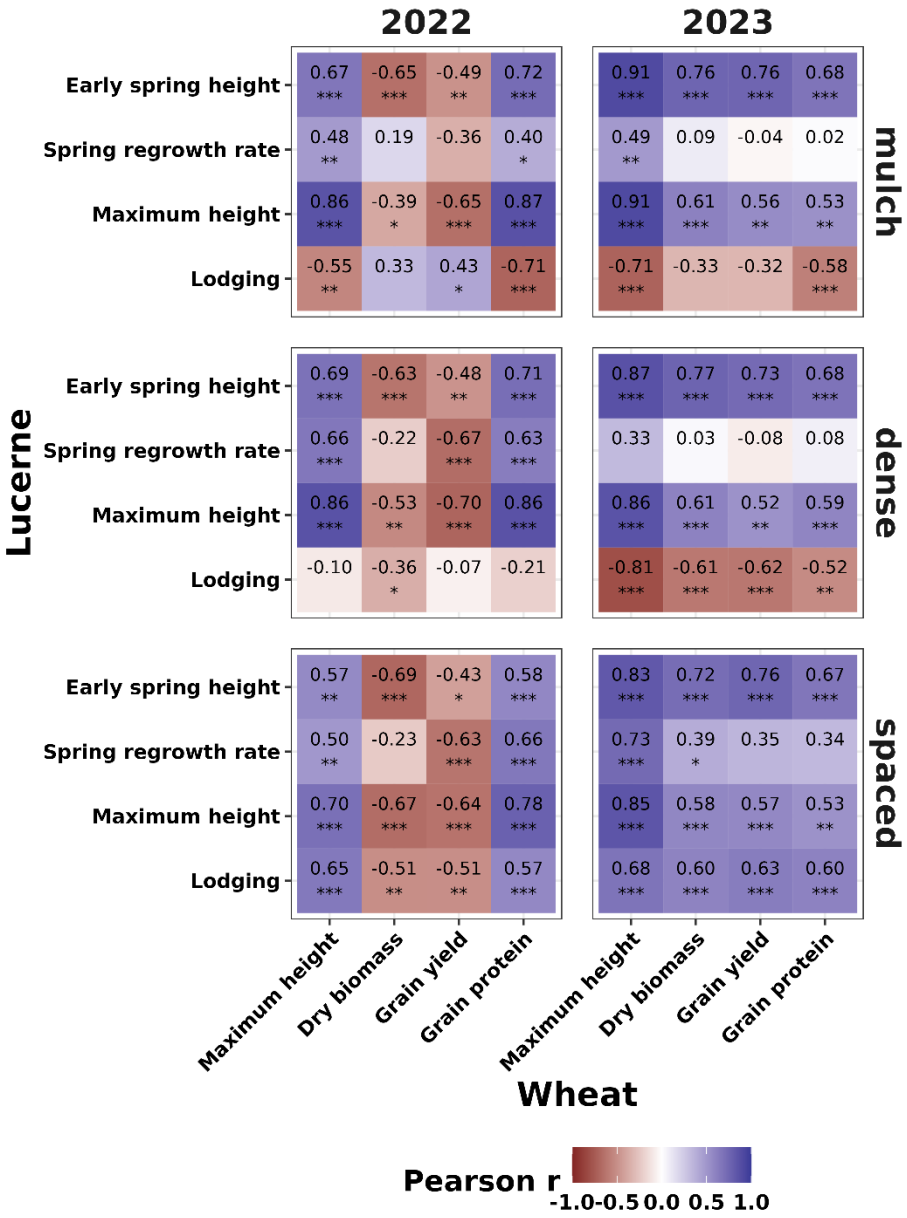

Figure 4. Phenotypic correlations between lucerne traits measured in the three modalities (*dense*, *mulch*, *spaced*) and wheat traits (maximum height, dry biomass, grain yield, grain protein content) measured in *mulch* in 2022 and 2023. (*** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$; ns = not significant).

4. Discussion

Our previous work showed that lucerne traits such as dormancy, height, and lodging strongly influence wheat development and competition in living mulch systems (El Ghazzal et al. 2025). Building on these findings, this study aimed at evaluating the expression of these traits across different cropping modalities, spaced plants, dense pure stands, and living mulch, and at assessing whether lucerne traits measured in pure stand conditions are related to wheat performance when both species grow together. To address these objectives, a diverse panel of lucerne accessions was evaluated over two years across the three cropping modalities, focusing on key lucerne traits as well as wheat yield and quality.

4.1. Lucerne traits correlation across modalities

Across the three cropping modalities, living mulch, dense pure stand, and spaced plants, the lucerne panel exhibited substantial variation for all key traits, accompanied by significant accession × modality (G × M) interactions, indicating pronounced trait plasticity. Lucerne plants in *mulch* consistently reached the greatest height, both in early spring and at maximum, and displayed the fastest spring regrowth rates, followed by those in *spaced* and lastly in *dense* in the first year, but plants in *dense* were taller than in *spaced* in 2023. These patterns reflect distinct growth dynamics shaped by both intra- and interspecific competition, as well as year effects in this perennial species.

In pure stands, plants grown in spaced conditions were taller than in dense conditions, a pattern also reported in other studies where reduced intraspecific competition allowed greater nutrient availability and individual growth (Liu et al. 2024). However, in the second year, lucerne biomass was exported and no fertilisation was applied, which reduced soil resources, so the effects of density became more evident, with plants in dense stands showing stronger stem

elongation driven by light competition (Postma et al. 2021). This vertical growth typically comes at the cost of stem thickness and shoot number (Hansen and Krueger 1973; Kephart et al. 1992), reducing mechanical stability and increasing lodging susceptibility (Guines et al. 2003), a pattern also observed in this study. Notably, lucerne in the living mulch grew even taller and lodged more than in other modalities, underscoring the additional influence of interspecific competition with wheat. As previously reported by El Ghazzal et al (2025), wheat often exceeded lucerne in height, particularly early in spring, which may have induced a light competition response lucerne, stimulating further stem elongation to avoid shading.

These G × M interactions not only reflect trait plasticity but also resulted in genotype re-ranking across modalities, indicating that accession performance in pure stands was not always predictive of its behaviour in the living mulch. This finding aligns with reports that varieties bred for monoculture often lack the attributes needed for mixed cropping, since selection in pure stand ignores the interspecific interactions (Bourke et al. 2021). Height traits showed strong genetic correlations between mulch and pure stand modalities, confirming that accession rankings for early spring height and maximum height remained highly stable across cropping modalities. These results are consistent with those of (Maamouri et al. 2017), who identified height as the most stable trait across lucerne–grass monoculture and mixture. Similarly, (Grieder et al. 2021) reported strong phenotypic correlations for lucerne forage yield between monoculture and grass-based mixtures, indirectly reflecting height stability as a major component of competitive ability and biomass. Such stability likely reflects the fact that height is a strongly heritable trait, making it a reliable indirect selection target.

However, the strength of height correlations does not extend to all related traits. Spring regrowth rate, while correlated with maximum height, showed variable genetic correlations across modalities, indicating sensitivity to planting density and species interactions. This

pattern was even more pronounced for lodging, where correlations between spaced and mulch were low or negative. This reflects both biological and methodological factors. In high-density, plants adopted a more or less erect growth due to neighbouring mechanical support and vertical light competition, even for inherently prostrate accessions. In contrast, in spaced conditions, the same genotypes may spread along the ground with no constraint, leading to low natural height and low natural-to-stretched ratios, interpreted as lodging, even if no structural collapse occurs.This mismatch in trait expression and measurement explains the strong genotype re-ranking for lodging between spaced and mulch. It reinforces that not all morphological traits can be reliably selected in pure stands, and that the efficiency of indirect selection is trait-dependent even among closely related traits (Bourke et al. 2021).

Interestingly, spring regrowth rate and lodging in *mulch* were both positively associated with early spring height and maximum height measured in spaced plants, suggesting that early architectural traits can serve as useful predictors of competitive responses. This provides a valuable breeding opportunity: by selecting in spaced design for easily measurable and heritable traits such as height, breeders can indirectly capture information about complex traits expressed under intercropping. This strategy enables the efficient screening of large numbers of genotypes under simple conditions in a first step, followed by targeted evaluations in living mulch systems to assess key context-dependent traits such as regrowth dynamics and lodging (Dubey et al. 2024).

4.2. Lucerne traits–wheat performance correlation

Lucerne–wheat correlations in the living mulch revealed a dynamic and year-dependent pattern of lucerne trait and wheat performance interdependence. In both years, non-dormant tall lucerne plants, characterised by great early spring and maximum heights, were consistently associated

with increased wheat height, likely related to vertical light competition, which stimulates wheat elongation, as previously explained. They were also associated with high grain protein content, because lucerne fixes atmospheric nitrogen in proportion to its growth, thus more vigorous lucerne fixes more nitrogen (Louarn et al. 2015), indirectly benefiting wheat. However, the correlation with wheat grain yield and lucerne biomass was negative in 2022. This may primarily be attributed to early competition, resulting in reduced tiller formation during the tillering stage (Hiltbrunner and Liedgens 2008), and a low proportion of tillers developing into ears (Carof et al. 2007). In contrast, these relationships turned positive in 2023. This shift may be due to legacy effects of the living mulch system, residual nitrogen from lucerne fixation, improved soil structure, and possibly better weed suppression could have compensated for competitive effects (Bullied et al. 2002; Cougnon et al. 2022). These findings confirm previous results on wheat morphological development and extend them to yield components (El Ghazzal et al. 2025).

This pattern was mirrored in the correlations between wheat traits and lucerne traits measured in dense and spaced pure stands. Early spring height and maximum height, the two traits with stable genetic correlations across modalities, measured in spaced conditions showed consistent positive correlations with wheat grain protein. Interestingly, early spring and maximum height shifted from negative to positive correlation with wheat yield between 2022 and 2023, reflecting the same environmental modulation observed in the mulch and reinforcing their predictive value for living mulch outcomes. In contrast, lodging had more variable and sometimes opposing effects, with strong negative correlations with wheat traits in the mulch modality, but positive or fluctuating correlations when measured in spaced conditions. This again confirms that traits such as lodging, shaped by dense canopy structure and neighbour effects, require direct evaluation under realistic living mulch conditions.

This study provides new insights into breeding lucerne ideotypes adapted for living mulch use. Height-related traits, such as early spring and maximum heights, showed high heritability and strong genetic correlations across modalities, making them reliable targets for early selection in spaced nurseries. This potential was further reinforced by their consistent associations with wheat traits when measured in spaced, confirming their predictive value for intercrop outcomes. In contrast, context-dependent traits such as lodging exhibited weak and unstable correlations across modalities, reflecting plasticity and strong G × M interactions. These traits require direct evaluation under intercropping to be reliably selected. Altogether, these results support a breeding strategy that begins with trait-based indirect selection in spaced pure stands to exploit heritable variation, followed by targeted evaluations in living mulch conditions to capture competition-driven traits. Such an approach offers a practical compromise between selection efficiency and ecological relevance.

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Supplementary materials

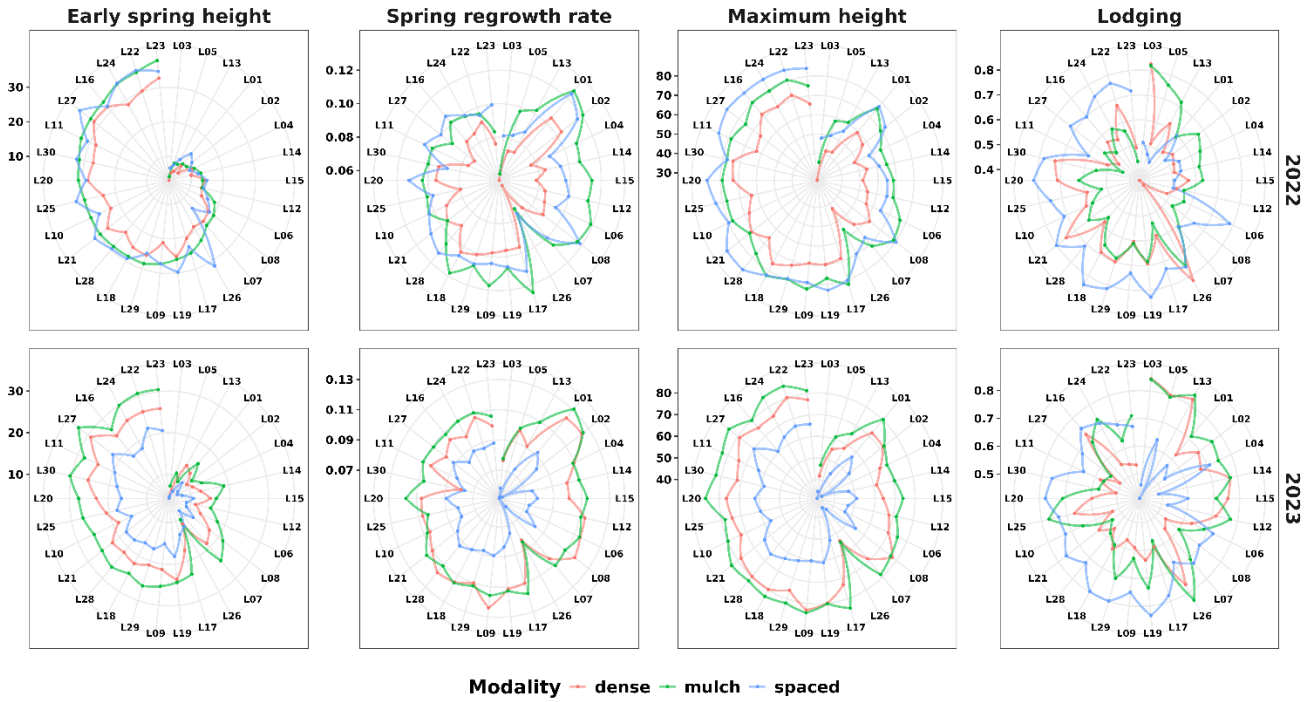

Figure 1S. *Phenotypic variation of lucerne accessions across cropping modalities (dense, mulch and spaced) in 2022 and 2023. The accession × modality interaction was significant for all traits. The concentric circles indicate increasing trait values, with inner circles showing lower values.*

