**Mixing varieties mitigates early root competition in wheat under water and nutrient limitation**

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# Keywords

varietal mixtures, agroecology, relative yield, stress-gradient hypothesis, complementarity, high-throughput root phenotyping, root area, competitive hierarchy

# Abstract

Ecological research suggest that plant diversity increases productivity, and that positive interactions between plants increase under harsh environments. Cultivating varietal mixtures instead of monovarietal stands could thus help optimizing resources while limiting chemical inputs. Mixing varieties indeed increase yield on average, but mixing effects are also highly variable. In this study, we tested whether diversity in root traits could promote positive interactions between varieties, especially under limiting resources. We grew 36 durum wheat (*Triticum turgidum* ssp. *durum*) varieties in pure stands and in 54 binary mixtures in a high-throughput root phenotyping platform under both controlled conditions (R+) and water and nutrient limitation (R-). We found that seedlings in mixed stands produced less biomass than predicted from their pure stands, particularly under R-. This biomass reduction reflected a relaxation of competition where competitive varieties benefited from having a less competitive neighbour than themselves in mixture. The competitive hierarchy between varieties was captured by a single trait, the 2D projected area of the root system which predicted ~50% of the biomass reduction in R-. Our results suggest that root area is a promising breeding targets to reduce intra-specific competition and a key trait to consider for mixture assembly.

# Introduction

Decades of experimental ecology have established a general positive relationship between plant diversity and ecosystem functioning (Tilman *et al.*, 1996, 2001; Hector *et al.*, 1999). Ecosystems with a higher number of species tend to be more productive, more efficient at regulating pathogens, at recycling nutrients, and at buffering abiotic stresses (Hooper *et al.*, 2005; Hector and Bagchi, 2007; Tilman *et al.*, 2014). Species diversity is generally thought to improve ecosystem functioning via two main effects: the complementarity effect, and the selection effect (Loreau and Hector, 2001). The complementarity effect results from differences in ecological niches between species that have different resource requirements and hence experience less competition, which ultimately translates into a more efficient conversion of resources into ecosystem functions (e.g., Roscher et al. 2008; Mueller et al. 2013). Communities with more species are also more likely to contain species which are the most efficient at performing a given function; such “efficient” species might be even more efficient in a diverse community than in a monospecific community (e.g., highly competitive species often benefit from relaxed competition in a mixture), which corresponds to the selection effect (e.g., Fargione et al. 2007; Li et al. 2018).

Similar ecological effects can be exploited in crops by mixing different species at the same time within the same field (i.e., intercropping, Vandermeer 1992). For example, one of the most ancient intercrop known as the “three-sisters” (maize, bean, squash) combines species with different root foraging strategies that complement each other and achieve greater yield in mixture than grown separately (Zhang *et al.*, 2014), which is commonly referred to as overyielding. However, intercropping also raises several challenges because the components of the intercrop can differ in many aspects of their life cycle (germination, growth rate, phenology, plant architecture, etc), which complicates agronomic management (Lemken *et al.*, 2017; Huss *et al.*, 2022). Moreover, they often have different harvest products that need to be separated from each other.

A more straightforward way to increase plant diversity is to cultivate varietal mixtures, that is a mixture of several varieties of the same species in the same field (Smithson and Lenné, 1996). This practice addresses most of the technical challenges of intercropping, but generally provides smaller benefits (Beillouin *et al.*, 2021). Historically, varietal mixtures have been shown to be very efficient at limiting pathogen spreads and disease severity (Borg *et al.*, 2018), especially in grain cereals (Wolfe, 1985; Mundt *et al.*, 1995; Zhu *et al.*, 2000; Finckh and Wolfe, 2006). This beneficial effect has recently renewed the interest in varietal mixtures, as they may help reduce pesticide use (Barot *et al.*, 2017; Borg *et al.*, 2018; Snyder *et al.*, 2020; Wuest *et al.*, 2021). Varietal mixtures could also help optimize resource use and ultimately increase crop yield through ecological mechanisms at play in natural ecosystems or in intercropping such as niche complementarity (Hajjar *et al.*, 2008; Barot *et al.*, 2017; Snyder *et al.*, 2020). In line with these expectations, most meta-analysis report slight yield increases in varietal mixtures compared to monogenotypic stands (on average 2-5%, Kiær et al. 2009; Reiss and Drinkwater 2018; Borg et al. 2018). In addition, ecological theory predicts that such positive effects of plant diversity should strengthen when resources become limiting, which is known as the stress-gradient hypothesis (SGH, Bertness and Callaway 1994; Maestre et al. 2009). Therefore, varietal mixtures could help maintain high productivity under low-input farming practices and more variable and extreme climates (Adu-Gyamfi *et al.*, 2015). However, how resource limitations affect the relative productivity of mixture remains poorly understood (Reiss and Drinkwater, 2018; Alsabbagh *et al.*, 2022).

