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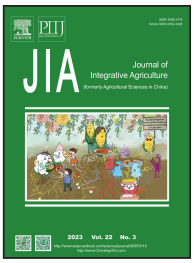

RESEARCH ARTICLE

Breeding against mycorrhizal symbiosis: Modern cotton (*Gossypium hirsutum* L.) varieties perform more poorly than older varieties except at very high phosphorus supply levels

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Abstract

Cotton (*Gossypium hirsutum* L.) is an important fiber cash crop, but its root traits related to phosphorus (P) acquisition, including mycorrhizal root traits, are poorly understood. Eight cotton varieties bred in northwestern China that were released between 1950 and 2013 were grown in pots with or without one arbuscular mycorrhizal fungal (AMF) species (*Funneliformis mosseae*) at three P supply levels (0, 50 and 300 mg P as KH_2PO_4 kg^{-1}). Eleven root traits were measured and calculated after 7 wk of growth. The more recent accessions had smaller root diameters, acquired less P and produced less biomass, indicating an (inadvertent) varietal selection for thinner roots that provided less cortical space for AMF, which then increased the need for a high P fertilizer level. At the two lower P levels, the mycorrhizal plants acquired more P and produced more biomass than non-mycorrhizal plants (3.2 *vs*. 0.9 mg P per plant; 1.8 *vs*. 0.9 g biomass per plant at P_0; 14.5 *vs*. 1.7 mg P per plant; and 4.7 *vs*. 1.6 g biomass per plant at P_{50}). At the highest P level, the mycorrhizal plants acquired more P than non-mycorrhizal plants (18.8 *vs*. 13.4 mg per P plant), but there was no difference in biomass (6.2 *vs*. 6.3 g per plant). At the intermediate P level, root diameter was significantly positively correlated with shoot biomass, P concentration and the P content of mycorrhizal plants. The results of our study support the importance of the outsourcing model of P acquisition in the root economics space framework. Inadvertent varietal selection in the last decades, resulting in thinner roots and a lower benefit from AMF, has led to a lower productivity of cotton varieties at moderate P supply (i.e., when mycorrhizal, the average biomass of older varieties 5.0 g per plant *vs*. biomass of newer varieties 4.4 g per plant), indicating the need to rethink cotton breeding efforts in order to achieve high yields without very high P input. One feasible way to solve the problem of inadvertent varietal selection for cotton is to be aware of the trade-offs between the root do-it-yourself strategy and the outsourcing towards AMF strategy, and to consider both morphological and mycorrhizal root traits when breeding cotton varieties.

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1. Introduction

Phosphorus (P) is an essential nutrient for plant growth, development and reproduction (Raghothama 1999). In many parts of the world, P deficiency is a major limiting factor for crop yield and hence a major cause of food insecurity and constrained economic development. P limitation can be addressed by the application of synthetic P fertilizer; however, due to strong sorption reactions of P with the soil mineral matrix (Zhang *et al.* 2008), supplying P fertilizer does not always translate into increased plant performance (Vance *et al.* 2003). Different plant species and varieties possess distinct strategies with different trait combinations to adapt to nutrient limitation (Lambers *et al.* 2006). These strategies have been captured in a conceptual model of the plant economics spectrum (Reich 2014), where plants are arranged in a one-dimensional spectrum from conservative to acquisitive plants. However, root traits do not fit easily in this one-dimensional framework. Recently, Bergmann *et al.* (2020) proposed a model for the root economics space, where root traits are classified along two dimensions. The first and most important dimension is the collaboration gradient, in which plants are classified ranging from a do-it-yourself strategy where the roots have a small diameter and high specific root length (SRL), to an outsourcing strategy where nutrient acquisition is outsourced to mutualistic arbuscular mycorrhizal fungi (AMF), while the second dimension reflects the spectrum from conservative to acquisitive roots. In order to accommodate AMF, outsourcing plants have thick roots with a large cortical area, and hence low SRL.

Root traits have an underlying genetic basis, but they also show environmental plasticity. Intraspecific variability in root traits has been observed in several crops including cereals and legumes (Fageria 2014; Fageria *et al.* 2016; Wang *et al.* 2020a), while genetic variation in the benefits that different plant varieties derive from the AMF symbiosis has been discussed by Kuyper *et al.* (2021). The ultimate goal of harnessing the genetic variation in root traits, including traits related to the AMF symbiosis (Bardgett *et al.* 2014), is to select for root phenotypes that provide substantial benefits to plant growth at a sustainable nutrient supply (Lynch 2019; Wang *et al.* 2020b). It is therefore important to screen crop varieties for P acquisition efficiency and to link this efficiency to (mycorrhizal) root traits, in order to select traits that predict plant performance under different conditions of P supply. Selecting root traits that are predictive for plant performance under varying conditions of supply is an important strategy to improve yield at the same P supply or to maintain yields at lower P supply, considering that P is a non-renewable resource and that the excessive use of P fertilizer has caused environmental problems worldwide (Liu *et al.* 2008; Yan *et al.* 2013; Blaas and Kroeze 2016).

Current research focuses on specific root traits or trait categories as indicators in screening programs. For example, Pang *et al.* (2015) observed that the rhizosphere carboxylate content and rhizosphere acid phosphatase activity of two herbaceous perennial pasture legumes (*Cullen australasicum* (Schltdl.) J.W. Grimes and *Kennedia prostrata* R. Br.) were highly correlated with P-uptake at low P availability. Zhu and Lynch (2004) suggested that enhanced lateral rooting under P deficiency may be harnessed as a useful trait for the selection and breeding of more P-efficient maize (*Zea mays* L.) genotypes. Other researchers have attempted to discover genetic loci that correlate with mycorrhizal responsiveness (Kaeppler *et al.* 2000; Galván *et al.* 2011).

Cotton (*Gossypium hirsutum* L.) is the main source of natural fiber worldwide and an important cash crop in China, India, Pakistan, USA and Australia. Cotton is often planted in semi-arid areas, where soils are low in available P (Gill *et al.* 2005). Cotton plants are usually colonized by diverse AMF species simultaneously (Zhang *et al.* 2019). In such areas, drought and salinity further adversely impact cotton growth (Feng *et al.* 2022), and AMF may alleviate these various stress factors (Eskandari *et al.* 2018; Kuyper *et al.* 2021). Cotton is poorly responsive to P fertilizer application and lacks the ability to acquire P from sparingly soluble P sources (Wang *et al.* 2010), making it likely that cotton is crucially dependent on the AMF symbiosis. In agreement with this, Damodaran *et al.* (2012) noted cotton cultivars with high mycorrhizal responsiveness. When testing different cotton varieties for P acquisition efficiency in a hydroponic system, Chen *et al.* (2019) noted that at a low P supply both fine-root ratio and specific root length were positively correlated with P acquisition efficiency, suggesting the higher efficiency of varieties with thinner roots. However, it is not known how cotton breeding during the last decades has affected its ability to acquire P either directly or *via* the AMF symbiosis.

We therefore conducted a pot experiment with eight cotton varieties released in different years (see Materials and methods) at three P supply levels. In this experiment, we addressed the role of (mycorrhizal) root traits and explored the interplay of root and mycorrhizal traits in enhancing P acquisition. We formulated three (null) hypotheses: 1) There is no directional trend in root traits for cotton varieties bred over time; 2) newer varieties do not differ in performance from older varieties at any P level; and 3) there is no difference in mycorrhizal growth and mycorrhizal P responsiveness between newer and older cotton varieties.

