1   # Mixing varieties mitigates early root competition in wheat

2   # under water and nutrient limitation

3   **Running title: mixing varieties mitigates root competition in wheat**

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Highlight

Asymmetric responses to belowground competition between varieties with high vs low root projected area generate positive selection effects in wheat varietal mixtures

Abstract

Competition between plants can lead to a Tragedy of the Commons where over-investments into resource harvesting organs reduce collective performance. Mixing different varieties could resolve such TOCs because different varieties might have different resource requirements (niche complementarity effect) or because the most competitive varieties benefit from being mixed with weaker competitors (selection effect). In the present study, we tested whether such ecological effects could mitigate belowground competition in durum wheat (*Triticum turgidum* ssp. *durum*). We grew 36 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-). Seedlings in mixed stands produced less biomass than predicted from their pure stands in R-. This biomass reduction reflected a relaxation of competition where competitive varieties benefited from having a weaker competitor than themselves (selection effect), and thus disengaged from an arms race for biomass accumulation. The competitive hierarchy between varieties was captured by a single trait, the 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.

Keywords

Agroecology, arms race, competitive hierarchy, high-throughput root phenotyping, niche complementarity, relative yield, root projected area, tragedy of the commons, varietal mixtures, wheat

Abbreviations

RY: Relative Yield; RYT: Relative Yield Total

Introduction

Competition for resources is a fundamental determinant of plant phenotypes and plant community dynamics (Tilman, 2020). A plant's survival, growth and reproduction can be reduced by highly competitive neighbours that absorb and use resources more quickly and/or more efficiently. To deal with such situations, plants have evolved various mechanisms to detect the presence of neighbours and/or the early exhaustion of resources, and to react by becoming more efficient at capturing resources (Pierik *et al.*, 2013). For example, plants can anticipate light competition by detecting changes in the red:far-red ratios of reflected and transmitted light on the leaves of their neighbours (Ballaré *et al.*, 1990). These changes in light quality in turn trigger a series of phenotypic changes, known as the shade avoidance syndrome, which ultimately lead to more vertical growth in order to out-compete neighbours, e.g., increased plant height or hyponastia of leaves (Ballaré and Pierik, 2017). Similarly, competition for belowground resources (water or nutrients) can lead to root proliferation in the presence of a competitor (Robinson *et al.*, 1999; Gersani *et al.*, 2001), which can be triggered either by early detection of resource depletion, or by the detection of chemical markers from the competitors (Schenk, 2006; Pierik *et al.*, 2013).

While such response primarily evolved to maximize individual plant fitness under competition, they can lead to a competitive arms race between individuals, and ultimately decrease fitness at the scale of the group, which is known as a Tragedy of the Commons (TOCs, Hardin, 1968). This reduction in collective performance results from two effects: resources allocated to competitive organs are diverted from reproduction, and all individuals suffer from an intense pressure on the resources from their neighbours. Such trajectories have important implications for agriculture, as intense competition between adjacent plants from the same species can reduce yield per unit area (Anten and Vermeulen, 2016). This was notoriously identified by agronomists and plant breeders in the context of the Green Revolution: intra-specific competition, especially for light, becomes a strong determinant of yield in a typical high density cereal cropping system with low weed pressure and high fertilizer and pesticide inputs (Donald, 1963, 1968; Jennings and de Jesus, 1968). This observation led to the emergence of a new paradigm for plant breeding based on the idea that selection should target varieties with weak

competitive abilities, because such varieties can be grown at high density without wasting resources into competition (Donald, 1968). The major yield gains achieved with the introduction of dwarfing genes contributed to validate this paradigm as these genes mainly reduced above ground plant stature and, *de facto*, the intensity of intra specific competition (Donald, 1968; Jennings and Herrera, 1968).

Transitioning towards low-input farming practices notably by reducing the use of fertilizers will reduce the amount of nutrients readily available for plants, and as such, will exacerbate the relative effect of belowground compared to aboveground intra-specific competition. When plants compete for soil resources, game theory models predicts that individuals will invest in root biomass beyond the payoff point where extra soil resource absorption compensate the marginal cost of the extra root biomass, leading to a Tragedy of the Commons (Gersani *et al.*, 2001; Anten and Vermeulen, 2016). Multiple empirical evidences support this prediction: overinvestment in roots in response to competition has been shown to reduce final aboveground biomass in wheat (Zhu *et al.*, 2019) and soybean (Gersani *et al.*, 2001). Topsoil root production also negatively correlates with grain yields in oats and barley (Vain *et al.*, 2023). Then, resolving belowground TOC might be key to maintain high yields under low input farming practices.

