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RESEARCH ARTICLE

Root exudates drive root avoidance of maize in response to neighboring wheat

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Abstract
Background and Aims Plant roots respond to soil nutrient availability, but also to the identity of neighbor plants, and root exudates play a role therein. However, how root exudates influence root behavior is not well understood.
Methods A sequence of eight experiments was designed to investigate whether and how root growth and distribution of maize (*Zea mays* L.) was affected when growing with neighboring wheat (*Triticum aestivum* L.). We focused on the role of 6-methoxy-benzoxazolin-2-one (MBOA), an important allelochemical in root exudates.
Results We found that maize roots distributed away from neighboring wheat roots but not from other roots. Root length of maize was reduced by 37%, 40%, and 64% when maize was grown with live wheat plants, with residuals of wheat root exudates, or when directly treated with wheat root exudates, respectively. MBOA concentration in root exudates of wheat/maize intercropping was 315% higher than in maize monoculture. The expression of IAA-related genes in maize roots was down-regulated by the MBOA treatment. MBOA addition decreased maize root length, but wheat root length was not affected under the same concentration.
Conclusions Our findings demonstrate that root exudate MBOA is an important specific mediator in maize-wheat interspecific interactions, providing new insights into the design and management of sustainable intercropping systems.

Keywords Belowground competition · Interspecific interactions · MBOA · Root distribution · Root exudates

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Xiao-Tong Yin, Fang-Fang Zhang and Rui-Peng Yu contributed equally to this work.

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Abbreviations

MBOA 6-Methoxy-benzoxazolin-2-one
DIMBOA 2,4-Dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one

Introduction

Interspecific interactions among plant species are important in influencing plant growth and co-existence across both natural and agricultural ecosystems (Callaway and Mahall 2007). Root-root interaction is a key belowground process of interspecific competition and facilitation among plants, whereby root distribution patterns may result from intrusive (approaching, over-proliferation), avoidance (repelling, underproliferation) or unresponsive interactions (Semchenko et al. 2007a, 2014). Root avoidance, occurs when plants adjust the growth of their primary and secondary roots to adapt to the biotic or abiotic environment by inhibition/promotion of primary/lateral root growth (Jacobsen et al. 2021; Khare et al. 2017; Wang et al. 2020). Evidence has been accumulating that root exudates mediate root behaviors, and could significantly affect plant growth (Li et al. 2016; Mommer et al. 2016), especially in species-diverse plant communities (Cabal et al. 2020; Griffiths et al. 2022).

Initially, root-root interactions were thought to mainly occur via nutrient competition whereby nutrients acquired by an individual plant would no longer be available to a neighboring plant (Chen et al. 2012; Fransen and De Kroon 2001; Hodge 2004). Root exudates are defined as the complex mixture of organic compounds that are actively secreted by plant roots into the surrounding rhizosphere soil (Bais et al. 2006). Recently, a growing body of studies have demonstrated that belowground facilitation and competition mediated by root exudates play an important role in influencing productivity (Jiao et al. 2021; Yu et al. 2021). For example, in an intercropping system with maize (*Zea mays* L.) and faba bean (*Vicia faba* L.), the acidification of the rhizosphere due to root exudation from faba bean plants contributes to releasing more soil phosphorus, which can greatly enhance the growth of maize (Li et al. 2007). Increased soil phosphorus availability induced by faba bean root exudation stimulates phosphorus uptake in neighboring maize (Zhang et al. 2016).

Yet the effects of root exudates can be very diverse and include also allelopathic effects whereby chemicals released in the rhizosphere inhibit the growth and development of neighboring plant species (Meiners et al. 2012). For example, increased momilactone B and tricin exudation by rice plants was detected in the presence of barnyard grass seedlings or barnyard grass root exudates, inhibiting the growth of neighboring plants (Li et al. 2020). Several studies also showed how individual plants exposed to root exudates can significantly increase root density and alter root morphology depending on the identity of the species producing the exudates (Ljubotina and Cahill 2019; Semchenko et al. 2014). The specificity of these rhizosphere interactions is intriguing, and more attention should be paid to the biochemical cues that mediate these interactions.

Benzoxazinoids (BXs) are allelochemicals exuded from the roots of gramineous species into the rhizosphere, where they can have multiple functions inhibiting the growth of neighboring plants and/or altering fungal and bacterial infections (Hazrati et al. 2020; Reiss et al. 2018). The benzoxazinoids 2,4-dihydroxy-(2H)-1,4-benzoxazin-3(4H)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-(2H)-1,4-benzoxazin-3(4H)-one (DIMBOA) are main allelochemicals of wheat (*Triticum aestivum* L.), maize, and rye (*Secale cereale* L.) (Belz and Hurle 2005). DIMBOA is rapidly transformed into 6-methoxy-benzoxazolin-2-one (MBOA) once released into aqueous solution or soil (Macias et al. 2004; Wu et al. 2000). DIMBOA and MBOA have a relatively broad spectrum of activity on weeds (Hazrati et al. 2020), and MBOA is more resistant to degradation in soil (Kong et al. 2019; Macias et al. 2004). Wheat releases more MBOA to effectively inhibit the growth of neighboring weeds (Chen et al. 2010; Kong et al. 2018).

Auxin plays an important role in regulating root growth and development, affecting root gravitropism, or the direction of plant root growth (Hu et al. 2021; Moret et al. 2020). In *Arabidopsis*, primary root elongation was inhibited by a model allelochemical benzoic acid (BA), the phenomenon was associated with increased expression of auxin biosynthesis and polar transporter genes *AUX1* and *PIN2* (Zhang et al. 2018). A recent experiment showed that MBOA reduced auxin levels and influenced lateral auxin distribution by suppressing expression of the auxin flux-facilitators-*PIN2* and the auxin-responsive reporter gene *DR5* ultimately determining root bending and affecting root-growth direction (Wang et al. 2023). Inhibited roots were consistent with the reduction of gene expression in auxin synthesis (*TAR/YUCs*), transport (*LAXs, PINs*), and signaling (*IAAs, ARFs*) under magnesium limitation (Ishfaq et al. 2021).

Our previous studies showed that wheat roots could spread under neighboring maize plants, whereas the lateral spread of maize roots under wheat plants was significantly limited in wheat/maize intercropping under field conditions (Li et al. 2006). Even in the presence of sufficient nutrient supply, maize roots distributed away from the wheat root zone (Liu et al. 2015).

This study was conducted to gain a thorough understanding of root exudate MBOA-mediated root avoidance of maize in response to neighboring wheat and their underlying mechanisms. For this purpose, we performed a series of eight multi-faceted experiments under controlled conditions to investigate the root placement and morphology of maize are affected by neighboring wheat. Furthermore, we addressed the tolerance levels of maize and wheat to MBOA. We verified the role of MBOA in determining root avoidance of maize by inhibiting root growth, and determined the action mechanisms through the gene expression of auxin biosynthesis, transport and signaling processes in primary and seminal roots.