In natural ecosystems, plant functional traits have been used to better understand and predict the effect of plant diversity on ecosystem processes, notably productivity (McGill *et al.*, 2006; Garnier *et al.*, 2015). These traits are defined as any morphological, physiological or phenological feature which impact fitness, and are measurable at the individual level without reference to the environment or any other level of organization (Violle *et al.*, 2007). They capture how individuals interact with their abiotic and biotic environment, and ultimately how they contribute to processes occurring at higher levels of organization (community, ecosystem). For example, the mean values of leaf functional traits of dominant species can be used to predict primary productivity or litter decomposition rates at the community level (Garnier *et al.*, 2004). Similarly, differences in functional traits between species or genotypes can capture differences in ecological niche and thus explain complementarity effects occurring at the community level (Violle and Jiang, 2009). Some functional traits reflect the competitive ability of a species or genotype for a limiting resource, in which case differences in trait values can lead to negative interactions or even competitive exclusion (e.g., taller plants can exclude shorter plants when light is limiting), which is known as competition-trait hierarchy (Kunstler *et al.*, 2012; Cadotte, 2017; Wagg *et al.*, 2017). Even if plant traits are commonly used in agriculture, for example to adapt management practices or to guide plant breeding programs, very few studies have applied trait-based approaches to investigate ecological mechanisms at play in varietal mixtures (e.g., Montazeaud et al. 2018, 2020; Kong and Zhao 2023).

While functional ecology initially focused mainly on aboveground plant traits, it has since been recognized that root traits also capture important plant functions that ultimately affect many ecosystem functions (Bardgett *et al.*, 2014; Fort, 2023). Similarly, previous trait-based studies of varietal mixtures have reported significant relationships between morphological and architectural root traits (both means and differences) and multiple facets of mixture performance, including productivity and grain quality (Montazeaud *et al.*, 2018, 2020*b*). However, the mechanisms underlying these relationships remains poorly understood, notably because root traits are difficult to measure. Moreover, ecological studies show that root traits-community performance relationships are strongly affected by growth conditions, especially by belowground resource availability (Fort *et al.*, 2014). A better understanding of the relationship between root traits and varietal mixture performance under contrasted resource conditions could thus help us identify the mechanisms underlying mixing effects and design rules to guide the assembly of optimal mixtures for low-input farming.

In the present study, we used durum wheat (*Triticum turgidum* ssp*. durum*) as a crop model to investigate the effect of root trait composition on mixture performance. We used a panel of 36 varieties previously shown to exhibit contrasted responses to combined water and nitrogen limitation, and grew them in monogenotypic pots and binary mixtures in a highly controlled high throughput root phenotyping platform under both non-limiting and limiting water and nutrient conditions. We hypothesized that (i) mixtures would produce more biomass than their monogenotypic counterparts (i.e., overyielding), (ii) overyielding would be higher under resource-limited conditions, (iii) resource-use complementarity would be the main effect driving overyielding under resource-limited conditions, (iv) complementarity effects would be explained by the trait composition of the mixtures.

# Material and Methods

## Plant material

This study made use of field data collected on a diversity panel of 250 durum wheat genotypes, which was assembled during the EU Project SolACE (<https://www.solace-eu.net/>) to analyse wheat responses to combined water and nitrogen limitations (Collet, 2022). This panel comprised genotypes from four different collections (Collet, 2022) (i) “CREA”, with Italian cultivars, worldwide cultivars, and breeding lines selected by CREA; (ii) “EPO”, an highly diverse collection of fixed lines derived from an evolutionary pre-breeding population (David et al 2014); (iii) “GPDUR”, with old and modern cultivars from various geographic areas including Western Europe; (iv) “UNIBO”, a diversity panel comprising genotypes from pre-breeding programs, elite lines, and representative genotypes of several worldwide breeding programs since the 1970s. Based on both field and platform data, a subset of 36 genotypes exhibiting contrasted responses to resource availability on a set of below- and aboveground traits (i.e., ranging from high to low plasticity) was selected by the European consortium to perform further experiments. Information on the 36 genotypes can be found in Supplementary Table 1.

## Experimental design

Based on the 36 genotypes, we designed a set of 54 binary mixtures selected at random, with each genotype observed in three different mixtures (Supplementary Table 2). Pure stands (here refereeing to groups of a single genotype, n = 36) and mixtures (here referring to groups of two genotypes, n = 54) were grown in RhizoTubes® (Jeudy *et al.*, 2016), transparent pots designed for high-throughput root phenotyping (Figure 1), with six plants per RhizoTube®. The six plants had the same genotype in pure stands, whereas two genotypes were grown in alternate positions in the mixtures (Figure 1). The 36 monocultures and the 54 mixtures were grown under combined water and nutrient limitation (R- treatment) or under optimal growth conditions (R+ treatment). All monocultures and mixtures were replicated three times within each treatment following a randomized complete block design within treatment, leading to a total of 540 RhizoTubes® (Figure 1). In addition, six RhizoTubes® per experimental block per treatment (i.e., 36 RhizoTubes® in total) were grown with the commercial variety ANVERGUR and were used as controls to check for environmental heterogeneity in the greenhouse.