Belowground TOC could be resolved by conducting direct selection on root architectural and morphological traits in order to select less competitive root systems. However, this option appears particularly challenging given the difficulty to measure root traits at high throughput on many candidates over several generations, and the little information available on their response to selection (Kuijken *et al.*, 2015). Alternatively, one could use the principles that prevent the evolution of TOC in natural ecosystems, notably group selection and kin selection, and apply them in plant breeding to select crops that have less competitive (or more cooperative) root systems, which is the core idea of Darwinian Agriculture and Evolutionary Agroecology (Denison *et al.*, 2003; Weiner *et al.*, 2017). Theoretically, these principles can be applied to avoid TOCs and to select more cooperative crops (Montazeaud *et al.*, 2020; Biernaskie, 2022). However, they are not straightforward to implement in practice, and they rely on high genetic relatedness between individuals, meaning that they produce varieties in which individuals are genetically homogeneous. Both ecological and agronomic research,

102   in contrasts, suggest that there are multiple benefits to increase genetic diversity within crop stands.

103   For example, mixing different varieties in the same field can be very efficient at limiting pathogen

104   spreads and disease severity (Wolfe, 1985; Mundt *et al.*, 1995; Zhu *et al.*, 2000; Finckh and Wolfe,

105   2006), and, on average, varietal mixtures yield 2-5% more than expected on the basis of their pure

106   stand components, which is known as overyielding (Kiær *et al.*, 2009; Borg *et al.*, 2018; Reiss and

107   Drinkwater, 2018; Beillouin *et al.*, 2021).

108   Reducing intra-specific competition for resources while maintaining genetic diversity in the field

109   could thus be the most optimal way to take advantage of intra-specific plant-plant interactions in

110   agriculture. Two ecological mechanisms are known to reduce competition in diversified plant

111   communities: the niche complementarity effect and the selection effect (Loreau and Hector, 2001).

112   The niche complementarity effect results from differences in ecological niches between species that

113   have different resource requirements and hence experience less competition (MacArthur and Levins,

114   1967), which ultimately translates into a higher biomass production in mixture (e.g., Roscher et al.

115   2008; Mueller et al. 2013). Through the selection effect, species or are the most efficient at

116   performing a given function are even more efficient at this function in a mixture than in a pure stand,

117   i.e., they are "selected" by the mixture, which they rapidly dominate (Loreau and Hector, 2001). For

118   example, competitive species can benefit from relaxed competition in mixture because the mixture

119   allow them to escape an arms race with themselves. If the dominant species gains more yield than the

120   sub-dominant loses, it can generate overyielding. Such an effect has for example been shown to

121   contribute to overyielding in tree communities (Schmid and Niklaus, 2017; Williams *et al.*, 2017) and

122   in inter-specific crop mixtures (Li *et al.*, 2018). Varietal mixtures could thus help escaping TOCs by

123   relaxing intra-genotypic competition through either complementarity or selection effects while

124   maintaining intraspecific genetic diversity in the field. However, the current literature provides little

125   insight into the ecological effects underlying varietal mixture performance under contrasted resource

126   levels (Borg *et al.*, 2018).

127   In the present study, we investigated the potential of varietal mixtures to reduce intra-specific

128   belowground competition at the seedling stage in durum wheat (*Triticum turgidum* ssp. *durum*). 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 under highly controlled climate condition in a high throughput root phenotyping platform under both non-limiting and limiting water and nutrient conditions. We hypothesized that (i) root responses to neighbours are already expressed at the seedling stage, (ii) such responses are more important under resource limited conditions, (iii) overinvestment in biomass at the seedling stage reflects a response to neighbour competition (iv) varietal mixtures can mitigate such response either through complementarity or selection effects

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 (Consiglio per la Ricercar in agricoltura e l'analisi dell'Economia Agraria, Italy); (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 yield responses to resource availability (i.e., ranging from stable to unstable yields) 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

155   to groups of a single genotype, n = 36) and mixtures (here referring to groups of two genotypes, n =

156   54) were grown in RhizoTubes® (Jeudy *et al.*, 2016), transparent pots designed for high-throughput