Methods

Pre-treatment and growth conditions of the three crop species

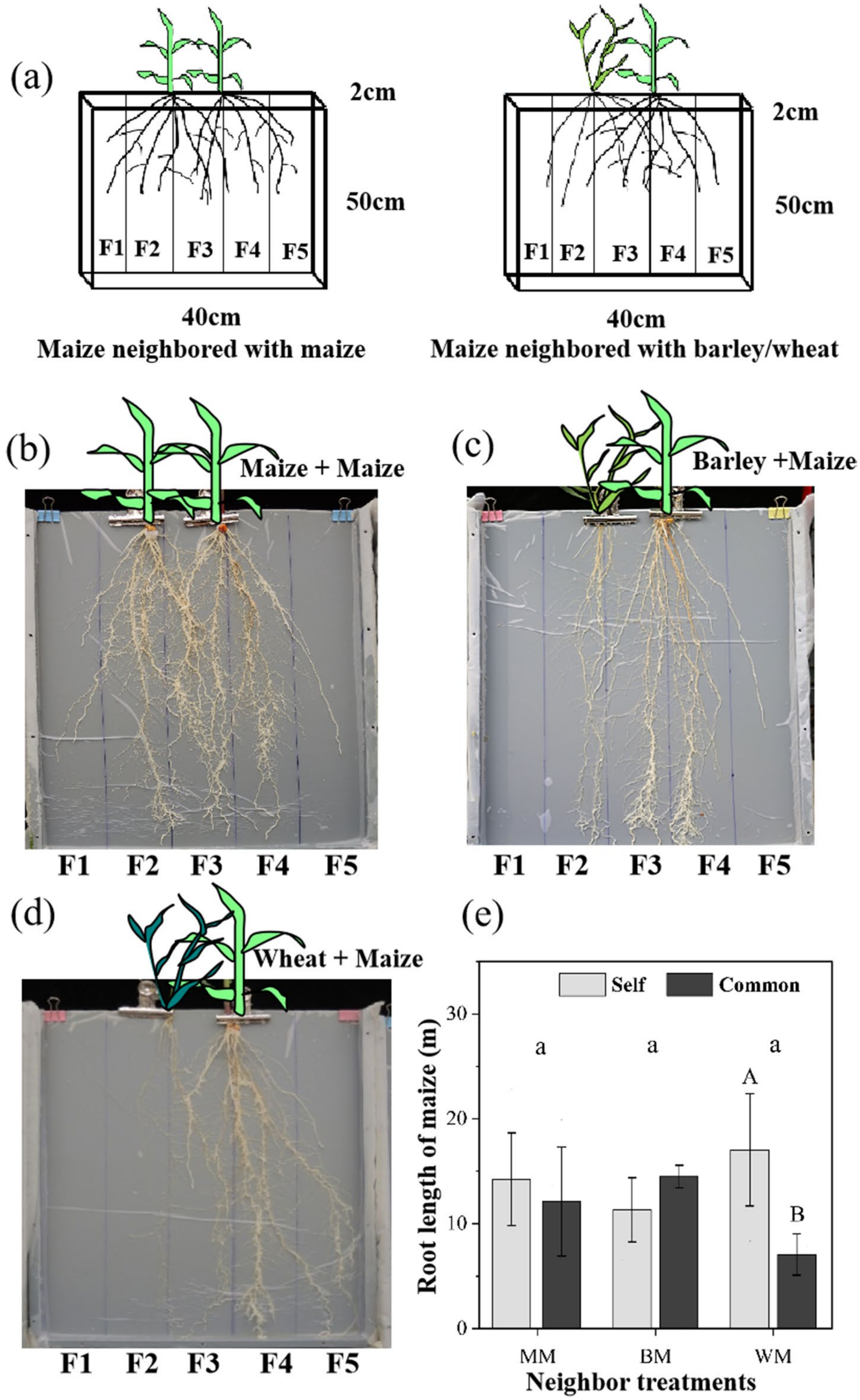
Seeds of wheat (cv. Yongliang No. 4), barley (cv. Ganpi No. 4) and maize (cv. Zhengdan No. 958) were ordered from a commercial seed company (Bosheng Inc., Wuwei, Gansu Province, China). Seeds of wheat and barley were surface-sterilized with 70% (v/v) alcohol for 2.5 min and rinsed with sterilized distilled water three times. Then, we sterilized the seeds with sodium hypochlorite (2.5% v/v) for 15 min and washed them three times in distilled water. Seeds of maize were firstly surface-sterilized with 70% (v/v) alcohol for 10 min and rinsed with sterilized distilled water three times; then, sterilized with hydrogen peroxide (15% v/v) for 20 min and washed three times in distilled water. In all experiments, seedlings were grown in a controlled glasshouse with daily cycles of 16 h.-light: 8 h.-dark; temperatures ranged between 18–22 ˚C at night and 25–30 ˚C during the day.

Seedlings received nutrient solution in all experiments: 0.65×10^{-3} mol L^{-1} $MgSO_4$, 0.75×10^{-3} mol L^{-1} K_2SO_4, 0.1×10^{-3} mol L^{-1} KCl, 2.0×10^{-3} mol L^{-1} $Ca(NO_3)_2$, 0.25×10^{-3} mol L^{-1} KH_2PO_4, 1.0×10^{-6} mol L^{-1} $MnSO_4$, 1.0×10^{-6} mol L^{-1} $ZnSO_4$, 5.0×10^{-9} mol L^{-1} $(NH_4)_6Mo_7O_{24}$ and 1.0×10^{-4} mol L^{-1} EDTA-Fe. The pH of the nutrient solution was adjusted to 6.0 daily using 0.01 mol L^{-1} HCl and 0.01 mol L^{-1} NaOH. In Experiment. 1, we used a sterile syringe to extract 200 ml of nutrient solution and evenly irrigate the area around the roots of the target plant. In experiment. 2, we regularly added deionized water to maintain the soil moisture, 200 ml of nutrient solution were added every day to ensure that the crops have sufficient nutrition with quartz substance. In Experiment. 6, 7, 8, the planting device used is divided into two parts: the upper part is the 1.25 L planting area, and the lower part is the 1 L nutrient solution area. To ensure sufficient nutrients, the nutrient solution was changed every 2 days.

Eight glasshouse experiments

Experiment. 1: Root distribution of maize with wheat or other neighbors

First, we examined the root distribution patterns of maize in response to different neighbors. Pre-germinated maize, barley and wheat seeds were planted into root boxes (each 40 cm × 2 cm × 50 cm) containing quartz sand (diameter: 0.7 mm; Fig. 1a). Each root box was made of 2 PVC panels. The front panel (panel A) could be removed to assess changes in root distribution and morphology. A fine nylon mesh (mesh size 37 μm) was put at the interface between the substrate and the unremovable PVC panel (panel B) to indicate the 5 vertical sections, each fraction consisted of the same volume of the substrate with 8 cm long, 2 cm wide and 50 cm deep. The root boxes were inclined 45 degrees to ensure roots would grow along the nylon mesh. We applied three treatments: (1) maize neighbored with maize (T1), (2) maize neighbored with barley (T2), and (3) maize neighbored with wheat (T3; Fig. 1a; n = 4). These treatments refer to field conditions where there are only intraspecific interactions between individual maize plants in monoculture, and both intraspecific


(a)
2cm
50cm
F1 F2 F3 F4 F5
40cm
Maize neighbored with maize
2cm
50cm
F1 F2 F3 F4 F5
40cm
Maize neighbored with barley/wheat
(b)
Maize + Maize
F1 F2 F3 F4 F5
(c)
Barley +Maize
F1 F2 F3 F4 F5
(d)
Wheat + Maize
F1 F2 F3 F4 F5
(e)
Self
Common
Root length of maize (m)
0
10
20
30
a
a
a
A
B
MM
BM
WM
Neighbor treatments

◂**Fig. 1** (**a**) Experimental setup of Experiment. 1. (**b–d**) Root distribution and (**e**) root length of maize grown neighbored with maize, barley or wheat in different locations (Experiment. 1). (a) Experiment. 1 included three treatments: (1) maize grown with maize, (2) maize grown with barley and (3) maize grown with wheat. Seedlings were planted 8 cm from each other. Root boxes were divided into 5 rooting zones (F1-F5). Roots in the five fractions (indicated by F1-F5) were separated for the two plants, respectively. Fraction 2 and fraction 3 contained all maize roots growing toward the neighbor (maize, barley or wheat) and we defined these areas as 'common-growing area'. While fraction 4 and fraction 5 contained all maize roots growing away from neighbors (maize, barley or wheat) and we defined these areas as 'self-growing area'. Values are means (n = 4) ± SE. Different letters denote significant differences among treatments ($P<0.05$). Uppercase letters refer to differences between 'self-growing area' and 'common-growing area'. Lowercase letters indicate difference between different neighbor treatments

between maize plants and interspecific interactions between maize and barley or wheat plants. Plants were harvested after 3 weeks, by removing PVC panel A and gently washing away the sand to ensure that the root distribution of plants was not affected. Sharp metal blades were used to separate roots into five vertical sections on the nylon mesh (F1 to F5 in Fig. 1a). Roots of both plants were visually separated. Maize roots are white, smooth and thick (diameter about 2 mm), while barley and wheat roots are yellow and smaller in diameter (< 0.5 mm), which made the separation of different species easy. For maize neighbored with maize treatment, because the root system of the maize plants is relatively coarse, we were able to disentangle the root systems of both maize plants. In the present experiment, roots grew in all sections (F1 to F5) and we defined F1-F2 as 'Self-growing area' while F3-F4 as 'Common-growing area' for the left seedling (maize, barley or wheat; Fig. 1a). Similarly, we defined F4-F5 sections as 'Self-growing area' and F2-F3 as 'Common-growing area' for the right seedling (always maize-Fig. 1a).