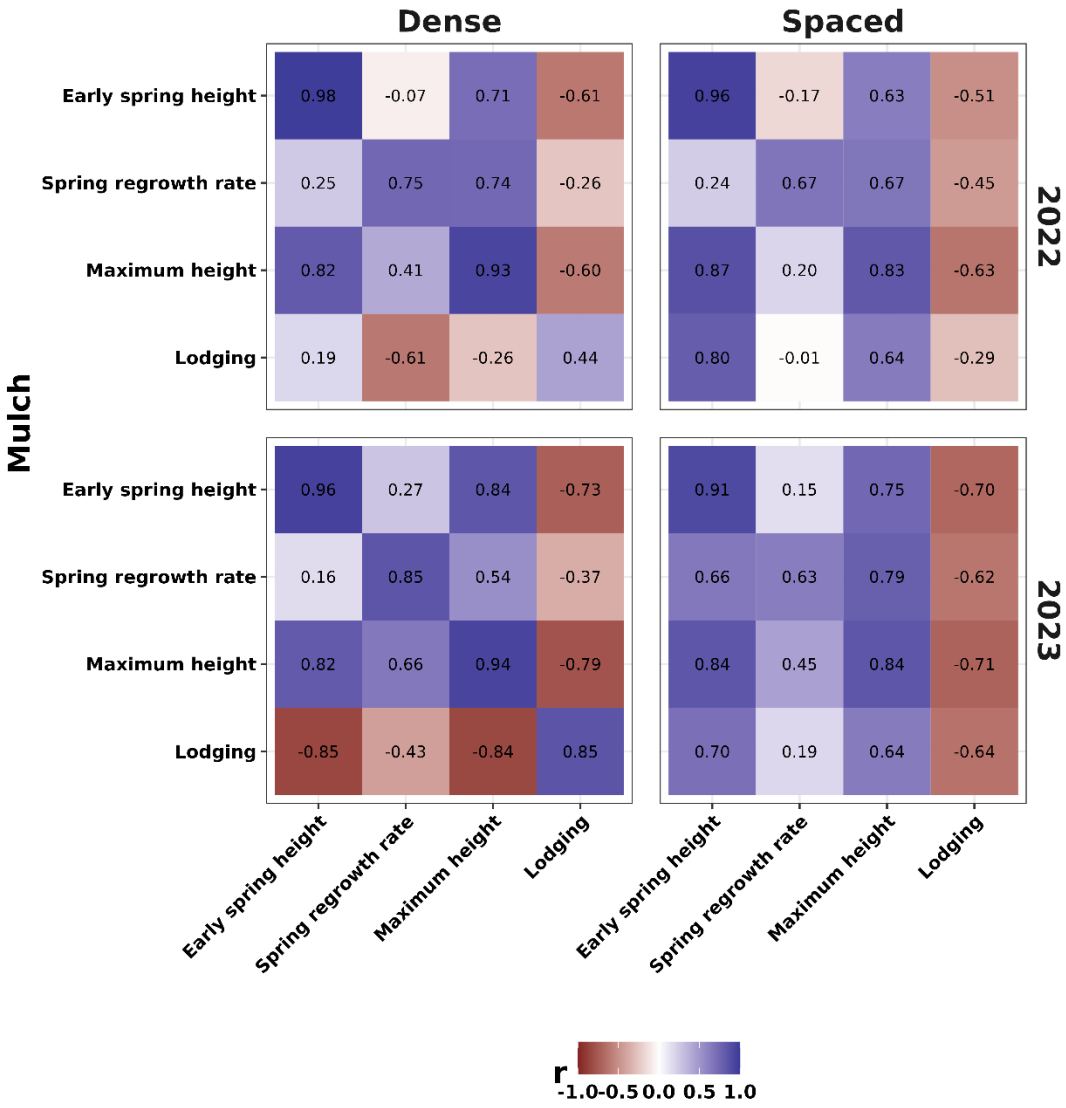

Figure S2. Genetic correlations of lucerne accession additive genetic values (BLUPs) between cropping modalities (dense vs. mulch and spaced vs. mulch) in 2022 and 2023.

Table S1. *List of lucerne accessions, including subspecies, ploidy level, types (cultivated or wild), and geographic origin.*

Accession ID	Accession name	Subspecies	Ploidy	Type	Origin
L03	Glomerata	*sativa*	*2x*	Wild	France
L13	Monte Oscuro	*sativa*	*4x*	Wild	Spain
L05	Malzeville	*falcata*	*4x*	Wild	France
L04	Maron	*falcata*	*4x*	Wild	France
L02	Quasifalcata	*falcata*	*2x*	Wild	Russia
L01	Romanica	*falcata*	*2x*	Wild	Russia
L14	Pancrudo	*sativa*	*4x*	Wild	Spain
L15	Villanueva de Jara	*sativa*	*4x*	Wild	Spain
L12	Greenmed	*sativa*	*4x*	Cultivated	Spain
L06	Krasnokutskaya	*falcata*	*4x*	Cultivated	Ukraine
L07	Camporegio	*falcata*	*4x*	Cultivated	Italy
L26	Gabès	*sativa*	*4x*	Cultivated	Tunisia
L17	Limory	*sativa*	*4x*	Cultivated	France
L08	L8988	*falcata*	*4x*	Cultivated	France
L19	Mezzo	*sativa*	*4x*	Cultivated	France
L09	Luzelle	*sativa*	*4x*	Cultivated	France
L18	Ludelis	*sativa*	*4x*	Cultivated	France
L29	L5323	*sativa*	*4x*	Cultivated	France
L28	L4332	*sativa*	*4x*	Cultivated	France
L10	Koalf 2-96	*sativa*	*4x*	Cultivated	Hungary
L21	Milky Max	*sativa*	*4x*	Cultivated	France
L25	Speeda	*sativa*	*4x*	Cultivated	France
L30	Miechowska	*sativa*	*4x*	Cultivated	Poland
L20	Timbale	*sativa*	*4x*	Cultivated	France
L11	Coussouls	*sativa*	*4x*	Cultivated	France
L27	Marais de Luçon	*sativa*	*4x*	Cultivated	France
L16	Wugong	*sativa*	*4x*	Cultivated	China
L24	Verdor	*sativa*	*4x*	Cultivated	France
L22	Milfeuil	*sativa*	*4x*	Cultivated	France
L23	Occitane	*sativa*	*4x*	Cultivated	France

Table S2. *Climate data from 2021 to 2023.*

Month	Temperature min / Temperature max (°C)			Rainfall (mm)		
	2021	**2022**	**2023**	**2021**	**2022**	**2023**
January	1.8/7.2	1.7/7.2	3.7/8.0	96.0	39.5	120.0
February	4.9/11.1	3.1/11.7	2.0 /10.5	80.0	31.0	47.5
March	2.9/13.5	4.8/14.4	5.7/13.5	16.0	30.5	117.5
Avril	3.8/16.1	6.2/16.3	6.1/16.0	18.5	60.5	34.0
May	7.9/17.8	10.5/23.4	9.8/20.8	90.5	12.0	28.5
June	13.6/24.5	14.1/26.2	14.8/27.2	98.0	98.5	86.0
July	14.1/24.4	14.8/29.3	14.2/25.8	59.5	5.5	26.5
August	13.4/24.1	16.3/29.5	15.1/26.8	23.5	46.5	43.5
September	13.1/24.2	11.6/22.6	15.3/27.2	38.0	65.5	70.0
October	7.4/18	12.5/20.9	10.7/20.8	52.5	51.0	203.0
November	3.5/9.9	6.9/14.1	7.1/12.6	34.0	88.5	214.5
December	3.9/9.1	3.6/8.5	5.4/10.0	120.0	92.0	119.5

Table S3. *Dates of trait measurements for lucerne across modalities (dense, mulch, and spaced) during 2022 and 2023.*

Trait	Treatment	Date 2022	Date 2023
Early spring height (cm)	Dense & Mulch	31/03/2022	30/03/2023
	Spaced	29/03/2022	28/03/2023
Spring regrowth rate (cm/GDD)	Dense & Mulch	31/03/2022, 14/04/2022, 26/04/2022, 10/05/2022	30/03/2023, 13/04/2023, 26/04/2023, 12/05/2023
	Spaced	29/03/2022, 11/04/2022, 28/04/2022, 12/05/2022	28/03/2023, 11/04/2023, 25/04/2023, 11/05/2023
Maximum height (cm)	Dense & Mulch	10/05/2022	12/05/2023
	Spaced	12/05/2022	11/05/2023
Lodging	Spaced	13/05/2022, 12/05/2022	12/05/2023, 11/05/2023

Table S4. *Summary of fixed and random effects from linear mixed models for lucerne traits across modalities and years, including estimated marginal means (EMMs).*

Trait	Effect	Test	Mean	P.value
Early spring height	Modality			6.82e-13
	Year			9.14e-07
	Modality × Year			<2e-16
	Accession × Modality (G×M)			<2e-16
	Accession × Year (G×Y)			<2e-16
	Accession × Modality × Year (G×M×Y)			7.52e-08
	dense - mulch 2022	EMM	-3.23 ± 0.52	2.55e-08
	spaced - mulch 2022	EMM	0.19 ± 0.51	8.90e-01
	dense - mulch 2023	EMM	-3.58 ± 0.52	1.37e-09
	spaced - mulch 2023	EMM	-8.25 ± 0.51	2.34e-10
Spring regrowth rate	Modality			<2e-16
	Year			1.84e-01
	Modality × Year			<2e-16
	Accession × Modality (G×M)			<2e-16
	Accession × Year (G×Y)			1.1e-11
	Accession × Modality × Year (G×M×Y)			3.15e-10
	dense - mulch 2022	EMM	-0.0183 ± 0.0018	2.86e-14
	spaced - mulch 2022	EMM	-0.0031 ± 0.0018	1.66e-01
	dense - mulch 2023	EMM	-0.0043 ± 0.0018	4.10e-02
	spaced - mulch 2023	EMM	-0.0311 ± 0.0018	2.80e-14
Maximum height	Modality			<2e-16
	Year			1.55e-04
	Modality × Year			<2e-16
	Accession × Modality (G×M)			<2e-16
	Accession × Year (G×Y)			3.08e-09
	Accession × Modality × Year (G×M×Y)			1.5e-04
	dense - mulch 2022	EMM	-11.68 ± 1.05	1.63e-10
	spaced - mulch 2022	EMM	2.56 ± 1.03	2.77e-02
	dense - mulch 2023	EMM	-5.74 ± 1.05	6.23e-07
	spaced - mulch 2023	EMM	-22.64 ± 1.03	2.34e-10
Lodging	Modality			3.19e-02
	Year			9.08e-04
	Modality × Year			1.40e-03
	Accession × Modality (G×M)			<2e-16
	Accession × Year (G×Y)			<2e-16
	Accession × Modality × Year (G×M×Y)			<2e-16
	dense - mulch 2022	EMM	-0.02 ± 0.03	7.13e-01
	spaced - mulch 2022	EMM	0.07 ± 0.03	2.14e-02
	dense - mulch 2023	EMM	-0.05 ± 0.03	6.84e-02
	spaced - mulch 2023	EMM	-0.01 ± 0.03	8.31e-01

Chapter 3

Genome-wide association study and genomic prediction of lucerne traits shaping living mulch performance

"This chapter was prepared as a research article for submission to Molecular Breeding"

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Résumé

La luzerne (*Medicago sativa*) présente un intérêt croissant en tant que mulch vivant dans les systèmes agroécologiques, en raison de sa capacité à fixer l'azote atmosphérique, à assurer une couverture permanente du sol et à limiter les adventices. Cependant, sa croissance vigoureuse engendre une forte compétition avec les cultures de rente pour la lumière et les nutriments, soulignant la nécessité de concevoir de nouveaux idéotypes plus compatibles à cet usage.

Cette étude visait à explorer la base génétique des caractères pertinents pour la sélection d'idéotypes de luzerne adaptés à un usage en mulch vivant. Les caractères étudiés incluaient la dormance, la repousse printanière, la hauteur, le port, la taille des folioles, le diamètre des tiges et la verse. Un panel de diversité composé de vingt-sept accessions, ainsi qu'une population synthétique, ont été phénotypés en plantes espacées. Plus de 100 000 marqueurs ont été utilisés pour le génotypage. Des analyses d'association à l'échelle du génome (GWAS) et de prédiction génomique ont été menées en tenant compte de la structure génétique des populations.

La GWAS a permis d'identifier quelques QTL à effets modérés (r^2 jusqu'à 0,27) pour la taille des folioles, la hauteur, le port et la verse, situés dans ou à proximité de gènes candidats impliqués dans la régulation de la croissance, la réponse au stress et les voies de signalisation. La prédiction génomique a montré une précision élevée dans le panel des accessions, où la large variabilité génétique a permis une estimation fiable des effets marqueurs, avec des précisions supérieures à 0,8 pour les caractères hautement héritables tels que le port et la taille des folioles. En revanche, les précisions sont restées faibles dans la population synthétique, en raison de sa diversité limitée et de sa petite taille, que les modèles soient entraînés à partir de cette population ou du panel des accessions.

Ces résultats mettent en évidence à la fois le potentiel et les limites de la sélection génomique chez la luzerne, et soulignent la nécessité de disposer de jeux d'entraînement représentatifs et suffisamment diversifiés pour renforcer l'efficacité de ces approches dans la création de nouveaux idéotypes de mulch vivant.

Mots-clés : luzerne, mulch vivant, GWAS, prédiction génomique.

Abstract

Lucerne is gaining interest as a living mulch in agroecological systems for its nitrogen fixation, soil cover, and weed suppression. However, its vigorous growth can lead to competition with cash crops for light and nutrients, necessitating new ideotypes. This study investigated the genetic basis of traits relevant to ideotype breeding: dormancy, spring regrowth, height, growth habit, leaflet size, stem diameter, and lodging. A diversity panel of 27 accessions and a synthetic population were phenotyped in a spaced plant nursery. Over 100,000 SNP markers were used for genotyping. Genome-wide association study (GWAS) and genomic prediction were conducted, considering the population structure. GWAS identified a few effect QTL (r^2 up to 0.27) for leaflet size, height, growth habit, and lodging, with candidate genes linked to growth, stress response, and signalling pathways. Genomic prediction was highly accurate in the diversity panel, where broad genetic variation allowed reliable estimation of marker effects, with prediction accuracies exceeding 0.8 for heritable traits including growth habit and leaflet size. In contrast, accuracies remained low in the synthetic population, reflecting its limited diversity and small size, whether training was based on the synthetic population itself or on the diversity panel. These results highlight the potential and the limits of genomic selection in lucerne, emphasising the need for representative and diverse training sets.

Keywords: *Medicago sativa*, living mulch, GWAS, genomic prediction.

1. Introduction

Lucerne (Medicago sativa L.) is often referred to as the "queen of forages" because of its high yield per unit area and exceptional nutritional quality. It is one of the most widely cultivated forage legumes worldwide, thanks to its adaptability to diverse climatic and soil conditions (Julier et al. 2017). As a perennial legume, lucerne provides long-term agronomic and ecological benefits. Its nitrogen-fixing capacity improves soil nitrogen content, while its deep root system accesses to subsoil moisture and enhances physicochemical soil properties (Rashmi et al. 1997; Song et al. 2019; Tucak et al. 2021). Furthermore, its dense canopy competes with weeds, providing significant weed suppression in the rotations (Meiss et al. 2010; Barilli et al. 2017). Lucerne is therefore gaining interest in agroecological systems as a service plant, particularly when used as a living mulch intercropped with cash crops (Cougnon et al. 2022).