157   root phenotyping (Figure 1a & 1b), with six plants per RhizoTube®. The six plants had the same

158   genotype in pure stands, whereas two genotypes were grown in alternate positions in the mixtures

159   (Figure 1c). The 36 monocultures and the 54 mixtures were grown under combined water and nutrient

160   limitation (R- treatment) or under optimal growth conditions (R+ treatment). All monocultures and

161   mixtures were replicated three times within each treatment following a randomized complete block

162   design within treatment, leading to a total of 540 RhizoTubes® (Figure 1c). In addition, six

163   RhizoTubes® per experimental block per treatment (i.e., 36 RhizoTubes® in total) were grown with

164   the commercial variety ANVERGUR and were used as controls to check for environmental

165   heterogeneity in the greenhouse.

166   **Growth conditions**

167   Seeds were first disinfected with a solution of 6 g/L of active chlorine (4 tablets/L of a standard

168   commercial chlorine) in which they were immersed, agitated for 15 min, and finally rinsed 10 times

169   with sterile water on the 22nd of June 2019. They were then soaked overnight, and sown into Petri

170   dishes on the 23rd. They stayed at 4°C during 24h before being transplanted on the into the

171   RhizoTubes® on the 24th. RhizoTubes® have a diameter of 17 cm and a depth of 49.5 cm (Jeudy *et*

172   *al.*, 2016). They were filled with a 25:75 mixture of sand (Biot B4, Silices et Refractaires de la

173   Méditerranée) and perlite. Temperatures were maintained around 20-25°C, relative humidity around

174   70-80%, and photoperiod was set to 16h, with an average PAR of 330 µmol.m^{-2}.s^{-1} during the day.

175   Seedlings were daily provided with a liquid nutrient solution that contained water, N, P, K and all

176   micronutrients required for plant growth (Supplementary Table 3). The water content of each

177   RhizoTube® was monitored each day, and the amounts of nutrient solution were adjusted to maintain

178   the RhizoTubes® at 100% of their water storage capacity (Supplementary Figure 1). In the R-

179   treatment, the provision of nutritive solution was stopped on June 28th (four days after seedling

180   transfer), causing the water content to decrease, ultimately reaching 55% of the full storage capacity

181   by the end of the experiment (Supplementary Figure 1). Plants were harvested between the 16th and

182 the 19th of July, i.e., about three weeks after transplantation, and were at the beginning of the tillering

183 stage.

Phenotyping

185 Root traits were measured for each RhizoTube® based on image analysis as described in Jeudy *et al.*,

186 (2016). We used images taken on the 15th of July. Because the roots of the different plants were

187 overlapping in most RhizoTube® at the end of the experiment, we were unable to measure root traits

188 for individual plants, and instead computed root traits at the level of the RhizoTube® as a whole.

189 Three root traits could be computed following this aggregation: root depth, corresponding to the

190 distance between the top of the RhizoTube® and the deepest root pixel; root length, the total length of

191 roots detected on the image; and root area, the 2D projected area of the total root system. Root area

192 combines information on both root length and root diameter. We did not consider root depth in our

193 analyses because root tips reached the bottom of the RhizoTubes® in most cases, leading to highly

194 left-skewed trait distribution and very low trait variability.

195 We also measured a set of aboveground plant traits in order to evaluate the relative importance of

196 belowground relative to aboveground traits in explaining mixture biomass. At harvest, we extracted

197 plants from the RhizoTubes® by carefully separating their rooting systems. For each plant, we

198 counted the number of leaves on the main tiller (hereafter "# leaves") and the total number of tillers

199 (hereafter "# tillers"). Above and belowground biomass were then separated and dried before

200 weighing to determine shoot biomass, root biomass, root:shoot ratio, and total biomass. Leaf nitrogen

201 content (hereafter "leaf N") was measured with Near-Infrared Spectrometry (NIRS). We measured

202 one NIR spectrum per leaf per plant in each RhizoTube® (i.e., six spectrum per RhizoTube®) using

203 the Fieldspec 2500© (Analytical Spectral Devices, Inc. (ASD), Boulder, CO, USA) spectrometer.

204 NIRS measurements were done one day before the harvest (i.e., on the 15th for the Rhizotubes

205 harvested on the 16th, on the 16th for the RhizoTubes® harvested on the 17th, etc). NIR spectra were

206 converted into nitrogen content using the calibration described in (Ecarnot *et al.*, 2013).