Experiment. 2: Root morphology of maize plants as affected by wheat/maize intercropping

This experiment aimed to test whether the growth and root morphology of maize can also be suppressed by neighbor wheat plants. Three cropping systems were included in this experiment, i.e. maize monoculture, wheat monoculture and maize/wheat intercropping, with four replicates. To test the effects of neighbor wheat plant on root morphology of maize under with and without soil microorganisms, two substrates, soils (with soil microorganisms) and quartz sands (without microorganisms), were adopted. The soil was a low-P soil collected from Shangzhuang experimental station in Beijing. The soil properties and additional nutrient supply were in line with Zhang et al. (2016). Pre-germinated maize and wheat seeds were planted into pots (1.25 L each) filled with 1L quartz sand (diameter: 0.7 mm) or 1 L soil. A fine nylon mesh (mesh size 37 μm) was put to divide the pot into two equal-volume compartments. Nylon mesh separation allows the flow of water and root exudates, which is a common method to examine the root-root interaction between plants. 10 wheat seedlings and 2 maize seedlings were cultivated in each compartment as a monoculture; 5 wheat seedlings were cultivated in the left compartment and 1 maize seedling was cultivated in the right compartment as intercropping. After 35 d of growth, shoot and root samples of each crop species were harvested separately. Shoot and root biomass, and root morphological parameters of maize and wheat were measured.

Experiment. 3: The effect of root exudates residue from wheat on root morphology of maize

Here, we further examined the role of root exudates of wheat on the root morphology of maize. Agar was used as a growth medium to determine the growth and morphology of maize roots in response to the presence/absence of root exudates from wheat and neighbored wheat plants. Thus maize was grown in a beaker (1 L) filled with agar (200 mL 0.4% agar and nutrient solution) under 3 different treatments: (1) maize growing alone, (2) maize growing with wheat after wheat grew there already for 7 days (i.e. in the presence of wheat roots), and (3) maize alone but after the initial wheat plants were removed (i.e. in the presence of wheat root exudates but not wheat roots) (Fig. 3a). At day 1, fifteen surface-sterilized and pre-germinated wheat seeds were selected and aseptically sown on one half of the agar surface with the embryo up (treatments 2 and 3). The beaker was wrapped with a parafilm membrane and placed in a sterile biochemical incubator. On day 7, one pre-germinated maize seed was sown in the other half of the agar surface (all treatments). For treatment 3 (root exudates only) whole wheat seedlings were removed while maize was sown on day 7. After sowing the maize, the beaker was again wrapped with a parafilm membrane and returned to the biochemical incubator (Fig. 3a). On day 21, the root morphological parameter of the 14-day-old maize seedling was measured.

Experiment. 4: The role of root exudates in interspecific root-root interactions

Experiment. 4 was designed to measure the response of maize roots to exudates from different cereal crop roots (Fig. 3c). Sixteen pre-germinated maize seeds were planted in 1.25 L pots (one maize seed per pot) filled with 1 L quartz sand (diameter: 0.7 mm)0.7-day-old maize seedlings received 4 different treatments for 5 weeks: (1) maize root exudates added to the nutrient solution (50 mL), (2) barley root exudates added to the nutrient solution (50 mL), (3) wheat root exudates added to the nutrient solution (50 mL) and (4) a nutrient solution (50 mL) only. In order to collect root exudates of maize (2 seedlings), barley (10 seedlings) and wheat (10 seedlings), we previously cultivated the different plants (7 day-olds) in nutrient solutions where roots were exposed to 50 mL nutrient solution for 2 h every day, and the root exudates were collected repeatedly on the same plants (Fig. 3c). After collection, different root exudates or nutrient solution (50 mL pot^{-1} day^{-1}) were immediately added to the maize seedlings pots with sterile syringes. Roots and shoots of maize seedlings were harvested separately 7 days after treatments. There were 4 replicates for each treatment.

Experiment. 5: The concentration of MBOA in root exudates of (a) maize grown with wheat or (b) maize grown with maize

MBOA is identified as an allelochemical inducing negative root-root interaction. We found that MBOA extracted in wheat exudates exhibited a similar inhibition on maize root growth compared with crude wheat exudates and MBOA standard substance in a pre-experiment (See Supplementary experiment, Table S8). Therefore, we mainly focused on the effects of MBOA. This experiment was specifically designed to detect changes in the exudation of MBOA in monoculture and wheat/maize intercropping. To detect early signals involved in the specific root response, we performed a pot experiment with three treatments including (1) maize grown with maize (2 seedlings in each pot), (2) wheat grown with wheat (10 seedlings per pot), and (3) maize grown with wheat (1 maize seedling and 5 wheat seedlings per pot). Each treatment was replicated 4 times. Pre-germinated wheat and maize seeds were cultivated in 2 L glass container filled with sterilized quartz sand (diameter: 0.7 mm). Seedlings were irrigated with nutrient solution at a rate of 100 mL day^{-1} (Fig. 3e). The maize and wheat seedlings were well established after 7 days.

In the present experiment, we adopted a hydrophobic root exudate trapping system (Tang and Young 1982) to constantly collect MBOA in root exudates of the 3 different treatments. XAD-7HP (Sigma-Aldrich Inc., St. Louis, MO, USA) was used as a synthetic adsorbent resin, because of its high affinity to phenolic compounds and benzoxazinoids (de Araujo Padilha et al. 2019; Larsen and Christensen 2000). The resin was pretreated with ethanol for 24 h, followed by 1 mol L^{-1} NaOH and 1 mol L^{-1} HCl, respectively for 4 h, and then washed with a large amount of distilled water. The XAD-7HP cleaned resin was packed in a glass column (volume: 60 mL) with following a well-described protocol for creating a hydrophobic root exudate trapping system. Each pot replicated under each of the three treatments (wheat-wheat, maize-maize and wheat–maize) was watered with distilled H_2O (8×250 mL). A XAD-7HP resin column and the circulating attachment were then connected to the pot (Fig. 3e). The nutrient solution was continuously circulated through the system at a rate of about 1 L h^{-1} by air pump to elute extracellular organics from the sand culture that maize or wheat seedlings were grown in. The nutrient solution was replenished twice daily to compensate for transpiration and evaporation losses. The XAD-7HP resin column was detached after 3 days, washed with 200 mL of distilled H_2O treated with MICROPUR (Katadyn Inc., Switzerland), and then eluted with 200 mL acetone (HPLC grade, Sigma-Aldrich Inc., St. Louis, MO, USA). The eluates containing MBOA were evaporated to dryness in a vacuum. Dry residues were dissolved in 2.5 mL acidified methanol (0.5% formic acid, HPLC grade, Sigma-Aldrich Inc., St. Louis, MO, USA) and filtered through a 0.22 μm filter. The solution was then injected into the HPLC for quantitative analysis.