2. Materials and methods

2.1. Experimental design

Our experiment was a three-factorial experiment: cotton varieties (eight varieties)×P supply (three levels: 0, 50, and 300 mg P kg^{-1} soil)×mycorrhiza (two levels: with and without one species of AMF). We executed a pre-experiment with five P levels to determine the best P application range for our experiment. We wanted to include a non-fertilized control, a P level where mycorrhizal plants would respond to increased P but non-mycorrhizal plants either would not or barely respond to increased P, in terms of both biomass and P concentration, and a P level where mycorrhizal and non-mycorrhizal plants would both achieve the same biomass (Appendix A). This pre-experiment resulted in our choice for three P supply levels of 0, 50, and 300 mg P kg^{-1} soil.

The experiment was conducted in a greenhouse at the China Agricultural University, Beijing (40°01´267´´N, 116°16´36´´E), in June–August 2015. The glasshouse temperature range was 25–30°C (day/night), and the average photosynthetically actively radiation was 380 μmol m^{-2} s^{-1}. There was no supplementary lighting. Pots of 15 cm height and 19 cm diameter were filled with 2 kg of soil. There were four replicates for each treatment, yielding 192 pots in total. The pots were arranged in a complete randomized block design, and the position of each block was re-randomized weekly.

2.2. Soil

A calcareous loamy soil was collected from field plots at the Changping Long-Term Fertilizer Station of China Agricultural University in Beijing, China (40°05´32´´N, 116°20´41´´E). The soil contained 17.8 g kg^{-1} organic matter, 2.9 mg kg^{-1} Olsen-P, 872 mg kg^{-1} N (C:N≈10), 156 mg kg^{-1} ammonium acetate-exchangeable K, and had a pH (in 0.01 mol L^{-1} $CaCl_2$) of 7.81. The soil was passed through a 2-mm sieve and sterilized by radiation with ^{60}Co γ-rays at 10 kGy.

2.3. Nutrient additions

The following mineral nutrients (per kg soil) were added uniformly before potting: 200 mg N (as KNO_3), 50 mg Mg (as $MgSO_4$), 5 mg Zn (as $ZnSO_4 \cdot 7H_2O$), and 2 mg Cu (as $CuSO_4$). The nutrients were fully mixed with the soil before pot filling. Three weeks after sowing, another 100 mg N (as KNO_3) was added to every pot. Three different levels of P fertilizer (as K_2HPO_4) were applied: 0 (P_0), 50 (P_{50}) and 300 (P_{300}) mg P kg^{-1} soil; these are equivalent to 0, 75 and 450 kg P ha^{-1} at a 15 cm soil depth. To achieve the same soil K level between the three P treatments, 377, 315 and 0 mg of extra K as K_2SO_4 was supplied before potting for P_0, P_{50} and P_{300}, respectively. The Olsen-P concentrations in the rhizosphere were 4.5, 9 and 76 mg kg^{-1}, respectively, for the three P levels after harvest.

2.4. Inoculum

An isolate of the AM fungal species *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & Schlüßler (formerly *Glomus mosseae*) was kindly supplied by Prof. Wang Youshan from the Bank of Glomeromycota of China, Institute of Plant Nutrition and Resources, Beijing Academy of Agriculture and Forestry Research, Beijing. The fungus was propagated in a 5:1 mixture (w/w) of zeolite and river sand with maize for 4 mon in a greenhouse, and the inoculum consisted of substrate containing spores, mycelium, and fine-root segments. An 80-g quantity of the inoculum was added to each mycorrhizal pot, and 80 g of sterilized inoculum was added to non-mycorrhizal pots. To minimize differences in the microbial communities of mycorrhizal and non-mycorrhizal treatments, 10 mL of AMF-free filtrate from the inoculum was added to each non-mycorrhizal pot, and 10 mL of deionized water was added to each mycorrhizal pot. A 50-g quantity of non-sterilized inoculum was submerged in 500 mL deionized water for 2 h, and the solution was filtered by two-layer filter paper with 30 μm filter pore size. We checked the filtrate under the stereomicroscope and confirmed the absence of AMF spores or hyphae. The filtrate was therefore regarded as AMF-free filtrate.

2.5. Cotton varieties

Eight cotton varieties were used in this experiment:

Suk202 (SK202), Xinluzao 1 (XLZ1), Junmian 1 (JM1), Xinluzao 2 (XLZ2), Xinluzao 13 (XLZ13), Xinluzhong 21 (ZM21), Xinluzhong 54 (ZM54), and Xinluzao 57 (XLZ57). These varieties were released in 1970, 1968, 1979, 1988, 2002, 2004, 2012, and 2013, respectively.

Seeds of each cotton variety were surface-sterilized in 10% (v/v) H_2O_2 for 10 min and rinsed five times in deionized water. Two germinated seeds were sown into each pot, and were thinned to one seedling per pot after emergence.

2.6. Harvest and analyses

At harvest after 7 wk, plants were separated into shoots and roots. Plant roots (first- and second-order roots) were carefully removed from the soil and shaken gently to remove loosely adhering soil. The soil in each pot was collected in a blender to obtain a uniform matrix for subsequent analyses. The shoots were oven-dried at 72°C for 72 h and thereafter ground to a fine powder by a grinding miller (size<1 mm). The shoot P concentration was determined by the vanado-molybdate method (Murphy and Riley 1962), after the ground shoots were digested in a H_2SO_4–H_2O_2 mixture at 360°C for 2 h.

The roots were washed with deionized water, weighed, and preserved at –20°C. Root length and diameter were measured using a WinRHIZO scanning and image-recording system (EPSON 1680, WinRHIZOPro2004b). Specific root length (m g^{-1}) was assessed as the ratio of root length over root dry weight. The proportion of fine-root (roots with a diameter≤0.3 mm) length over total root length was determined by the WinRHIZO Software automatically. We calculated root tissue density, assuming the roots to be perfect cylinders (Ostonen *et al.* 2007). After scanning, a weighed subsample of the root system was cleared and stained for assessment of mycorrhizal colonization intensity by the method of McGonigle *et al.* (1990). Roots were cut into 1-cm segments, thoroughly mixed, and a 0.5-g subsample was cleared with 10% (w/v) KOH at 90°C for 2 h and stained with trypan blue. The rest of the roots were oven-dried at 70°C for 3 d and weighed. Total root dry weight was determined taking the subsample for mycorrhizal assessment into account. The root/shoot ratio was calculated based on dry weight. Soil samples from each pot were taken for the determination of hyphal length density (HLD, meters of hyphae per gram of dry soil). HLD was determined according to Jakobsen *et al.* (1992).

The alkaline phosphatase activity of the rhizosphere (p-nitrophenylphosphate (p-NPP); μmol p-NPP h^{-1} g^{-1} dry soil) was measured according to Alvey *et al.* (2001). The roots with tightly adhering rhizosphere soil were transferred into 200-mL vials containing a measured amount of 0.2 mmol L^{-1} $CaCl_2$ solution depending on root volume (Veneklaas *et al.* 2003). Roots were repeatedly dipped (about 60 s) into the solution until the rhizosphere soil was removed as much as possible. Two 0.5-mL aliquots of soil suspension were transferred into 2-mL centrifuge tubes for the measurement of alkaline phosphatase activity (Alvey *et al.* 2001; Neumann 2006). The pH value of Na-Ac buffer (200 mmol L^{-1}) was adjusted to the average pH value (8.4) of the rhizosphere soil. The rhizosphere soil in the $CaCl_2$ suspension was separated by centrifugation for 10 min at 12 000×g, dried at 60°C, and then weighed. The concentration of para-nitrophenol (p-NP) in the supernatant was measured spectrophotometrically at 405 nm. The acid phosphatase activity at the root surface (μmol p-NPP h^{-1} g^{-1} root fresh weight) was measured according to the method of Neumann (2006). Excised cotton root segments were washed 3–5 times in acetate buffer (0.2 mol L^{-1}, pH 5.2) and immersed in a solution containing 0.5 mL water, 0.4 mL acetate buffer (0.2 mol L^{-1}, pH 5.2) and 0.1 mL 0.15 mol L^{-1} p-NPP substrate. After reaction for 10 min at 25°C, 0.8 mL of the reaction mixture was transferred to a new tube, and 0.4 mL of 0.5 mol L^{-1} NaOH was added to terminate the reaction. The concentration of p-NP was measured spectrophotometrically at 405 nm.