Statistical analysis

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

209 We first tested the effect of the treatment (R+ *vs* R-) on the different traits and biomass components

210 (above and belowground) using only pure stand data summed per Rhizotube® (except leaf N which

211 was averaged per Rhizotube®). We used mixed linear models with a given trait or biomass

212 component as the response variable, treatment as a fixed effect, and genotype identity as a random

213 intercept and random treatment slope. We also included two covariates as fixed effects: block, and

214 harvest date or measurement date (except for root traits which were all measured on the same day).

215 We assessed the significance of the fixed effects with standard analyses of variance and *F* statistics

216 computed with Kenward-Roger's approximations for the degrees of freedom (Supplementary Table 4).

217 We fitted the mixed model with the *lmer()* function (package *lme4*), and checked significance with the

218 *anova()* function (package *lmerTest*).

219 To compare the relative biomass of mixed *vs* pure stands, we computed the Relative Yields (RYs, de

220 Wit and van den Bergh 1965) of the varieties in mixed stands for each biomass component

221 (aboveground, belowground, and total biomass):

222 (1)
$$RY_{ijk} = \frac{B_{ijk}}{B_{iik}},$$

223 Where RY_{ijk} is the Relative Yield of the variety *i* grown in mixture with the variety *j* in the treatment

224 *k*, B_{ijk} is the biomass (aboveground, belowground, or total biomass) of the variety *i* grown in mixture

225 with the variety *j* in the treatment *k* and, and B_{iik} is the pure stand reference biomass of the variety *i* in

226 treatment *k*. To compute RYs, we first separated our dataset between the pure and mixed stands. Then,

227 we summed the biomass of all plants of the same genotype within each RhizoTube® (i.e., 6 plants in

228 pure stands, 3 plants in mixed stands). We used the pure stands dataset to compute pure stand

229 reference biomass. To do so, we fitted a linear mixed model with biomass as the response variable,

230 measurement date, block, and treatment as fixed effects, and variety identity as a random effect (here

231 we included only the random effect of the variety on the intercept, as adding the random effect on the

232 treatment slope led to singular models due to very low variance on the slope). We then summed the

233   Best Linear Unbiased Predictor (BLUP) of each variety with the estimated fixed effect of the

234   treatment (R+ or R-) to obtain the pure stand reference biomass values adjusted for the effects of

235   block and measurement date within each treatment (i.e., $B_{iik.}$). For mixed stands, we also fitted a

236   linear mixed model with each variety biomass as the response variable, measurement date, block, and

237   treatment as fixed effects, and variety pair identity as a random effect on the intercept and on the

238   treatment slope. Pair identity was constructed as the concatenation of the identity of the focal and

239   neighbor variety, such that variety 1 and variety 2 in a mixed stand had pair identities variety1-

240   variety2, and variety2-variety1, respectively. We then summed the BLUP of pair identity (intercept in

241   the R+ treatment, intercept + slope in the R- treatment) with the estimated fixed effect of the treatment

242   (R+ or R-) to obtain the biomass of each variety in mixed stands adjusted for the effects of the block

243   and measurement dates within each treatment (i.e., B_{ijk}). We then divided these mixed stand values

244   by the pure stand reference values to obtain RYs for each variety within each mixture following

245   formula (1). Under the null hypothesis that the variety produced equal amount of biomass in mixed

246   than in pure stand, RY = 0.5 because there are half the number of plants of a variety in mixed stands

247   (n = 3) compared to pure stands (n=6). RY > 0.5 means that the variety produced more biomass in

248   mixed than in pure stand, and RY < 0.5 means that the variety produced less biomass in mixed than in

249   pure stand.

250   In a second step, we summed the RYs of the two varieties in the mixed stand to obtain the Relative

251   Yield Total (RYT, de Wit and van den Bergh 1965) of the mixture:

252   (2)$$RYT_{(ij)k} = RY_{ijk} + RY_{jik},$$

253   Where $RYT_{(ij)k}$ is the Relative Yield Total of the mixture containing varieties *i* and *j* in treatment *k*.

254   RYT = 1 means that the mixture as a whole produced similar amount of biomass than expected from

255   the productivity of the varieties grown in pure stands, whereas RYT > 1 means that the mixture

256   produced more biomass than expected, and RYT < 1 means that the mixture produced less biomass

257   than expected.