The quantification of MBOA was performed by liquid extraction-solid-phase extraction followed by

HPLC. The quantitative analysis of MBOA was carried out with an HPLC instrument (HP-1260; Agilent Inc., Santa Clara, CA, USA) equipped with a C_{18} reverse-phase column (Hypersil 250 mm×4.6 mm, 5 μm) with a diode array UV detector. The detection wavelength was 280 nm. The injection volume of samples was 5 μL. Elution was performed with a mixture of 0.5% acetic acid for quantitative analysis of aqueous solution and methanol (65:35, v/v) at a constant flow rate of 0.6 mL min^{-1} at a temperature of 40 °C. The peak of allelochemical MBOA was identified by its retention time (via coelution with MBOA standard, ca 27.1 min). MBOA was quantified by regression analysis of the peak areas against standard concentrations.

Experiment. 6: Effects of different concentrations of MBOA on root morphology of maize and wheat

Experiment. 6 was conducted to test whether different MBOA concentrations inhibited root growth of maize seedlings and wheat seedlings in a dose-dependent manner. Two pre-germinated maize or wheat seeds were planted into a 1.25 L pot containing 1 L fine quartz sand (diameter: 0.7 mm). When two leaves were visible, one maize or wheat individual with uniform size was left, avoiding intraspecific interaction. In a pre-experiment, we showed that 0.3 μg L^{-1} MBOA exhibited a negative effect on the root growth of maize. Here, five MBOA concentration was selected along a gradient, i.e. 0 μg L^{-1},0.3 μg L^{-1}, 1 μg L^{-1}, 5 μg L^{-1}, 10 μg L^{-1}. We used MBOA standard solution (Sigma-Aldrich, Inc., St. Louis, MO, USA) to prepare different concentrations of MBOA. Every seedling was treated daily with 20 mL MBOA with different concentrations by using sterile syringes for 30 days; 20 mL ultrapure water was used as control (0 μg L^{-1} MBOA). Each treatment had five replicates. After 30 d of growth, plant shoots and roots were harvested, and the biomass and root morphological parameters of maize and wheat plants were measured.

Experiment. 7: Effect of MBOA on root morphology of maize

Experiment. 7 was conducted to further test whether MBOA inhibited the primary root or seminal root of maize. Based on Experiment. 6, we selected 0.3 μg L^{-1} MBOA in this experiment. The experimental device and operation are the same as those in Experiment. 6. There were two treatments (0 μg L^{-1} and 0.3 μg L^{-1} MBOA as standard substance) with four replicates. After 16 d of growth, plant shoots and roots were harvested, the primary root and seminal roots were carefully separated for each plant's roots and the biomass and root morphological parameters of maize plants were measured.

Experiment. 8: Effect of MBOA on the expression of IAA-related genes in maize roots

Based on Experiment. 7, primary roots and seminal roots from each root were sampled to further analyze the expression of auxin-related genes. At 6, 12, 24, 48, 96 and 192 h after adding 0.3 μg L^{-1} MBOA, harvesting root and washing with ultrapure water until no quartz sand is attached to root, root samples were wrapped by tinfoil and immediately put in liquid nitrogen. Total RNA was extracted from 100 mg powdered (in liquid nitrogen) root samples using the Trizol reagent according to the manufacturer's protocol. The quality of extracted RNA was checked using a NanoDrop Spectrophotometer. After digestion with RNase-free DNase I (Takara Biomedicals, Kyoto, Japan) to avoid potential DNA contamination, reverse transcription of RNA samples into cDNA was conducted by M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA United States). Quantitative PCR (RT-qPCR) was performed using the Bio-Rad iCycler iQ5 system (Bio-Rad, Hercules, CA, United States) to quantify relative gene expression by using cDNA with the SYBR Premix Ex Taq™ (Takara). The designed primers of genes are listed in Table S1. The experiment for each gene was performed with four biological replications. The relative gene expression was determined via the Eq. 2ˆ-(ΔΔCt) method (Livak and Schmittgen 2001).

Measurement of root and shoot biomass across all experiments

All root samples in the above experiments were scanned with an Epson Perfection V750 Pro (Epson Inc., Nagano, Japan). Root morphological parameters, such as root length and root surface area were then obtained from the scanned images using WinRHIZO root analysis software (Régent Instrument Inc., Québec, Canada). Roots were dried at 60 ºC for 4 days prior to weighing. Specific root length was calculated as total root length divided by root biomass. The shoot samples were initially heated at 105 °C for 30 min, followed by drying at 80 °C until a constant weight was achieved. The dried samples were then weighed to determine the shoot biomass.

Statistical analyses

We performed analysis of variance (ANOVAs) to test for the potential effects of neighbor plant identity on the root growth of maize. Before conducting the one-way ANOVAs analysis, we tested the assumption of normal distribution and transformed the data to meet this requirement if needed. We also conducted a homogeneity of variance test and determined that the variance was homogeneous. In Experiment. 1, we performed ANOVA to test for the effects of (a) neighboring plants and (b) root zonation (i.e. F1-F5; Fig. 1a) on four parameters (i.e. root length, root biomass, root surface area and specific root length). In Experiment. 2, we conducted one-way ANOVAs to test the effects of cropping systems on biomass and root morphology of maize and wheat. In Experiment. 3, we also performed ANOVA to examine the effects of root exudates collected from wheat on root traits of maize. In Experiment. 4, we used one-way ANOVAs to investigate the effect of root exudates from wheat, barley and maize on four parameters as described above. In Experiment. 5, we tested for potential root-root interaction effects (using one-way ANOVAs) on the concentration of MBOA in root exudates. In Experiment. 6, we tested for the effect of MBOA with different concentrations on four parameters of maize and wheat as described above. In Experiment. 7, we conducted one-way ANOVAs to test the effects of MBOA on lateral root number of primary root and seminal root of maize. We compared the expression of IAA-related genes through one-way ANOVAs in Experiment. 8. Mean values were compared by least significant difference (LSD) at the 5% level. Statistical analyses were performed with R version 4.1.2 (R Development Core Team 2021).

Results

Root distribution and root morphology of maize with wheat or other neighbors

We found that the roots of the two maize seedlings growing in the same rhizobox were distributed uniformly between root zones (Experiment. 1; common-growing area *vs*. self-growing area; i.e. F2 + F3 vs. F4 + F5; $F_{1,\ 6} = 0.22$, $P = 0.66$; Fig. 1b, e). Similarly, maize roots were distributed uniformly between root zones when they encountered barley roots ($F_{1,\ 6} = 2.13$, $P = 0.19$; Fig. 1c, e). However, when maize was neighbored with wheat, root length, root biomass and surface area of maize were 59%, 54%, and 58% lower in the zone adjacent to wheat, respectively (i.e. F4 + F5 vs. F2 + F3; Fig. 1d, e; Fig. S1), suggesting that maize roots distributed 'away' from wheat roots. Root length of the target maize was similar in the 'self' area in response to different neighbors. When maize neighbored with wheat, maize root length in the common growing area (F2 + F3) was the lowest among the three treatments (Fig. 1e), indicating avoidance of maize roots to wheat roots.

Then, maize and wheat were grown in soil and quartz sand to investigate the effect of plant growth and root morphology (Experiment. 2). The root length, root surface area, root volume and specific root length of the maize intercropped with wheat were lower than those in monoculture under quartz sand culture, and decreased by 13%, 16%, 18% and 13%, respectively (Fig. 2a; Table S2). Similar results for maize were observed in soil culture (Fig. 2c; Table S2). On the contrary, the root growth and morphology of intercropped wheat increased more than monoculture under both mediums (Fig. 2b, d; Table S3).