Living mulches, defined as cover crops sown prior to the cash crop, persist as a living ground cover throughout and beyond the crop growing season (Hartwig and Ammon 2002; Carof et al. 2007a; Leoni et al. 2022). To maintain their role as service plants, living mulches must ensure rapid and dense ground coverage for effective weed suppression (Leoni et al. 2020) and contribute to nitrogen enrichment, as biomass accumulation is closely linked to nitrogen fixation (Louarn et al. 2015). Perennial legumes, such as lucerne, are the most common species used as living mulches in cereal systems, as their ability to fix atmospheric nitrogen improves soil fertility and reduces the need for fertilisers (Cougnon et al. 2022). They can also support the reduction of intensive tillage practices, thereby saving energy and preserving biodiversity (Vincent-Caboud et al. 2019; Fonteyne et al. 2020). Despite its potential benefits, lucerne faces several challenges when used as a living mulch. Current varieties, which have been selected for forage production, exhibit vigorous growth, leading to significant competition with cash crops for key resources, particularly light and nutrients (Carof et al. 2007b; Cougnon et al. 2022; El Ghazzal et al. 2025). This competition often results in yield losses for the cash crop; for example, wheat yield has been shown to decrease by an average of 45% (Humphries et al., 2004), reaching up to 50% in some cases (Shili-Touzi 2009).

Different strategies can be employed to manage the competitive effects of lucerne used as a living mulch. While mechanical and chemical methods are available, they often raise concerns regarding cost, labour, and environmental impact (Cougnon et al. 2022). A promising alternative involves the selection of less competitive lucerne varieties. However, defining an

effective ideotype requires balancing two contradictory objectives: minimising competition with the cash crop while maintaining enough competitiveness to suppress weeds. Several key traits must be considered to optimise lucerne growth and compatibility with cash crop, by enhancing its ability to suppress weeds during the off-season or early development of the cash crop, while avoiding light competition during the cash crop growth cycle (Carof et al. 2007b). An appropriate ideotype would combine late spring regrowth, high autumn dormancy, and slow spring regrowth to limit competition (Cougnon et al. 2022). This aligns with findings by Humphries et al. (2004), reporting reduced wheat yield losses when using winter-dormant lucerne varieties (dormancy classes 0.5 and 2) compared with winter-active ones (classes 6 and 10). Moreover, El Ghazzal et al. (2025) showed that varieties combining intermediate dormancy, moderate height, slow spring regrowth, and high lodging resistance provide the best balance between ecological services, such as weed suppression, and compatibility with the cash crop.

A better understanding of the genetic basis of key traits is essential to accelerate breeding efforts and effectively develop lucerne ideotypes adapted for living mulch use. Lucerne exhibits extensive phenotypic and genetic diversity, shaped by multiple biological and ecological factors, including both natural and artificial selection. While domestication and selection have narrowed the genetic base used in cultivated populations (Muller et al. 2006), wild accessions retain broad variation, reflecting adaptation to climates ranging from cold to arid (Julier et al. 1995). Complex population structure and significant relatedness among accessions have been observed (Li and Brummer 2009; Pégard et al. 2023). This diversity makes the *Medicago sativa* complex, comprising mainly the *sativa* and *falcata* subspecies, a valuable reservoir for breeding programmes targeting sustainable agricultural practices, particularly ideotypes for living mulch systems (Cougnon et al. 2022). In addition, as an autotetraploid (2n = 4x = 32) and an outcrossing (allogamous) species, lucerne maintains high levels of heterozygosity and genetic variation within a population or variety (Julier et al. 2000; Musial et al. 2002; Flajoulot et al. 2005; Alfaifi et al. 2013).

Genome-wide association studies (GWAS) and genomic selection (GS) are proposed to exploit lucerne diversity and identify the genetic basis of complex traits (Pégard et al. 2023). The genetic structure within a population used for GWAS or GS can introduce confounding effects in GWAS, resulting in the identification of spurious associations (false positives) due to

ancestry differences and cryptic relatedness (Kaler et al., 2020; Sul et al., 2018; Thornton et al., 2012). To address this issue, mixed linear model (MLM) based GWAS methods have been widely adopted in structured plant populations, including the multi-locus mixed model (MLMM) (Segura et al. 2012). These models are effective at controlling false positives by considering relatedness with a kinship matrix and sequentially incorporating associated markers as cofactors through forward and backward selection (Alemu et al. 2021; Alamin et al. 2022). Genomic selection, on the other hand, is increasingly used in plant breeding programmes to predict the genomic estimated breeding values (GEBV) of individuals based on the cumulative effects of genome-wide markers. Its effectiveness lies in capturing both large- and small-effect loci across the genome, including those often missed in GWAS (Crossa et al. 2017). Prediction accuracy depends on several factors, such as marker density, training population size, trait heritability, and, critically, the genetic relatedness between training and target populations (Pégard et al. 2020). Accuracy generally increases when these factors are optimised. In lucerne, genomic selection has shown moderate to high predictive abilities for complex traits (Li et al. 2015a; Annicchiarico et al. 2015b; Pégard et al. 2023), which is encouraging given the similarly complex nature of the traits of interest for living mulch.

GWAS and GS studies in lucerne have employed a range of population designs, each with specific strengths and limitations in terms of allelic diversity, mapping resolution, and prediction accuracy. Diversity panels composed of landraces, cultivars, and wild accessions capture broad allelic variation and enable high-resolution mapping, although they require careful correction for population structure (Pégard et al. 2023, 2025). Breeding panels, while genetically narrower, are highly relevant to applied breeding and often yield moderate to high prediction accuracies due to close relatedness between training and target populations (Li et al. 2015b). F_1 full-sib or half-sib populations allow precise segregation analyses and detection of rare alleles, though they capture only a limited fraction of diversity (Murad Leite Andrade et al. 2022). Synthetic populations derived from multiple parents or commercial cultivars offer intermediate diversity and reduced structure, which can increase the power to detect true associations in GWAS (Biazzi et al. 2017).

Each of these designs provides complementary insights. In this context, a diversity panel and a synthetic population were analysed in this study to investigate the genetic basis of traits relevant to lucerne ideotype breeding for living mulch systems. Specifically, autumn dormancy, spring

regrowth rate, plant height, growth habit, leaflet size, stem diameter, and lodging resistance. Both populations were phenotyped and genotyped using high-density SNP markers, with trait heritability and genetic correlations estimated. GWAS was conducted to identify associated loci, and genomic prediction models were tested within and across populations to evaluate the potential of GS for improving lucerne ideotypes. The study also assessed how prediction accuracy depends on heritability and genetic relatedness, providing insights for designing effective breeding strategies for lucerne varieties suited for living mulch use.

2. Materials and Methods

2.1. Plant material

In this study, two populations, differing for composition and diversity range, were studied. An "accession panel" was composed of 27 tetraploid *Medicago sativa* accessions selected for their high morphological variability, ranging from wild prostrate to tall, erect cultivated types, each represented by 40 individuals (Table S1). These accessions belonged to two subspecies within the *M. sativa* complex: subsp. *sativa* and subsp. *falcata*. A synthetic population (further named as "Syn1"), representing the first-generation progeny of a polycross between three populations, Krasnokutskaya (subsp. *falcata*), Mezzo (subsp. *sativa*), and LPIII (subsp. *sativa*), was included. Each parental population was represented by 30 genotypes. The first two were selected for their morphological differences and where also part of the accession panel, and LPIII was included for its high seed production. The accession panel is supposed to cover a wider range of diversity and to show larger genetic structure than the Syn1.

2.2. Experimental design

Seeds were scarified to enhance germination, then incubated at 25 °C in the dark in 10 cm Petri dishes lined with moist filter paper. After germination, seedlings were transplanted into plastic pots and grown in a greenhouse for one month. For each accession of the panel, 40 healthy plants were transplanted into the field on 21 April 2021. The same procedure was applied to 228 plants from the Syn1 polycross progeny. The field received an initial irrigation of approximately 25 mm following transplanting, and then relied solely on natural rainfall (Table S2). The experiment was conducted from April 2021 to June 2023 at the INRAE research station in Lusignan, France (46°26′08″N, 0°07′25″E), on clay loam soil with an acidic pH of 6.5. The field was organised into four blocks. Within each block, 10 plants per accession of the

diversity panel were planted in 10 columns spaced 70 cm apart, with each row corresponding to a distinct accession. The accession "Milky Max" was included four times per block in different spatial positions to assess spatial variability. The Syn1 was planted in the same field, at the end of each block after the accession rows (Figure S1). Weed control was performed mechanically between plants, with repeated passes as needed throughout the duration of the trial.

2.3. Genotyping and SNP filtering

Leaflet sampling, DNA extraction, and GBS library construction were performed at the plant level, following the method outlined by Julier et al. (2018), with a modified digestion protocol involving a double digestion using the restriction enzymes *PstI* and *MseI*. SNP calling and allele frequency determination followed the pipeline described by Pégard et al. (2023). Briefly, raw reads were processed using the GBprocesS pipeline, which included demultiplexing, trimming, merging, quality filtering, and mapping to the haploid *Medicago sativa* 'B' reference genome (Chen et al. 2020). Genotyping was performed by extracting read counts for each nucleotide position, retaining only those with a read depth between 10 and 1200. Only biallelic SNP with a minor allele frequency (MAF) above 1% were selected.

Quality filtering was conducted separately for the accession panel and Syn1 to account for differences in allele frequencies. For both datasets, individuals with more than 60% missing data were removed, as were SNP with more than 20% missing values or with MAF below 5%. After filtering, 111,486 SNP were retained for the accession panel, including 42,894 without missing data and an overall missing rate of 2.7%. For Syn1, 106,750 SNP were retained, among which 57,040 showed no missing data, with a missing rate of 2.4%. Missing genotype data were imputed separately for the accession panel and Syn1, using the mean allele frequency of each SNP. For the accessions, imputation was performed at the accession level: missing genotypes for a given individual were imputed using the mean allele frequency of that SNP among other individuals from the same accession.

2.4. Genetic diversity

To characterise the genetic structure of the accession panel, a principal component analysis (PCA) was first performed using the R package "*FactoMineR"* (Lê et al. 2008), based on 39,400

SNP common to both the accession panel and Syn1, without missing data. The first principal components explaining 80% of the total genetic variation (551 PCs) were retained for subsequent clustering. Syn1 individuals were then projected onto the same PCA using the supplementary individual approach to determine their genetic position relative to the accession panel. Subsequently, a discriminant analysis of principal components (DAPC) was conducted using the R package "*adegenet"* (Jombart and Ahmed 2011). The optimal number of genetic clusters was identified using the "*find.clusters*" function, based on the Bayesian information criterion (BIC), by selecting the first local minimum of the BIC. Genetic differentiation among clusters was estimated using pairwise F_{ST} (Weir and Cockerham 1984). Linkage disequilibrium (LD) decay was assessed by calculating the squared partial correlation coefficient (r^2) for each intrachromosomal SNP pair (Mangin et al. 2012; Lin et al. 2012), to evaluate the extent of LD in the populations and to estimate the expected resolution of association mapping.

2.5. Phenotypic Data

Autumn regrowth was used as a proxy for dormancy, where lower regrowth indicates higher dormancy, following the 'Fall Dormancy' standard test (Teuber et al. 1998). After the summer harvest on 16/09/2021, plant height was measured three times from the soil to the top of the tallest stretched stem. These three measurements were used to calculate the slope of autumn regrowth over time, expressed in cumulative thermal time (growing degree days (GDD), base 0 °C, in cm ·GDD^{-1}. The stretched height was used to track growth independently of plant growth habit. Spring regrowth was characterised by three traits. First, the regrowth rate was calculated as the slope of stretched height (cm·GDD^{-1}) recorded every 15 days from March to May 2022. The last measurement in May was taken as the maximum height. Finally, leaflet size was visually assessed using a scale from 1 to 9. Growth habit was also visually assessed in June and August 2021, with ratings ranging from 1 (upright growth) to 9 (prostrate growth), before any occurrence of lodging. Lodging resistance was described by the ratio of maximum stretched height to maximum natural height, both measured on 13/05/2022. This index reflects not only lodging but also the influence of growth habit. In prostrate accessions, which develop close to the soil surface, the combination of a naturally short standing height and a much greater stretched height may yield values similar to lodged plants, although these accessions are not truly lodged. Stem diameter (mm) was calculated as the average diameter of three randomly selected stems per plant, measured at the lower third of the stem in May 2022 and 2023. This

trait was included as a key determinant of lodging resistance, reflecting the mechanical support provided by stem tissues (Guines et al. 2003).

To correct the microenvironmental heterogeneity within the experimental trial, phenotypic data for each trait were independently adjusted for spatial variability using R package "breedR" (Munoz and Rodriguez 2020). The adjustment was carried out using a linear mixed model (Eq. 1), that incorporated both genetic effects, spatial effects across rows, columns, and blocks within the plot.

$$Y = \mu + B\beta + Ss + Gg + \epsilon \quad (1)$$

Where Y was the vector of phenotypic observations, and $\boldsymbol{\mu}$ was the overall mean. $B\beta$ represented the fixed effects of blocks, where B was the incidence matrix for blocks and β was the vector of block effects. Ss represented the random spatial effect across rows and columns within the field, following $s \sim \mathbb{N}(0, S\sigma_s^2)$, where S was the covariance matrix derived from a two-dimensional tensor product of B-splines along rows and columns, and σ_s^2 was the spatial variance. Gg represented the random additive genetic effects, $g \sim \mathbb{N}(0, G\sigma_g^2)$, where G was the Genomic Relationship Matrix (GRM), and σ_g^2 was the additive genetic variance. Finally, ϵ represented the residual error, where $\epsilon \sim \mathbb{N}(0, I\sigma_e^2)$, with I was the identity matrix and σ_e^2 was the residual variance. The GRM was computed following (Eq.2).

$$G = \frac{MM\prime}{K} \quad (2)$$

Where M was the $n \times q$ matrix (n: number of individuals, q: number of SNP), centred by allele frequency following VanRaden (2008) method. Only SNP without missing values were used to compute M. The matrix was scaled so that the average of the diagonal elements equalled 1, following Forni et al. (2011) (Eq.3).

$$K = \frac{trace(MM\prime)}{n} \quad (3)$$

The same mixed model (Eq. 1) described above was used to estimate narrow sense heritability (h^2) of each trait (Eq. 4), as well as the genetic correlation between traits using the adjusted phenotypic data (Eq. 5).

$$h^2(\mathrm{A}) = \frac{{\sigma_g}^2(\mathrm{A})}{\sigma_g^2(\mathrm{A}) + {\sigma_e}^2(\mathrm{A})} \quad (4)$$

$$r_g = \frac{Cov_g(\mathrm{A,B})}{\sqrt{{\sigma_g}^2(A) + {\sigma_g}^2(B)}} \quad (5)$$

where $Cov_g(A, B)$ was the genetic covariance between trait A and trait B.

The effect of genetic group on phenotypic variation was assessed using analysis of variance (ANOVA). When significant differences among groups were detected, Tukey's Honest Significant Difference (HSD) test was applied to perform pairwise comparisons between genetic groups. Phenotypic correlation among traits was also estimated on adjusted phenotypic data, using Pearson coefficient.