2.7. Calculations and statistical analysis

In order to assess the effect of breeding on varietal performance, we calculated the correlations between year of release, root traits, and plant performance. We used one-way analysis of variance to test for significant differences between two variety groups of equal size (older varieties released before 1990 *vs*. younger varieties released after 2000).

Absolute mycorrhizal growth/P responsiveness (AMGR/AMPR) refers to mycorrhiza-induced increases in shoot biomass and shoot P content. AMR was calculated according to eq. (1) (Janos 2007):

$$AMG(P)R=M–NM \quad (1)$$

where M and NM refer to the shoot biomass or shoot P content of mycorrhizal and non-mycorrhizal plants, respectively.

Relative mycorrhizal growth/P responsiveness (MGR/MPR) refers to mycorrhiza-induced fractional increases in shoot biomass and shoot P content. MR was calculated according to eq. (2) (Janos 2007):

$$MG(P)R=((M–NM)/M)×100 \quad (2)$$

where M and NM refer to the shoot biomass or shoot P content of mycorrhizal and non-mycorrhizal plants, respectively.

Three-way ANOVA was done for shoot dry biomass, shoot P concentration, shoot P content, root biomass, root/shoot ratio, root length, specific root length, ratio of fine-root length, root diameter, root tissue density, and phosphatase activity in the rhizosphere and on the root surface. Two-way ANOVA was done for mycorrhizal colonization, hyphal length density, and the absolute and relative mycorrhizal growth/P response ratios. Because of the large number of comparisons that were partly non independent, we applied Benjamini-Hochberg corrections with a false-discovery rate of 5%. One-way ANOVA with Tukey's honestly significant differences test at the 5% level was done for shoot biomass, shoot P concentration and P content across varieties under six conditions (2 mycorrhizal treatments×3 P levels). Correlations between biomass, shoot P content and 12 root traits at three P levels, and between mycorrhizal colonization and mycorrhizal responsiveness, were determined using Pearson correlation coefficients. All analyses were performed with SPSS 20.0 (IBM Corp., Armonk, NY., USA).

3. Results

3.1. Cotton breeding and root traits

Averaged over all mycorrhizal and P-level treatments, year of varietal release and root diameter were significantly negatively correlated (r=–0.73, P=0.04; Fig. 1-A), whereas year of varietal release and fine-root fraction and specific root length were significantly positively correlated (r=0.74, P=0.03; r=0.81, P=0.02, respectively; Fig. 1-B and C), demonstrating that plant breeding had directionally changed the root traits towards thinner roots with more of a do-it-yourself strategy at the expense of the (mycorrhizal) collaboration strategy. Other root traits were not significantly correlated with year of release.

3.2. Plant performance

The results of the three-way ANOVA for plant performance are shown in Table 1 and Appendix B. For shoot biomass, shoot P concentration and shoot P content, all the main factors were significant sources of variation. The two-way interactions of mycorrhiza×P and variety×mycorrhiza were also significant, while the two-way interaction of variety×P was not significant. The highly significant interaction of mycorrhiza×P in shoot biomass was due to the fact that plants responded strongly to mycorrhiza addition at P_0 and P_{50}, but they did not respond at P_{300}. A comparable pattern was observed for plant P content and

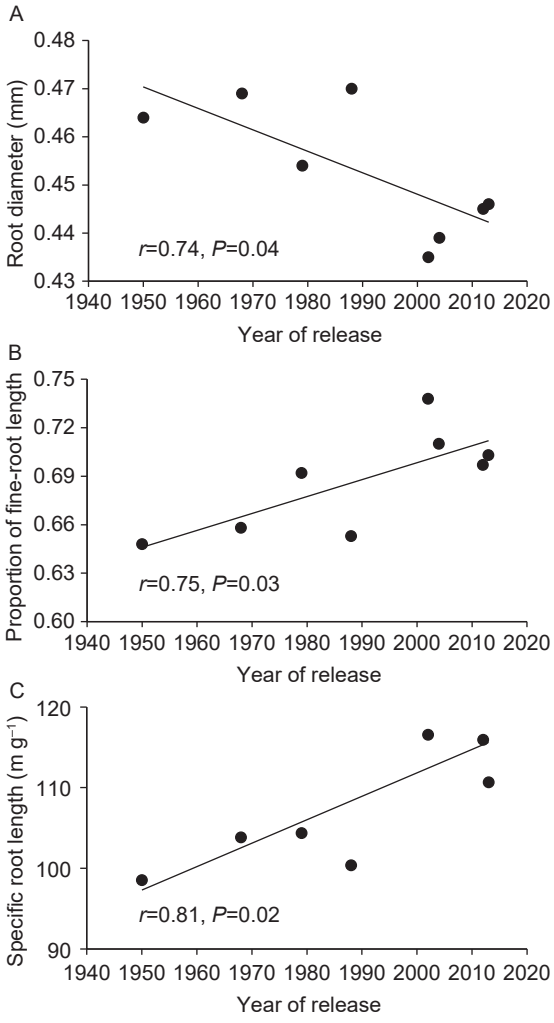

Fig. 1 Correlations between year of release of eight cotton varieties and root diameter (A), proportion of fine-root length (B), and specific root length (C). The r values are presented (Pearson coefficient at P<0.05).

P concentration, where the mycorrhiza effect declined at P_{300}. Both shoot biomass and shoot P content of older cotton varieties were significantly higher than those of younger varieties at P_0 and P_{50}, but not at P_{300} (Fig. 2; Appendix B).

Averaged over all varieties, AMF increased plant biomass by 111 and 193% at P_0 and P_{50}, and reduced biomass by 1% at P_{300} (Appendix C). For shoot P concentration, the mycorrhizal effect was the largest at P_{50} (188% increase), followed by P_0 and P_{300} (79 and 41% increases, respectively; Appendix C). Total shoot P content also showed the largest mycorrhizal effect at P_{50} (744% increase), followed by P_0 (278% increase) and then P_{300} (39% increase). Mycorrhizal plants at P_0 had the same biomass and a significantly higher P content than non-mycorrhizal plants at P_{50}. Mycorrhizal plants

Table 1 Three- or two-way ANOVA results with cotton varieties (*df*=7), mycorrhiza (*df*=1) and P levels (*df*=2) as independent variables