## Growth conditions

Seeds were sown into Petri dishes on the 22nd of June 2019 and kept at 4°C during two days. Seedlings were then transferred into the RhizoTubes® on the 24th. RhizoTubes® have a diameter of 17 cm and a depth of 49.5 cm (Jeudy *et al.*, 2016). They were filled with a 25:75 mixture of sand (Biot B4, Silices et Refractaires de la Méditerranée) and perlite. Temperatures were maintained around 20-25°C, relative humidity around 70-80%, and photoperiod was set to 16h, with an average PAR of 330 µmol.m-².s-1 during the day. Seedlings were daily provided with a liquid nutrient solution that contained water, N, P, K and all micronutrients required for plant growth (Supplementary Table 3). The water content of each RhizoTube® was monitored each day, and the amounts of nutrient solution were adjusted to maintain the RhizoTubes® at 100% of their water storage capacity (Supplementary Figure 1). In the S treatment, the provision of nutritive solution was stopped on June 28th (four days after seedling transfer), causing the water content to decrease, ultimately reaching 55% of the full storage capacity by the end of the experiment (Supplementary Figure 1). Plants were harvested between the 16th and the 19th of July, i.e., about three weeks after transplantation, and were at the beginning of the tillering stage.

## Phenotyping

Root traits were measured for each RhizoTube® based on image analysis as described in Jeudy *et al.*, (2016). We used images taken on the 15th of July. Because the roots of the different plants were overlapping in most RhizoTube®, we were unable to measure root traits for individual plants, and instead computed root traits at the level of the RhizoTube® as a whole. Three root traits were computed following image processing: root depth, corresponding to the distance between the top of the RhizoTube® and the deepest root pixel; root length, the total length of roots detected on the image; and root area, the 2D projected area of the total root system. Root area combines information on both root length and root diameter. We did not consider root depth in our analyses because root tips reached the bottom of the RhizoTubes® in most cases, leading to highly left-skewed trait distribution and very low trait variability.

At harvest, we extracted plants from the RhizoTubes® by carefully separating their rooting systems. For each plant, we counted the number of leaves on the main tiller (hereafter “# leaves”) and the total number of tillers (hereafter “# tillers”). Above and belowground biomass were then separated and dried at XX °C during XX days before weighing to determine shoot biomass, root biomass, root:shoot ratio, and total biomass. Leaf nitrogen content (hereafter “leaf N”) was measured with Near-Infrared Spectrometry (NIRS). We measured one NIR spectrum per leaf per plant in each RhizoTube® (i.e., six spectrum per RhizoTube®) using the Fieldspec 2500© (Analytical Spectral Devices, Inc. (ASD), Boulder, CO, USA) spectrometer. NIRS measurements were done one day before the harvest (i.e., on the 15th for the Rhizotubes harvested on the 16th, on the 16th for the RhizoTubes® harvested on the 17th, etc). NIR spectra were converted into nitrogen content using the calibration described in (Ecarnot *et al.*, 2013).

## Statistical analysis

We performed all statistical analyses with R v. 4.3.2 (R Core Team, 2019).

We first tested the effect of the treatment (R+ *vs* R-) on the different traits and biomass components (above and belowground) using only pure stand data summed per Rhizotube® (except leaf N which was averaged per Rhizotube®). We used mixed linear models with a given trait or biomass component as the response variable, treatment as a fixed effect, and genotype identity as a random intercept and random treatment slope. We also included two covariates as fixed effects: block, and harvest date or measurement date (except for root traits which were all measured on the same day). We assessed the significance of the fixed effects with standard analyses of variance and *F* statistics computed with Kenward-Roger's approximations for the degrees of freedom (Supplementary Table 4). We fitted the mixed model with the *lmer()* function (package *lme4*), and checked significance with the *anova()* function (package *lmerTest*).

To compare the relative performance of mixed *vs* pure stands, we computed the Relative Yields (RYs, de Wit and van den Bergh 1965) of the varieties in mixed stands for each biomass component (aboveground, belowground, and total biomass):

