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Mixing cultivars reduces early root competition between wheat seedlings under resource limited conditions

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# Keywords

Cultivar mixtures, relative yield, stress-gradient hypothesis, agroecology, selection effect, root phenotyping, …

# Abstract

# 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). That is, ecosystems with a higher number of species tend to be more productive, more efficient at regulating pathogens, at recycling nutrients, or 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, and such “efficient” species might be even more efficient in a diverse community than in a monoculture (e.g., highly competitive species often benefit from relaxed competition in a mixture compared to a monoculture), 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. Mixing species that get their nitrogen from the soil (e.g., cereals) with species that can fix nitrogen from the atmosphere (e.g., legumes) is another way to achieve complementarity effects and ultimately overyielding (Bedoussac et al. 2015). 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.

Cultivar mixtures, that is the sowing of a mixture of genotypes of the same species in the same field, allows to address most of these challenge by mobilizing plant diversity at the intra-specific level (Smithson and Lenné 1996). Historically, cultivar mixtures have been shown to be very efficient at limiting pathogen spread and disease severity compared to monogenotypic cultivars, especially in grain cereals (Wolfe 1985; Mundt et al. 1995; Zhu et al. 2000; Finckh and Wolfe 2006). This advantage has recently renewed the interest in cultivar mixtures as they could help reduce the use of pesticides and as such support the agroecological transition (Barot et al. 2017; Borg et al. 2018; Snyder et al. 2020; Wuest et al. 2021). Extensive research in phytopathology has helped identify the different epidemiological and evolutionary mechanisms that underly the protective effect of cultivar mixtures against pathogens (Finckh and Mundt 1992; Mundt et al. 1995; Finckh et al. 2000; Vidal et al. 2017). For example, recent results suggest that some cultivars might be able to improve the basal immunity of their neighbors, a phenomenon referred to as neighbor-modulated susceptibility (NMS, Pélissier et al. 2021, 2023).

In addition to their beneficial effects on pathogen control, cultivar mixtures could also help optimize resource use and ultimately increase crop yield (Hajjar et al. 2008; Barot et al. 2017; Snyder et al. 2020). Ecological mechanisms at play in natural ecosystems or in intercropping such as niche complementarity could similarly improve resource use efficiency by reducing competition intensity between the different cultivars. Moreover, ecological theory predicts that such positive effects of plant diversity should strengthen as the environment becomes harsher, which is known as the stress-gradient hypothesis (SGH, Bertness and Callaway 1994; Maestre et al. 2009). According to the SGH, cultivar mixture could thus help maintain high productivity under low-input farming practices and more variable and extreme climates. In line with these theoretical expectations, most meta-analysis report slight yield increases in cultivar mixtures compared to monogenotypic cultivars (on average 2-5%, Kiær et al. 2009; Reiss and Drinkwater 2018; Borg et al. 2018). However, contrary to their beneficial effect on pathogen control, the positive effect of varietal mixtures on productivity and its interaction with resource availability are highly variable and remains poorly understood (Reiss and Drinkwater 2018; Alsabbagh et al. 2022).

In nature, plant functional traits can be 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 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 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 can capture differences in ecological niche (Violle and Jiang 2009) or in competitive ability (Wagg et al. 2017; Cadotte 2017), and in turn inform us on the ecological effects that drive productivity at the community level (e.g., complementarity or selection effects).

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 cultivar mixtures (Montazeaud et al. 2018, 2020; Kong and Zhao 2023). Preliminary results from these studies suggest that the traits with the strongest effect on mixture performance are not necessarily the ones commonly measured in agricultural applications. In particular, morphological and architectural root traits have been shown to determine multiple facets of mixture performance (Montazeaud et al. 2018, 2020), which echoes recent findings on the central role of root traits in natural ecosystems (Bardgett et al. 2014). However, the relationship between root traits and mixture performance remains poorly understood, notably because root traits are difficult to measure and highly plastic in response to both biotic and abiotic factors. A better understanding of this relationship could help us identify the mechanisms underlying the variability of mixing effects on productivity, as well as provide us with measurable features that could be used to guide mixture assembly.

In the present study, we used durum wheat (*Triticum turgidum* ssp*. durum*) as a crop model to investigate the effect of root trait diversity on mixture performance. We grew 54 binary mixtures obtained from 36 contrasted cultivars in a highly controlled high throughput root phenotyping platform under both non-limiting and limiting water and nutrient conditions. Based on ecological theory, we hypothesized that (i) mixtures would produce more biomass than their monoculture counterparts (i.e., overyielding) (ii) overyielding would be higher under resource-limited conditions, (ii) resource-use complementarity would be the main effect driving overyielding, (iii) complementarity effects would arise from differences in root traits between cultivars.