258 We compared RYTs between the R+ and R- treatment using a linear mixed model with RYT as the

259 response variable, treatment as a fixed effect, and varietal mixture identity (i.e., non-oriented

260 concatenation of the names of the two varieties grown in mixture) as a random effect on the intercept.

261 We assessed the significance of the fixed effects as detailed above (Supplementary Table 5). Finally,

262 we checked whether RYTs significantly differed from 1 within each treatment using two-sided *t*-tests.

263 To assess whether trait composition (above and belowground traits) of the mixture explained RYT

264 variability, we used traits measured in pure stands to predict mixed stands RYTs. This approach is

265 based on the hypothesis that pure stand traits are the information available to agronomists and plant

266 breeders when designing varietal mixtures. First, we computed reference trait values for pure stands

267 that we corrected for design effects and measurement dates. As described above, we fitted a linear

268 mixed model with pure stand trait as the response variable, block, measurement date, and treatment as

269 fixed effects, and varietal identity as a random effect. Varietal identity random effect was specified

270 both on the intercept and the treatment slope, except for # leaves where adding the random slope led

271 to singular models due to very low variance on the slope. We then computed pure stand reference trait

272 values as the sum of the BLUP of each variety (intercept in the R+ treatment, intercept + slope in the

273 R- treatment when random slope was specified) with the estimated fixed effect of the treatment (R+ or

274 R-). Then, for each mixed stand, we computed both the average and the absolute trait difference

275 between the two varieties using the pure stand reference trait values. Finally, we fitted a full linear

276 model with RYT as the dependent variable and all trait averages and all trait differences as

277 independent variables (*lm()* function from the *stats* package). We standardized all dependent and

278 independent variables ($\mu = 0$, $\sigma = 1$) and ran a backward model selection (*glmulti()* function from the

279 *glmulti* package). We used the second-order Akaike Information Criterion (AIC$_C$, Sugiura 1978) to

280 rank the models and performed model-averaging inference based on the top 10 models using the

281 *coef()* function (*glmulti* package). We report parameter estimates and their 95% unconditional

282 confidence interval computed as ±1.96 unconditional sampling standard deviation, variable

283 importance, and adjusted R-squared averaged over the top 10 models (Burnham and Anderson, 2002).

284 Detailed information on the top 10 models is available in Supplementary Table 6.

285   Finally, to further investigate the ecological mechanisms behind the significant relationship between

286   RYT and trait composition, specifically root area, we assessed the relationship between pure stand

287   biomass and pure stand root area, and between RYs and pure stand root area using simple linear

288   regressions between these variables within each treatment. We used trait and biomass values adjusted

289   for block effects and measurement date effects as explained above, such that we had one value per

290   variety per treatment. We also checked the relationship between RYs and the hierarchical distance in

291   root area between the variety and its neighbour in pure stands. Hierarchical distance was the

292   difference between the focal root area and the neighbour root area, divided by the focal root area (i.e.,

293   positive values mean that the focal had higher root area that its neighbor, and *vice versa*). Finally, we

294   tested whether root area plasticity affected RYT in the two treatments. We computed root area

295   plasticity as the difference between the average root area of the pure stands of the two varieties and

296   the observed root area of their mixture, divided by the average of the pure stands. (i.e., positive values

297   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

300   The limitation of water and nutrients strongly reduced total plant biomass in pure stands (1947.35 mg

301   in the R- treatment vs 2364.71 in the R+ treatment, $F_{1,87.77} = 144.49$, $p < 0.001$, Figure 2a). Such

302   biomass reduction was accompanied by a reallocation of resources from the aboveground to the

303   belowground compartment: while plants produced less above-ground biomass (Figure 2b), less leaves

304   (Figure 2d), and less tillers (Figure 2e), they produced higher root biomass (Figure 2c), longer roots

305   (Figure 2h), and had higher root areas (Figure 2i) in the R- treatment. Consequently, the root:shoot

306   ratio increased from 0.48 in the R+ treatment to 0.82 in the R- treatment (+71%, $F_{1,87.78} = 3966.58$, $p <$

307   0.001, Figure 2g). Leaf nitrogen concentration also increased in the R- treatment (3.32 vs 2.95%,

308   $F_{1,87.19} = 525.03$, $p < 0.001$, 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 contrast, 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 R^2 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 (R^2 = 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). Traits had higher explanatory power on root biomass RYT in the R+ treatment (Supplementary Figure 2c, average adjusted R^2 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). Trait differences between varieties, either above or belowground, and either in the R- or R+ treatment, did not explain mixture biomass (Figure 4, Supplementary Figure 2).