The role of wheat root exudates in the root morphology of maize

Agar and quartz sands were used as a growth medium to determine the growth and morphology of maize in response to root exudates from different plants including wheat in experiments 3 and 4, respectively. We found that the root length of maize was reduced significantly by 37% when neighbored by a wheat seedling, and by 40% when growing on substrates enriched with wheat root

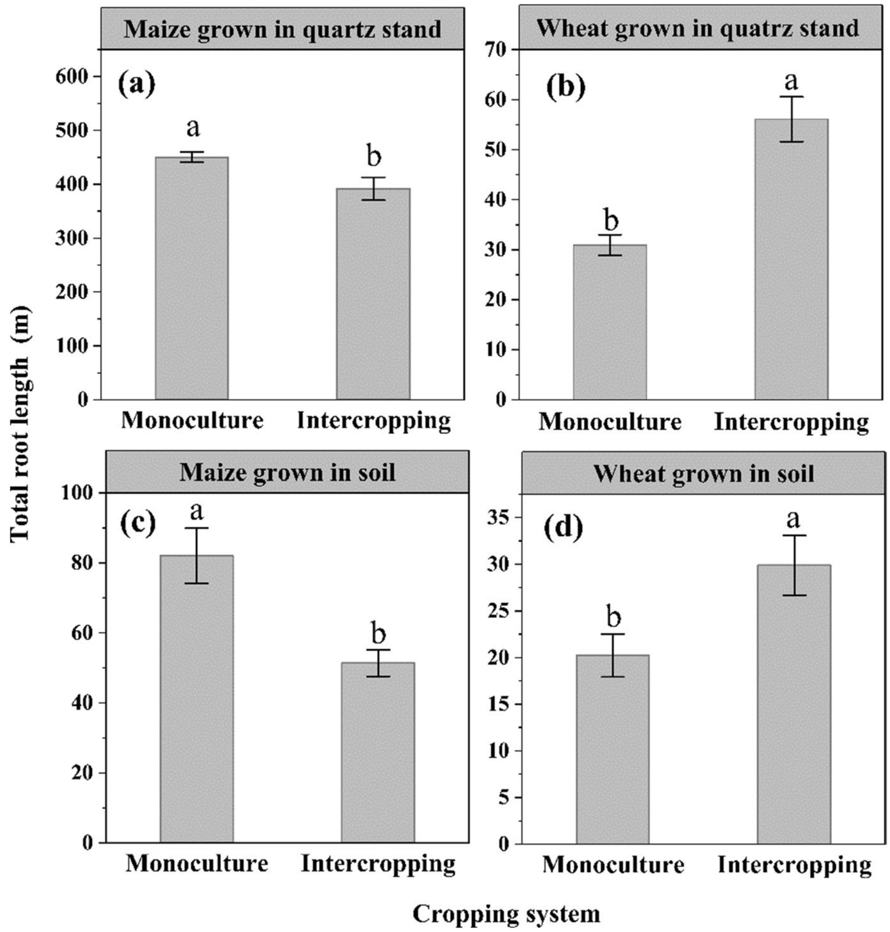


Fig. 2 Root length of maize and wheat plants as affected by interspecific interactions between wheat and maize under (**a**, **b**) quartz sand culture and (**c**, **d**) soil culture (Experiment. 2). Values are mean ± SE (n = 4), different small letters denote significant difference among treatments ($P < 0.05$)

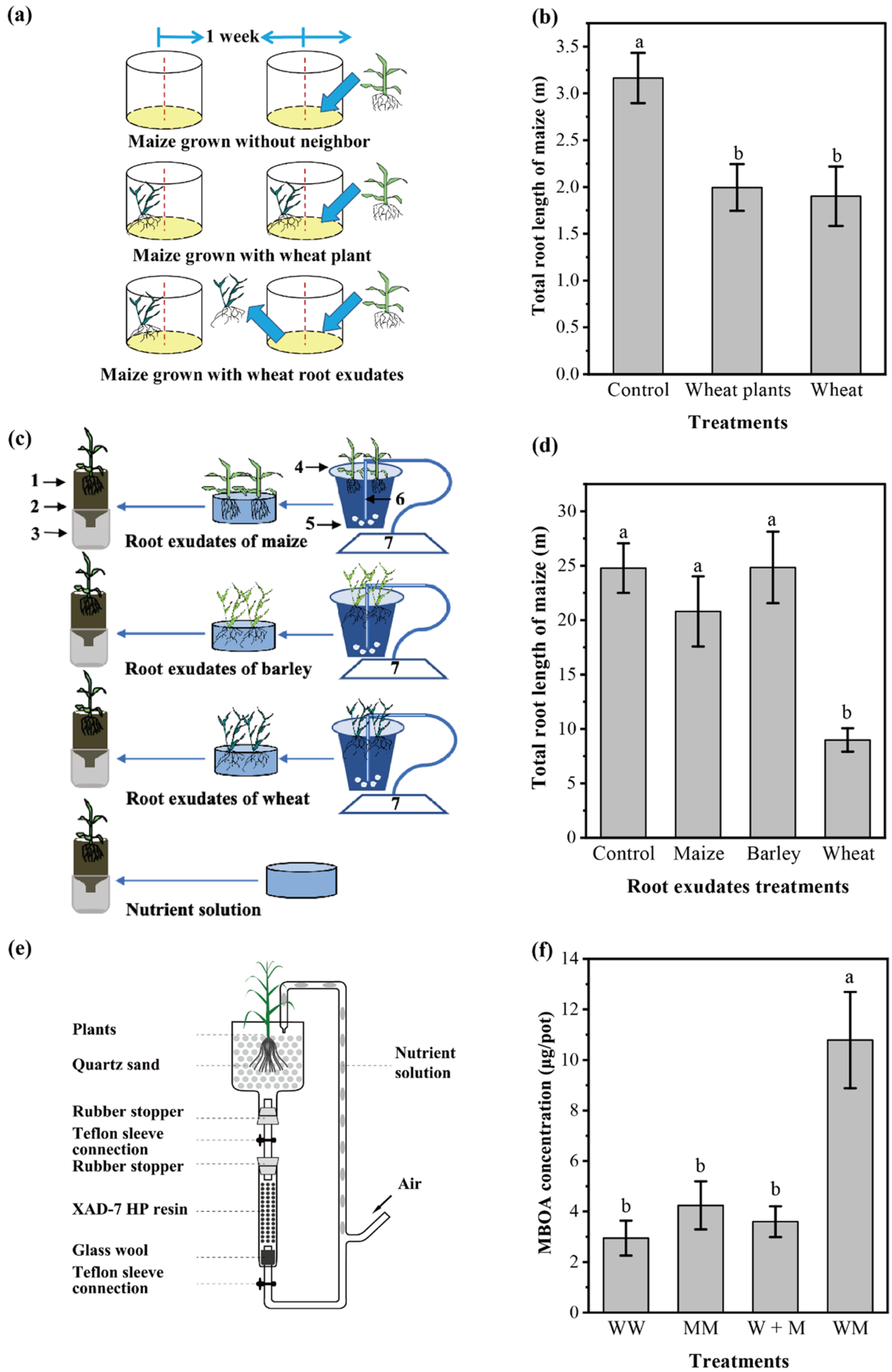
exudates, compared with maize grown alone under sterile conditions (Experiment. 3; Fig. 3b). Similarly, maize roots treated with wheat root exudates had 64% shorter root length and 59% lower root biomass than maize roots that were only treated with the nutrient solution (Experiment. 4; Fig. 3d; Table S5). Moreover, maize or barley root exudates did not affect the maize root length, root biomass and root surface area.

Depressed root branching was also found in maize grown in the presence of wheat root exudates or near wheat (Fig. S2). In addition, the specific root length of maize was significantly reduced by 56% and 55% when growing with wheat plant and with wheat root exudates compared with the control, respectively (Table S4).

Effect of MBOA on root morphology of maize and wheat

We firstly aimed to detect the specific chemical that was responsible for the root response to wheat presence. The chemical MBOA was detected in the root exudates of wheat, maize grown alone and wheat and maize mixture (Experiment. 5). The concentration of MBOA in the mixture (maize + wheat) was significantly higher when compared to wheat grown alone (+477%); maize grown alone (+224%), and the weighted means of wheat and maize grown alone (+315%), based on plant numbers in pots (Fig. 3f).