Variable	Varieties (V)	Mycorrhiza (M)	P level (P)	V×M	V×P	M×P	V×M×P
Shoot biomass	3.5**	315***	1423***	2.5*	1.9 ns	157***	1.6 ns
Shoot P concentration	5.2***	1474***	515***	5.9***	1.8 ns	157***	1.3 ns
Shoot P content	3.9**	1019***	1439***	6.4***	1.8 ns	210***	2.3**
Root biomass	11***	188***	816***	2.9**	1.9 ns	126***	2.3*
Root shoot ratio	2.4*	8.7**	8.0***	2.1 ns	1.4 ns	10***	0.52 ns
Root length	4.0***	132***	504***	2.2 ns	1.8 ns	57***	2.5**
Mean root diameter	6.0***	0.03 ns	0.75 ns	0.44 ns	1.1 ns	0.81 ns	0.96 ns
Root tissue density	0.61 ns	6.9***	2.1 ns	0.39 ns	1.1 ns	1.0 ns	1.0 ns
Proportion of fine root length	10***	0.56 ns	6.3**	2.7**	1.6 ns	4.4*	1.2 ns
Specific root length	5.5***	6.7***	2.9 ns	1.2 ns	1.9 ns	1.4 ns	1.6 ns
Phosphatase activity on root surface at pH 5.2	3.2**	7.3**	30***	2.1 ns	0.36 ns	0.10 ns	0.15 ns
Phosphatase activity of rhizosphere soil at pH 8.2	2.2*	7.1**	22***	2.4 ns	0.93 ns	4.3*	1.2 ns
Mycorrhizal colonization	86***		14509***		69***		
Hyphal length density	36***		147***		163***		
Mycorrhizal growth response	3.3**		188***		2.3*		
Mycorrhizal P response	5.8**		286***		2.0*		
Absolute mycorrhizal growth responsiveness	3.4**		175***		2.0*		
Absolute mycorrhizal P responsiveness	7.1***		238***		2.9**		

F-values are shown. *, **, *** and ns represent significance at $P<0.05$, $P<0.01$, $P<0.001$, and no significance, respectively, after Benjamini-Hochberg correction with a false-discovery rate of 5%.

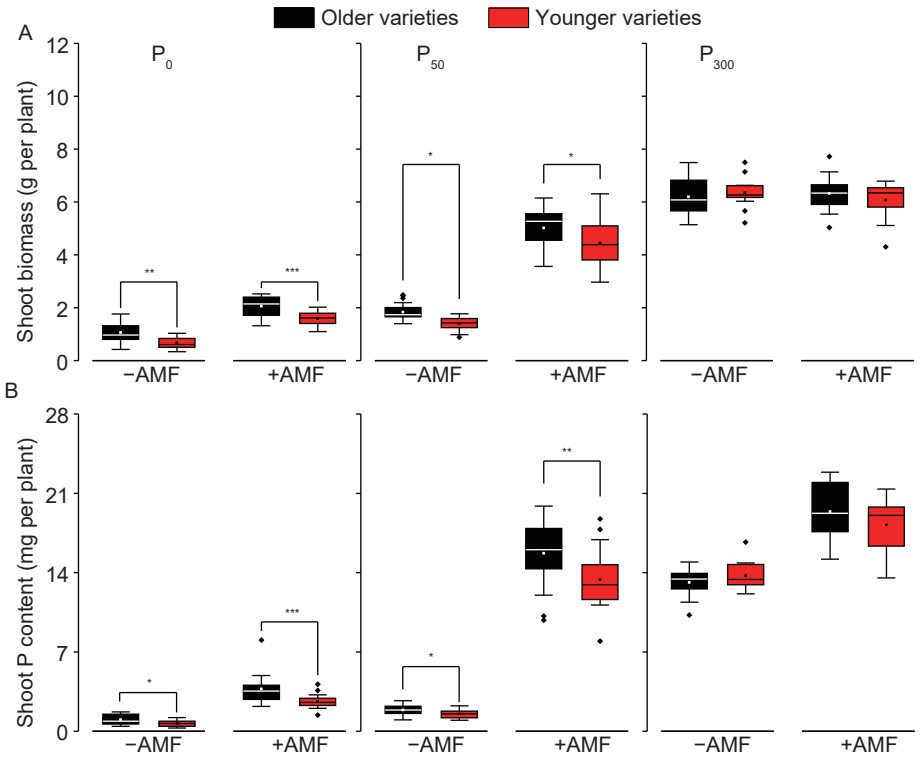

Fig. 2 Shoot biomass (A) and shoot P content (B) of older and younger cotton varieties as influenced by P application (P_0, P_{50}, and P_{300}), and arbuscular mycorrhizal fungi (AMF). P_0, P_{50} and P_{300} represent P addition rates of 0, 50 and 300 mg kg^{-1}, respectively. Boxes show first quartile, median and third quartile; whiskers and bars extend to the most extreme points within 1.5× box lengths. *, ** and *** indicate significant differences between older and younger cotton varieties at $P<0.05$, $P<0.01$ and $P<0.001$, respectively (*t*-test).

at P_{50} had a lower biomass than non-mycorrhizal plants at P_{300}, whereas the P contents of both groups of plants were not significantly different. These comparisons suggest considerable potential for P fertilizer savings with adequate mycorrhizal management and varietal selection.

Root biomass and root length showed the same main

effects as shoot performance (Table 1; Appendix D). Root biomass and root length increased with increasing P levels (Fig. 3). Root biomass of older varieties was significantly larger than that of more modern varieties, irrespective of P level (Fig. 3). In the presence of mycorrhiza, root biomass and root length were also significantly higher than in the non-mycorrhizal condition at P_0 and especially at P_{50}, similar to the strong mycorrhiza×P interaction for shoots. At P_{300}, mycorrhiza had no significant effect on either root biomass or root length (Fig. 3).

3.3. Mycorrhizal responsiveness

Absolute and relative mycorrhizal responsiveness and mycorrhizal P responsiveness were significantly affected by variety, P level and the interaction variety×P level (Table 1). All four parameters were the highest at P_{50}, an effect that was stronger for absolute responsiveness than for relative responsiveness (Appendix E). At P_{300}, absolute and relative mycorrhizal P responsiveness were still positive, indicating that cotton plants benefitted from AMF in terms of P acquisition even at the highest P level (Appendix E). At P_0 and P_{50}, relative mycorrhizal responsiveness, mycorrhizal P responsiveness, and absolute mycorrhizal growth responsiveness were significantly negatively correlated with shoot biomass and P content of non-mycorrhizal plants (Appendix F). Relative mycorrhizal growth and P responsiveness did not differ between older and younger varieties at any of the P levels (Fig. 4-A and B). AMGR was also not significantly different between older and younger varieties (Fig. 4-C), whereas AMPR was somewhat higher for older varieties than for younger varieties at P_{50} and P_{300}, but the differences were not significant (Fig. 4-D).

3.4. Root trait variation

For the morphological trait root diameter, only variety was a significant source of variation, whereas for root tissue density (RTD) only mycorrhiza was a significant source of variation (Table 1). Root diameter and root tissue diameter were not correlated. Specific root length was significantly affected by variety and mycorrhiza. Non-mycorrhizal plants had a significantly higher RTD than mycorrhizal plants, 0.059 *vs*. 0.056 g cm^{-3}, respectively. The proportion of fine-root length, which is determined by both average root diameter and the degree of root branching, was significantly affected by variety, mycorrhiza, P level, and the interactions

