

Root-exuded benzoxazinoids can alleviate negative plant–soil feedbacks

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Summary

- Plants can suppress the growth of other plants by modifying soil properties. These negative plant–soil feedbacks are often species-specific, suggesting that some plants possess resistance strategies. However, the underlying mechanisms remain largely unknown.
- Here, we investigated whether benzoxazinoids, a class of dominant secondary metabolites that are exuded into the soil by maize and other cereals, allow maize plants to cope with plant–soil feedbacks.
- We find that three out of five tested crop species reduce maize (*Zea mays* L.) performance via negative plant–soil feedbacks relative to the mean across species. This effect is partially alleviated by the capacity of maize plants to produce benzoxazinoids. Soil complementation with purified benzoxazinoids restores the protective effect for benzoxazinoid-deficient mutants. Sterilization and reinoculation experiments suggest that benzoxazinoid-mediated protection acts via changes in soil biota. Substantial variation of the protective effect between experiments and soil types illustrates context dependency.
- In conclusion, exuded plant secondary metabolites allow plants to cope with plant–soil feedbacks. These findings expand the functional repertoire of plant secondary metabolites and reveal a mechanism by which plants can resist negative effects of soil feedbacks. The uncovered phenomenon may represent a promising avenue to stabilize plant performance in crop rotations.

Introduction

Plants constantly interact with their soil environment. They change the biotic and abiotic soil attributes, which then, in turn, alters the performance of other plants. These plant–soil feedbacks are involved in many ecological processes, including vegetation succession, plant invasion, and maintenance of species diversity (van der Putten *et al.*, 1993, 2013; Klironomos, 2002; Teste *et al.*, 2017). In agriculture, they have been used for centuries to mitigate negative impacts of monocropping or to promote positive heterospecific feedbacks. Legumes, for example, can increase soil fertility through their symbiosis with nitrogen-fixing bacteria and thereby boost the productivity of subsequent crops (Stagnari *et al.*, 2017). More efforts to translate ecological knowledge from plant–soil feedback research into improved crop rotations are now needed (Mariotte *et al.*, 2018). Such evidence-based crop rotation design represents a promising avenue toward more sustainable agriculture (Dias *et al.*, 2015; Mariotte *et al.*, 2018).

Crop rotations are commonly employed in agriculture to stabilize crop performance. Over years of cultivation, crop rotations are capable of increasing soil health and suppressing weeds, pathogens, pests, and insects (Brust & King, 1994; Karlen *et al.*, 1994; Chen *et al.*, 2001; McDaniel *et al.*, 2014; Tiemann *et al.*, 2015; Leandro *et al.*, 2018). In addition, making cropping

systems more diverse also makes them more resilient against adverse growth conditions and weather extremes (Bowles *et al.*, 2020). Soil conditioning by a given crop species (referred to as ‘precrop’) can alter the growth, defense, yield, and soil processes of the following crop plant (Sieling & Christen, 2015; McDaniel *et al.*, 2016; Benitez *et al.*, 2017). Benitez and colleagues, for example, showed that precrop identity alters the microbial communities in the rhizosphere of maize seedlings and affects their performance. Given that plant-associated microbes are known to be important determinants for plant health (Berendsen *et al.*, 2012), it is tempting to hypothesize that changes in plant seedling performance are driven by precrop-dependent microbiomes.

Plant–soil feedbacks can be neutral, positive, or negative. Negative plant–soil feedbacks can for instance be triggered by reduced nutrient availability, accumulation of soil-borne pathogens, depletion of beneficial microbes, and changes in soil chemistry (Bennett & Klironomos, 2019; Schandry & Becker, 2020). The design of crop rotation schemes is thus often constrained to certain sequences that avoid negative plant–soil feedbacks.

Plant–soil feedbacks often depend on the environmental context (Smith-Ramesh & Reynolds, 2017). Furthermore, the direction and magnitude of plant–soil feedbacks in general is species- and variety-specific (Bever, 1994; Cadot *et al.*, 2021a; Awodele

& Bennett, 2022). Thus, it is conceivable that certain plants possess resistance strategies against negative plant–soil feedbacks. How plants resist negative plant–soil feedbacks is largely unknown. Understanding this process may help to predict the strength and direction of plant–soil feedbacks on plant performance and thereby increase the stability of crop rotations.

Root-exuded secondary metabolites can shape the rhizosphere microbiome (Sasse *et al.*, 2018) and can thereby influence subsequent plant performance (Hu *et al.*, 2018b; Yu *et al.*, 2021). Through the same mechanism, these compounds can also determine plant performance in the field across two generations (Gfeller *et al.*, 2023a,b). Benzoxazinoid exudation from maize roots for instance provokes neutral to negative feedbacks on conspecific maize plants (Hu *et al.*, 2018b; Cadot *et al.*, 2021a) and negative to positive feedback on heterospecific wheat plants (Cadot *et al.*, 2021a; Gfeller *et al.*, 2023a,b). Considering that secondary metabolites such as maize benzoxazinoids can structure the rhizosphere microbiome at the seedling stage (Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019), that they can suppress soil pathogens (Wilkes *et al.*, 1999; Martyniuk *et al.*, 2006), and that they can attract beneficial soil bacteria (Neal *et al.*, 2012; Neal & Ton, 2013), they may also increase crop rotation stability for the exuding plant itself by alleviating negative plant–soil feedbacks. To date, the potential of root secondary metabolites in plant–soil feedback resistance, that is in protecting the exuding plants from negative feedbacks, remains unexplored. This mechanism could be exploited, for example by growing varieties with high benzoxazinoid exudation in crop rotations.

So far, we studied how benzoxazinoid exudation affects the performance of other plants growing in benzoxazinoid-conditioned soils (Hu *et al.*, 2018b; Cadot *et al.*, 2021a; Gfeller *et al.*, 2023a,b). Here, we investigate how benzoxazinoid exudation affects the performance of the exuding maize plants growing in soils conditioned by other plants. Benzoxazinoids, a class of indole-derived plant secondary metabolites, are well known for their bioactivity (Niemeyer, 2009). They are most prevalent in grasses, including agronomically important crops such as maize, wheat, and rye (Frey *et al.*, 2009). Besides their effects on microbes, they are well known as defense metabolites against insects and pathogens (Niemeyer, 2009), as signaling molecules (Ahmad *et al.*, 2011), and iron chelators (Hu *et al.*, 2018a). Apart from increasing resistance and plant nutrition, benzoxazinoids can also benefit specialized pathogens and herbivores (Saunders & Kohn, 2009; Robert *et al.*, 2012) further emphasizing the importance of studying the advantages and disadvantages of these compounds in soil conditioned by different precrops.

By growing wild-type (WT) and benzoxazinoid-deficient *bx1* mutant maize in different conditioned soils, we examined how benzoxazinoids alter soil legacy effects of a diverse selection of precrops commonly cultivated in crop rotations with maize. In several plant–soil feedback experiments, we tested whether the direction and/or magnitude of these feedbacks change with different precrops, soils, and/or response maize lines. Through chemical complementation and sterilization experiments, we further assessed the direct effect of benzoxazinoids and the role of soil biota in these feedbacks. We found that, despite substantial

variability between experiments, benzoxazinoids consistently increase maize resistance to negative plant–soil feedbacks through root exudation and soil biota-dependent mechanisms.