2.6. GWAS and QTL Analysis

GWAS was conducted separately for the accession panel (111,486 SNP) and the Syn1 (106,750 SNP). Analyses were performed using the MLMM approach (Segura et al. 2012), accounting for population structure and genetic relatedness through a kinship matrix computed using the VanRaden method (VanRaden 2008), and implemented with the R package "*mlmm*". Each trait was analysed using stepwise mixed-model regression. Significant associations were identified based on a Bonferroni-adjusted threshold (0.1 / number of SNP). The proportion of phenotypic variance explained (r^2) by each QTL was estimated by comparing mixed linear models with and without the focal QTL using likelihood ratio tests. A combined r^2 was also calculated by including all significant QTL. Finally, candidate genes located near significant QTL were identified using the *Medicago sativa* reference genome (Chen et al., 2020) through the "*myGenomeBrowser*" platform (Carrere and Gouzy 2017).

2.7. Genomic prediction

To evaluate the predictive ability of genomic selection, four scenarios (S1–S4) were tested using the Genomic Best Linear Unbiased Prediction (GBLUP) model, implemented in the R package "*breedR*" (Muñoz & Rodríguez, 2020). The first two scenarios assessed within-population prediction. In Scenario 1 (S1), prediction was performed within the accession panel

(n = 1025) using 111,486 SNP and stratified cross-validation. A fixed 20% (n = 205) of individuals was randomly selected as a test set, while the remaining 80% was split into training subsets ranging from 20% to 80% of the population and validation subsets. Stratification across genetic clusters was applied to preserve population structure, as this improves predictive ability when genetic subgroups are represented in the training set (Pégard et al. 2023). This scenario simulated selection within structured germplasm and evaluated prediction performance across a genetically diverse panel of accessions. Scenario 2 (S2) assessed prediction within the Syn1 population (n = 228) using 106,750 SNP. Here, 30% of individuals (n = 68) were used as a fixed test set, while the remaining 70% formed training subsets of increasing size (20% to 80% of Syn1 plants), maintaining balanced representation from the three Syn1 parental accessions. This scenario reflects early-stage selection in a synthetic breeding pool and enables the identification of superior genotypes within targeted diversity.

The last two scenarios addressed across-population prediction. Scenario 3 (S3) used the full accession panel as the training population, with the Syn1 as the test population, based on the 104,250 SNP common to both datasets. Stratified sampling was again applied to training subsets (20% to 80%) to retain population structure. This scenario examines the feasibility of predicting phenotypes in less diverse materials using a diverse germplasm panel, which is relevant for pre-breeding to reduce phenotyping costs. Scenario 4 (S4) followed the same structure as S3 but limited the training population to cultivated accessions from three genetic clusters, including two of the Syn1 parents. This design represents a more realistic breeding scenario with moderate genetic relatedness between training and test populations and helps assess the impact of relatedness on prediction ability. All scenarios were replicated 30 times to ensure robust estimation. Predictive ability was evaluated as the Pearson correlation between genomic estimated breeding values (GEBV) and adjusted phenotypes. In addition, the correlation between trait heritability and mean prediction ability was calculated using prediction ability from the test set when 80% of the individuals of the training population were used. One data point per trait was used to visualise this relationship.

3. Results

3.1. Population Structure and Genetic Relatedness

In the principal component analysis (PCA) conducted to assess the genetic structure of the accession panel, the first two principal components explained 10.4% (PC1) and 2.2% (PC2) of

the total genetic variation. Clustering analysis of the accessions identified six distinct genetic groups (K = 6; Figure S2), which broadly aligned with subspecies and cultivation status (Figure 1A). Groups G1 and G2 comprised cultivated *M. sativa* subsp. *sativa* accessions, although individuals were dispersed between these two clusters. Group G3 comprised non-dormant cultivated *sativa* types, including the Tunisian landrace 'Gabès' and the variety 'Speeda'. Group G4 contained three wild *sativa* accessions from Spain ('Mielga'), the turf-type variety 'GreenMed' (derived from 'Mielga'), and a Chinese cultivated accession ('Wugong'). Group G5 consisted of cultivated *falcata*, while G6 included wild *falcata* accessions. When the Syn1 individuals were projected onto the PCA (Figure 1A), they occupied an intermediate position between cultivated *sativa* (G1, G2, G3) and *falcata* (G5), reflecting their mixed background.

Pairwise F_{ST} estimates among the six clusters revealed a consistent pattern of increasing genetic differentiation from cultivated to wild types, and from *sativa* to *falcata*. F_{ST} values ranged from 0.002 between G1 and G2 (both cultivated *sativa*) to 0.093 between G6 (wild *falcata*) and G3 (non-dormant cultivated *sativa*) (Figure 1B). Syn1 also showed differentiation from all clusters, with F_{ST} values ranging from 0.005 with G1 (which shared a *sativa* parent) to 0.056 with G6 (*falcata*).

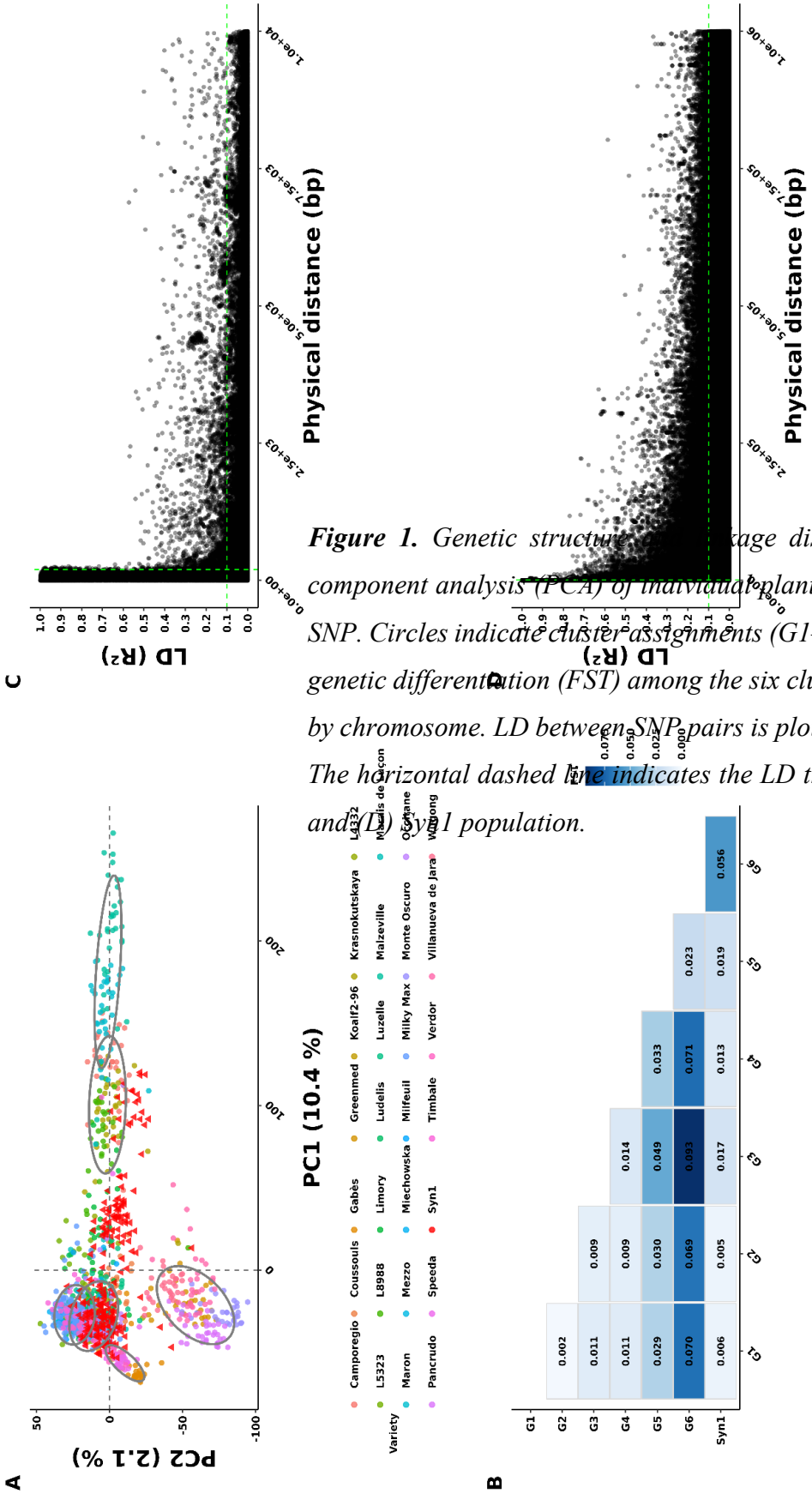

Figure 1. *Genetic structure and linkage disequilibrium in the lucerne accession pane component analysis (PCA) of individual plants from the 27-accession panel with projectio SNP. Circles indicate cluster assignments (G1–G6), and ellipses represent 80% confidence genetic differentiation (FST) among the six clusters and between each cluster and Syn1. (C by chromosome. LD between SNP pairs is plotted against physical distance, with all chrom The horizontal dashed line indicates the LD threshold (r^2 = 0.1), and the vertical dashed and (D) Syn1 population.*

3.2. Phenotypic variation

Phenotypic traits varied across both the accession panel and the Syn1 population, with significant differences observed among the six genetic clusters (G1–G6) (Figure 2, Table S3). Dormancy, expressed as autumn growth rate, was 0.037 ± 0.016 cm GDD^{-1} in the accession panel and 0.041 ± 0.011 cm GDD^{-1} in Syn1. Growth habit scores were 4.65 ± 1.97 in the accession panel and 4.90 ± 1.00 in Syn1. Leaflet size was 5.30 ± 1.34 in the accession panel and 5.65 ± 0.79 in Syn1. Spring regrowth rate was 0.099 ± 0.023 cm GDD^{-1} in the accession panel and 0.102 ± 0.021 cm GDD^{-1} in Syn1. Maximum height reached 74.2 ± 17.0 cm in the accession panel and 78.4 ± 14.1 cm in Syn1. Stem diameter was 2.45 ± 0.95 mm in the accession panel and 2.76 ± 0.77 mm in Syn1. Lodging scores were 0.66 ± 0.18 in the accession panel and 0.81 ± 0.11 in Syn1. Although outlier values extended to similar ranges in both groups (Table S3), the diversity in Syn1 was generally narrower (Figure 2).

Analysis of variance (ANOVA) confirmed that genetic cluster had a significant effect on all traits ($p < 0.001$), with pairwise differences identified via Tukey's HSD test (Figure 2). Cultivated *sativa* accessions (G1–G3) were characterised by great height, large leaflets, fast regrowth, thick stems, and good lodging resistance. They also exhibited erect architecture (low growth habit scores) and intermediate dormancy. Among them, G3 displayed the lowest autumn growth rate, corresponding to the highest dormancy. In contrast, G5 (cultivated *falcata*) exhibited intermediate values, and G4 and G6 (wild *sativa* and *falcata*, respectively) showed the lowest trait values overall. The Syn1 population broadly showed a similar range of variation as the cultivated *sativa* groups, but its variation did not cover that included in the wild accessions of the diversity panel.

Trait heritability estimates (Table S3) ranged from moderate to high in the accession panel (h^2 = 0.36 for spring growth rate to 0.70 for growth habit). In contrast, Syn1 showed lower heritability across all traits (h^2 = 0.17 to 0.33), largely reflecting reduced genetic variance, while stronger spatial or residual effects also contributed for some traits.

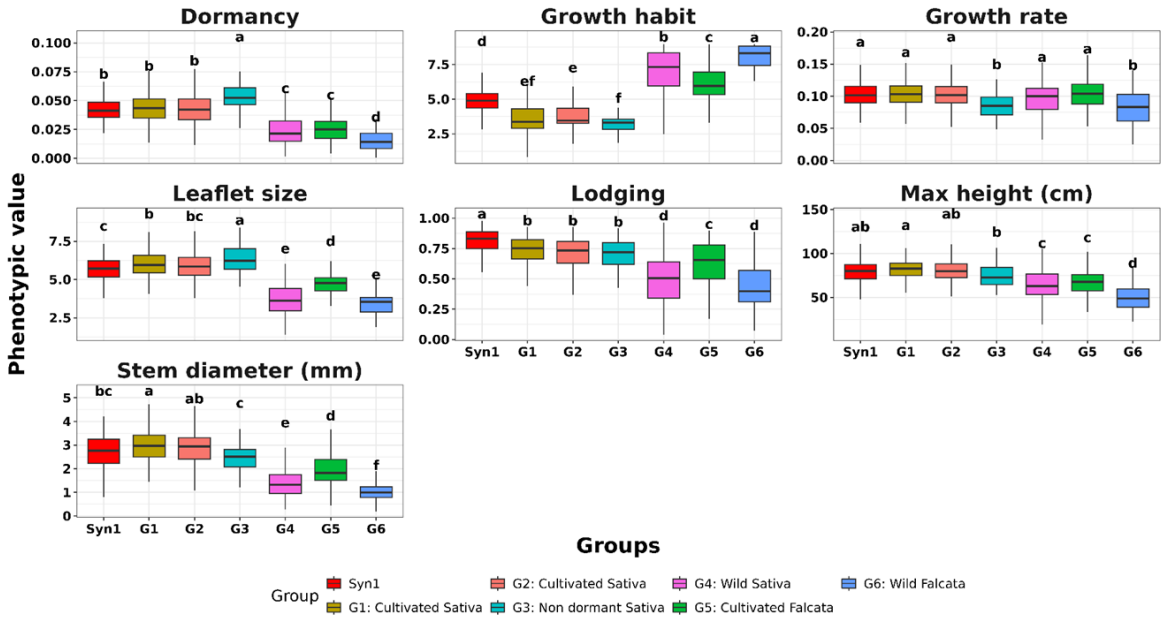

Figure 2. *Boxplots illustrating the distribution of phenotypic traits across the six genetic clusters (G1–G6) of the accession panel and the Syn1 population. The letters above the boxplots denote the statistically significant differences among groups based on Tukey's HSD test ($p < 0.05$) following ANOVA.*

3.3. Genetic and phenotypic correlations

Genetic and phenotypic correlations among traits were estimated separately for the accession panel and the Syn1 population (Figure 3). In the accession panel (Figure 3A), growth habit and leaflet size were strongly negatively correlated (genetic r = –0.62; phenotypic r = –0.73), indicating that prostrate genotypes tended to have small leaflets. Dormancy (as slow autumn growth) was positively correlated with leaflet size (genetic r = 0.60; phenotypic r = 0.52), while spring regrowth rate and maximum height were highly positively correlated (genetic r = 0.78; phenotypic r = 0.79). In contrast, stem diameter exhibited near-zero genetic correlation with height (r = –0.05) but a strong positive phenotypic correlation (r = 0.69), and also showed strong phenotypic correlations with leaflet size (r = 0.70) and growth habit (r = -0.67). Lodging resistance was moderately negatively correlated with growth habit (genetic r = –0.47; phenotypic r = –0.56), indicating that more erect genotypes were better able to resist lodging.