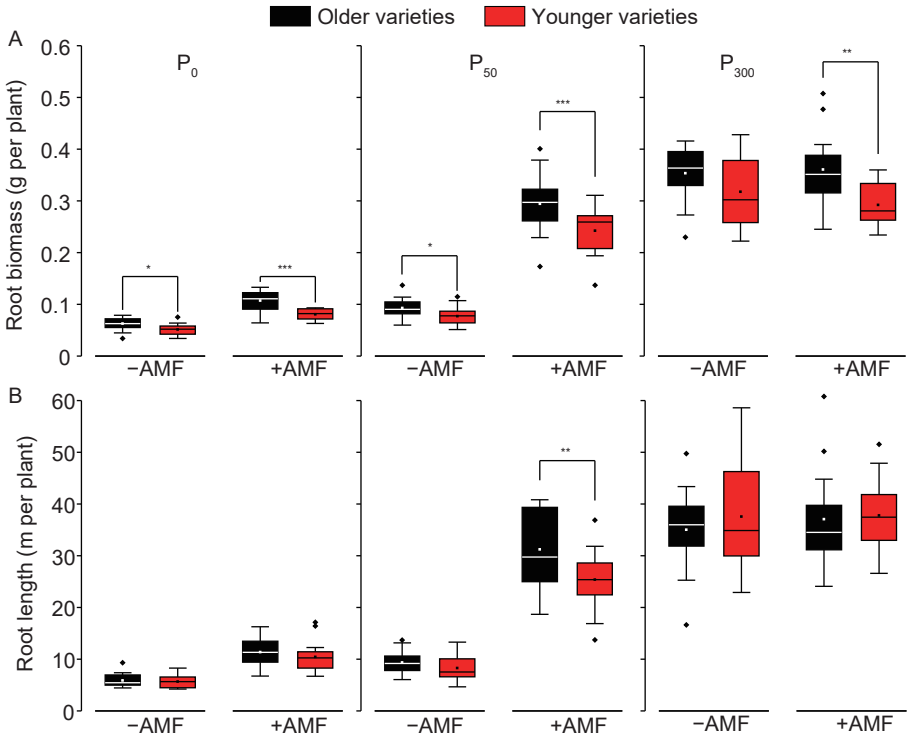

Fig. 3 Root biomass (A) and root length (B) of older and younger cotton varieties as influenced by P application (P_0, P_{50} and P_{300}) and arbuscular mycorrhizal fungi (AMF). P_0, P_{50} and P_{300} represent P addition rates of 0, 50 and 300 mg kg^{-1}, respectively. Boxes show first quartile, median and third quartile; whiskers and bars extend to the most extreme points within 1.5× box lengths. *, **, and *** indicate significant differences between older and younger cotton varieties at $P<0.05$, $P<0.01$ and $P<0.001$, respectively (*t*-test).

variety×mycorrhiza and mycorrhiza×P level (Table 1; Appendix G). Younger varieties had higher SRL and a larger proportion of fine-root length than older varieties (Fig. 5).

Both physiological root traits, phosphatase activity in the rhizosphere and on the root surface, were significantly

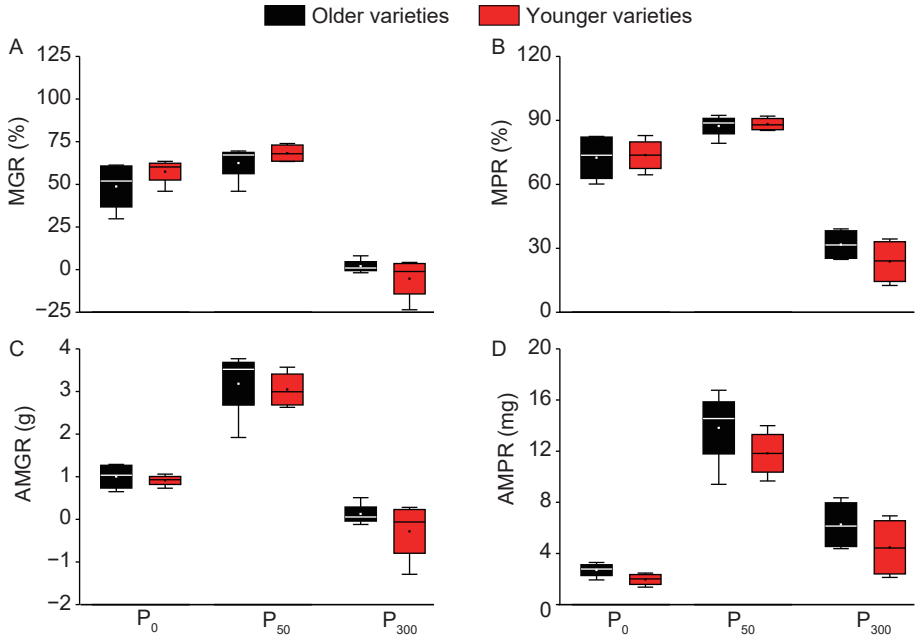

Fig. 4 Mycorrhiza-induced changes in relative (A and B) and absolute (C and D) shoot biomass (A and C) and shoot P content (B and D), of older and younger cotton varieties as influenced by P application (P_0, P_{50} and P_{300}). P_0, P_{50} and P_{300} represent P addition rates of 0, 50 and 300 mg kg^{-1}, respectively. Boxes show first quartile, median and third quartile; whiskers and bars extend to the most extreme points within 1.5× box lengths. MGR, mycorrhizal growth responsiveness; MPR, mycorrhizal P responsiveness; AMGR, absolute mycorrhizal growth responsiveness; AMPR, absolute mycorrhizal P responsiveness.

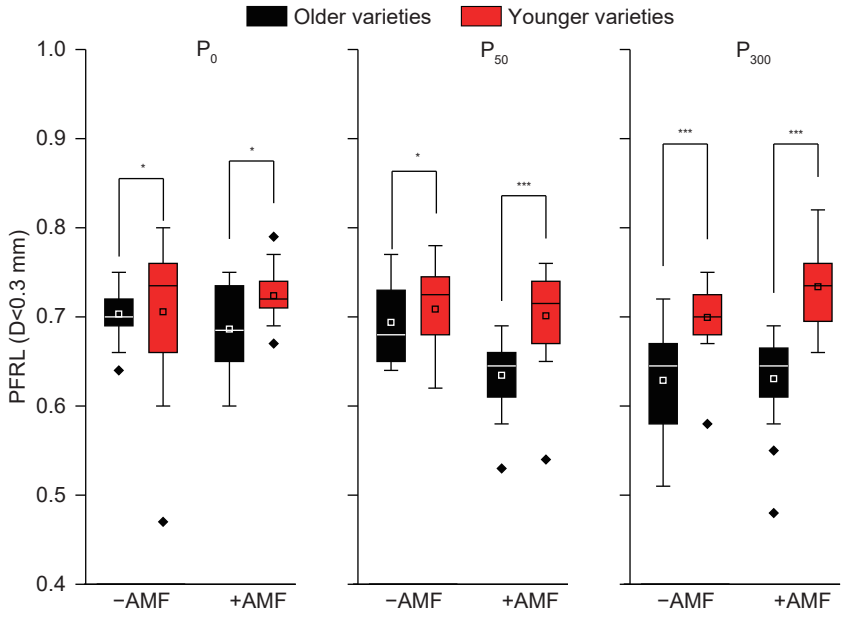

Fig. 5 Proportions of fine-root length (D<0.3 mm) (PFRL) of older and younger cotton varieties as influenced by P application (P_0, P_{50} and P_{300}) and arbuscular mycorrhizal fungi (AMF). P_0, P_{50} and P_{300} represent P addition rates of 0, 50 and 300 mg kg^{-1}, respectively. Boxes show first quartile, median and third quartile; whiskers and bars extend to the most extreme points within 1.5× box lengths. * and *** indicate significant differences between older and younger cotton varieties at P<0.05 and P<0.001, respectively (t-test).

affected by variety, mycorrhiza and P level (Table 1). Both enzyme activities decreased with increasing P levels (Appendix H). Some varieties had higher phosphatase levels when mycorrhizal, while other varieties showed an opposite pattern (Appendix H).