(1) ,

Where is the Relative Yield of the variety *i* grown in mixture with the variety *j* in the treatment *k*, is the biomass (aboveground, belowground, or total biomass) of the variety *i* grown in mixture with the variety *j* in the treatment *k* and, and is the pure stand reference biomass of the variety *i* in treatment *k*. To compute RYs, we first separated our dataset between the pure and mixed stands. Then, we summed the biomass of all plants of the same genotype within each RhizoTube® (i.e., 6 plants in pure stands, 3 plants in mixed stands). We used the pure stands dataset to compute pure stand reference biomass. To do so, we fitted a linear mixed model with biomass as the response variable, measurement date, block, and treatment as fixed effects, and variety identity as a random effect (here we included only the random effect of the variety on the intercept, as adding the random effect on the treatment slope led to singular models due to very low variance on the slope). We then summed the Best Linear Unbiased Predictor (BLUP) of each variety with the estimated fixed effect of the treatment (R+ or R-) to obtain the pure stand reference biomass values adjusted for the effects of block and measurement date within each treatment (i.e., ). For mixed stands, we also fitted a linear mixed model with each variety biomass as the response variable, measurement date, block, and treatment as fixed effects, and variety pair identity as a random effect on the intercept and on the treatment slope. Pair identity was constructed as the concatenation of the identity of the focal and neighbor variety, such that variety 1 and variety 2 in a mixed stand had pair identities variety1-variety2, and variety2-variety1, respectively. We then summed the BLUP of pair identity (intercept in the R+ treatment, intercept + slope in the R- treatment) with the estimated fixed effect of the treatment (R+ or R-) to obtain the biomass of each variety in mixed stands adjusted for the effects of the block and measurement dates within each treatment (i.e., ). We then divided these mixed stand values by the pure stand reference values to obtain RYs for each variety within each mixture, cf formula (1). Under the null hypothesis that the variety produced equal amount of biomass in mixed than in pure stand, RY = 0.5 because there are half the number of plants of a variety in mixed stands (n = 3) compared to pure stands (n=6). RY > 0.5 means that the variety produced more biomass in mixed than in pure stand, and RY < 0.5 means that the variety produced less biomass in mixed than in pure stand.

In a second step, we summed the RYs of the two varieties in the mixed stand to obtain the Relative Yield Total (RYT, de Wit and van den Bergh 1965) of the mixture:

(2) ,

Where is the Relative Yield Total of the mixture containing varieties *i* and *j* in treatment *k*. RYT = 1 means that the mixture as a whole produced more biomass than expected from the productivity of the varieties grown in pure stands, whereas RYT > 1 means that the mixture produced more biomass than expected, and RYT < 1 means that the mixture produced less biomass than expected.

We compared whether RYTs differed between the R+ and R- treatment using a linear mixed model with RYT as the response variable, treatment as a fixed effect, and varietal mixture identity (i.e., non-oriented concatenation of the names of the two varieties grown in mixture) as a random effect on the intercept. We assessed the significance of the fixed effects as detailed above (Supplementary Table 5). Finally, we checked whether RYTs significantly differed from 1 within each treatment using two-sided *t*-tests.

To assess whether trait composition of the mixture explained RYT variability, we used traits measured in pure stands to predict mixed stands RYTs. This approach is based on the hypothesis that pure stand traits are the information available to agronomists and plant breeders when designing varietal mixtures. First, we computed reference trait values for pure stands that we corrected for design effects and measurement dates. As described above, we fitted a linear mixed model with pure stand trait as the response variable, block, measurement date, and treatment as fixed effects, and varietal identity as a random effect. Varietal identity random effect was specified both on the intercept and the treatment slope, except for # leaves where adding the random slope led to singular models due to very low variance on the slope. We then computed pure stand reference trait values as the sum of the BLUP of each variety (intercept in the R+ treatment, intercept + slope in the R- treatment when random slope was specified) with the estimated fixed effect of the treatment (R+ or R-). Then, for each mixed stand, we computed both the average and the absolute trait difference between the two varieties using the pure stand reference trait values. Finally, we fitted a full linear model with RYT as the dependent variable and all trait averages and all trait differences as independent variables (*lm()* function from the *stats* package). We standardized all dependent and independent variables (*μ* = 0, *σ* = 1) and ran a backward model selection (*glmulti()* function from the *glmulti* package). We used the second-order Akaike Information Criterion (AICC, Sugiura 1978) to rank the models and performed model-averaging inference based on the top 10 models using the *coef()* function (*glmulti* package). We report parameter estimates and their 95% unconditional confidence interval computed as ±1.96 unconditional sampling standard deviation, variable importance, and adjusted R-squared averaged over the top 10 models (Burnham and Anderson, 2002). Detailed information on the top 10 models is available in Supplementary Table 6.

Finally, to further investigate the ecological mechanisms behind the significant relationship between RYT and trait composition, specifically root area, we assessed the relationship between pure stand biomass and pure stand root area, and between RYs and pure stand root area using simple linear regressions between these variables within each treatment. We used trait and biomass values adjusted for block effects and measurement date effects as explained above, such that we had one value per variety per treatment. We also checked the relationship between RYs and the hierarchical distance in root area between the variety and its neighbour in pure stands. Hierarchical distance was the difference between the focal root area and the neighbour root area, divided by the focal root area (i.e., positive values mean that the focal had higher root area that its neighbor, and *vice versa*). Finally, we tested whether root area plasticity affected RYT in the two treatments. We computed root area plasticity as the difference between the average root area of the pure stands of the two varieties and the observed root area of their mixture, divided by the average of the pure stands. (i.e., positive values mean that root area increased in the mixed relative to the pure stands, and *vice versa*).