Then, we tested the effects of different MBOA concentrations on root morphological traits of maize and wheat (Experiment. 6). When maize


(a)
1 week
Maize grown without neighbor
Maize grown with wheat plant
Maize grown with wheat root exudates
(b)
Total root length of maize (m)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
Control
Wheat plants
Wheat
Treatments
(c)
1
2
3
4
5
6
7
Root exudates of maize
Root exudates of barley
Root exudates of wheat
Nutrient solution
(d)
Total root length of maize (m)
30
25
20
15
10
5
0
a
a
a
b
Control
Maize
Barley
Wheat
Root exudates treatments
(e)
Plants
Quartz sand
Rubber stopper
Teflon sleeve connection
Rubber stopper
XAD-7 HP resin
Glass wool
Teflon sleeve connection
Nutrient solution
Air
(f)
MBOA concentration (µg/pot)
14
12
10
8
6
4
2
0
b
b
b
a
WW
MM
W + M
WM
Treatments

◂**Fig. 3** Experimental setup and corresponding results of (**a-b**) Experiment. 3, (**c-d**) Experiment. 4, (**e-f**) Experiment. 5.(**a**) In Experiment 3, we applied three treatments in beakers filled with agar: (1) maize grown alone, (2) maize grown with wheat, and (3) maize grown alone with root exudates of wheat. In each pot, wheat seedlings were planted on the left half, while maize seedlings were planted on the right half. (**b**) The effects of control, wheat plants and wheat root exudates treatment on root length of maize in Experiment. 3. Control treatment refers to maize grown alone. (**c**) Experiment. 4 had four treatments: (1) maize root exudates with nutrient solution added to the maize seedling, (2) barley root exudates with nutrient solution added to the maize seedling, (3) wheat root exudates with nutrient solution added to the maize seedling, (4) nutrient solution added to the maize seedling. 1: Quartz sand; 2: Silver paper; 3: Nutrient solution; 4: Shading cover; 5: Nutrient solution; 6: Vent pipe; 7: Vent pump. (**d**) The effects of root exudates of different crop species on root length of maize in Experiment. 4. Control refers to 50 ml nutrient solution only, the other three treatments represent maize root exudates, barley root exudates and wheat root exudates added to the nutrient solution (50 ml). (**e**) In Experiment. 5, the nutrient solution was continuously circulated through the system to elute extracellular organics from the sand culture in which maize or wheat seedlings were grown. (**f**) The concentration of MBOA in the root exudates of maize and wheat seedlings in Experiment. 5. WW represents wheat grown with wheat (i.e. wheat grown with intraspecific neighbors, 10 wheat seedlings/pot), MM refers to maize grown with maize (i.e. maize grown with the intraspecific neighbor, 2 maize seedlings/pot), W + M represents weighted means of maize and wheat grown with corresponding intraspecific neighbors (i.e. 1/2 × 10 wheat seedlings/pot + 1/2 × 2 maize seedlings/pot, and WM indicates wheat and maize grown together (i.e. 5 wheat seedlings + 1 maize seedling/pot). Values (mean ± SE, n = 5 (b), 4 (d, e)) with the same letter are not significantly different at the $P = 0.05$ level

seedlings were treated with 0.3 μg L^{-1} MBOA standard substance, a 30% reduction ($P < 0.01$) of root length of maize was observed compared with the control (i.e. 20 mL ultrapure water). Root length of maize was inhibited by 32% following exposure to 1 μg L^{-1} MBOA and by up to 44% under 10 μg L^{-1} MBOA treatment ($P < 0.01$, Fig. 4a). Likewise, significant root biomass and root surface area inhibition of maize with MBOA exposure was observed (Table S6). However, the root length, root biomass and root surface area of wheat were not affected by different concentrations of MBOA (Fig. 4b; Table S7). Next, under 0.3 μg L^{-1} MBOA treatment (Experiment. 7), we showed that total root length, primary root length and seminal root length of maize were inhibited by 30%, 20% and 34% after 16 days (Fig. 5).

The expression of IAA-related genes in maize roots in response to MBOA treatment

The results showed that 0.3 μg L^{-1} MBOA led to a significant down-regulation of *PIN2* by 69%, 69%, 67%, 49%, 55%, and 38% of the control at 6, 12, 24, 48, 96, and 192 h, respectively, in the primary root (Fig. 6a). In the seminal root, transcription of *PIN2* was also significantly depressed compared with the control at most sampling points (Fig. S3a). Similar results were found in the expression of *GH3* (Fig. 6e, Fig. S3e). The expression of *IAA2, IAA21*, and *ARF2* decreased after 6 h, with varying results observed at earlier stages. For example, the relative expression of *IAA2* decreased by 76%, 69%, 75%, and 49% compared to the control at 6, 48, 96, and 192 h, while the opposite result was observed in the seminal root after 6 h (Fig. 6b, Fig. S3b). However, *IAA2, IAA21*, and *ARF2* expression was significantly repressed by 49%, 73%, and 79% compared to the control at 192 h in the primary root (Fig. 6b, c, d). In the seminal root, the expression of *IAA2, IAA21*, and *ARF2* was inhibited by 23%, 24%, and 9%, respectively, at 192 h (Fig. S3b, c, d).

Discussion

Root avoidance of maize roots to wheat roots

Root avoidance occurs when root growth of a plant species is inhibited by the presence of neighboring plant roots (Homulle et al. 2021). This root response phenomenon has been observed in various plant species and can be beneficial for plants to avoid competition for resources. For example, the roots of tobacco plants (*Nicotiana tabacum*) avoided growing towards the roots of sagebrush (*Artemisia tridentata*) in the same soil (Falik et al. 2005). *Salvinia molesta*, a metal tolerant species, shows a clear nickel avoidance response suggesting that the avoidance responses of roots may be involved in the adaptation of metal tolerant species to soils rich in nickel (Tognacchini et al. 2020). Other research studies found that the roots of grasses actively avoided growing towards the roots of other grass species, and that it may be an important mechanism for promoting species diversity in grasslands (Semchenko et al. 2007b).