Materials and Methods

Plant material

To investigate the effect of maize benzoxazinoids in resistance to negative plant–soil feedbacks, we selected five plant species as precrops and two maize (*Zea mays* L.) lines with their corresponding benzoxazinoid-deficient mutant as response plants. We selected a genetically diverse set of precrops belonging to four different families, all of them commonly cultivated in crop rotations with maize: *Glycine max* L. cv green shell (soybean), *Medicago sativa* L. (alfalfa), *Brassica napus* L. (rapeseed), *Phacelia tanacetifolia* Benth. (lacy phacelia), and *Triticum aestivum* L. cv Claro (winter wheat). *G. max*, *M. sativa*, and *P. tanacetifolia* seeds were obtained from Sativa Rheinau AG (Rheinau, Switzerland), *B. napus* seeds were purchased online (www.saemereien.ch), and *T. aestivum* seeds were kindly provided by Saatucht Düringen (Düringen, Switzerland). To ensure nodulation, *G. max* seeds were inoculated with rhizobia (LegumeFix; Sativa Rheinau AG) according to the supplier's recommendations. The maize lines W22 and B73 were selected as response plants, since for them benzoxazinoid-deficient *bx1* mutants are available (Tzin *et al.*, 2015; Maag *et al.*, 2016).

Soil material

Feedback experiments were conducted in field soil (clay loam) collected in three batches at the Agroscope field station in Changins (Switzerland). For the initial precrop screening, soil was sampled on field parcel 29, hereafter referred to as Changins-29 soil. For all the other experiments, soil was sampled in two batches on another field, parcel 30, hereafter referred to as Changins-30 soil. An additional soil (silt loam), referred to as Q-Matte, was collected from a grassland site near Bern (Switzerland) and was used to test for soil-specific effects. Collected soil was sieved (10 mm mesh size), completely homogenized, and stored at 4°C before utilization. Soils were characterized in previous publications (Hu *et al.*, 2018b; Cadot *et al.*, 2021a).

Plant growth

Experiments were performed in walk-in climate chambers under controlled conditions (day length: 14 h; temperature: 22°C: 18°C; humidity: 60%; light: $c. 550 \mu\text{mol m}^{-2} \text{s}^{-1}$). In the conditioning phase, the precrops were grown in 21 pots (Rosentopf Soparco 2.0 l; Hortima, Hausen, Switzerland) for 6 wk, followed by the maize feedback phase in either 2 or 11 pots (Rosentopf Soparco 1.0 l; Hortima) for 6 or 4 wk (Supporting Information Fig. S1). To prevent the roots from growing out of the pot, fleece (Geotex; Windhager, Thalgau, Austria) was placed at the bottom of each pot, before filling with soil. Pots were subsequently put in the climate chamber to acclimatize for at least 1 d before sowing. For each precrop, an excess of seeds was sown and thinned out to

two plants per plot after 1 wk, except for the fast-growing soybean, where we only kept one plant. Plants were watered as needed, and once a week, 100 ml of a nutrient solution (0.2% (w/v); Plantaaktiv Typ K; Hauer, Grossaffoltern, Switzerland) supplemented with iron (1‰ (w/v); Sequestrene rapid, Maag) was supplied in the conditioning phase and increased to 0.02% (w/v) Sequestrene in the feedback phase (unless otherwise stated). Fertilizer concentrations were based on previous work in the laboratory, ensuring that both WT and *bx1* plants have sufficient iron supply for normal growth, in order to avoid biases because of differential biomass accumulation. Pot positions within the climate chamber were randomized on a weekly basis. At the end of the soil conditioning phase, precrop shoots and root systems were removed and the remaining soil was sieved (10 mm mesh size), pooled and homogenized within each precrop (in all but one experiment, Fig. S1) to obtain a uniform soil composition, and used for the feedback phase. In the feedback phase, pot preparation was performed identical to the conditioning phase and maize was grown under the same climate chamber conditions as the precrops. See Fig. S1 for an overview of the details of all the performed plant–soil feedback experiments.

Effect of benzoxazinoid exudation on plant growth in conditioned soils

To examine whether benzoxazinoids improve resistance to negative plant–soil feedbacks, five precrops commonly cultivated in rotations with maize were grown for soil conditioning, followed by a feedback phase with WT B73 and benzoxazinoid-deficient *bx1* mutant maize. Precrops were grown for 6 wk in 2 l pots filled with Changins-29 soil. At harvest, shoot biomass was collected and dried until constant weight at 80°C, before dry weight was determined on a microbalance. After harvesting, conditioned soils were sieved and mixed before sowing WT and mutant seeds for the feedback phase. Plants were grown for 6 wk in 2 l pots. At harvest, plant height and shoot dry biomass were determined. To assess benzoxazinoid exudation by WT and *bx1* plants, we sampled the soils of three WT pots and one *bx1* pot per precrop species at the end of the experiment. We sieved the soil through a 10 mm sieve, again sieved a subset of this soil through a test sieve (5 mm; Retsch, Haan, Germany), and filled 25 ml soil into a 50-ml centrifuge tube and stored at –80°C until further processing. The frozen 50-ml centrifuge tubes containing the soil were thawed before the soil was dissolved in 25 ml acidified MeOH : H₂O (70 : 30 v/v; 0.1% formic acid). The suspension was placed on a rotary shaker for 30 min at room temperature, followed by sedimentation of the soil by centrifugation (5 min, 2000 g). The supernatant was filtered (Filter paper, Grade 1; Size: 185 mm; Whatman, GE Healthcare Live Sciences, Chicago, IL, USA); a 1 ml aliquot of the filtrate was transferred into a 1.5-ml centrifuge tube and centrifuged (10 min, 19 000 g, 4°C); and the supernatant was sterile filtered (Target2™, Regenerated Cellulose Syringe Filters, Pore size: 0.45 µm; Thermo Scientific, Waltham, MA, USA) into a glass tube for analysis. Benzoxazinoid analysis was performed as described previously (Gfeller *et al.*, 2023b). MBOA (6-methoxy-benzoxazolin-2(3H)-one), DIMBOA-Glc

(2-O-β-D-glucopyranosyl-2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), HDMBOA-Glc (2-O-β-D-glucopyranosyl-2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one), DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), HMBOA (2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), and AMPO (2-amino-7-methoxy-3H-phenoxazin-3-one) were quantified through standard curves of pure compounds. For DIM2BOA-Glc (2-O-β-D-glucopyranosyl-2,4-dihydroxy-7,8-dimethoxy-2H-1,4-benzoxazin-3(4H)-one) where no analytical standard was available, the compound was quantified on the closely related DIMBOA-Glc.