Non-inoculated treatments remained free from mycorrhizal colonization. Mycorrhizal colonization and HLD were significantly affected by variety, P level and the interaction variety×P level (Table 1). Mycorrhizal colonization was lower at the extreme P levels (P_0 and P_{300}) than at P_{50} (Appendix I). HLD decreased with increasing P (Appendix I). For both mycorrhizal root traits, there were significant varietal differences; however, these differences varied with P levels and no clear pattern was observed. There were no significant differences between older and younger varieties in terms of mycorrhizal colonization at any of the three P levels (Fig. 6-A). Compared with younger varieties, older varieties had a significantly higher HLD (5.1 *vs*. 4.4 m g^{-1}) at P_{50} but a significantly lower HLD (4.2 *vs*. 5.0 m g^{-1}) at P_{300} (Fig. 6-B).

3.5. Correlations between plant performance (shoot biomass and shoot P content) and root traits

Correlations between plant performance (shoot biomass and P content) and root traits are provided in Tables 2

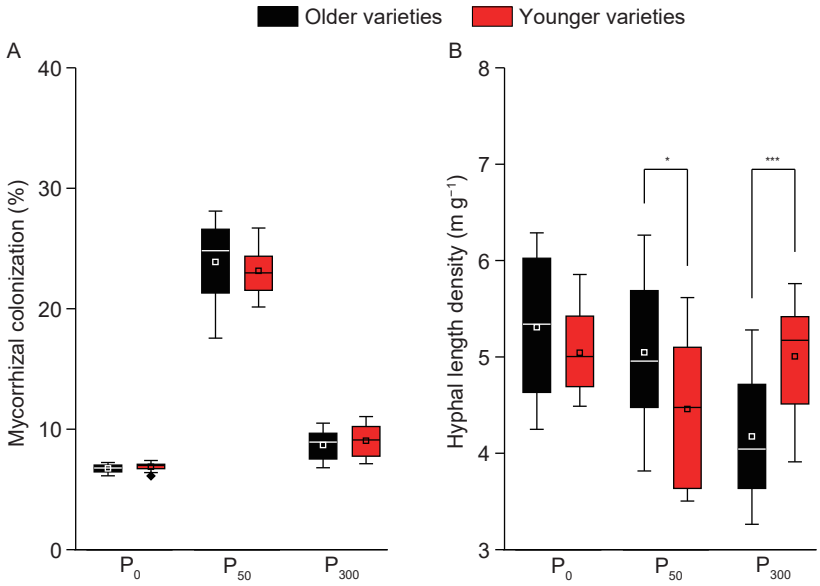

Fig. 6 Mycorrhizal colonization (A) and hyphal length density (B) of older and younger cotton varieties as influenced by P application (P_0, P_{50} and P_{300}). P_0, P_{50} and P_{300} represent P addition rates of 0, 50 and 300 mg kg^{-1}, respectively. Boxes show first quartile, median and third quartile; whiskers and bars extend to the most extreme points within 1.5× box lengths. * and *** indicate significant differences between older and younger cotton varieties at P<0.05 and P<0.001, respectively (t-test).

Table 2 Correlations (r values) between shoot biomass and root traits at different P application levels (P_0, P_{50} and P_{300}), in plants without and with arbuscular mycorrhizal fungus (AMF), *Funneliformis mosseae*, inoculation (n=8)[1)]

Variable	P_0		P_{50}		P_{300}	
	–AMF	+AMF	–AMF	+AMF	–AMF	+AMF
Root biomass	0.85**	0.71*	0.71*	0.79*	0.25	0.50
Root shoot ratio	–0.92**	–0.12	–0.77*	0.16	–0.27	0.14
Root length	0.27	0.11	0.51	0.49	0.54	0.43
Specific root length	0.42	0.35	0.53	0.45	0.62	0.37
Proportion of fine root length	0.00	–0.76*	–0.32	–0.12	–0.02	–0.05
Mean root diameter	0.66	0.46	0.46	0.91**	–0.14	0.30
Root tissue density	0.26	0.69	0.20	–0.47	–0.69	0.04
Phosphatase activity of rhizosphere soil	0.08	–0.39	–0.36	–0.04	0.74*	0.25
Phosphatase activity on root surface	0.77*	–0.07	0.66	0.27	–0.14	–0.14
Mycorrhizal colonization		–0.27		0.60		0.38
Hyphal length density		0.19		0.39		0.40

[1)] P_0, P_{50} and P_{300}, P addition rates of 0, 50 and 300 mg kg^{-1}, respectively.
* and ** represent significant correlations at P<0.05 and P<0.01, respectively (Pearson coefficient at P<0.05).

and 3. At P_0, the performance of non-mycorrhizal plants was significantly correlated with phosphatase activity on the root surface. There were no significant correlations between mycorrhizal-plant performance and root traits at P_0. At P_{50}, the performance of non-mycorrhizal plants was not significantly correlated with any root trait. For mycorrhizal plants, there were significantly positive correlations between root diameter and shoot biomass and P content (Table 2). At P_{300}, there were no significant correlations between root traits and plant performance. The physiological root traits, phosphatase activity on the root surface and in the rhizosphere, were not correlated with shoot P content at P_{50} or P_{300} (Table 3).

At P_{50}, mycorrhizal colonization was marginally positively correlated with MGR, MPR, AMGR and AMPR (P=0.1; Fig. 7), suggesting that the intensity of mycorrhizal colonization is important for plant biomass and plant P uptake. However, at P_0 and P_{300}, the correlations were not significant (data not shown). In addition, there were marginally significant negative correlations between shoot biomass of non-mycorrhizal plants and AMGR at each soil P level (r=–0.54, 0.05<P<0.1; r=–0.57, 0.05<P<0.1;

Table 3 Correlations (r values) between shoot P content and root traits at different P application levels (P_0, P_{50}, and P_{300}), in plants without and with arbuscular mycorrhizal fungus (AMF), *Funneliformis mosseae*, inoculation (n=8)[1)]

Variable	P_0		P_{50}		P_{300}	
	–AMF	+AMF	–AMF	+AMF	–AMF	+AMF
Root biomass	0.79*	0.55	0.46	0.76*	0.04	0.32
Root shoot ratio	–0.77*	–0.14	–0.74*	0.16	–0.04	–0.01
Root length	–0.077	0.12	0.38	0.48	0.32	0.13
Specific root length	0.10	0.29	0.32	0.41	0.15	0.13
Proportion of fine-root length	–0.22	–0.59	–0.05	–0.01	0.36	–0.05
Mean root diameter	0.67	0.39	0.05	0.87**	–0.67	0.27
Root tissue density	0.53	0.47	0.20	–0.47	0.16	0.13
Phosphatase activity of rhizosphere soil	–0.20	–0.33	–0.27	–0.07	0.10	0.03
Phosphatase activity on root surface	0.80*	–0.06	0.42	0.29	–0.14	–0.33
Mycorrhizal colonization		–0.64		0.62		0.22
Hyphal length density		0.47		0.51		0.09

[1)] P_0, P_{50} and P_{300}, P addition rates of 0, 50 and 300 mg kg^{-1}, respectively.
* and ** represent significant correlations at P<0.05 and P<0.01, respectively (Pearson coefficient at P<0.05).