Relationship between root area and mixture biomass

We hypothesized that the negative relationship between RYT and the average root projected area of varieties in the mixtures reflected a relaxation of belowground competition for varieties with high root area in monocultures. Under this hypothesis, varieties with high root area in monocultures are highly competitive varieties that reach such high root areas through root proliferation in response to their neighbour in pure stand, which 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 varieties, when grown in mixtures, are more likely to be paired with varieties 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, $p < 0.001$). At the opposite, varieties which had lower root areas than their neighbours (i.e., negative hierarchical distance to their neighbour on Figure 5c) produced more biomass in mixture than in pure stands (RY > 0.5). This effect was not symmetrical: for a similar hierarchical distance, varieties which were lower in the hierarchy gained less biomass than the biomass loss observed for varieties placed higher in the hierarchy (e.g., at hierarchical distance = -0.2, estimated RY = 0.53 while at hierarchical distance = 0.2, estimated RY = 0.40, Figure 5c). 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

362   area in mixtures (Figure 5d). Again, the relationship between RYT and root area plasticity was

363   stronger in the R- treatment (Pearson's $R = 0.80$, $p < 0.001$, slope $= 1.93$ $\%^{-1}$) than in the R+ treatment

364   (Pearson's $R = 0.54$, $p < 0.001$, slope $= 0.91$ $\%^{-1}$). We obtained identical results when doing these

365   analyses on shoot and root biomass separately, except that the differences between the R+ and R-

366   treatments were less marked for root biomass (Supplementary Figures 3 & 4). Altogether, these results

367   support the idea that low RYTs in the R- treatment resulted from relaxed belowground competition in

368   mixed relative to pure stands.

369   Discussion

370   Shift in root allocation under water and nutrient limitation

371   Wheat seedling growth was strongly affected by the limitation of water and nutrients in our

372   experiment. Resource limitation triggered an overall reduction in biomass, along with a shift in

373   biomass allocation from the aboveground to the belowground compartment. This result is in line with

374   the optimal allocation theory, which states that plants prioritize allocation to increase their uptake of

375   the most limiting resources (Bloom *et al.*, 1985; Weiner, 2004). In our case, the high allocation to

376   roots suggests that belowground resource limitations were much stronger than aboveground resource

377   limitations, a pattern consistent with what we intended to induce with our experimental protocol. In

378   wheat, plasticity of the root-to-shoot ratio and high reallocation to roots have been shown to be

379   advantageous under drought stress (Bacher *et al.*, 2021, 2022). Allocating carbon to the roots

380   enhances access to water through deeper, longer, and more branched roots, which in turns helps

381   maintain high stomatal conductance and physiological activity. We can thus hypothesize that higher

382   root allocation in response to water and nutrient limitation reflected an adaptive response of the

383   varieties.

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

On average, mixture biomass was not significantly different from pure stand biomass under optimal growth conditions, indicating no significant interactions between varieties. However, when water and nitrogen were limiting, mixtures produced significantly less biomass than expected from their pure stand components. Individual variety responses to mixture cultivation (i.e., RYs) combined with root trait data (incl. hierarchical distance and plasticity in root area) together suggest that such reduction in biomass was the consequence of intense competition in pure stands: water and nutrient limitation triggered an arms race between plants of highly competitive varieties in pure stands, which manifested through root proliferation and ultimately overinvestment in both below and aboveground biomass. According to previous study on root responses to the presence of competitor plants, such proliferation could have been triggered either by a direct perception of the presence of a competitive neighbour through the detection of chemical cues in the rhizosphere, or by the perception of resource depletion caused by the neighbour (Schenk, 2006; Pierik *et al.*, 2013). Such highly competitive varieties experienced reduced competition in mixed stands and thus disengaged from this competitive arms race and produced less biomass. Conversely, their less competitive neighbour experienced stronger competition in mixed than in pure stands, and hence produced more biomass. The net balance between these two effects was an overall reduction in biomass because the biomass gain from the weaker competitor was lower than the biomass loss from the stronger competitor, which explains the average negative RYT observed in the R- treatment. All together, these results suggest a positive selection effect where the dominant genotype (here most competitive) gain more than what is lost by the sub-dominant genotype. Similar ecological mechanisms have been shown to be responsible for the overyielding in forest trees, with larger tree species able to benefit from reduced light competition from their smaller neighbours (Schmid and Niklaus, 2017; Williams *et al.*, 2017). In our case, a "gain" is seen as a reduction of competition intensity which translates into a reduction of biomass. This is because in a cereal such as wheat, at the seedling stage, biomass typically reflects competitive ability. For example, seedling growth rate and early vigour are typically targeted by plant breeders as