Influence of soil type and maize genetic background

Plant–soil feedbacks are known to depend on the growth environment and plant genotype (Smith-Ramesh & Reynolds, 2017). To test whether benzoxazinoid-mediated resistance to negative plant–soil feedbacks varies with soil type and maize genetic background, we performed a feedback experiment comparing B73 and W22 maize in Changins-30 soil. For the W22 genetic background, we included a second soil, Q-Matte, that differs in chemical properties and has been shown to eliminate benzoxazinoid-dependent feedback effects in previous studies (Cadot *et al.*, 2021a). Based on their strong positive and negative effects on maize growth, we focused on *M. sativa* and *T. aestivum* as representative precrops for these experiments. Precrops were grown for 6 wk in 2 l pots filled with Changins-30 soil. After harvesting, conditioned soils were sieved and homogenized for each pot separately to evaluate whether we find back the same effects as with soil pooling. In the feedback phase, WT and mutant plants were grown for 6 wk in 2 l pots. In this experiment, the fertilization was maintained at low levels during precrop conditioning and maize response. One hundred milliliter of a nutrient solution (0.2% (w/v); Plantaaktiv Typ K; Hauer, Grossaffoltern, Switzerland) supplemented with iron (1‰ (w/v); Sequestrene rapid, Maag) was supplied on a weekly basis. To get an impression of the feedback resistance over time, we measured plant height after 3 and 6 wk. Shoot dry weight was determined at harvest. Based on the results of this experiment, we decided to work in the W22 maize background for further experiments.

Temporal dynamics of feedback effects

To assess the temporal dynamics of the feedback resistance, we conducted a time course experiment in *T. aestivum* conditioned Changins-30 soil. Soils were conditioned as described above, and shoot dry biomass was determined at harvest. In the feedback, four WT or *bx1* mutant seeds were sown in 1 l pots filled with the conditioned soil and germination was assessed by inspecting the length of the emerging shoots 2 and 4 d after sowing. On Day 6, we thinned out to one plant per pot and measured plant height every 3 d. Twenty-seven days after sowing, chlorophyll content was determined by averaging nine measurements equally distributed along the youngest fully opened leaf by means of a SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan) and shoot dry weight was determined. Based on the results of this

experiment, plants were grown for 4 wk in all following experiments.

Benzoxazinoid complementation experiments

To evaluate whether benzoxazinoid exudation into the soil protects maize plants against negative plant–soil feedbacks, we externally applied a mixture of benzoxazinoids to *bx1* mutant plants growing in *T. aestivum* conditioned soil. To test different benzoxazinoid concentrations (0.05, 5.5, and 1.6 mg per pot and week) and capture possible variation between experiments, three experiments were conducted each of them with an independent soil conditioning and feedback phase. Experiments 1 and 2 were performed in the Changins-30 soil batch used before, for Experiment 3, we collected fresh soil from the same location, as the initial soil batch was depleted (Fig. S1). The soil was conditioned as described above and shoot dry weight was measured at harvest. In all three experiments, feedback experiments were conducted in 11 pots. Benzoxazinoid levels in the soil were determined at the end of each experiment, to estimate the effectiveness of our treatment. Because benzoxazinoids have been shown to rapidly degrade to the stable degradation product AMPO in soil (Gfeller *et al.*, 2023a), this compound was used as a marker for complementation success. AMPO was increased in the soil following benzoxazinoid complementation. All other compounds were only detected in trace amounts in complemented soils, suggesting rapid degradation in the absence of a constant emitter (Fig. S2c–e). Compared with levels in the soil of WT plants, AMPO concentrations in complemented *bx1* mutant soil were very low in the first experiment, where we applied 0.05 mg of benzoxazinoids per week and pot. We thus increased our complementation dose in the second experiment to 5.5 mg, resulting in AMPO concentrations that were higher than in soils of WT plants. For the third experiment, we thus used an intermediate dose, 1.6 mg, resulting in WT levels (Fig. S2c–e). It should be noted that mimicking natural exudation dynamics via exogenous complementation and verifying complementation success is challenging; based on previous studies, the molar concentration of the low benzoxazinoid complementation is comparable to MBOA accumulation in the soil during 4 wk of maize growth (Hu *et al.*, 2018b) while the highest concentration is comparable to DIMBOA–Glc exudation into the rhizosphere during the first 4 wk of hydroponically grown plants (Hu *et al.*, 2018a).

To purify benzoxazinoids for the complementation experiment, 40 g of maize seeds (var. Akku) was placed in a 1 l glass beaker and soaked in autoclaved water for 14 h. Kernels were washed twice a day and harvested after 4 d. Soaking and growth took place in the dark at 26°C. During harvest, kernels were immediately put into a blender (MioStar Beld 600s; Migros, Zürich, Switzerland) prefilled with 600 ml methanol (MeOH), blended at maximum speed for 5 min, and passed through a filter paper (Grade 1; Whatman, GE Healthcare Live Sciences). Next, we removed MeOH and water in the extracts by evaporation (40°C; rotary evaporator), followed by freeze-drying. The dry material was dissolved in MeOH, bound on silica (0.062–0.2 mm), and evaporated to dryness, and compounds were separated on a flash chromatography purification

system (CombiFlash RF+; Teledyne ISCO, Lincoln, NE, USA) in two subsequent runs, where benzoxazinoids were detected at wavelength 254 nm. The first run was performed on a 120 g RediSep Silica column at a flow rate of 85 ml min⁻¹, with chloroform (stab./EtOH; solvent A) and MeOH (solvent B) as solvents. The elution profile was as follows: 0–2 min, 0–13% B; 2–6 min, 13–16% B; 6–7.5 min, 16% B; 7.5–9.6 min, 16–33.6%; 9.6–12.7 min, 33.6–58% B, and kept at 58% B. The second run was performed on a 40 g RediSep Silica column at a flow rate of 40 ml min⁻¹ with the same solvents and the following elution profile: 0–2 min, 0–15% B; 2–3 min, 15% B; 3–8.7 min, 25–30% B, and kept at 30% B. The fractions containing benzoxazinoids were evaporated on a rotary evaporator (40°C), sterile filtered through a PTFE 0.20 (ChromafilXtra; Macherey-Nagel, Düren, Germany) filter, and evaporated to dryness. To crystallize the benzoxazinoid mixture, the compounds were dissolved in deionized water and lyophilized. The benzoxazinoid composition of the resulting white powder was characterized as described above.

For complementation, the purified benzoxazinoids were dissolved in deionized water, and 5 ml of this solution was pipetted to the *bx1* plants every 3 d, starting 2 d after sowing (at germination). Control *bx1* plants and WT plants were supplied with the same amount of deionized water. After 4 wk of growth, plant height, chlorophyll content, and shoot dry biomass were determined. To investigate benzoxazinoid accumulation in the pots, soil was sampled as described above for a random subset of plants.

Sterilization and soil reinoculation experiments

To evaluate whether the feedback resistance conferred by benzoxazinoids depends on soil biota, we performed sterilization and reinoculation experiments. The soils were conditioned with *T. aestivum* in three batches together with the complementation experiments as described above. To test for precrop-specific inoculation effects, in the third batch soil was also conditioned by *M. sativa*. For all three feedback experiments, a part of the conditioned soil was X-ray sterilized (20–60 kGy; Steris, Däniken, Switzerland). In the feedback phase, WT and *bx1* mutant plants were grown in unsterilized, sterilized, and reinoculated soil. Reinoculation was achieved by complementing 95% of sterilized soil with 5% of unsterilized (living) *T. aestivum* conditioned soil and homogenizing thoroughly. All soils were acclimatized for 1 wk in the climate chamber before sowing. All plants were watered with autoclaved tap water. To further investigate the relative contribution of the soil biota and abiotic soil attributes, we also tested for precrop-specific inoculation effects. Therefore, we included four additional soil conditions consisting of unsterilized *M. sativa* soil, sterilized *M. sativa* soil, and sterilized *M. sativa* soil inoculated with either unsterilized *M. sativa* or *T. aestivum* soil. In all experiments after 4 wk of growth, plant height, chlorophyll content, and shoot dry biomass were determined.