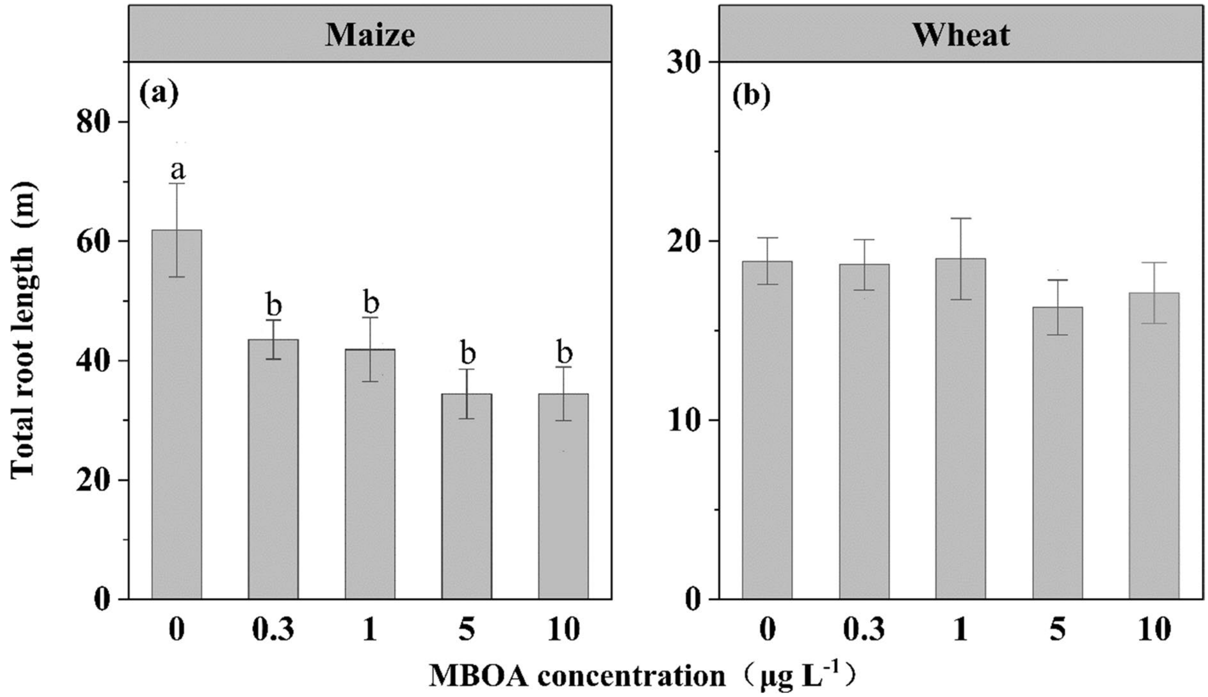
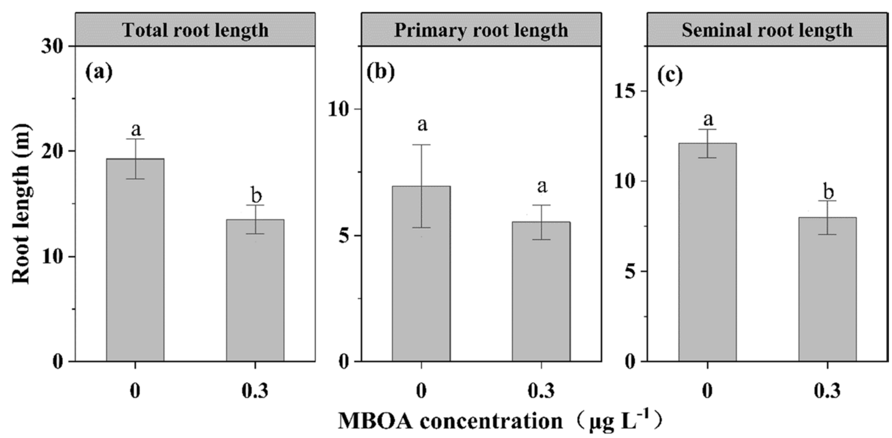


Fig. 4 Effects of different concentrations of MBOA on (**a**) root length of maize and (**b**) wheat in Experiment. 6. MBOA concentration including 0, 0.3, 1, 5 and 10 μg L^{-1}; 0 μg L^{-1} MBOA means 20 ml distilled water. Values (mean ± SE, n = 5) with the same letter are not significantly different at the *P* = 0.05 level

Fig. 5 Effect of 0.3 μg L^{-1} MBOA on (**a**) total root length, (**b**) primary root length and (**c**) seminal root length of maize under 16 days of treatment (Experiment. 7). Values (mean ± SE, n = 4) with the same letter are not significantly different at the *P* = 0.05 level



Root competition can affect soil resource availability by resource depletion or by mechanisms that inhibit access of other roots to a specific resource (Mommer et al. 2016; Schenk 2006). Generally, resource depletion-driven competition is difficult to distinguish from competition induced by root exudates. In the present study, we aimed to separate interspecific competition induced by root exudates from resource depletion-driven competition by supplying a nutrient solution (Experiments 1–3) and by adding root exudates to maize roots (Experiment. 4). Based on previous estimates of maize plant nutrition (Li et al. 2011), the amount of N and P that were supplied met to a great extent the N and P requirements for the growth of a maize seedling in the present study.

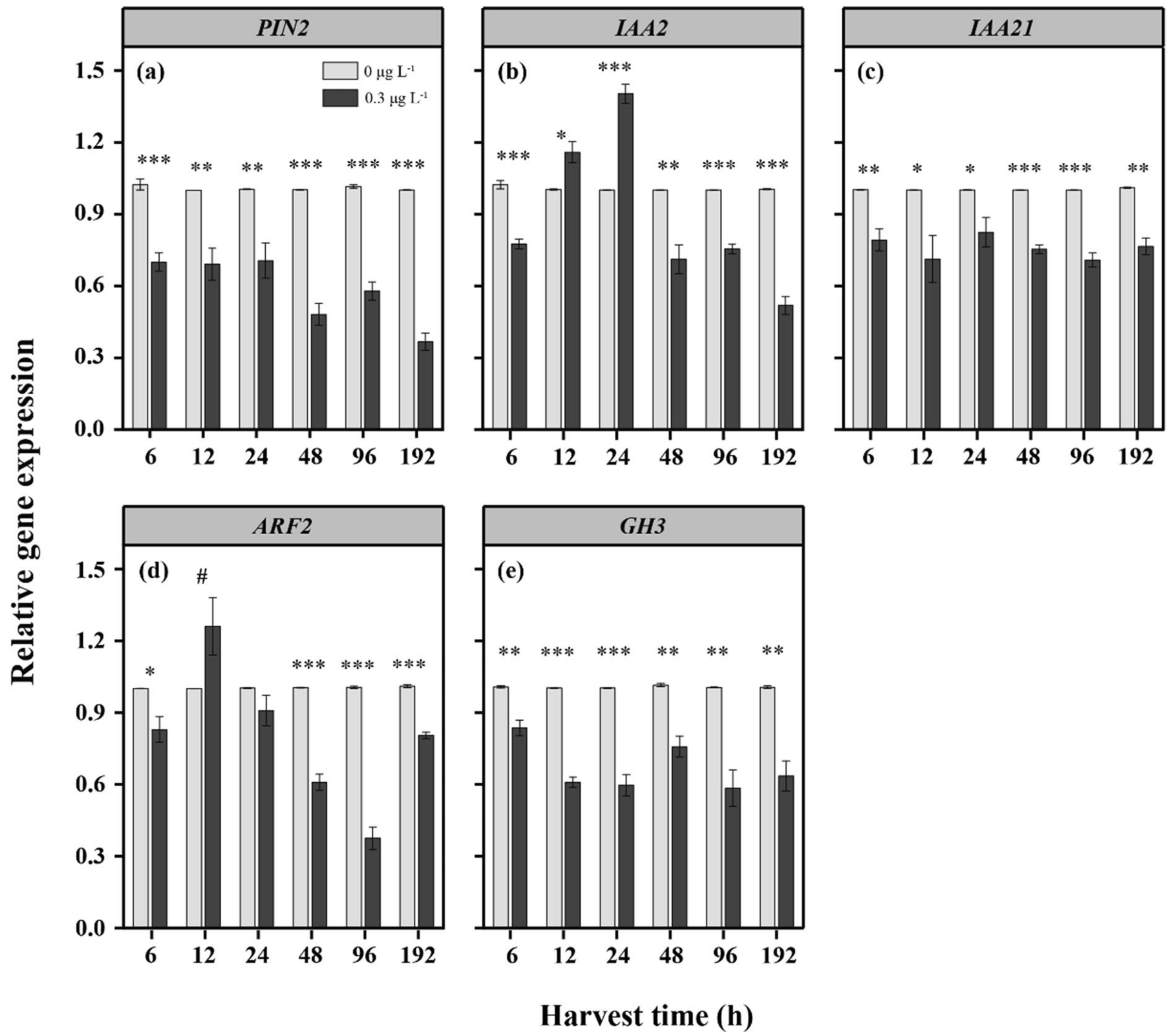


Fig. 6 Expression of (**a**) *PIN2*, (**b**) *IAA2*, (**c**) *IAA21*, (**d**) *ARF2* and (**e**) *GH3* genes in the primary root of maize at 6, 12, 24, 48, 96 and 192 h under 0 and 0.3 μg L^{-1} MBOA (Experiment. 8). Shown are values (mean ± SE, n = 4) of four independent biological replicates. Asterisks indicated a significant difference at.#$P < 0.01$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

Field observations show that the roots of intercropped wheat expanded in the soil area under maize plants, exhibiting much greater root length density than in monocultures (Li et al. 2001). Unlike intercropped wheat, there were similar spatial root distributions of intercropped maize across the different N application rates (Liu et al. 2019). Increased N fertilization completely relieved the inhibition of barley on maize plants but did not completely relieve inhibition of wheat on maize plants (Li et al. 2011). Moreover, the root distribution of maize changed when grown with one wheat seedling, but not when grown with barley seedling in the present study, where nutrient supply was sufficient (Fig. 1). This suggests that root exudates played an important role in controlling avoidance of maize roots between wheat and maize plants, whereas resource depletion-driven competition was not the main driver for the root avoidance.