# Results

## Effect of resource availability on plant growth and traits

The limitation of water and nutrients strongly reduced total plant biomass (1947.35 mg in the R- treatment vs 2364.71 in the R+ treatment, *F*1,87.77 = 144.49, *p* < 0.001,Figure 2a). Such biomass reduction was accompanied by a reallocation of resources from the aboveground to the belowground compartment: while plants produced less above-ground biomass (Figure 2b), less leaves (Figure 2d), and less tillers (Figure 2e), they produced higher root biomass (Figure 2c), longer roots (Figure 2h), and had higher root areas (Figure 2i) in the R- treatment. Consequently, the root:shoot ratio increased from 0.48 in the R+ treatment to 0.82 in the R- treatment (+71%, F1,87.78 = 3966.58, *p* < 0.001, Figure 2g). Leaf nitrogen concentration also increased in the R- treatment (3.32 vs 2.95%, F1,87.19 = 525.03, *p* < 0.001, Figure 2f).

## Relative biomass of mixtures

RYTs were significantly different between the R+ and R- treatments for all biomass components (Figure 3 and Supplementary Table 5). In the R+ treatment, the biomass production of the mixtures did not significantly differ from the biomass expected from their pure stand components (average RYT for total biomass = 0.99, *t*53 = -0.46, *p* = 0.0.6473, Figure 3a; average RYT for shoot biomass = 1.00, *t*53 = -0.05, *p* = 0.9632, Figure 3b; average RYT for root biomass = 0.99, *t*53 = -0.75, *p* = 0.4568, Figure 3c). In contrasts, in the R- treatment, mixtures produced significantly less biomass than expected from the biomass of their components grown in pure stands (average RYT for total biomass = 0.92, *t*53 = -6.18, *p* < 0.001, Figure 3a; average RYT for shoot biomass = 0.94, *t*53 = -4.29, *p* < 0.001, Figure 3b; average RYT for root biomass = 0.94, *t*53 = -6.83, *p* < 0.001, Figure 3c).

## Effect of trait composition on mixture biomass

RYTs were highly variable in both treatments (Figure 3). The trait composition of the mixtures poorly explained RYT variability in total biomass observed in the R+ treatment (Figure 4a, average adjusted R² over the top ten models = 0.10). In contrast, trait composition explained up to 49% of RYT variation in the R- treatment (Figure 4b). Most of this variability was explained by the average root area of the two varieties grown in mixture (R² = 47% in a model with average root area as the single explanatory variable), which had a negative effect on RYT (Figure 4b, Supplementary Table 6): mixing two genotypes with higher average root area resulted in a decrease in biomass production in mixture compared to pure stands. We obtained similar results when performing the analysis on shoot biomass alone (Supplementary Figure 2a and 2b, Supplementary Table 6). In contrast, traits had higher explanatory power on root biomass RYT in the R+ treatment (Supplementary Figure 2c, average adjusted R² over the top ten models = 0.34), with strong negative effects of average leaf number and average root area. In the R- treatment, however, as for the other biomass components, average root area was the main explanatory trait with a negative effect on RYT (Supplementary Figure 2d).

## Relationship between root area and mixture biomass

To further investigate the potential mechanisms underlying the strong negative relationship between RYT and average root area in the R- treatment, we tested whether our results fitted with the hypothesis that root area might be a proxy of competitiveness under resource limited conditions. Under this hypothesis, the most competitive genotypes are the ones that reach the highest root area values in pure stands under resource limited conditions. They reach such high root areas through phenotypic plasticity, as a result of competition with a neighbour that has the same genotype, i.e., that is himself very competitive. Such strong investment into competition in turn results in an overinvestment in biomass (above and belowground) to outgrow the neighbour Those genotypes, when grown in mixtures, are more likely to be paired with genotypes that are less competitive, leading to relaxed competition in mixtures compared to pure stands. Such relaxed competition in turn leads to a disengagement from the “arms race” between plants, potentially resulting in lower root area and lower above and belowground biomass production, and thus low RYTs. In line with this hypothesis, there was a strong positive relationship between the total biomass of the pure stands and their root area (Figure 5a). This relationship was stronger in the R- treatment (Figure 5a, Pearson’s *R* = 0.87, slope = 0.079 mg.mm-2, *p* < 0.001) than in the R+ treatment (Pearson’s *R* = 0.74, slope = 0.0446 mg.mm-2, *p* < 0.001). Additionally, consistent with our hypothesis, varieties with the highest root areas in pure stands and the highest hierarchical distances in root area with their neighbours were the ones with the strongest biomass reduction in mixed stands in the R- treatment (Figure 5b, Pearson’s *R* = -0.70, *p* < 0.001; Figure 5c, Pearson’s *R* = -0.51, *p* < 0.001). These relationships were not significant in the R+ treatment (Figures 5b and 5c). Finally, the strongest biomass reductions occurred in mixtures where the observed root area was lower than the root area predicted from the pure stands, i.e., where phenotypic plasticity led to reduced root area in mixtures (Figure 5d). Again, the relationship between RYT and root area plasticity was stronger in the R- treatment (Pearson’s *R* = 0.80, *p* <0.001,slope = 1.93 %-1) than in the R+ treatment (Pearson’s *R* = 0.54, *p* <0.001,slope = 0.91 %-1). We obtained identical results when doing these analyses on shoot and root biomass separately, except that the differences between the R+ and R- treatments were less marked for root biomass (Supplementary Figures 3 & 4). Altogether, these results support the idea that low RYTs in the R- treatment resulted from relaxed belowground competition in mixed relative to pure stands.