In Syn1, the correlation matrix was very different. Many genetic correlations were reduced or reversed relative to the accession panel, and the alignment between genetic and phenotypic correlations was less consistent (Figure 3B). The strong negative phenotypic and genetic correlation between growth habit and leaflet size observed in the accessions was reduced to a weak correlation in Syn1 (r = –0.19 and r = –0.01, respectively). More generally, all correlations involving growth habit were weak in Syn1, consistent with the reduced variability of this trait compared to the accession panel. The dormancy–leaflet size correlation also declined to r = 0.29 genetically and r = 0.11 phenotypically. Stem diameter showed a strong negative genetic correlation with dormancy (r = –0.61). It was also strongly negatively correlated with height at the genetic level (r = –0.76), in contrast with the positive phenotypic correlation between stem diameter and height (r = 0.55), which reflects the expected structural relationship between these traits. Lodging resistance, which had been largely independent of height in the accession panel, became strongly positively correlated (genetic r = 0.63; phenotypic r = 0.60). The correlation between growth habit and lodging also shifted from strongly negative in the accessions to near-zero in Syn1 (genetic r = 0.07; phenotypic r = –0.13).

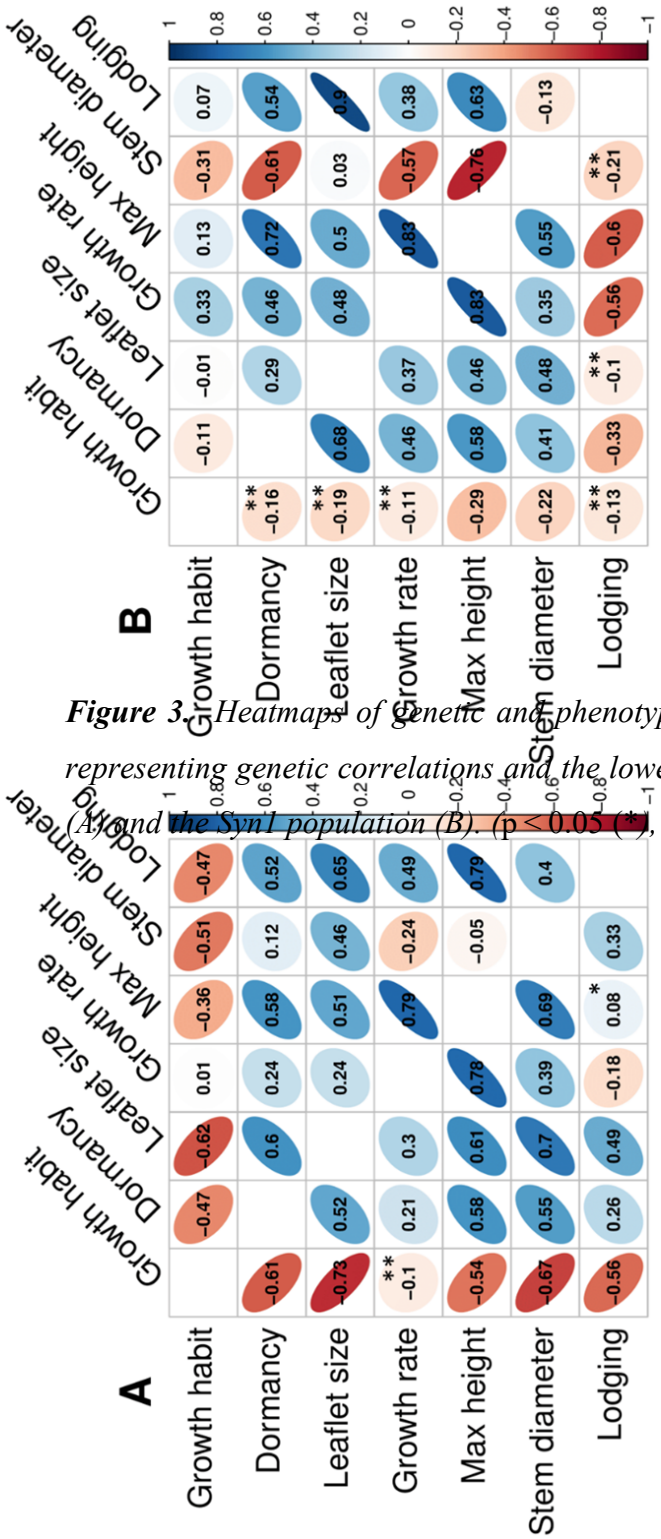

Figure 3. *Heatmaps of genetic and phenotypic correlation coefficients between traits, with the u representing genetic correlations and the lower triangle showing phenotypic correlations, in the ac (A) and the Syn1 population (B). (p < 0.05 (*), p < 0.01 (**); p < 0.001 shown without asterisks).*

3.4. Genome-wide association studies (GWAS)

Linkage disequilibrium (LD) decayed rapidly in both populations, but at different scales. In the accession panel, mean r^2 dropped below 0.1 within approximately 200 base pairs across all

chromosomes (Figure 1C), with over 99.95% of SNP pairs showing r^2 values below 0.1. In contrast, LD decay was slower in the Syn1, with mean r^2 falling below 0.2 at ~1000 bp (Figure 1D). Based on these patterns, candidate gene search windows were set to ±2 kb in the accession panel and ±5 kb in the Syn1.

In the accession panel, a major QTL on chromosome 7 was associated with both growth habit and leaflet size, explaining 27.3% and 27.6% of phenotypic variance, respectively (Table 1, Figure S3). This locus lies 795 bp upstream of MS.gene036920, a RING-type zinc finger protein involved in protein ubiquitination. A minor QTL for spring regrowth rate was identified on chromosome 8 (3.8% of phenotypic variance), located near MS.gene61257, a calcium-binding protein. Lodging resistance was associated with a QTL on chromosome 6 (15.6% of phenotypic variance), near MS.gene031124, encoding a ubiquitin conjugation factor E4. In Syn1, three QTL for leaflet size were detected on chromosomes 1, 2, and 4, jointly explaining 31% of phenotypic variance. Only the QTL on chromosome 4 was near a gene (MS.gene91452), though its function was not annotated. For plant height, multiple QTL were found on chromosomes 1, 2, 3, 4, 5, and 8, cumulatively explaining 60% of phenotypic variance. A major QTL on chromosome 8 (30% of phenotypic variance) was located in MS.gene000456, encoding a protein involved in ubiquitin signalling. Other loci were associated with defence, chromatin organisation, and growth pathways (Table 1, Figure S4). Overall, GWAS revealed distinct QTL architectures between the two populations, with no overlapping loci identified.

Population	Trait	QTL (Chr_Position)	r^2	Candidate gene	Length	Functional annotation	InterPro
Accession panel	Growth habit	Chr.7_30942343	0.273	MS.gene036920 (7900 pb)	3983 bp	RING-type zinc finger protein	IPR027370
	Leaflet size	Chr.7_30942343	0.276	MS.gene036920 (7900 pb)		RING-type zinc finger protein	IPR027370
	Growth rate	Chr.8_22962823	0.038	MS.gene61257	2953 bp	Calcium-binding protein containing EF-hand domain	IPR002048 5
	Lodging	Chr.6_8657463	0.156	MS.gene031124	6334 bp	Ubiquitin conjugation factor E4	IPR019474
Syn1	Leaflet size	Chr.4_66375176	0.116	MS.gene91452 (1840 pb)	5334 bp	—	—
		Chr.1_54376907	0.117	—	—	—	—
		Chr.2_27450832	0.171	—	—	—	—
		all	0.313				
	Max height	Chr.8_30807660	0.29	MS.gene000456	3674 bp	UBA and ubiquitin domain-containing protein	IPR015940
		Chr.1_6626985	0.15	MS.gene016175	2200 bp	—	
		Chr.4_88805862	0.193	MS. gene79508	2859 bp	GTD-binding domain-containing protein	IPR007656
		Chr.8_12075031	0.12	MS.gene041445	1872 bp	RING-type zinc finger domain-containing protein	IPR001841
		Chr.5_74425872	0.147	MS.gene016862	3755 bp	LRR and protein kinase domain-containing protein	IPR003591
		Chr.5_58666533	0.135	MS.gene44845	10550 bp	Ribonuclease E/G and carbohydrate-binding domain-containing protein	IPR019307
		Chr.2_2981857	0.125	MS.gene051935	6499 bp	Serine/threonine protein phosphatase domain-containing protein	IPR013235
		Chr.4_9096745	0.13	MS.gene57386	6409 bp	—	—
		Chr.3_89965817	0.107	MS.gene42281	8757 bp	WD40-repeat domain-containing protein	IPR001680
		all	0.603				

d in the accession panel and the Syn1 population. For each QTL, the chromosome and henotypic variance explained (r^2), are reported. Gene information includes the gene ID outside the gene), gene length, predicted functional annotation, and associated InterPro

3.5. Genomic prediction

Prediction ability varied considerably across the four genomic prediction scenarios (S1–S4) (Figure 4A). In S1, conducted within the accession panel, prediction ability (PA) was generally high. Traits such as growth habit and leaflet size achieved PA values above 0.80 even with small training sets, while others, including plant height, stem diameter, and lodging resistance, reached PA between 0.50 and 0.70 with increasing training set size. Spring regrowth rate exhibited the lowest prediction ability in this panel (PA = 0.33 with the largest training set). S2, conducted within the Syn1 population, showed lower PA than S1. Prediction improved with training set size, but still at moderate levels. The most predictable traits were stem diameter and growth habit (PA = 0.37 at 80%), while dormancy was essentially non-predictable (PA < 0.05), regardless of training set size. Across-population prediction scenarios showed lower accuracy than within-population scenarios. In S3, where the accession panel was used to predict Syn1 performance, PA values were generally comparable to those observed in S2 for some traits such as growth habit, and only slightly reduced for stem diameter and leaflet size. In contrast, lodging showed a clear reduction in predictive ability, and PA of other traits that were already poorly predicted in S2 (e.g. dormancy, growth rate) remained low. Restricting the training set to cultivated accessions in S4 produced a similar pattern.

The correlation between trait heritability and PA at 80% training size also differed markedly among scenarios (Figure 4B). In S1 where heritability ranged from 0.35 and 0.69 PA was strongly and positively correlated with heritability ($r^2 = 0.89$, $p < 0.05$). In contrast, this correlation was not observed in S2, S3, and S4 ($r^2 = 0.43$, 0.019, and 0.004, respectively; $p > 0.05$), where the heritability ranged from 0.17 to 0.51.

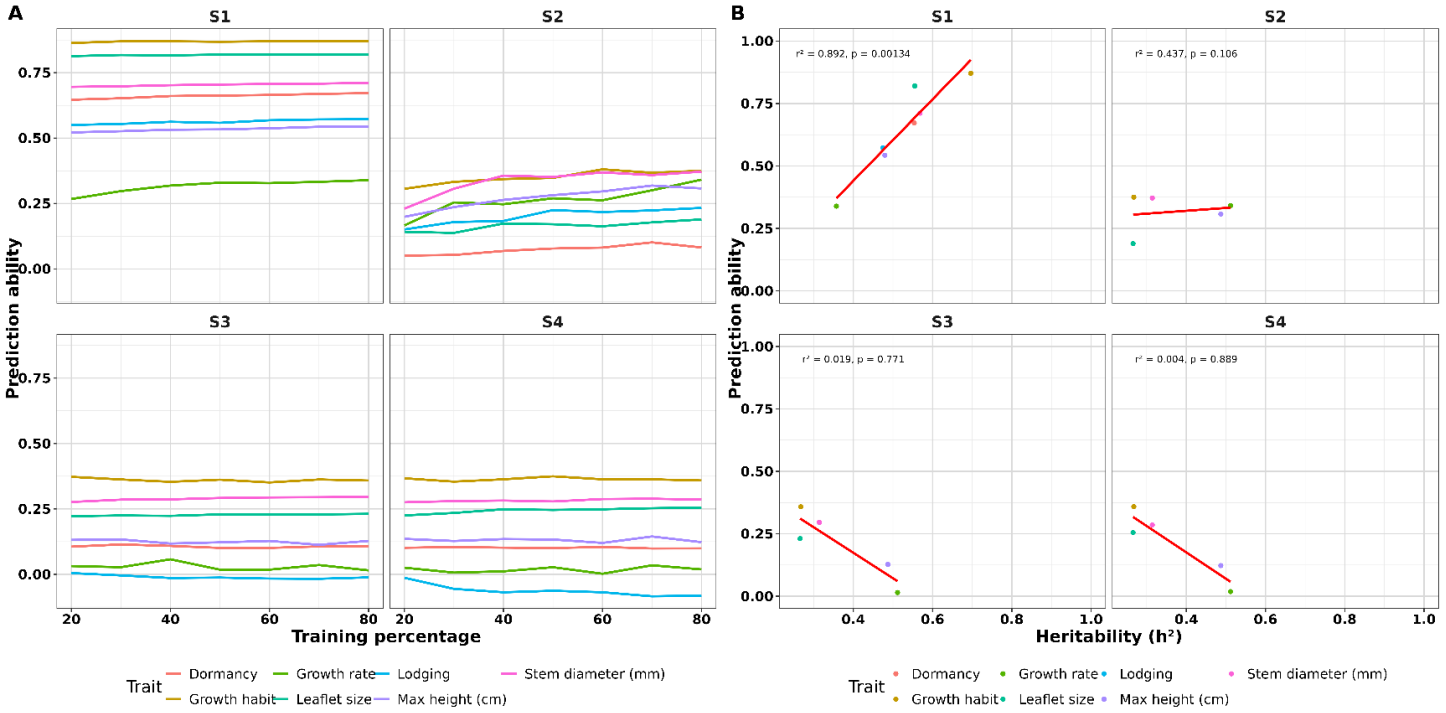

Figure 4. *Genomic prediction across scenarios. (A) prediction ability for the phenotypic traits across four prediction scenarios (S1–S4). evaluated at increasing training set sizes (20% to 80%). Each line represents the mean prediction ability for each trait. In Scenario 1 (S1). predictions were performed within the accession panel. Scenario 2 (S2) prediction in the syn1 population. Scenario 3 (S3) tested across-population prediction using the accession panel to predict the Syn1. while Scenario 4 (S4) restricted training to cultivated accessions (G1. G2. G4). (B) Correlation between trait heritability and genomic prediction ability at 80% training set size across the four genomic prediction scenarios (S1–S4). Each dot represents a trait. and the red line indicates the linear regression fit.*

4. Discussion

This study investigated the genetic determinants of lucerne traits linked to dual role in living mulch systems, balancing reduced competition with wheat while maintaining ecosystem services. A diverse panel of accessions and a synthetic population (Syn1) were used to explore genetic variation and assess genomic prediction performance under different population structures.