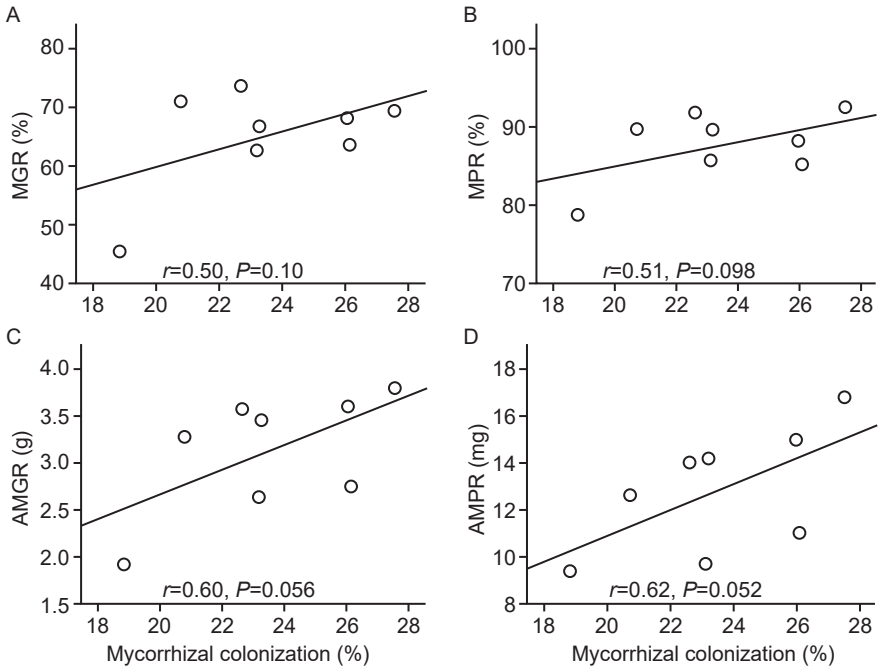

Fig. 7 Correlations between mycorrhizal colonization of eight cotton varieties and various relative or absolute mycorrhizal responses at P addition rate of 50 mg kg^{-1} (P_{50}). A, mycorrhizal growth responsiveness (MGR). B, mycorrhizal P responsiveness (MPR). C, absolute mycorrhizal growth responsiveness (AMGR). D, absolute mycorrhizal P responsiveness (AMPR). The r values are presented (Pearson coefficient at P<0.05).

r=–0.64, P<0.05; at P_0, P_{50}, and P_{300}, respectively; Appendix F), indicating that larger plants in the non-mycorrhizal condition exhibited lower mycorrhizal responsiveness.

4. Discussion

This study demonstrated that cotton breeding had directionally changed root traits, as younger varieties have thinner roots, higher SRL, and a higher fine-root fraction than older varieties on average (Fig. 1). Our first null hypothesis therefore was rejected. A consequence of this varietal selection is that modern cotton varieties exhibit more of a do-it-yourself P acquisition strategy and rely less on a collaborative strategy with AMF. As cotton is strongly dependent on and responsive to the AMF symbiosis, such a likely inadvertent and indirect selection against mycorrhizal symbiosis suggests the need for reconsidering and redirecting breeding efforts, considering the need to reduce the currently unsustainable level of P application in cotton cultivation (Singh *et al.* 2006). Our second null hypothesis was largely rejected as well, as older varieties performed better on average than younger varieties in the mycorrhizal condition, especially at the two lower P levels (Figs. 2 and 3). Differences in performance between older and younger varieties were largely absent only at P_{300}. Our third null hypothesis was not rejected for relative and absolute mycorrhizal growth responsiveness and relative mycorrhizal P responsiveness (Fig. 4), however, absolute mycorrhizal P responsiveness tended to be larger for older varieties.

4.1. Mycorrhizal benefits of cotton plants

Cotton was highly responsive to AMF. Compared with non-mycorrhizal plants, mycorrhizal cotton plants increased biomass by 111–193% (at P_0 and P_{50}) and plant P content by 39–744%, depending on P level. Mai *et al.* (2018) showed that cotton yield is closely connected to P uptake (and hence shoot P content), and so the larger positive effect of AMF on P content than on shoot biomass in this study is highly relevant for cotton production. Large beneficial effects of AMF on biomass, shoot P concentration and P content were also reported by Nehl and McGee (2010) and Wang *et al.* (2021). Root traits of cotton roots confirm their strong interaction with AMF. Our cotton varieties had roots with an average diameter (0.45 mm), which was somewhat smaller than the data in Guerrero-Ramirez *et al.* (2021) that indicated a diameter of 0.61 mm for domesticated plants and 0.73 mm for the wild ancestors of cotton. Wang *et al.* (2021) did not report average root diameter, but provided data for different diameter classes. In that study, 75–80% of the roots were less than 0.3-mm diameter, a number that fits well with our proportion of fine-root length (roots≤0.3 mm) of 65–75% (Fig. 5). The roots had a very low tissue density (0.06 g cm^{-3}), implying large cortical cells that provide space for AMF. Martin-Robles *et al.* (2019) similarly indicated a very low RTD for cotton (0.086 g cm^{-3}), which was slightly higher than in our cotton varieties. The reported colonization intensities of cotton roots show large variation, related to soil properties, cotton variety, AMF species, and possibly management, so it is therefore difficult to unequivocally support our findings of a large physical space for AMF with actual data on colonization. Mycorrhizal colonization was the highest at the intermediate P level, consistent with the unimodal relation between soil P availability and root colonization reported by Eskandari *et al.* (2017). A large variation in mycorrhizal colonization of roots of different cotton varieties was reported by Damodaran *et al.* (2012), who noted mycorrhizal colonization among 10 varieties varying between 37 and 74%. Variations in colonization can additionally be linked to the selectivity of cotton roots for certain species of AMF. Salgado *et al.* (2017) compared five species of AMF and noted root colonization of around 20–30% by four fungal species, similar to the colonization by *F. mosseae* in our study, but around 90% by *Rhizophagus clarus*, however, the levels of colonization were not correlated with plant biomass. Another factor that determines the variability in colonization in different studies could be inoculum limitation to achieve maximum colonization. Zak *et al.* (1998) reported much higher colonization levels when cotton was grown in fields in which wheat was previously grown than in fields with cotton under conventional tillage. Cely *et al.* (2016) noted colonization levels of 80% when cotton was inoculated with *R. clarus*, but only 50% in the field without inoculum addition. Inoculum limitation is a possible issue in plant breeding arenas, and this topic deserves to receive more attention. Hyphal length density varied between 3 and 6 m g^{-1} soil, comparable to data by Wang *et al.* (2021), who reported values between 2 and 6 m g^{-1} soil. They reported a lower hyphal length density at the higher P level, similar to our study.

At P_{50}, the shoot P concentration with AMF was significantly higher than that without AMF at P_{300} (Appendix C), and the shoot P content with AMF at P_{50} was not significantly different from that without AMF at P_{300} (Appendix C), suggesting the potential for considerable P fertilizer savings when cultivating cotton under adequate mycorrhizal management. Other studies have shown similar possibilities for fertilizer savings with adequate mycorrhizal management. Wang *et al.* (2020b) showed that the performance of normal mycorrhizal maize plants

at low P availability was almost equal to that of reduced-mycorrhizal plants at much higher P availability. For cotton, Mai *et al.* (2018) found that a P application rate of 75 kg P ha^{-1} increased both root length and hyphal length to the same extent as a P application rate of 150 kg P ha^{-1}, and while saving 50% of the P, these plants gave 90% of the yield of the high application rate. Considering that older varieties in the mycorrhizal condition at P_{50} outperformed younger varieties (Fig. 2), a mycorrhizal focus both at the management level and in varietal choice for cotton seems imperative.