# Discussion

## Shift in root allocation under water and nutrient limitation

Wheat seedling growth was strongly affected by the limitation of water and nutrients in our experiment. Resource limitation triggered an overall reduction in biomass, along with a shift in biomass allocation from the aboveground to the belowground compartment. This result is in line with the optimal allocation theory, which states that plants prioritize allocation to increase their uptake of the most limiting resources (Bloom *et al.*, 1985; Weiner, 2004). In our case, the high allocation to roots suggests that belowground resource limitations were much stronger than aboveground resource limitations, a pattern consistent with what we intended to induce with our experimental protocol. In wheat, plasticity of the root-to-shoot ratio and high reallocation to roots have been shown to be advantageous under drought stress (Bacher *et al.*, 2021, 2022). Allocating carbon to the roots enhances access to water through deeper, longer, and more branched roots, which in turns helps maintain high stomatal conductance and physiological activity. We can thus hypothesize that higher root allocation in response to water and nutrient limitation reflected an adaptive response of the varieties.

## Biomass reduction in mixed relative to pure stands reflects a relaxation of competition

Contrary to our expectations, mixing varieties did not result in increased seedling biomass. In fact, we observed the opposite pattern: mixed stands produced less biomass than expected based on their pure stand components, especially under water and nutrient limitations. At first glance, we might conclude that this result contradicts ecological theory, notably the Stress Gradient Hypothesis that predicts a shift towards more positive interactions as abiotic conditions become less favorable (Bertness and Callaway 1994; Maestre et al. 2009). However, individual variety responses to mixture cultivation combined with root trait data together suggest that such reduction in biomass in mixtures was the consequence of intense competition in pure stands, rather than evidence for decreased performance in mixed stands. This interpretation is based on the hypothesis that early biomass does not reflect final community performance that would better be approximated by latter established biomass components, but rather individual plant competitiveness. In line with this interpretation, seedling growth rate and early vigour are traditionally targeted by plant breeders as favourable traits for competitiveness against weeds (Lemerle *et al.*, 1996; Bertholdsson, 2005; Hendriks *et al.*, 2022). Hence, our results suggest an overall relaxation of competition intensity in mixed relative to pure stands at the seedling stage, especially under limiting water and nutrient resources.

## No evidence for niche complementarity between seedlings

We found no evidence of complementarity effects, and no effect of functional trait diversity on mixed stand biomass, including under resource limitation. This could be explained by the fact that functional differences between varieties were not large enough 3-weeks after sowing to generate complementarity effects. A second hypothesis is that we did not measure the “right” traits to detect complementarity effects, notably belowground. Indeed, even if root functional traits are involved in many ecosystem processes (Bardgett *et al.*, 2014), diversity in architectural and morphological root traits have rarely been shown to associate with complementarity effects in ecological studies (Fort *et al.*, 2014; Bakker *et al.*, 2018). In varietal mixtures, previous studies also failed to find evidence of niche complementarity based on root trait differences (Montazeaud *et al.*, 2018). Overall, this study confirm that root trait differences alone are less likely to generate complementarity effects in varietal mixtures than they are for example in intercropping systems where differences in root foraging strategies between species can be more significant (Zhang *et al.*, 2014; Homulle *et al.*, 2022; Schmutz and Schöb, 2023).

## Root area as the main driver of competitive hierarchy

We identified root area as the main driver of plant biomass and mixing effects in our experiment. The average root area of the two varieties in pure stands could explain up to 50% of RYT variability under resource-limiting conditions. A higher root area in pure stand was associated with a greater biomass reduction in mixture, especially when the mixture partner had a lower root area. These results support the idea of an early-stage belowground competitive hierarchy between varieties (Kunstler *et al.*, 2012), where higher root area is associated with higher competitive ability. In line with these results, root functional traits associated with root foraging and absorption potential have already been shown to shape early-stage competitive hierarchies between grassland species (Fort *et al.*, 2014; Ravenek *et al.*, 2016; Wagg *et al.*, 2017). The very high explanatory power of root area found in our study might be explained by the fact that this trait integrates several functional dimensions of the root system that are classically captured by distinct traits (e.g., root length density, specific root length, or root diameter).