Statistical analysis

All statistical analyses were conducted in R v.4.1.2. (R Core Team, 2021). Data management and visualization was facilitated

with the TIDYVERSE package collection (Wickham *et al.*, 2019). Phenotypic data were analyzed by analysis of variance (ANOVA) unless otherwise stated. For that, statistical assumptions such as normal distribution and homoscedasticity of error variance were visually checked. If treatments showed unequal variance, a generalized least squares model was fitted using the `gls()` function of the NLME package (Pinheiro *et al.*, 2021). Differences in estimated marginal means (EMMs) were analyzed by pairwise comparison with the `emmeans()` function of the EMMEANS package and false discovery (FDR)-corrected *P* values were reported (Benjamini & Hochberg, 1995; Lenth, 2022). To evaluate the effect of individual precrop soil conditioning on the subsequent maize performance, one-sample *t*-tests were performed for every precrop, comparing its biomass production to the overall mean of all precrops. To test for differences in benzoxazinoid production between WT and *bx1* mutant maize as well as validation of complementation success, Wilcoxon rank-sum tests were performed. Within the conditioning phase differences in biomass accumulation between the precrops, *T. aestivum* and *M. sativa* were tested with Welch's two-sample *t*-test. To test at what point in time the growth increase in WT plants relative to *bx1* mutant plants became statistically significant, Welch's two-sample *t*-tests were performed and FDR-corrected *P* values were reported. The end-point analysis of the time series experiment was also analyzed by Welch's two-sample *t*-tests.

Results

Benzoxazinoids enhance resistance to negative plant–soil feedbacks

To test whether benzoxazinoids enable maize plants to cope with negative plant–soil feedbacks, we grew five crop species for 6 wk under controlled conditions, followed by a feedback phase with WT (B73) and benzoxazinoid-deficient *bx1* mutant maize. Biomass accumulation of the different precrops after 6 wk ranged from 3.4 to 10.5 g dry weight (Fig. S3a). After harvesting the conditioning plants, conditioned soils were sieved, and WT and *bx1* mutant maize seeds were sown (Fig. S1). After 6 wk of growth, we observed marked differences in biomass accumulation of maize depending on the precrop (Fig. 1a). Maize plants accumulated significantly less biomass on soils conditioned by *T. aestivum* (winter wheat), *P. tanacetifolia* (lacy phacelia), and *B. napus* (rapeseed) compared with the overall mean of all precrops ($P < 0.001$), indicating a negative plant–soil feedback. By contrast, maize plants accumulated significantly more biomass on soils conditioned by *G. max* (soybean) and *M. sativa* (alfalfa) compared with the overall mean of all precrops ($P < 0.001$), indicating a positive plant–soil feedback. A literature survey confirmed the validity of the definition of positive and negative feedbacks for maize across a wider range of precrops (Benitez *et al.*, 2017; Koyama *et al.*, 2022; Wojciechowski *et al.*, 2023). The growth suppression by *T. aestivum*, *P. tanacetifolia*, and *B. napus* soils was less pronounced in WT plants compared with *bx1* mutant plants. A similar pattern was observed for plant height (Fig. S3b). Analyzing soil benzoxazinoid concentrations and their degradation products in the soil after

harvest confirmed that WT plants released significantly more benzoxazinoids than *bx1* mutant plants (Fig. 1b). Thus, benzoxazinoid production in maize can convey partial resistance against negative plant–soil feedbacks.

Benzoxazinoid-dependent resistance to negative plant–soil feedbacks vary across time, soil type, and experiments

Plant–soil feedbacks can be highly context and genotype dependent (Smith-Ramesh & Reynolds, 2017). We thus conducted an additional experiment with a *bx1* mutant in a different genetic background (W22). We also grew WT and mutant plants in two different soils (Changins-30 and Q-Matte). We also included B73 plants grown in Changins-30 soil as a positive control. Two plant species which triggered opposite feedback effects, *T. aestivum* and *M. sativa*, were used to condition the soils (Fig. S4a). Three weeks after sowing, the height of WT plants was increased compared with *bx1* mutants on *T. aestivum* conditioned Changins-30 soil in both the B73 and the W22 genetic background. Maize plants grew similarly on *M. sativa* conditioned Changins-30 soil, thus confirming that benzoxazinoids increase resistance to negative plant–soil feedbacks (Fig. 2a). In contrast to the Changins-30 soil, no difference between WT and *bx1* mutants in the W22 background was found in Q-Matte soil, illustrating that the suppressive effect induced by *T. aestivum* depends on the soil type (Fig. 2a).

Six weeks after sowing, the differences in height between WT and mutant plants were less pronounced and even reversed in the B73 background in the Changins-30 soil (Fig. S4b). Dry weight patterns for W22 were as expected from the early height data, with the *bx1* mutant accumulating less biomass than the WT in *T. aestivum* conditioned Changins-30 soil, and no difference in *M. sativa* conditioned Changins-30 soil as well as Q-Matte soil (Fig. 2b). No clear conditioning effects on biomass were observed in the B73 background. Thus, while benzoxazinoids increase resistance to negative plant–soil feedbacks, the strength of the effects varies across time, different soil types and experiments.

To better capture the variation in feedback resistance, we conducted further experiments with *T. aestivum* conditioned Changins-30 soil (Figs S1, S5). First, we performed a detailed time course analysis to better understand the temporal variation in feedback resistance. No differences in germination were observed between genotypes (Fig. 3a). After 18 d of growth, WT plants grew significantly taller than *bx1* mutant plants in *T. aestivum* conditioned soil. The effect was most pronounced 27 d after sowing. Chlorophyll contents and dry weight were increased in WT compared with *bx1* mutant plants at day 27 (Fig. 3b,c). Thus, feedback effects appear 2 wk after sowing maize and are clearly visible 4 wk after sowing. Based on these results, we set the feedback phase to 4 wk in all further experiments.