The specific role of root exudates in mediating root-root interactions between wheat and maize plants

We demonstrate that the roots of maize avoided the roots of wheat (Fig. 1) and that maize root growth was significantly inhibited when wheat roots or wheat root exudates were present (Fig. 3). These findings suggest that root exudates determined changes in root distribution under controlled glasshouse conditions, changes that were also previously observed under field conditions in wheat/maize intercropping systems (Li et al. 2006).

We also found evidence that root exudates had specific effects on root behavior. A previous study showed how the root response of perennial grass, *Deschampsia caespitosa* was affected by the application of root exudates (Semchenko et al. 2014). Also rice roots tend to reduce the secretion of allelochemicals through intraspecific recognition, and intraspecific kin recognition may have consequences for interspecific allelopathic interference (Xu et al. 2021). In our study, we demonstrate species-specificity in root responses by maize because roots of other cereals (e.g., barley) did not induce the same root behavior in maize roots (Figs. 1, 3d). These results suggest that maize plants can respond differently when encountering different neighbors.

MBOA in wheat root exudates caused root avoidance by inhibiting root growth of maize

Previous studies show that root exudates have a selective effect in altering the structure of the rhizosphere microorganisms (Lin et al. 2022). Benzoxazolinones (BXs), for example, are important allelochemicals released by roots of cereals such as wheat that can inhibit plant growth (Hu et al. 2018). However, crops such as barley, rice, oat and sorghum do not contain any detectable amounts of BXs and not all crops are equally susceptible to them (Tang et al. 1975). A previous experiment demonstrated that allelochemical DIMBOA concentrations in roots of wheat could vary significantly depending on the identity of 100 different plant neighbors (Kong et al. 2018).

In our study, we observed that MBOA levels in wheat/maize mixtures were significantly higher than those in each of the two monocultures (Fig. 3f). Several studies reported increased levels of allelochemicals in the presence of competing weeds (Dayan 2006; Kong et al. 2006, 2004). For example, it has been shown that DIMBOA production from wheat was induced by the presence of weed species and was then effective in inhibiting the growth of these weeds (Li et al. 2016). Furthermore, several other studies demonstrated that wheat seedlings could detect the presence of neighboring weeds and respond by producing increased amounts of MBOA in the rhizosphere (Chen et al. 2010; Lu et al. 2012). Thus, wheat is very likely responsible for the greater MBOA production observed in wheat/maize intercropping.

Additionally, the sensitivity of different plants to exposure to MBOA was found to relate to plant species identity and applied MBOA concentrations (Reigosa et al. 2010). Neighboring plants need to recognize the existence of allelopathic species and adjust root placement accordingly to evade the influence of these allelochemicals. Failure to adjust root placement would result in the inhibition of their roots by allelochemicals (Wang et al. 2021). Our evidence shows that MBOA inhibited maize root growth but had no effect on wheat roots under the same concentration of MBOA (Fig. 4), therefore, the avoidance of maize root is to avoid the inhibition of allelochemical MBOA.

Auxin may be involved in the MBOA-mediated inhibition of maize root elongation

Several key genes of auxin are essential for root elongation in response to MBOA. Auxin transport gene of *PIN2* participates in root development and plays a critical role in root system development (Hu et al. 2021). During allelochemical benzoic acid exposure, the inhibition of *Arabidopsis* primary root elongation was associated with *PIN2* and *AUX1* genes (Zhang et al. 2018). Aux/IAA proteins and ARFs are key proteins in auxin signal transduction, with *ARF2* upregulating related cell expansion genes to increase organ size (Liu et al. 2017; Wang et al. 2016). *GH3* genes play an important role in auxin conjugation and response to stress (Feng et al. 2015). In our study, the expression of *IAA2, IAA21, ARF2* and *GH3* were downregulated in the primary root and seminal root of maize under MBOA treatment at most time points (Fig. 6, Fig. S3). Given the important role of auxin in modulating root growth, we suggest that MBOA can inhibit auxin-related functioning, including biosynthesis, transport, accumulation, and signalling in the root growth of maize thus suppressing root elongation. Allelochemicals can alter gravitropism by reducing starch grains and auxin levels, which may affect root placement and growth (Yan et al. 2018).

Implications for crop diversity systems

The multifunctional toxicity of MBOA makes this compound a natural pesticide against pathogens, insects, and weeds (Belz and Hurle 2005; Metlen et al. 2009). Specific species combinations can reduce pests and diseases, reduce autotoxicity, improve productivity and reduce the demand for agricultural fertilizer through allelopathy. A better understanding of root-root interactions can promote the sustainable development of agriculture and forestry and achieve the purpose of protecting natural resources (Scavo and Mauromicale 2021). Interspecific competition driven by resource depletion has been well-studied (Schenk 2006).

Here, we show that, beyond the mechanism of nutrient competition, root exudates play a vital role in root inhibition mediated by auxin in species-diverse agroecosystems, i.e. interference competition. Our evidence also suggests that the identification of key effective compounds, signaling, hormones and microbes that are involved in root-root competition would help understand the mechanisms of species coexistence and the establishment of species-diverse plant communities (Chen et al. 2020; Kong et al. 2018; Ruijven et al. 2020). Plants first may detect and potentially recognize their neighbors, and then initiate allelopathic interference to regulate inter-specific or intra-specific interactions. Allelobiosis refers to the transmission of signaling chemicals in plant-plant interactions, where the responses of neighboring plants influence their growth, defense strategies, and interactions with herbivores (Kong et al. 2024). However, allelopathy and allelobiosis are usually studied separately, despite these plant responses are interconnected in nature (Kong et al. 2024), and future studies should focus more on the combined effects of allelopathy and allelobiosis across different agroecosystems.

There is evidence to suggest that root avoidance is actually a cooperative strategy (Cabal et al. 2020), because when maize plants avoid a neighbor, they can improve biomass allocation into reproduction. Understanding the mechanisms of root avoidance can improve the predictability of interspecific interactions and will help design sustainable crop-diverse food-production systems.

Conclusions

Our results highlight the significant role of root exudate MBOA in mediating root interactions in wheat/maize intercropping systems. This avoidance behavior of maize is likely driven by the down-regulation of IAA-related genes in maize roots in response to MBOA, which inhibits maize root growth while leaving wheat roots unaffected. The elevated MBOA levels observed in intercropping scenarios further underscore the specificity of this allelopathic interaction. These findings provide new insights into interspecific root interactions and suggest that MBOA could be strategically utilized in designing sustainable intercropping systems to regulate belowground competition, potentially enhancing crop productivity and ecosystem sustainability.

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Author contributions LL, XTY, FFZ and RPY designed the study, XTY executed Experiments. 6, 7 and 8, FFZ executed Experiments. 1, 2, 4 and 5, NL executed Experiment. 3, XTY, FFZ and RPY analyzed data and drafted the manuscript, LL, WPZ, DF, LM and XXL revised the manuscript.

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Data availability All data supporting the findings of this paper are available within the paper and within its supplementary materials. Original data used here can be obtained directly from the authors.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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