4.2. Breeding cotton for mycorrhizal symbiosis

Differential performance among different varieties of the same plant species with respect to mycorrhizal benefit, either absolute or relative, has been reported for many crops (Kuyper *et al.* 2021). The older varieties frequently exhibited a larger relative mycorrhizal responsiveness and such observations have given rise to the hypothesis that plant breeding under the current, often nutrient-rich, conditions has inadvertently selected against mycorrhizal responsiveness; and that, therefore, mycorrhizal responsiveness has to be re-introduced in plant breeding for sustainable agriculture. It has become clear, however, that the use of relative mycorrhizal responsiveness as a potential selection target for breeding is highly problematic (Galván *et al.* 2011; Kaeppler *et al.* 2000; Sawers *et al.* 2010). Because the performance of the non-mycorrhizal plant is in the formula of relative responsiveness (eq. (2)), the negative relationship is partially mathematical rather than strictly biological, with the highest values for relative mycorrhizal responsiveness being associated with plants that perform most poorly when non-mycorrhizal. These properties make the relative mycorrhizal responsiveness an unlikely desirable trait for plant breeding. We found highly significant negative correlations at P_0 and P_{50} between performance of the non-mycorrhizal cotton plant and relative mycorrhizal growth responsiveness (at P_0 and P_{50}, r=–0.92 and r=–0.86, respectively; Appendix F), confirming the unsuitability of the relative mycorrhizal growth responsiveness as a selection target.

One alternative, proposed by Janos (2007) and Galván *et al.* (2011), could be to use absolute mycorrhizal responsiveness (eq. (1)). Applying that formula showed a marginally significant negative relationship between the performance of the non-mycorrhizal plant and absolute mycorrhizal growth responsiveness (at P_0 and P_{50}, r=–0.54 and –0.57, respectively; Appendix F). Thus, that parameter provides no clear criterion for the selection for enhanced benefit from the mycorrhizal symbiosis, as plant breeders are ultimately interested in larger plants with higher P content under normal field conditions, i.e., when plants are mycorrhizal. Wang *et al.* (2021) also tested cotton varieties for absolute mycorrhizal growth and P responsiveness. They observed no effect of year of varietal release on absolute mycorrhizal growth or P responsiveness at high P, and no effect of year of release on absolute mycorrhizal P responsiveness at low P, but a positive effect of year of release on absolute mycorrhizal growth responsiveness at low P. However, they also showed a negative correlation between the performance of the non-mycorrhizal plant and absolute mycorrhizal growth responsiveness (r=–0.53, P=0.008), confirming that selection for absolute mycorrhizal growth responsiveness is problematic.

Finally, Sawers *et al.* (2010) proposed a method to differentiate between what they referred to as dependence- and non-dependence-based variation in responsiveness, by regressing the performance of non-mycorrhizal against mycorrhizal plants and partitioning the variance, with the dependence-based part of responsiveness being equal to the r^2 of the regression, and the non-dependence part (which is the target for selection) being the residual variation. The application of this method at P_{50}, as the more likely soil fertility condition for the selection arena, indicated that the performance of non-mycorrhizal and mycorrhizal plants was uncorrelated (r=–0.08; data not shown), and that therefore selection based on this method would equate to selection for the best performing plants in the mycorrhizal condition, independent of the (absolute or relative) benefits that the plants derive from being mycorrhizal.

There was no relation between mycorrhizal colonization and the year of variety release, which is consistent with An *et al.* (2010), who found that modern plant breeding programs in maize did not lead to the suppression of colonization. Mycorrhizal colonization is often an important predictor for mycorrhizal responsiveness (Lekberg and Koide 2005; Lehmann *et al.* 2012). In our study, mycorrhizal colonization at P_{50} was marginally significantly positively correlated with absolute and relative mycorrhizal responsiveness as well as mycorrhizal P responsiveness (Fig. 7), suggesting that varietal selection for high mycorrhizal colonization could be an additional strategy to improve cotton growth and P efficiency. Mycorrhizal colonization is correlated with root diameter and especially cortical area (Valverde-Barrantes *et al.* 2016), and so the positive relationship between mycorrhizal colonization and plant performance could be mediated by root diameter, implying that root diameter as a reflection of the underlying strategy in the root economics space might be a better direct selection target. Wang *et al.* (2021) suggested that hyphal length density at low P supply could be a selection target, because of its positive correlations with shoot

biomass and shoot P content.

Plant biomass was greater for the four varieties that were released before 2000 than for the four varieties released after 2000 (Fig. 2). Those older varieties also had thicker roots (and root diameter was the best predictor for plant performance at P_{50}; Tables 2 and 3), lower SRL, and lower fine-root fraction than the more modern varieties. Younger varieties had a smaller root diameter but retained the low root tissue density of the older varieties. Similarly, Martín-Robles *et al.* (2019) observed that plant breeding did not result in changes in RTD in cotton. We therefore conclude that breeding had resulted in cotton plants with thinner roots and reduced collaboration with AMF (Bergmann *et al.* 2020) that, consequently, performed more poorly except under the conditions of a very high P supply. At that high P supply (P_{300}), differences in performance between the older and younger varieties disappeared.

We do not know why cotton breeding has resulted in plants with thinner roots. Cotton plants with thicker roots (and with more mycorrhizal association) not only acquired more P, but other studies have shown that cotton plants with thicker roots are more tolerant of drought (McMichael *et al.* 1985) and have higher levels of mycorrhizal colonization, contributing to the alleviation of salinity stress (Liu *et al.* 2016). One possible explanation for breeding for thinner roots could be that cotton plants with thinner roots and higher SRL were more P-efficient in hydroponic systems (Chen *et al.* 2019). For example, Martin-Robles *et al.* (2019) compared 30 domesticated plants with their wild progenitors. While they noted no consistent pattern of domestication on root diameter, the data for cotton indicated that breeding has resulted in a significant shift towards thinner roots than its wild progenitor (from 0.73 to 0.61 mm; data in Guerrero-Ramirez *et al.* 2021). One possible explanation for this selection effect could be domestication towards a more annual life style, with possible consequences of selection for a larger reproductive output and a shift towards a larger investment in aboveground tissues and a lower investment in roots. The lower root biomass of younger compared to older varieties, irrespective of P level (Fig. 3), supports this explanation. A similar shift towards a more annual life style, in combination with a larger reproductive output, a lower root/shoot ratio, thinner roots and a lower mycorrhizal responsiveness, has been noted for the invasive plant *Hypericum perforatum* L. in North America compared to conspecific plants in its native area in Europe. This selection towards an annual life style could have been especially successful in a more nutrient-rich and/or AMF-depleted environment (Seifert *et al.* 2009). The breeding arena for cotton potentially reflects comparable environmental conditions of regular disturbance and high nutrient levels. An alternative explanation is suggested through the work by Xiao *et al.* (2020). They noted a positive correlation between root diameter and root life span, and also observed that drought induced enhanced root turnover. Therefore, they suggested that plants with thinner roots are in an advantageous position under drought, a suggestion that contrasts with the results of McMichael *et al.* (1985). Further studies are recommended regarding the selection criteria and the breeding arenas applied by cotton breeders, and the possible consequences for mycorrhizal root traits.

5. Conclusion

All eight cotton varieties in this study were responsive to AMF. Cotton plants exhibited the collaborative strategy in the root economics space as defined by Bergmann *et al.* (2020). Over time, cotton breeding has resulted in plants with thinner roots, implying a shift towards a do-it-yourself strategy. However, that switch resulted in reduced performance of these younger varieties, except at the highest P level. In order to achieve good cotton yields while reducing fertilizer P application, to achieve better environmental sustainability and to reduce the negative environmental impacts from the overuse of fertilizer, breeding efforts should be directed toward the interplay of root traits and mycorrhiza-related traits, in order to move away from what can best be described as inadvertent plant breeding against mycorrhizal symbiosis.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendices associated with this paper are available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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