Benzoxazinoids in the soil increase resistance to negative plant–soil feedbacks

To test whether benzoxazinoids act via the soil, we complemented the soil of *bx1* mutant plants with a benzoxazinoid mixture

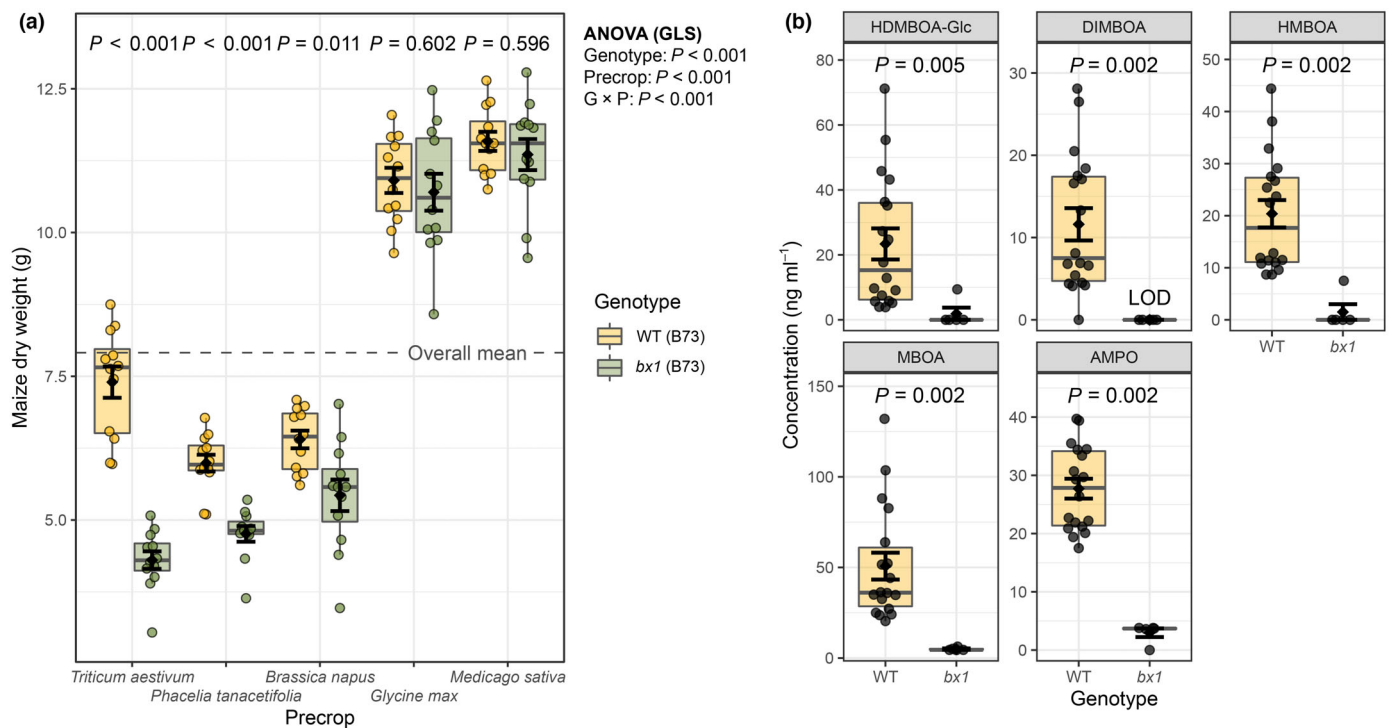


Fig. 1 Benzoxazinoid production is associated with resistance to negative plant–soil feedbacks. (a) Dry weight of wild-type (WT) or benzoxazinoid-deficient *bx1* mutant maize grown for 6 wk in soils conditioned by five precrop species. Means \pm SE, boxplots, and individual datapoints are shown ($n = 11$ – 12). ANOVA table and pairwise comparisons of estimated marginal means within each precrop (FDR-corrected P values) are provided. The overall mean of all precrops is indicated by the dashed line. (b) Soil benzoxazinoid concentration after maize growth of WT or benzoxazinoid-deficient *bx1* mutant plants indicated in ng per ml of soil. Means \pm SE, boxplots, and individual datapoints are shown (WT: $n = 18$, *bx1*: $n = 5$). ‘ $G \times P$ ’, interaction between genotype and precrop; GLS, generalized least squares (linear model); LOD, below limit of detection.

typical for young maize seedlings (Fig. S2a,b). We then compared the performance of WT and *bx1* mutant plants grown in *T. aestivum* conditioned soil with and without benzoxazinoid supplementation. Three different complementation concentrations were applied in three consecutive experiments.

There was considerable variation in the observed phenotypic effects across experiments. In each experiment, at least one out of three measured plant performance parameters was enhanced in WT plants compared with *bx1* mutant plants growing in *T. aestivum* conditioned soil (Fig. 4). In each case, benzoxazinoid supplementation rescued the WT phenotypes, either fully or partially. The clearest effect was observed for chlorophyll contents (Fig. 4). Interestingly, even though the applied benzoxazinoid concentrations varied by two orders of magnitude, we observed complementation effects in all three experiments (Fig. 4). Taken together, these results suggest that despite considerable variation, resistance to negative plant–soil feedbacks can be explained by benzoxazinoid release into the soil.

Soil biota drive benzoxazinoid-dependent resistance to negative plant–soil feedbacks

To investigate the role of soil biota in benzoxazinoid-mediated resistance against negative plant–soil feedbacks, we X-ray sterilized part of the conditioned soil and grew WT and *bx1* mutant plants in the soils. A reinoculation treatment with living soil was

included to control for changes in soil chemical and physical properties that may result from the sterilization treatment (Berns *et al.*, 2008). As expected, WT plants outperformed *bx1* mutants across experiments in one or several performance parameters when growing in *T. aestivum* conditioned soil (Fig. 5). All resistance effects were lost in sterilized soil; in some cases, *bx1* mutants performed even better than WT plants. Reinoculation restored all resistance effects in Experiment 2. In Experiments 1 and 3, only tendencies for restored resistance effects were found in reinoculated soil. Thus, benzoxazinoid-mediated resistance is mediated by elements that are labile to sterilization. At least in some cases, soil biota can account for these effects, as reinoculation with a small quantity of soil is sufficient to restore benzoxazinoid-mediated resistance.

To further examine the role of soil biota in benzoxazinoid-dependent plant–soil feedbacks, we conducted an additional inoculation experiment. We sterilized *M. sativa* conditioned soil and inoculated it with either *M. sativa* or *T. aestivum* soil. In unsterilized *M. sativa* conditioned soil, WT and *bx1* mutant plants grew similarly well, as observed before (Fig. 6). In sterilized soils, the *bx1* mutant outperformed WT plant growth. This effect disappeared when the soil was inoculated with *M. sativa* soil (Fig. 6). When the soil was inoculated with *T. aestivum* biota, WT plants outperformed *bx1* mutant plants. This reciprocal transplant experiment shows that the negative effects of *T. aestivum* soil biota can be overcome by benzoxazinoids.