# Material and Methods

## Cultivars

We first screened 250 durum wheat genotypes from different research programs in Europe in the field in 2018 (Southern France, INRAE – UE DIASCOPE – 43°360N, 3°590E). Each genotype was grown under two conditions: no water and nitrogen limitation vs water and nitrogen limitation. We then selected a subset of 36 genotypes with contrasted responses to resource availability (going from very stable to very unstable genotypes). 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 such that each genotype was observed in three different mixtures. Monocultures (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®, transparent pots designed for high-throughput root phenotyping (Figure 1), with six plants per RhizoTube. The six plants were the same genotype in monocultures, whereas two genotypes were grown in alternate positions in the mixtures (Figure 1). The 36 monocultures and the 54 mixtures were grown under a combined water and nutrient stress (S treatment) or without stress (C treatment). All monocultures and mixtures where replicated three times within each treatment following a randomized complete block design within treatment (Figure 1).

## Growth conditions

Seeds were sown into XXX media on the 22nd of June 2019 and kept at XX °C during two days. Seedlings were then transferred into the RhizoTubes on the 24th and harvested about three weeks later between the 16th and the 19th of July. 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 (see the description of the solution in Supplementary Table 2). 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 the 28th of June (four days after seedling transfer) such that the water content progressively decreased to reach 55% of the full storage capacity by the end of the experiment (Supplementary Figure 1).

## Phenotyping

Root traits were measured at the Rhizotube® level based on image analysis. Images of each RhizoTube® were taken on the 15th of July (i.e., one day before harvest). Three traits were computed following image processing: root depth, which was the distance between the top of the RhizoTube® and the deepest root pixel; root length, which was the total length of roots detected on the image; and root surface which was the 2D projected area of the total root system and combined information on root length and root diameter. We did not consider root depth in our analyses because seedling roots often reached the bottom of the Rhizotubes®, leading to highly left-skewed trait distribution with very low variability.

At harvest, plants were extracted from the RhizoTubes® and we counted the number of leaves on the main tiller (hereafter “# leaves”) and the total number of tillers (hereafter “# tillers”) on each plant. Above and belowground biomass were 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®). Because root traits could only be computed at the RhizoTube® level, we pooled aboveground traits by either summing (# leaves, # tillers, shoot biomass, root biomass, total biomass) or averaging (leaf N) the trait values of the six plants to get one trait value per RhizoTube®. Root:shoot ratio was also computed at the RhizoTube® level by dividing the total root biomass by the total shoot biomass of the RhizoTube®.

## Statistical analysis

# Results

## Effect of the stress on plant growth and architecture

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

## Comparison of pure vs mixed stands

We did not detect any significant effect of the stand type (pure vs mixed) or its interaction with resource availability (C vs S treatment) on seedling traits or biomass (Supplementary Figure 2 and Supplementary Table 3). However, RYT computations showed that the mixtures produced less biomass than expected from the biomass of their components grown in pure stands (average RYT for total biomass = 0.97, *t*107 = -4.10, *p* < 0.001, Figure 3c). This was true for both above and below-ground biomass (average RYT for above-ground biomass = 0.97, *t*107 = -3.03, *p* = 0.0031, Figure 3a; average RYT for below-ground biomass = 0.97, *t*107 = -4.53, *p* < 0.001, Figure 3b). RYTs were significantly lower in the S than in the C treatment for the three biomass components (shoot biomass: RYT = 0.94 vs 1.00, *F*1,53 = 26.23 , *p* < 0.001, Figure 3a; root biomass: RYT = 0.94 vs 0.99, *F*1,53 = 32.56, *p* < 0.001, Figure 3b; total biomass: RYT = 0.94 vs 0.99, *F*1,53 = 34.87, *p* < 0.001, Figure 3c).

## Effect of cultivar traits on mixed stand biomass

As expected from the literature, RYTs were highly variable in both treatments (Figure 3). We aimed to explain this variability using a trait-based approach. The trait composition of the mixtures poorly explained RYT variability on total biomass in the C treatment (Figure 4a, average adjusted R² over the top ten models = 0.09). In contrasts, the traits could explain 49% of RYT variation in the S treatment (Figure 4b). Most of this variability was explained by the average root surface of the two cultivars (R² = 47% in a model with average root surface as the single explanatory variable), which had a negative effect on RYT (Figure 4b, Supplementary Table 5). We obtained identical results when performing the analysis on shoot biomass alone (Supplementary Figure 3a and 3b, Supplementary Table5). In contrasts with aboveground biomass, traits had higher explanatory power on root biomass RYT in the C treatment (Supplementary Figure 3c, average adjusted R² over the top ten models = 0.33), with strong negative effects of average leaf number and average root surface. In the S treatment, however, as for the other biomass components, average root surface was the main explanatory trait with a negative effect on RYT (Supplementary Figure 3d).