4.1. Phenotypic variation and trait correlations differed between accessions and Syn1

The accession panel exhibited broad phenotypic variation for all traits. Cultivated *M. sativa subsp. Sativa* groups (G1–G3) were characterised by tall and erect plants with large leaflets, fast regrowth, and thick stems, consistent with traits targeted in forage breeding (Annicchiarico et al. 2015a). In contrast, wild accessions (G4, G6) exhibited prostrate growth, reduced size, and finer morphology, reflecting their adaptation to natural environments that allow them to escape grazing. Syn1 displayed a narrower overall phenotypic range than the accession panel, consistent with its limited number of parents. However, some individuals showed intermediate or atypical values, particularly for growth rate and lodging resistance. These patterns suggest that recombination among divergent parents may have generated unusual phenotypic profiles, even though overall diversity was reduced. These phenotypic patterns aligned with the genetic results. The accession panel was structured into six genetic clusters reflecting subspecies and cultivation status, whereas Syn1 occupied an intermediate genetic position between cultivated *sativa* and *falcata*.

Trait correlation patterns differed substantially between the accession panel and Syn1, reflecting both the underlying genetic background and the range of diversity captured in each population. In the accession panel, taller plants exhibited stronger lodging resistance, although height and stem diameter were not genetically correlated. This likely reflects the measurement of maximum stretched height, which captures potential elongation rather than growth habit. Some plants may have long stems yet remain prostrate or semi-erect, whereas truly erect plants tend to develop thick stems to maintain structural support. Leaflet size is a relevant breeding trait due to its influence on canopy structure and photosynthetic efficiency (Simkin 2019). Erect growth habit and large leaflet size were positively associated with plant height, reflecting co-selection for traits promoting light interception and forage yield (Jia et al. 2022) .

In Syn1, correlations between traits were generally weaker than in the accession panel. For example, the strong association between leaflet size and growth habit observed in the accessions was reduced or absent, while taller individuals tended to have thinner stems but still resisted lodging. This attenuation can be explained partly by the narrower range of variation in Syn1, which limits the detectability of phenotypic relationships. In addition, recombination among divergent parents may have disrupted historical associations between traits, generating novel combinations not present in the accession panel. Syn1 also displayed longer linkage

disequilibrium, as expected for an early-generation synthetic population with limited recombination (Li et al. 2014). While this feature does not explain the reduced phenotypic correlations, it is advantageous for GWAS because extended LD facilitates the detection of QTL even with a moderate marker density, as observed in this study.

4.2. GWAS identified context-dependent QTL

In the accession panel, several QTL were detected for traits of interest. A major locus on chromosome 7 was associated with both growth habit and leaflet size, explaining more than 27% of the variance for each trait. This SNP was located near MS.gene036920, encoding a RING-type zinc finger protein. Homologues in *Arabidopsis thaliana* (L.) Heynh. and rice (e.g. AtRZFP34, OsRZFP34) regulate stomatal aperture and ABA responses (Hsu et al. 2014), suggesting a potential role in morphogenesis and water-use efficiency. To date, no previous lucerne studies have directly reported QTL for growth habit, although F. Zhang et al. (2019) identified several QTL for shoot diameter and branching on linkage group 7, traits potentially related to plant architecture. For leaflet traits, prior studies have used detailed measurements (length, width, area) to dissect size variation. He et al. (2019) reported 13 QTL for leaflet area, while Jiang, Yang, et al. (2022) identified seven candidate genes through combined QTL mapping and RNA-seq. None of their candidate genes overlapped physically with the loci identified in this study, suggesting novel regions of interest.

A QTL for lodging resistance was detected on chromosome 6 in the accession panel, explaining 15.6% of the phenotypic variance. The candidate gene, MS.gene031124, encodes a ubiquitin conjugation factor E4, involved in protein degradation and cell survival (Koegl et al. 1999). In Arabidopsis, E4 family proteins such as AtUAP2 participate in ABA-mediated stress responses (Park et al. 2024). Previous work by McCord et al.(2014) identified two QTL for lodging (≥14% of phenotypic variation each) in a backcross population. However, the loci did not overlap physically with those identified here, likely due to differences in mapping populations and marker resolution. For growth rate, a minor QTL (3.8% of phenotypic variation) was identified on chromosome 8, near MS.gene61257, which encodes a calcium-binding protein with an EF-hand domain. EF-hand proteins are widely known to mediate calcium signalling during growth reactivation following stress (Lewit-Bentley and Réty 2000; Grabarek 2006).

In Syn1, several QTL were detected for plant height, distributed across chromosomes 1, 2, 3, 4, 5, and 8, jointly explaining of 60% of phenotypic variance. The locus with the strongest effect was located on chromosome 8 within MS.gene000456, a ubiquitin-associated domain protein, which may influence hormonal or structural growth pathways. Other candidate genes were implicated in signalling, chromatin organisation, and defence-related processes. Previous studies using biparental populations reported height QTL across all linkage groups, often co-localising with yield loci (Zhang et al. 2019; He et al. 2020; Jiang et al. 2022b). For leaflet size, QTL were identified on chromosomes 1, 2, and 4, jointly explaining 31% of the variance. Two QTL were not located close to genes but the QTL on chromosome 4 was located near MS.gene91452, though no functional annotation was available. Prior leaflet-related QTL studies, including the QTL detected in the accession panel, did not report overlapping loci with these regions (Jiang, Yang, et al., 2022). Unlike in the accession panel, where leaflet size and growth habit co-localised to a major QTL on chromosome 7, no overlapping QTL were detected for these two traits in Syn1. This decoupling supports that recombination among divergent parents disrupted historical associations between traits.

Overall, QTL sets in the accession panel and Syn1 did not overlap, underlining the strong dependence of trait–marker associations on genetic background, allele frequencies, and population history. The lack of overlap also reflects differences in phenotypic variance, Syn1 displayed reduced variability for several traits, while in the accession panel, correction for strong population structure could have limited the detection of associations. Marker density (>100,000 SNP) provided sufficient resolution for both populations, but cannot rule out the presence of undetected loci with small effects.

4.3. Genomic prediction accuracy depended on genetic relatedness and trait heritability

Genomic prediction varied substantially across the four prediction scenarios. Within the accession panel, prediction was highly effective for several traits. Growth habit and leaflet size achieved prediction abilities exceeding 0.8, even with small training subsets, while plant height, stem diameter, and lodging resistance reached moderate to high prediction abilities (0.5- 0.7). These findings are consistent with previous studies reporting high prediction ability for traits such as plant height (PA = 0.65; (Jia et al. 2018)), autumn dormancy (PA > 0.75; in Pégard et al. (2023) and PA > 0.64 in F. Zhang et al. (2023)). For spring regrowth rate, prediction ability

was weak (PA ≈ 0.30), which can be explained by its low heritability and polygenic nature, as also observed by Jia et al.(2018).

Within the Syn1 population, prediction ability was generally lower than in the accession panel but improved with increasing training set size. The highest performance was achieved for stem diameter and growth habit (PA = 0.37), while dormancy showed near-zero predictability. This reduced accuracy is likely attributable to both the limited genetic variability within Syn1 and its small population size. Although Syn1 was derived from three different accessions, recombination remains incomplete, and genetic diversity is narrower than in the accession panel. These factors restrict the model capacity to estimate reliable marker effects, a known limitation in early-generation breeding populations H. Wang et al., 2024). A larger or more recombined synthetic population (e.g. Syn2) would likely improve performance.

Across-population predictions gave prediction abilities comparable to those obtained within Syn1, generally below 0.30. Using a large and diverse training set to predict a narrow synthetic population did not improve accuracy, but also did not perform worse than within-population prediction. This indicates that accession panels can provide predictive information across populations, although accuracy remains limited by the small size and reduced variance of Syn1.

The relationship between trait heritability and prediction ability varied depending on the scenario. In the accession panel, prediction accuracy correlated strongly with trait heritability ($r^2 = 0.89$, $p < 0.05$). However, this relationship was not observed in Syn1 or across-population predictions. This breakdown suggests that high heritability alone does not ensure prediction success when population size and diversity are limited or when the training set does not represent the target population (Alemu et al. 2024).

5. Conclusion

This study investigated the genetic determinants of lucerne traits relevant for ideotype breeding in living mulch systems, using both a diverse accession panel and a synthetic population. The accession panel captured broad phenotypic and genetic diversity structured by subspecies and cultivation status, while Syn1 provided a complementary, less structured population where recombination generated novel trait combinations. Correlation patterns reflected these differences: strong associations in the accession panel illustrated historical selection and the

maintenance of broad diversity, whereas weaker correlations in Syn1 highlighted the potential to decouple traits through recombination. QTL identified through population-specific GWAS confirmed the polygenic nature of key architectural traits and their dependence on genetic background. Genomic prediction was highly effective in the accession panel, with accuracies above 0.80 for several traits, and moderately promising in Syn1 despite its reduced size and diversity. Across-population predictions were comparable to those within Syn1, showing that accession panels can inform prediction across populations, though accuracy remained constrained by Syn1 limited size and variance. Overall, prediction ability depended not only on trait heritability but also on the diversity and relatedness between training and test sets

From a breeding perspective, these findings provide guidance for the development of lucerne ideotypes adapted to living mulch systems. Traits such as growth habit, leaflet size, and lodging resistance can be efficiently improved through genomic prediction within well-characterised and sufficiently large populations, while QTL may assist in selection within specific contexts. Synthetic populations underline the opportunity to recombine and decouple traits. Breeding ideotypes that combine reduced vigour with sufficient lodging resistance and robust canopy traits remains a key challenge, and will require larger, more recombined populations evaluated across multiple environments.

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Supplementary materials

Table S3. *Characteristics of the 27 lucerne accessions, including subspecies, type (wild or cultivated), and geographical origin.*

Accession name	Subspecies	Type	Origin
Camporegio	falcata	Cultivated	Italy
Coussouls	sativa	Cultivated	France
Gabès	sativa	Cultivated	Tunisia
Greenmed	sativa	Cultivated	Spain
Koalf 2-96	sativa	Cultivated	Hungary
Krasnokutskaya	falcata	Cultivated	Ukraine
L4332	sativa	Cultivated	France
L5323	sativa	Cultivated	France
L8988	falcata	Cultivated	France
Limory	sativa	Cultivated	France
Ludelis	sativa	Cultivated	France
Luzelle	sativa	Cultivated	France
Malzeville	falcata	Wild	France
Marais de Luçon	sativa	Cultivated	France
Maron	falcata	Wild	France
Mezzo	sativa	Cultivated	France
Miechowska	sativa	Cultivated	Poland
Milfeuil	sativa	Cultivated	France
Milky Max	sativa	Cultivated	France
Monte Oscuro	sativa	Wild	Spain
Occitane	sativa	Cultivated	France
Pancrudo	sativa	Wild	Spain
Speeda	sativa	Cultivated	France
Timbale	sativa	Cultivated	France
Verdor	sativa	Cultivated	France
Villanueva de Jara	sativa	Wild	Spain
Wugong	sativa	Cultivated	China

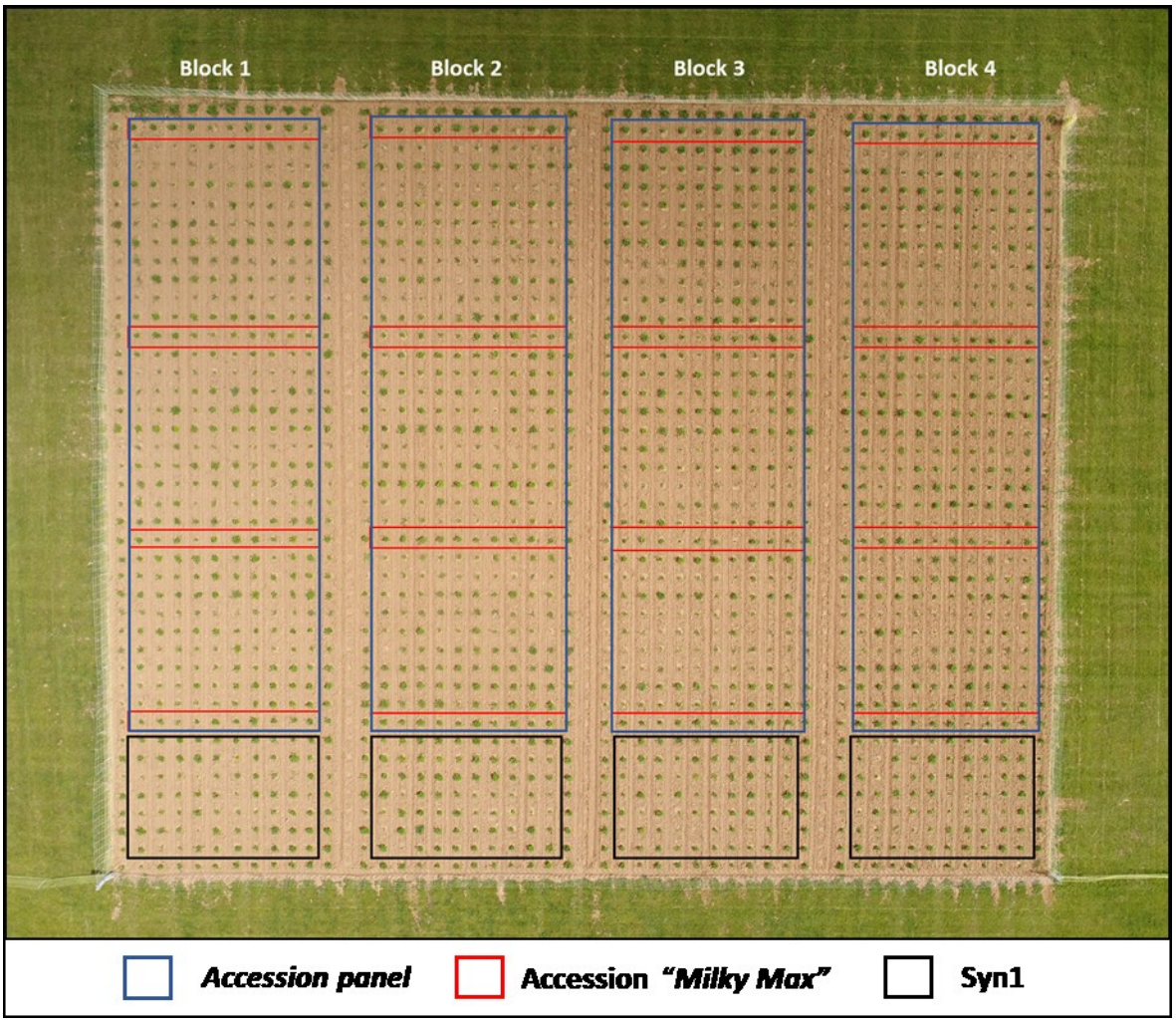

Figure S1. *Field layout showing the four blocks, each containing 10 plants per accession; The "Milky Max" accession was repeated four times per block to assess spatial variability. The Syn1 population was planted at the end of each block. following the accession rows. All remaining plants surrounding the experimental plots served as border plants.*