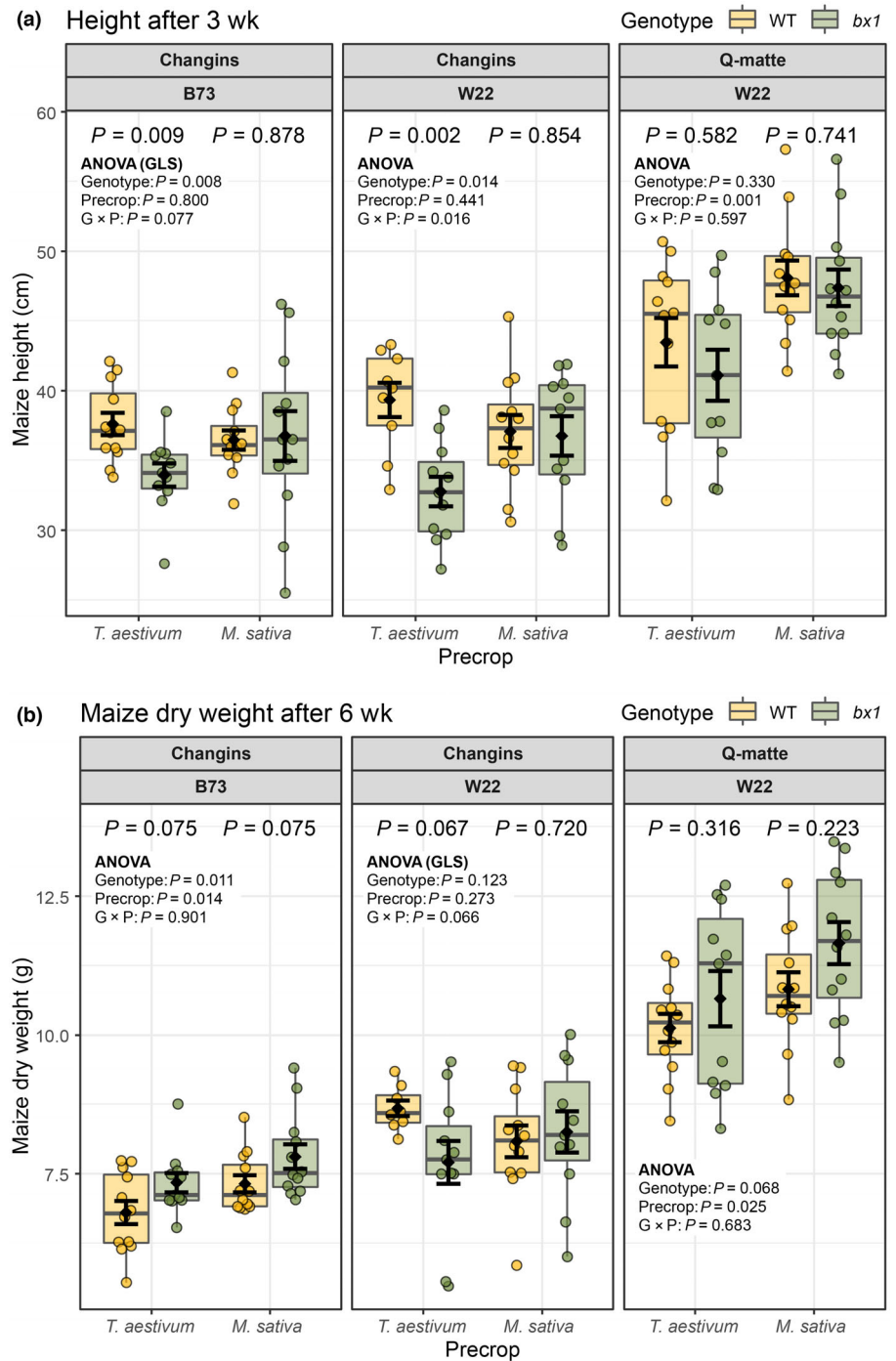


Fig. 2 Benzoxazinoid-dependent resistance to negative plant–soil feedbacks is soil-specific and transient. (a) Height after 3 wk of growth and (b) dry weight at harvest (6 wk) of wild-type (WT) or benzoxazinoid-deficient *bx1* mutant plants of the maize lines B73 or W22 growing in different soils (Changins-30, Q-matte) that were previously conditioned by *Triticum aestivum* or *Medicago sativa*. Means \pm SE, boxplots, and individual datapoints are shown ($n = 8–12$). ANOVA table and pairwise comparisons of estimated marginal means within each precrop (FDR-corrected P values) are provided. ‘ $G \times P$ ’, interaction between genotype and precrop; GLS, generalized least squares (linear model).

Discussion

Plant–soil feedbacks have a major impact on plant performance. How plants resist negative plant–soil feedbacks is not well known. In this study, we demonstrate that exuded secondary metabolites can help plants to cope with variable negative plant–soil feedbacks. This effect is, at least partially, mediated by the interaction between secondary metabolites and soil biota. Below, we discuss the underlying mechanisms and agroecological implications of our findings.

Plant–soil feedbacks can be triggered through exuded secondary metabolites and their capacity to change root-associated microorganisms. In maize, for example, such changes in the root microbiota through flavonoids and benzoxazinoids have been shown to affect the performance of the next maize plant grown in the same soil (Hu *et al.*, 2018b; Yu *et al.*, 2021). To what extent root secondary metabolites can protect plants from negative plant–soil feedbacks is unknown. Our results demonstrate that benzoxazinoid exudation into the rhizosphere can mitigate negative plant–soil feedbacks. This effect was found in two maize lines

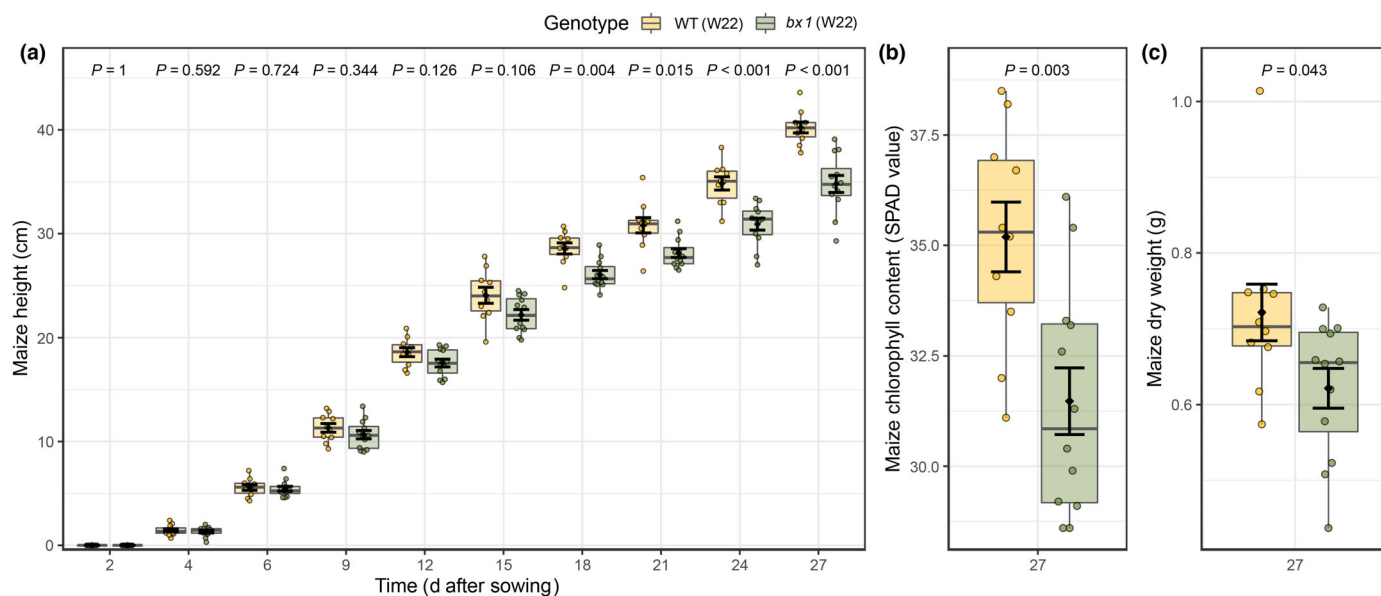


Fig. 3 Benzoxazinoid-dependent resistance to negative plant–soil feedbacks appears in early seedling growth. (a) Time series of plant height, (b) endpoint chlorophyll content, and (c) dry weight at harvest of wild-type (WT) or benzoxazinoid-deficient bx1 mutant plants grown in soils that were conditioned by *Triticum aestivum*. Means \pm SE, boxplots, and individual datapoints are shown ($n = 10$ – 12). Statistical significance is indicated as P values computed by Welch's two-sample t -test. P values were adjusted for multiple testing (FDR) in (a).