411 favourable traits for competitiveness against weeds (Lemerle *et al.*, 1996; Bertholdsson, 2005;

412 Hendriks *et al.*, 2022).

No evidence for niche complementarity between seedlings

414 We found no evidence of complementarity effects that would be driven by trait differences between

415 varieties, including under resource limitation. This could be explained by the fact that functional

416 differences between varieties were not large enough 3-weeks after sowing to generate

417 complementarity effects. A second hypothesis is that we did not measure the "right" traits to detect

418 complementarity effects, notably belowground. Our phenotyping method did not allow us accessing

419 traits such as root diameter or root tissue density. Other traits could not be computed due to root

420 overlapping between adjacent plants (e.g., root angle) or to the reduced size of the Rhizotube® (e.g.,

421 root depth). However, such traits have rarely been found to associated with complementarity effects in

422 ecological studies (Fort *et al.*, 2014; Bakker *et al.*, 2018). Previous studies in rice varietal mixtures

423 also failed to find evidence of belowground niche complementarity driven by differences in

424 morphological and architectural root traits such as rooting depth, root diameter, or specific root length

425 (Montazeaud *et al.*, 2018). Overall, this study confirm that root trait differences alone are less likely to

426 generate complementarity effects in varietal mixtures than they are for example in intercropping

427 where differences in root foraging strategies between species can be more significant (Zhang *et al.*,

428 2014; Homulle *et al.*, 2022; Schmutz and Schöb, 2023).

Root area as the main driver of competitive hierarchy

430 We identified root area as the main driver of plant biomass and mixing effects in our experiment. The

431 average root area of the two varieties in pure stands could explain up to 50% of RYT variability under

432 resource-limiting conditions. A higher root area in pure stand was associated with a greater biomass

433 reduction in mixture, especially when the mixture partner had a lower root area. These results support

434 the idea of an early-stage belowground competitive hierarchy between varieties (Kunstler *et al.*,

435 2012), where higher root area is associated with higher competitive ability. In line with these results,

436 root functional traits associated with root foraging and absorption potential have already been shown

17

437   to shape early-stage competitive hierarchies between grassland species (Fort *et al.*, 2014; Ravenek *et*

438   *al.*, 2016; Wagg *et al.*, 2017). The very high explanatory power of root area found in our study might

439   be explained by the fact that this trait integrates several functional dimensions of the root system that

440   are classically captured by distinct traits (e.g., root length density, specific root length, or root

441   diameter).

Root plasticity contributes to mixing effects

443   We found that root area in mixed stands deviated from root area observed in pure stands, indicating a

444   plastic response of the root system to neighbour genotype identity. On average, root area decreased in

445   mixed relative to pure stands, and the stronger the decrease, the lower the RYT especially in the R-

446   treatment. In accordance with ecological theory, our results thus support the view that phenotypic

447   plasticity contributes to mitigate competition intensity in diverse plant communities (Callaway *et al.*,

448   2003; Schiffers *et al.*, 2011; Burns and Strauss, 2012). In varietal mixtures, previous studies have

449   already shown that plasticity in aboveground traits such as plant height, specific leaf area, or canopy

450   cover contribute to overyielding, notably by increasing trait divergence between varieties (Kong and

451   Zhao, 2023; Tschurr *et al.*, 2023; Su *et al.*, 2024). We here show that plastic root changes may also

452   contribute to mixture performance, although it may not be by increasing differences between varieties,

453   but rather by changing the average trait value.