Table S2. *Climate data from April 2021 to June 2023.*

Month	Temperature min / Temperature max (°C)			Rainfall (mm)		
	2021	2022	2023	2021	2022	2023
January	1.8/7.2	1.7/7.2	3.7/8.0	96.0	39.5	120.0
February	4.9/11.1	3.1/11.7	2.0 /10.5	80.0	31.0	47.5
March	2.9/13.5	4.8/14.4	5.7/13.5	16.0	30.5	117.5
Avril	3.8/16.1	6.2/16.3	6.1/16.0	18.5	60.5	34.0
May	7.9/17.8	10.5/23.4	9.8/20.8	90.5	12.0	28.5
June	13.6/24.5	14.1/26.2	14.8/27.2	98.0	98.5	86.0
July	14.1/24.4	14.8/29.3	14.2/25.8	59.5	5.5	26.5
August	13.4/24.1	16.3/29.5	15.1/26.8	23.5	46.5	43.5
September	13.1/24.2	11.6/22.6	15.3/27.2	38.0	65.5	70.0
October	7.4/18	12.5/20.9	10.7/20.8	52.5	51.0	203.0
November	3.5/9.9	6.9/14.1	7.1/12.6	34.0	88.5	214.5
December	3.9/9.1	3.6/8.5	5.4/10.0	120.0	92.0	119.5

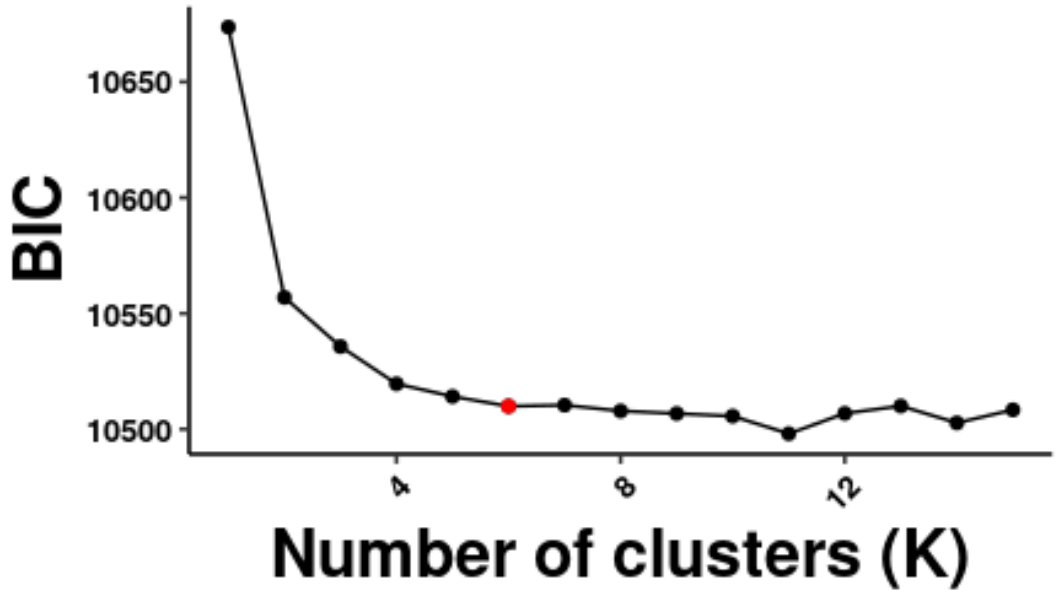

Figure S2. *Bayesian Information Criterion (BIC) values for different numbers of clusters (K).*

Table S3. *Summary of descriptive statistics (minimum, maximum, and mean) and heritability (h^2) estimates for studied traits measured in the accessions panel and the Syn1 population. Heritability estimates were derived using the model described in Eq.1. Variance components (spatial. genetic. and residual) associated to these traits are provided.*

Trait	Panel	Data description				Spatial	Genetic	Residual	h^2
		Min	Max	Mean	sd				
Dormancy	Accession panel	0.0006	0.099	0.037	0.016	2.314e-05	9.890e-05	7.968e-05	0.553
	Syn1	0.0044	0.069	0.041	0.011	8.254e-05	2.025e-05	9.760e-05	0.171
Growth habit	Accession panel	0.788	8.972	4.647	1.968	0.031e-01	8.767e-01	3.820e-01	0.696
	Syn1	2.716	8.669	4.903	0.998	2.375e-01	3.183e-01	6.343e-01	0.334
Leaflet size	Accession panel	1.401	8.783	5.300	1.335	1.830e-01	4.896e-01	3.924e-01	0.555
	Syn1	1.762	8.031	5.645	0.785	0.978e-01	1.940e-01	4.387e-01	0.306
Growth rate	Accession panel	0.008	0.182	0.099	0.023	0.350e-04	1.929e-04	3.471e-04	0.357
(cm·GDD-1)	Syn1	0.004	0.174	0.102	0.021	0.005e-04	2.409e-04	2.294e-04	0.512
Max height (cm)	Accession panel	13.038	137.041	74.207	17.044	0.202e+02	1.047e+02	1.136e+02	0.479
	Syn1	7.667	123.172	78.388	14.076	0.002e+02	0.983e+02	1.033e+02	0.487
Stem diameter (m	Accession panel	0.184	5.001	2.448	0.946	0.357e-01	2.886e-01	2.191e-01	0.568
m)	Syn1	0.794	4.991	2.757	0.765	0.089e-01	1.838e-01	3.923e-01	0.319
Lodging	Accession panel	0.040	0.964	0.660	0.178	0.126e-02	1.137e-02	1.257e-02	0.474
	Syn1	0.360	0.979	0.811	0.106	0.885e-03	2.489e-03	8.823e-03	0.220

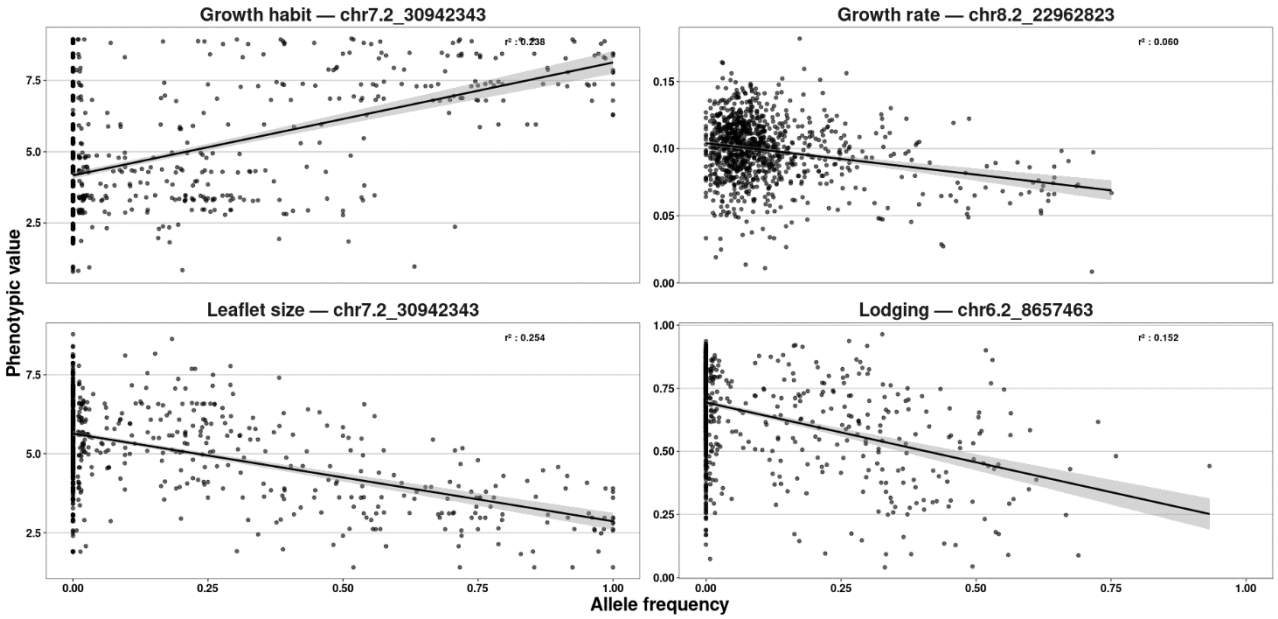

Figure 3S. *Allele frequency–phenotype associations for significant QTL identified in the accession panel.*

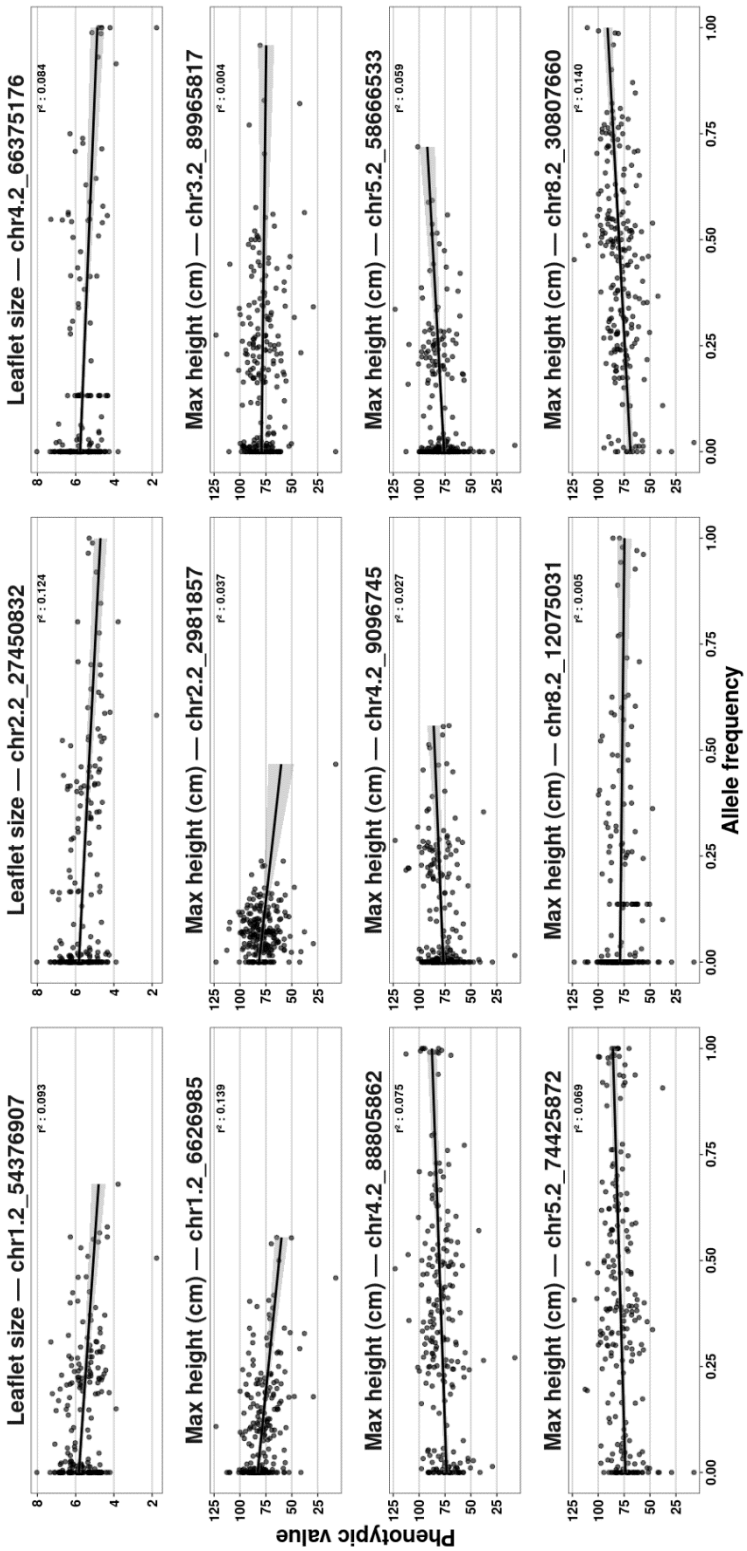

Figure 4S. *Allele frequency–phenotype associations for significant QTL identified in the syn1 population.*

Discussion générale

L'utilisation de la luzerne comme mulch vivant pour des cultures céréalières illustre à elle seule les difficultés de l'agroécologie, marquées par la recherche d'un compromis entre les services écosystémiques. Cette légumineuse pérenne combine de nombreux atouts écologiques, tels que la fixation symbiotique de l'azote, l'amélioration de la structure du sol, la couverture durable et l'appui au contrôle des adventices, mais ses variétés actuelles, issues de la sélection fourragère, se révèlent trop compétitives vis-à-vis des cultures de rente comme le blé. Ce déséquilibre entre la fourniture de services écologiques et de production constitue l'un des principaux verrous techniques limitant l'adoption à grande échelle des cultures en mulch vivant.

Face à ce constat, cette thèse avait pour objectif de poser les bases conceptuelles et méthodologiques d'une sélection de la luzerne orientée vers l'usage en mulch vivant. L'hypothèse de départ reposait sur l'idée que le complexe *Medicago sativa*, extrêmement large et diversifié, recèle la variabilité nécessaire pour identifier et combiner des traits permettant, en limitant la compétition entre la luzerne et le blé, de concilier les services écosystémiques fournis par la luzerne et la productivité du blé. L'objectif général était de concevoir un tel idéotype de luzerne et de proposer des outils et méthodes pour la création de variétés ciblant cet idéotype.

Pour répondre à cette question, située à l'interface entre génétique, écophysiologie et sélection, trois volets complémentaires ont été développés : (i) l'identification des traits morphologiques et phénologiques de la luzerne influençant la performance du blé et la régulation des adventices pour la conception d'un idéotype ; (ii) l'évaluation de l'efficacité de la sélection directe en association par rapport à la sélection indirecte en cultures pures, qu'elles soient menées sur plantes espacées ou en peuplements denses ; (iii) l'analyse des bases génétiques et du contrôle moléculaire des caractères définissant l'idéotype.

1. Redéfinir l'idéotype pour le mulch vivant : concepts et méthodes

L'évolution des objectifs de l'amélioration des plantes, désormais orientés vers la multifonctionnalité et la durabilité des agroécosystèmes, impose une redéfinition profonde des objectifs de sélection (Brummer et al. 2011; Lammerts van Bueren et al. 2018). Le concept

d'idéotype, formulé par Donald (1968), visant la maximisation du rendement en monoculture, a permis d'importants gains de productivité, mais il reste inadapté aux cultures associées où les interactions entre plantes deviennent déterminantes. La valeur d'une variété réside alors autant dans sa capacité à coexister que dans sa productivité propre (Litrico and Violle 2015; Bourke et al. 2021). Cette thèse s'inscrit dans ce changement de paradigme, en particulier pour le mulch vivant, en adoptant la notion d'idéotype orienté vers les services écosystémiques, sélectionné non pas pour sa production propre, mais pour sa contribution au fonctionnement écologique au service de la culture de rente : couverture du sol, fixation symbiotique, régulation des adventices.

Les résultats obtenus confirment la pertinence de cette approche idéotypique. L'idéotype de luzerne adapté au mulch vivant repose sur une combinaison morpho-phénologique originale, caractérisée par une dormance intermédiaire, une croissance limitée au printemps et une bonne résistance à la verse. Cette combinaison de traits réduit la compétition avec le blé, favorise sa production de talles et assure une couverture du sol pour limiter les adventices. Les phénotypes intermédiaires s'avèrent ici plus adaptés à un usage en mulch vivant, car ils maintiennent un équilibre entre différences de niches, liées à l'utilisation des ressources et à l'occupation de l'espace, et différences de valeurs sélectives, reflétant les écarts de performance compétitive. Cet équilibre limite la domination d'une espèce et favorise une coexistence stable (DeMalach et al. 2022).