at early plant growth, an important trait in crop cultivation (Ellis, 1992; ter Steege *et al.*, 2005; Shi *et al.*, 2020). Benzoxazinoids are known to shape the root and rhizosphere microbiome and suppress particular soil pathogens (Wilkes *et al.*, 1999; Martyniuk *et al.*, 2006; Cadot *et al.*, 2021b); therefore, benzoxazinoid-mediated resistance could be driven by decreasing the harm of soil-borne plant pathogens, which can strongly reduce seedling performance (Packer & Clay, 2000). Indeed, sterilization eliminated the negative effect of *T. aestivum* conditioned soil as well as the capacity of benzoxazinoids to improve plant performance, and (re)inoculation with soil biota partially restored these effects. Given the wide range of metabolites plants can employ to modulate root microbiota and establish their own, often beneficial microbial communities (Pang *et al.*, 2021), we propose that this form of soil conditioning may be a widespread mechanism that protects plants from growth suppression by other plants. To what extent such conditioning may be costly by reversing positive feedback effects remains to be established.

Selecting a suitable precrop can improve crop performance through positive plant–soil feedback effects (Koyama *et al.*, 2022). In this study, we found striking effects of the precrop soil conditioning on maize growth. Soil conditioning by the two selected Fabaceae species, *G. max* (soybean) and *M. sativa* (alfalfa), led to increased maize growth, relative to the three other precrops. This could, for example, be driven by increased soil fertility as a result of biological nitrogen fixation of rhizobia in their symbiosis with legume precrops (Peoples *et al.*, 2009) or a reduction in the abundance of soil-borne pathogens (Nemadodzi *et al.*, 2023). A positive effect of benzoxazinoids on maize growth was only found in soils conditioned by *T. aestivum*, *P. tanacetifolia*, and *B. napus* that all triggered an overall reduction in maize growth compared with the overall mean across all

precrops, and we therefore defined these feedbacks as negative. All tested precrops are commonly grown at a global scale (Leff *et al.*, 2004) and capture important crop families. Recent studies examining precrop soil feedbacks on maize growth found similar overall effects for these precrops, including most negative effects for *T. aestivum* (Koyama *et al.*, 2022). Other precrops such as *Pisum sativum* (pea) and *Helianthus annuus* (sunflower) showed even stronger positive effects compared with soybean (Benitez *et al.*, 2017). A recent meta-analysis on cover crop effects further reported that under field conditions, Fabaceae show the most positive effects on maize performance, followed by Brassicaceae and Poaceae (Wojciechowski *et al.*, 2023). Thus, the definition of negative plant–soil feedbacks used here is likely valid even if additional precrops would be taken into account. Future experiments could include additional crop species to test how widespread the positive effect of benzoxazinoids on plant performance in different precrop conditioned soils is. Such experiments will also improve our understanding on when benzoxazinoids help to resist negative feedbacks or promote positive feedback.

Soil conditioning by benzoxazinoids from maize influences subsequent maize growth in a negative to neutral manner (Hu *et al.*, 2018b; Cadot *et al.*, 2021a). This effect differs from the positive effects observed here, where the exuding plant benefits from benzoxazinoid exudation when growing in soils after negative conditioning by other plants. This difference is likely due to the fact that benzoxazinoid patterns differ greatly between the root surface of the exuding plant (as investigated here) and in the conditioned soil in which the next plant grows. While the total benzoxazinoid concentrations around the exuding roots are very high (hundreds of ng per ml soil), only trace amounts of benzoxazinoids remain by the time the next plant generation grows in this soil (Macías *et al.*, 2004; Etzerodt *et al.*, 2008; Gfeller