## Root plasticity contributes to mixing effects

We found that root area in mixed stands deviated from root area observed in pure stands, indicating a plastic response of the root system to neighbour genotype identity. On average, root area decreased in mixed relative to pure stands, and the stronger the decrease, the lower the RYT especially in the R- treatment. In accordance with ecological theory, our results thus support the view that phenotypic plasticity contributes to mitigate competition intensity in diverse plant communities (Callaway *et al.*, 2003; Schiffers *et al.*, 2011; Burns and Strauss, 2012). In varietal mixtures, previous studies have already shown that plasticity in aboveground traits such as plant height, specific leaf area, or canopy cover contribute to overyielding, notably by increasing trait divergence between varieties (Kong and Zhao, 2023; Tschurr *et al.*, 2023; Su *et al.*, 2024). We here show that plastic root changes may also contribute to mixture performance, although it may not be by increasing differences between varieties, but rather by changing the average trait value.

## Practical implications for plant breeding

Crop performance, including grain yield, is affected by trade-offs between individual competitiveness and group performance. As early pointed out by plant breeders (Donald, 1968), and more recently revisited with the concepts of Darwinian Agriculture, or Evolutionary Agroecology, these trade-offs can hold promising opportunities to increase productivity, notably by reversing past selection for individual competitiveness (Denison *et al.*, 2003; Weiner, 2019). This can be achieved either by selecting directly on group performance, i.e., selecting on population yield (Montazeaud *et al.*, 2020*a*), or by targeting key traits related to competitive ability. Our study suggests that selecting genotypes with reduced root area in pure stands and reduced root area proliferation in presence of neighbours could be a way to reduce competition intensity between seedlings, which could then translate into higher yields. This is a classical prediction of game theory models: investment in root biomass beyond the payoff point where soil resource absorption compensate the cost of the root system only happens at cost for neighbouring plants, leading to a Tragedy of the Commons (Hardin, 1968; Gersani *et al.*, 2001; Anten and Vermeulen, 2016). In line with this prediction, a previous study that used the same phenotyping platform found multiple negative correlation between above and belowground seedling biomass measured in the platform and yield components measured in the field in both bread and durum wheat (Colombo *et al.*, 2022). Interestingly, modern wheat varieties that have been subject to selection for increased population yield for many generations display reduced root biomass at the seedling stage (Colombo *et al.*, 2022), and reduced root proliferation in presence of a neighbour compared to old landraces (Zhu *et al.*, 2019). In oat and barley, excessive growth of the root system in the topsoil is also associated with yield reductions (Vain *et al.*, 2023).

Mitigating competition for resources between varieties is also key to design varietal mixtures that are able to perform under low input conditions. Our results suggest that this can be achieved by choosing varieties with low average root area. This means that promoting complementarity effects through functional differences between varieties is not the only way to reduce competition and may only work for some traits, such as phenological traits (Fletcher *et al.*, 2019; Lowry *et al.*, 2020) or traits related to light interception (Su *et al.*, 2024), but for others, more attention should be paid to the average trait value.

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# Figure legends

**Figure 1: Experimental design.** (**a**) Schematic representation of the experiment, each dark square representing a RhizoTube®. The green squares are the control RhizoTubes® and are all grown with the same wheat variety to check environmental heterogeneity. (**b**) and (**c**) close-up views on RhizoTubes® showing wheat seedlings and their roots. (**d**) Spatial distribution of the seedlings within the RhizoTubes® in both pure (up) and mixed (bottom) stands. Different colours represent different genotypes.

**Figure 2: Effect of resource limitation on plant growth and traits.** Comparison of total biomass (**a**), shoot biomass (**b**), root biomass (**c**), number of leaves (**d**), number of tillers (**e**), leaf nitrogen concentration (**f**), root:shoot ratio (**g**), root length (**h**), and root area (**i**) between the R+ (blue) and the R- (red) treatments. Only pure stand data were used, and trait values were summed per Rhizotube® (except for leaf N for which we averaged trait values per Rhizotube®). Points and error bars represent the mean ± standard deviation. The number of observations in each treatment is reported below each violin plot. Symbols above the plots represent the significance of the treatment effect (\*: *p* < 0.05, \*\*: *p* < 0.01, \*\*\*: *p* < 0.001, complete analysis of variance is reported in Supplementary Table 4).

**Figure 3: Effect of resource limitation on interactions between varieties.** Comparison of Relative Yield Total (RYT) indices on total biomass (**a**), shoot biomass (**b**), and root biomass (**c**) between the R+ (blue) and the R- (red) treatments. Points and error bars represent the mean ± standard deviation. The number of observations in each treatment is reported below each violin plot. Symbols above violins represent the significance of a two-sided *t*-test testing if the mean RYT within treatment is significantly different from 1 (\*\*\*: *p* < 0.001). ANOVAs testing the significance of treatment effect on RYTs are reported in Supplementary Table 5).

**Figure 4: Relationships between the trait composition of the mixtures and their performance.** Standardized effects of traits on mixture RYTs measured on total biomass in the R+ treatment (**a**), and R- treatment (**b**). Backward model selection was performed on a full model with RYT as the response variable and all trait means and differences as explanatory variables. Based on AICC, the top-ten models were retained to compute model-averaged estimates reported on the left side of the panels with their 95% unconditional confidence intervals (Supplementary Table 6). Empty symbols represent trait differences and filled symbols represent trait means. The relative importance of the variables are reported on the right side of the panels and can be interpreted as the probability that the variable appears in the best model. Hatched bars represent trait differences and filled bars represent trait means. Colours refer to the type of traits, with aboveground traits and belowground traits represented in green and brown, respectively. Adjusted R-squared averaged across the top-ten models () are also reported.