## Relationship between root surface and mixed stand biomass

The strong negative relationship between RYT and average root surface in the S treatment indicates that mixing two genotypes with high average root surface resulted in a decreased biomass production in mixed compared to pure stands. We speculated that this relationship could be explained by considering root surface as a proxy of competitiveness under resource limited conditions. Cultivars with high root surface experience strong inter-seedling competition in pure stands, because their neighbor also has high root surface. Such strong competition result in an overinvestment in biomass to outgrow the neighbor. Those genotypes, when grown in mixed stands, are more likely to be grown with genotypes that have lower than higher root surface than themselves, and hence, to experience relaxed competition compared to their pure stands. Such relaxed competition might in turn lead to a disengagement from the arms race between seedlings, and thus to reduced above and belowground biomass production. To validate this scenario, we first checked the relationship between the biomass of the pure stands and their root surface. As expected under our hypothesis, there was a strong positive relationship between the total biomass of the pure stands and their root surface. This relationship was stronger in the S treatment (Figure 5a). Also in line with our scenario, the average biomass of the cultivars in pure stands was a very good predictor of mixture RYT, especially in S treatment: mixing cultivars with high biomass production in pure stands resulted in decreased RYT in mixture (Figure 5b). We also found that cultivars with the highest root surface and highest biomass production in pure stands were the ones with the sharpest biomass reduction in mixture, again only in the S treatment.

# Figure legends

**Figure 1: Experimental design.** (**a**) Schematic representation of the experiment, each dark square representing a Rhizothube®. (**b**) and (**c**) close-up views on Rhizotubes® showing wheat seedlings and their roots. (**d**) Positions of the seedlings within the Rhizotubes® in both pure (up) and mixed (bottom) stands. Different colors represent different genotypes.

**Figure 2: Effect of resource limitation on seedlings growth and architecture.** Comparison of number of leaves (**a**), number of tillers (**b**), leaf nitrogen content (**c**), shoot biomass (**d**), root biomass (**e**), total biomass (**f**), Root:Shoot Ratio (**g**), root length (**h**), and root surface (**i**) between the control (C, blue) and the stressed (S, red) treatments. Points and error bars represent the mean ± standard deviation. The number of observations in each treatment are reported below each violin plot. Symbols above the plots represent the significance of the treatment effect (\*\*: *p* < 0.01, \*\*\*: *p* < 0.001, complete analysis of variance is reported in Supplementary Table 3).

**Figure 3: Effect of resource limitation on cultivar interactions.** Comparison of Relative Yield Total (RYT) indices on shoot biomass (**a**), root biomass (**b**), and total biomass (**c**) between the control (C, blue) and the stressed (S, red) treatments. Points and error bars represent the mean ± standard deviation. The number of observations in each treatment are reported below each violin plot. Symbols in the title of the plots represent the significance of a two-sided *t*-test testing if the mean RYT is significantly different from 1 (\*\*: *p* < 0.01, \*\*\*: *p* < 0.001).Symbols above the plots represent the significance of the treatment effect on RYTs (\*\*\*: *p* < 0.001, complete analysis of variance is reported in Supplementary Table 4).

**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 C treatment (**a**) and S 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 5). 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. Colors 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 surface, resource availability, and biomass. (a)** relationship between the total biomass of the pure stands and their root surface (n = 36 per treatment), **(b)** relationship between mixture RYT computed on total biomass and the average total biomass produced by the two components in pure stands (n = 54 per treatment), **(c)** relationship between RY computed on total biomass and root surface measured in pure stands (n = 108 per treatment), **(d)** relationship between RY computed on total biomass and total biomass measured in pure stands (n = 108 per treatment). Pearson correlation coefficients (*R*) and *p-*values (*p*) refer to simple linear models fitted independently in the C (blue, circle) and S (red, triangles) treatments.

# Legends for Supplementary Materials

**Supplementary Table 1: Cultivar information.**

**Supplementary Table 2: Description of the nutrient solution.**

**Supplementary Table 3: Analysis of Variance (ANOVA) of traits and biomass.** 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 both the intercept and the slope of the treatment effect (C vs S 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 4: 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 both the intercept and the slope of the treatment effect (C vs S treatment). 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 5: Ten best fitting models between RYT 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”) are reported for the ten models. 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 C treatment (blue) and the S 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 harvested form the 16thto the 19th of July 2019.

**Supplementary Figure 2: Effect of the stand type on seedlings growth and architecture.** Comparison of number of leaves (**a**), number of tillers (**b**), leaf nitrogen content (**c**), shoot biomass (**d**), root biomass (**e**), total biomass (**f**), Root:Shoot Ratio (**g**), root length (**h**), and root surface (**i**) between pure and mixed stands. Points and error bars represent the mean ± standard deviation. The number of observations in each stand type are reported below each violin plot. Symbols above the plots represent the significance of the treatment effect (n.s.: not significant, complete analysis of variance is reported in Supplementary Table 3).

**Supplementary Figure 3: 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 C treatment (**a** and **c**) and S 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 5). 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. Colors 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.