Practical implications for plant breeding

455   Crop performance, including grain yield, is affected by trade-offs between individual competitiveness

456   and group performance. As early pointed out by plant breeders (Donald, 1968), and more recently

457   revisited with the concepts of Darwinian Agriculture, or Evolutionary Agroecology, these trade-offs

458   can hold promising opportunities to increase productivity, notably by reversing past selection for

459   individual competitiveness (Denison *et al.*, 2003; Weiner, 2019). This can be achieved either by

460   selecting on group performance, i.e., selecting on population yield (Montazeaud *et al.*, 2020), or by

461   targeting key traits related to competitive ability. Our study suggests that selecting genotypes with

462   reduced root area in pure stands and reduced root area proliferation in presence of neighbours could

463 be a way to reduce competition intensity between seedlings, which could then translate into higher

464 yields. This result aligns with classical prediction of game theory models: investment in root biomass

465 beyond the payoff point where soil resource absorption compensate the cost of the root system only

466 happens at cost for neighbouring plants, leading to a Tragedy of the Commons (Hardin, 1968; Gersani

467 *et al.*, 2001; Anten and Vermeulen, 2016). It is also supported by empirical data measured in the same

468 phenotyping platform: Colombo *et al.* (2022) found that early seedling biomass measured in the

469 platform was negatively correlated with yield components measured in the field in multiple wheat

470 panels. Interestingly, modern wheat varieties that have been subject to selection for increased

471 population yield for many generations display reduced root biomass at the seedling stage (Colombo *et*

472 *al.*, 2022), and reduced root proliferation in presence of a neighbour compared to old landraces (Zhu

473 *et al.*, 2019). In oat and barley, excessive growth of the root system in the topsoil is also associated

474 with yield reductions (Vain *et al.*, 2023).

475 Beyond direct selection on root traits in pure stand varieties, which could be challenging to

476 implement, mixing varieties could be another solution to resolve belowground TOCs. This solution

477 would allow to benefit from other known positive effects of genetic diversity at the same time (e.g.,

478 improved disease control, Wolfe, 1985; Mundt *et al.*, 1995; Zhu *et al.*, 2000; Finckh and Wolfe,

479 2006)). Our study suggests that highly competitive varieties could benefit from relaxed belowground

480 competition in varietal mixtures, which could translate into overyielding through positive selection

481 effects. Conveniently, the choice of the varieties could be guided by their root projected area

482 measured in monoculture. Of course, the effect of relaxed competition at the seedling stage on final

483 grain yield would still need to be validated in field trials. This means that promoting complementarity

484 effects between varieties is not the only way to reduce competition and may only work for some traits,

485 such as phenological traits (Fletcher *et al.*, 2019; Lowry *et al.*, 2020) or traits related to light

486 interception (Su *et al.*, 2024).

Supplementary Data

488 **Supplementary Table 1:** List of the 36 wheat varieties used in the experiment

489 **Supplementary Table 2:** List of the 54 binary mixtures used in the experiment

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

491 **Supplementary Table 4:** Analysis of Variance (ANOVA) of biomass components and traits in pure

492 stands to test the treatment effect

493 **Supplementary Table 5:** Analysis of Variance (ANOVA) to test the effect of the treatment on RYTs

494 **Supplementary Table 6:** Ten best fitting models between RYTs on aboveground, belowground, and

495 total biomass and mixture trait composition

496 **Supplementary Figure 1:** RhizoTubes® monitoring

497 **Supplementary Figure 2:** Relationships between the trait composition of the mixtures and their

498 above and belowground RYTs

499 **Supplementary Figure 3:** bivariate plots illustrating the interaction between root area, resource

500 availability and shoot biomass

501 **Supplementary Figure 4:** bivariate plots illustrating the interaction between root area, resource

502 availability and root biomass

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Author contributions

509 Conception of the research and design of the experiment: C. J., C. S, H. F., P. R.

510 Implementation and monitoring of the experiment: C. J., C. S.

511 Root image analysis: M. L.

512   Near-Infrared Spectrometry: M. E.

513   Harvest and trait measurements: H. F., L. M. C., M. E., P. R.

514   Data analysis: G. M.

515   Redaction of the manuscript: G. M. with inputs from all authors.

516 Conflict of interest

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521 Data availability

522   Data and code used for analysis are available at https://doi.org/10.5281/zenodo.14163518.

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Figure legends

Figure 1: Experimental design. (**a**) and (**b**) close-up views on RhizoTubes® showing wheat seedlings and their roots. (**c**) Spatial distribution of the seedlings within the RhizoTubes® in both pure (up) and mixed (bottom) stands. Different colours represent different genotypes. (**d**) 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.

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 AIC_C, 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 ($\overline{R^2_{adj}}$) 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. ΔAICc ("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 16th to 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 AIC_C, 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 ($\overline{R^2_{adj}}$) 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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