En parallèle à la définition des traits, les modalités de culture dans lesquelles ils sont mesurés s'avèrent déterminantes pour la sélection de cet idéotype. Cette thèse a comparé plusieurs modalités d'évaluation, plantes espacées, peuplements denses et mulch vivant blé-luzerne, afin d'analyser la transférabilité et la robustesse des critères selon la modalité de culture. Elle apporte une validation expérimentale aux hypothèses formulées par Dubey et al. (2024), selon lesquelles la prédictibilité des performances en culture associée dépend fortement de la stabilité des traits à travers les modalités de culture et les stades de développement. Nos résultats confirment que certains caractères à fonction écologique stable, tels que la dormance et la hauteur, peuvent être sélectionnés efficacement en conditions simplifiées (plantes espacées), tandis que d'autres, comme la verse, requièrent une évaluation directe en association.

Cette approche expérimentale, articulée sur différents niveaux de compétition, a permis aussi de comparer la réponse des accessions selon des contextes intra- (entre luzernes) et

interspécifiques (entre luzerne et blé). Elle offre ainsi un cadre pertinent pour analyser la plasticité des traits et leur contribution à la performance du blé. L'intégration des outils de la génétique quantitative dans l'étude des caractères définissant l'idéotype renforce la portée du travail en permettant de mieux caractériser la gamme de variation des traits, d'identifier leurs relations génétiques et d'évaluer leur héritabilité. Ces analyses ouvrent de nouvelles perspectives pour la sélection.

Le panel de diversité génétique étudié, combiné à la population synthétique Syn_1, constitue une ressource génétique structurée et exploitable, permettant de concevoir des schémas de croisements ciblés pour créer des combinaisons de traits complémentaires adaptées à l'usage en mulch vivant. Enfin, l'intégration de marqueurs moléculaires, qu'ils soient associés à des caractères clés ou inclus dans des équations de prédiction génomique, offre la possibilité d'accélérer la création de variétés dédiées à un usage de mulch vivant. Des sélections sont d'ailleurs déjà en cours à partir de ce matériel.

2. Analyse critique et limites de l'étude

Cette étude a été conçue comme une exploration visant à obtenir des premiers éléments pour identifier les caractères phénotypiques clés à prendre en compte dans la définition d'un idéotype de luzerne adapté à un usage en mulch vivant, et à en étudier le contrôle génétique en vue de la création de cet idéotype, très peu exploré à ce jour (Cougnon et al. 2022). Le dispositif expérimental a ainsi été construit pour cibler un nombre restreint de leviers à explorer, en fixant volontairement certains paramètres. Ce positionnement méthodologique, cohérent avec une approche exploratoire, en délimite néanmoins la portée et les possibilités de généralisation.

Les essais ont été conduits sur un seul site (Lusignan) pendant deux années consécutives, avec une seule variété de blé, 'Gény', utilisée comme variété testeur, c'est-à-dire une culture de référence permettant d'évaluer la réponse relative des accessions de luzerne (Annicchiarico et al. 2021). Ce choix méthodologique assure une comparaison cohérente entre accessions, mais limite la représentativité environnementale et génétique de l'étude (Demie et al. 2022). L'observation du comportement contrasté de certaines accessions illustre cette sensibilité : 'Gabès', fortement pénalisée par le froid, a favorisé la dominance du blé. Un essai conduit dans une région avec des hivers plus tièdes, ou l'emploi d'un blé à pailles plus hautes, présentant un pouvoir couvrant supérieur au stade fin tallage (≥ 6 au lieu de 4,5 dans cette étude ;

(agriobtentions 2024)), aurait probablement modifié la dynamique d'interaction. Parmi le large panel de luzerne, issu majoritairement de matériel européen, toutes les combinaisons de traits n'étaient pas disponibles, notamment aucune n'avait une dormance élevée et un port dressé. Ainsi, l'idéotype, avec une combinaison de traits identifiés comme les plus adaptés, n'existe pas dans le panel d'accessions testées, même si ce panel a été conçu dans cet espoir. Toutefois, il est important de noter que l'étude ne visait pas à tester un idéotype abouti, mais à identifier les axes de variation et les compromis à mobiliser pour sa conception future.

Sur le plan agronomique, plusieurs paramètres du dispositif ont pu accentuer la compétition entre les deux espèces, en défaveur du blé. Concernant la luzerne, la densité de semis appliquée (10 kg ha^{-1}) sur la ligne était équivalente à celle couramment recommandée pour les cultures fourragères pures (Julier et al. 2017). Combinée à une configuration spatiale alternée (un rang de luzerne pour un rang de blé), cette disposition a réduit la lumière disponible pour le blé et accru la compétition. De plus, le blé a été semé en fin d'automne dès la première année d'implantation du couvert, alors que la luzerne n'avait pas encore atteint l'âge d'un an à partir duquel elle commence à relarguer de l'azote dans le sol (Louarn et al. 2015). Le choix d'un apport restreint d'azote en fin d'hiver, limité à 40 kg ha^{-1}, sans restitution de la biomasse de la luzerne, a renforcé l'effet de la compétition de la luzerne sur le blé, déjà pénalisé par la forte densité du couvert qui réduisait son accès à la lumière. L'ensemble de ces choix et conditions expérimentales a également influencé une autre composante majeure de l'association : la dynamique des adventices. La maîtrise des adventices s'est révélée techniquement difficile dans ce dispositif et n'a pas été totalement efficace, ce qui a amplifié la compétition au sein du mélange. Malgré ces contraintes, le dispositif a offert un cadre d'observation pertinent pour tester la robustesse du système et révéler les principaux traits de coexistence entre le blé et la luzerne.

En plus de ces aspects expérimentaux, la compréhension du fonctionnement global du mulch vivant reste limitée par le manque d'informations sur les processus physiologiques sous-jacents. La partie aérienne des associations a été correctement caractérisée à travers un ensemble de traits morphologiques et phénologiques pertinents, permettant de distinguer clairement les accessions du point de vue de l'acquisition de la lumière. Toutefois, ces mesures se limitent à la composante phénotypique et ne renseignent pas directement sur les mécanismes physiologiques, notamment ceux liés à la symbiose. Bien qu'aucune donnée spécifique n'ait

été collectée sur la partie racinaire ou sur la disponibilité en azote du sol, certains indicateurs indirects, tels que les teneurs en azote des parties aériennes du blé, constituent des proxys utiles pour appréhender le fonctionnement de l'association. Ces éléments restent néanmoins insuffisants pour caractériser pleinement les processus de compétition et de complémentarité entre le blé et la luzerne (Zhao et al. 2023; Salinas-Roco et al. 2024). Enfin, l'étude s'est centrée sur l'effet de la luzerne sur le blé, sans analyser les données liées à la réponse de la luzerne à l'association, ainsi que sa croissance post-récolte, ce qui limite l'interprétation globale du fonctionnement de l'association.

3. Perspectives

Cette thèse a permis d'identifier les bases d'un idéotype de luzerne adapté à un usage en mulch vivant, conciliant la fourniture de services écosystémiques et la compatibilité avec la culture de blé. Elle a également conduit à proposer des outils et approches de sélection pour orienter la création de ce type de matériel. Les résultats obtenus constituent une première validation expérimentale solide de la faisabilité d'une sélection dédiée à cet usage. Bien que menés dans un dispositif expérimental ciblé, ils offrent des enseignements généralisables et ouvrent la voie à de nouvelles perspectives de recherche.

Sur le plan agronomique, les observations réalisées mettent en évidence plusieurs leviers d'ajustement de l'association. La réduction de la densité de semis de la luzerne, combinée à un écartement de rangs plus large, apparaît comme une voie prioritaire pour limiter la compétition lumineuse. Un compromis reste néanmoins nécessaire : la densité doit être suffisamment faible pour réduire la pression sur le blé, mais assez élevée pour maintenir les services attendus du mulch vivant, notamment la couverture du sol et la régulation des adventices. Le semis du blé à l'automne, plus précoce qu'au cours de cette étude, renforcerait son implantation avant la reprise de croissance de la luzerne et améliorerait son accès à la lumière et aux ressources, tout en lui permettant de mieux concurrencer les adventices durant la phase d'installation de l'association. Le choix d'un blé à fort pouvoir couvrant accentuerait cet effet (Corre-Hellou et al. 2011). De même, la restitution de la biomasse de luzerne après la coupe pourrait contribuer à limiter la levée d'adventices et à enrichir le sol en azote par minéralisation (Gaudin et al. 2013; Louarn et al. 2015). La maîtrise des adventices demeure un facteur clé pour la réussite du blé sur un mulch vivant, mais elle reste complexe en raison de la dynamique de croissance différentielle entre le blé et la luzerne. Une approche combinant plusieurs méthodes de

régulation pourrait être présentée comme un compromis pour stabiliser le mélange. Avec un idéotype de luzerne moins compétitif, tel que celui identifié dans cette étude, il serait envisageable de réduire la fréquence des interventions mécaniques de régulation de la luzerne dans les inter-rangs de blé, estimée à trois ou quatre passages selon Arvalis (2022), à une ou deux opérations seulement, conciliant maîtrise des adventices, stabilité de l'association et diminution des intrants grâce à la restitution de la biomasse de luzerne qui enrichit le sol en azote.

La suite du travail consistera à confirmer la pertinence des traits identifiés et à préciser les gammes de valeurs à rechercher pour concevoir un idéotype pleinement opérationnel avec des pratiques agronomiques (densité, date de semis, fertilisation) qui peuvent évoluer. Cette étape pourrait être amorcée par des simulations *in silico*, à l'aide de modèles de croissance et de compétition interspécifique (Gaudio et al. 2019), afin d'évaluer virtuellement l'effet de différentes combinaisons de traits et d'orienter les expérimentations futures. Ces validations devront ensuite être conduites dans des contextes pédoclimatiques contrastés et avec plusieurs variétés de blé, voire d'autres céréales, pour évaluer la robustesse et la transférabilité des résultats. Parallèlement, une analyse plus fine de la performance propre de la luzerne est nécessaire. Les données déjà acquises au cours de cette expérimentation, issues du suivi par drone et des mesures post-récolte du blé, n'ont pas été exploitées dans cette thèse. Leur analyse permettra de mieux caractériser la dynamique de croissance, la production de biomasse et la contribution de la luzerne aux entrées d'azote, afin de compléter la compréhension du fonctionnement du système.

Sur le plan de la sélection, il serait également pertinent d'intégrer les concepts d'aptitude générale et spécifique au mélange (GMA/SMA) lors des étapes de sélection. L'aptitude générale au mélange (GMA) permet d'identifier des accessions de luzerne performantes, quel que soit le partenaire, favorisant ainsi le développement de variétés robustes et prévisibles dans divers contextes. L'aptitude spécifique au mélange (SMA) permet de mettre en évidence des combinaisons particulièrement efficaces avec une variété d'une céréale donnée (Michalitsis et al. 2024). Enfin, les approches de sélection génomique et l'identification de QTL associés à des caractères clés constituent des leviers puissants pour accélérer la création de matériel adapté aux mulchs vivants. Leur intégration dans les programmes de sélection en mélange s'est déjà révélée plus efficace que les approches conventionnelles (Bančič et al. 2021).

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Titre de thèse : Conception d'un idéotype de luzerne pour un usage de mulch vivant au service des cultures de rente et étude génétique des caractères impliqués**.**

Mots-clés : luzerne, mulch vivant, association luzerne-blé, diversité génétique, GWAS, prédiction génomique, idéotype, agriculture durable.

Résumé : La luzerne, légumineuse pérenne reconnue pour sa fixation de l'azote, sa persistance et sa capacité à couvrir le sol, présente un fort potentiel en tant que mulch vivant pour les cultures céréalières. Cependant, sa croissance vigoureuse induit souvent une compétition excessive avec les cultures de rente telles que le blé, limitant ainsi son utilisation à grande échelle. Cette thèse visait à fournir les bases génétiques et phénotypiques pour la création d'idéotypes de luzerne adaptés à un usage en mulch vivant, en (i) identifiant les traits morphologiques et phénologiques déterminant les interactions luzerne–blé, (ii) évaluant quels dispositifs de sélection prédisent le mieux la performance en mulch vivant pour ces caractères, et (iii) analysant leur déterminisme génétique et leur prédictibilité génomique. Un panel diversifié de trente accessions sauvages et cultivées a été évalué pendant deux ans dans trois modalités de culture, plantes espacées, peuplements denses et mulch vivant luzerne–blé, en combinant phénotypage et analyses génomiques. La dormance, le port, la hauteur et la verse ont été identifiés comme des déterminants majeurs de l'équilibre entre la dominance du blé, la suppression des adventices et la contribution azotée. Les corrélations génétiques entre modalités ont révélé que la hauteur de début de printemps et maximale mesurées sur plantes espacées prédisent de manière fiable les performances en association, tandis que la verse nécessite une évaluation directe en mélange. Les analyses d'association ont détecté plusieurs QTL à effets modérés, tandis que la prédiction génomique a atteint une haute précision (> 0,8) pour des caractères héritables tels que le port. L'ensemble de ces résultats établit un cadre conceptuel et méthodologique pour la conception d'idéotypes de luzerne soutenant la transition agroécologique des systèmes céréaliers.

Thesis Title: Designing a lucerne ideotype for use as a living mulch for cash crop production and genetic analysis of key underlying traits.

Keywords: lucerne, living mulch, wheat intercropping, genetic diversity, GWAS, genomic prediction, ideotype breeding, sustainable agriculture.

Abstract: Lucerne, a perennial legume valued for its nitrogen fixation, persistence, and soil-cover capacity, offers strong potential as a living mulch to support cereal-based agroecosystems. However, its vigorous growth often induces excessive competition with companion crops such as wheat, limiting its practical use. This thesis aimed to provide the genetic and phenotypic basis for breeding lucerne ideotypes adapted to living-mulch systems by (i) identifying the morphological and phenological traits governing lucerne-wheat interaction outcomes, (ii) evaluating which selection designs best predict living-mulch performance for these traits, and (iii) analysing their genetic determinism and genomic predictability. A diverse panel of thirty wild and cultivated accessions was evaluated over two years under three cropping modalities, spaced plants, dense monocultures, and lucerne-wheat living mulch, combining field phenotyping and genomic analyses. Dormancy, growth habit, plant height and lodging were identified as major determinants of the balance between wheat dominance, weed suppression, and nitrogen contribution. Genetic correlations among modalities revealed that early-spring and maximum height measured in spaced nurseries reliably predict intercrop outcomes, whereas lodging requires direct evaluation in mixtures. Genome-wide association analyses detected several moderate-effect QTL, while genomic prediction reached high accuracy (> 0.8) for heritable traits such as growth habit. Together, these results establish a conceptual and methodological framework for designing lucerne ideotypes that support the agroecological transition of cereal-based systems.