**Figure 5: Interactions between root area, resource availability, and biomass. (a)** relationship between the total biomass of the pure stands and their root area (n = 36 per treatment), **(b)** relationship between RY computed on total biomass and root area measured in pure stands (n = 108 per treatment), **(c)** relationship between RY computed on total biomass and the hierarchical distance on root area, i.e. the difference between the root area of the focal and the root area of the neighbour, both measured in pure stands (n = 108 per treatment), **(d)** relationship between RYT computed on total biomass and root area plasticity, i.e. the difference between the expected (based on pure stands) and the observed root area (n = 54 per treatment). Pearson correlation coefficients (*R*) and *p-*values (*p*) refer to simple linear models fitted independently in the R+ (blue, circle) and R- (red, triangles) treatments.

# Legends for Supplementary Materials

**Supplementary Table 1: Variety information.**

**Supplementary Table 2: List of the 54 binary mixtures.**

**Supplementary Table 3: Description of the nutrient solution.**

**Supplementary Table 4: Analysis of Variance (ANOVA) of biomass components and traits in pure stands.** Pure stand data was summed per Rhizotube® (except leaf N which was averaged per Rhizotube®). We used Type III analysis of Variance using the Kenward-Roger’s method on mixed models where the identity of the variety was used as a random effect on both the intercept and the slope of the treatment effect (R+ vs R- treatment). For each fixed effects, we report the sum of squares (“Sum Sq”), the mean squares (“Mean Sq”), the numerator degrees of freedom (“NumDF”), the denominator degrees of freedom (“DenDF”), the value of the *F* statistic (“F value”), and the *p*-value (“Pr(>F)”).

**Supplementary Table 5: Analysis of Variance (ANOVA) of RYTs.** Type III analysis of Variance using the Kenward-Roger’s method on mixed models where the identity of the genotypic pair (concatenation of the identity of the two genotypes in a RhizoTube®) was used as a random effect on the intercept. We report the fixed effect of the treatment with the sum of squares (“Sum Sq”), the mean squares (“Mean Sq”), the numerator degrees of freedom (“NumDF”), the denominator degrees of freedom (“DenDF”), the value of the *F* statistic (“F value”), and the *p*-value (“Pr(>F)”).

**Supplementary Table 6: Ten best fitting models between RYT on aboveground, belowground, and total biomass and mixture trait composition.** The top-ten models are ranked according to their AICc. ∆AICc (“delta\_AICc”), model weights (“weight”), and adjusted R-squared (“R2\_adj”). The “avg” and “diff” suffixes refer to trait averages and trait differences, respectively.

**Supplementary Figure 1: RhizoTubes® monitoring.** Measurements of nutrient solution inputs (**a**) and water status (**b**) of the RhizoTubes® in the R+ treatment (blue) and the R- treatment (red) over the course of the experiment. Values are averaged over all RhizoTubes®. Seedlings were transferred in the RhizoTubes on the 24th of June, and plants were harvested from the 16thto the 19th of July 2019.

**Supplementary Figure 2: Relationships between the trait composition of the mixtures and their above and belowground RYTs.** Standardized effects of traits on mixture RYTs measured on above- (**a** and **b**) and below- (**c** and **d**) ground biomass in the R+ treatment (**a** and **c**) and R- treatment (**b** and **d**). Backward model selection was performed on a full model with RYT as the response variable and all trait means and differences as explanatory variables. Based on AICC, the top-ten models were retained to compute model-averaged estimates reported on the left side of the panels with their 95% unconditional confidence intervals (Supplementary Table 6). Empty symbols represent trait differences and filled symbols represent trait means. The relative importance of the variables are reported on the right side of the panels and can be interpreted as the probability that the variable appears in the best model. Hatched bars represent trait differences and filled bars represent trait means. Colours refer to the type of traits, with aboveground traits and belowground traits represented in green and brown, respectively. Adjusted R-squared averaged across the top-ten models () are also reported.

**Supplementary Figure 3: Interactions between root area, resource availability, and shoot biomass. (a)** relationship between the shoot biomass of the pure stands and their root area (n = 36 per treatment), **(b)** relationship between RY computed on shoot biomass and root area measured in pure stands (n = 108 per treatment), **(c)** relationship between RY computed on shoot biomass and the hierarchical distance on root area, i.e., the difference between the root area of the focal and the root area of the neighbour, both measured in pure stands (n = 108 per treatment), **(d)** relationship between RYT computed on shoot biomass and root area plasticity, i.e. the difference between the expected (based on pure stands) and the observed root area (n = 54 per treatment). Pearson correlation coefficients (*R*) and *p-*values (*p*) refer to simple linear models fitted independently in the R+ (blue, circle) and R- (red, triangles) treatments.

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