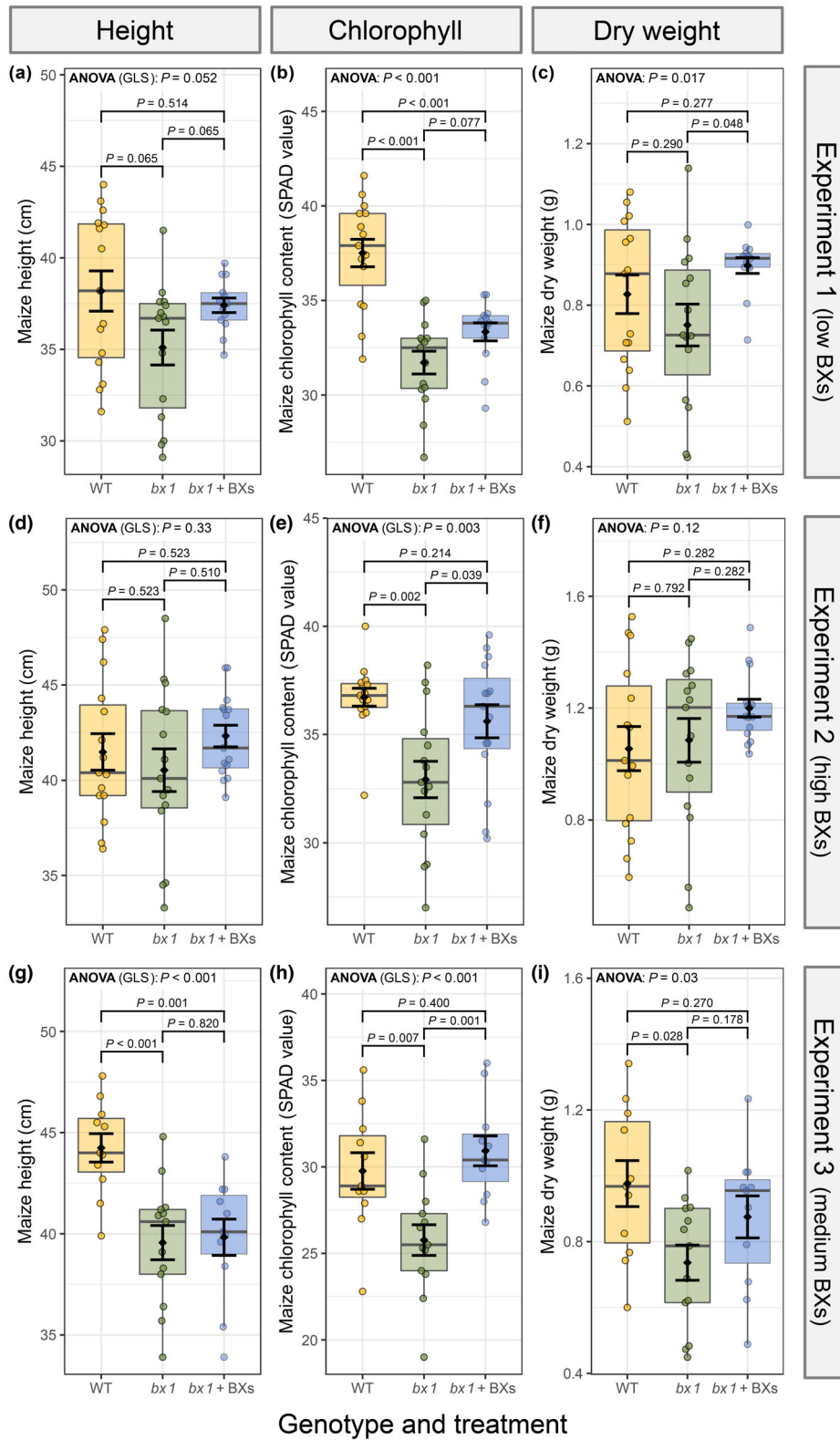


Fig. 4 Benzoxazinoid-dependent resistance to negative plant–soil feedbacks is partially associated with benzoxazinoids in the rhizosphere. For all three replications of this experiment height, chlorophyll content, and dry weight of wild-type (WT) plants, benzoxazinoid-deficient *bx1* mutant plants, or *bx1* plants complemented with benzoxazinoids (BXs) grown for 4 wk in soils that were previously conditioned by *Triticum aestivum*. Means \pm SE, boxplots, and individual datapoints are shown. ANOVA table and pairwise comparisons of estimated marginal means between all three treatments (FDR-corrected *P* values) are provided. Experiment 1 (a–c), 2 (d–f) and 3 (g–i) were complemented with low (0.05 mg), high (5.5 mg), and medium (1.6 mg) amounts of benzoxazinoids per week and pot. Experiment 1: *n* = 13–15, Experiment 2: *n* = 15, Experiment 3: *n* = 11–13. GLS, generalized least squares (linear model).

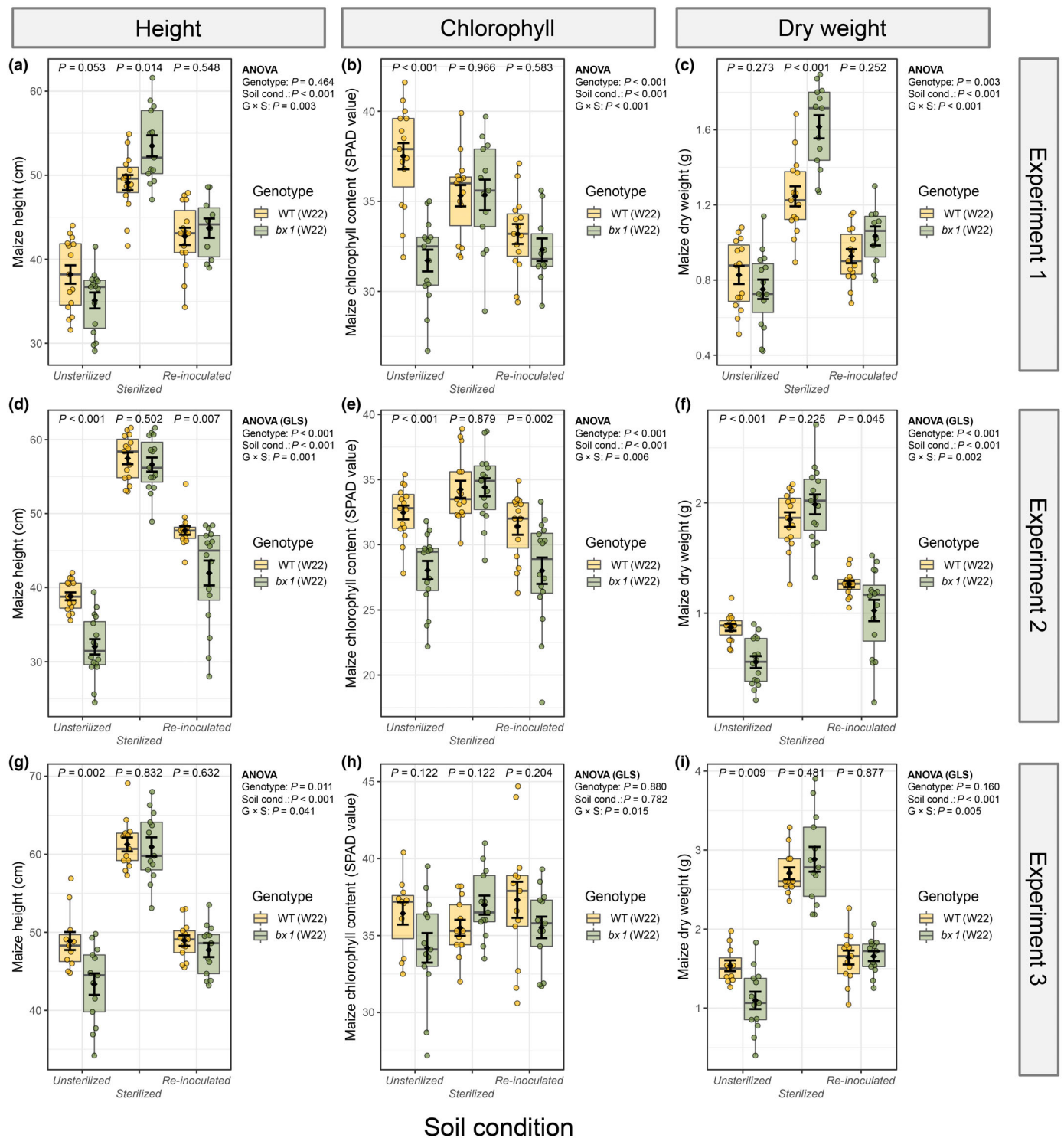


Fig. 5 Benzoxazinoid-dependent resistance to negative plant–soil feedbacks can depend on soil biota. For all three replications of this experiment height, chlorophyll content, and dry weight of wild-type (WT) or benzoxazinoid-deficient *bx1* mutant plants grown for 4 wk in *Triticum aestivum* conditioned soil that was either unsterilized, sterilized, or sterilized and reinoculated with unsterilized soil. Means \pm SE, boxplots, and individual datapoints are shown. ANOVA table and pairwise comparisons of estimated marginal means between all three treatments (FDR-corrected P values) are provided. Experiment 1 (a–c): $n = 10$ – 15 , Experiment 2 (d–f): $n = 15$ – 16 , Experiment 3 (g–i): $n = 11$ – 13 . ‘ $G \times S$ ’, interaction between genotype and soil condition; GLS, generalized least squares (linear model).

et al., 2023a). Furthermore, the identity and bioactivity of the compounds is also very different: While DIMBOA-Glc, DIMBOA, and HDMBOA-Glc are abundant on the root surface of

the exuding plant, the breakdown products MBOA and AMPO are the dominant compounds in the soil by the time the next plant generation grows (Hu *et al.*, 2018b; Gfeller *et al.*, 2023a).

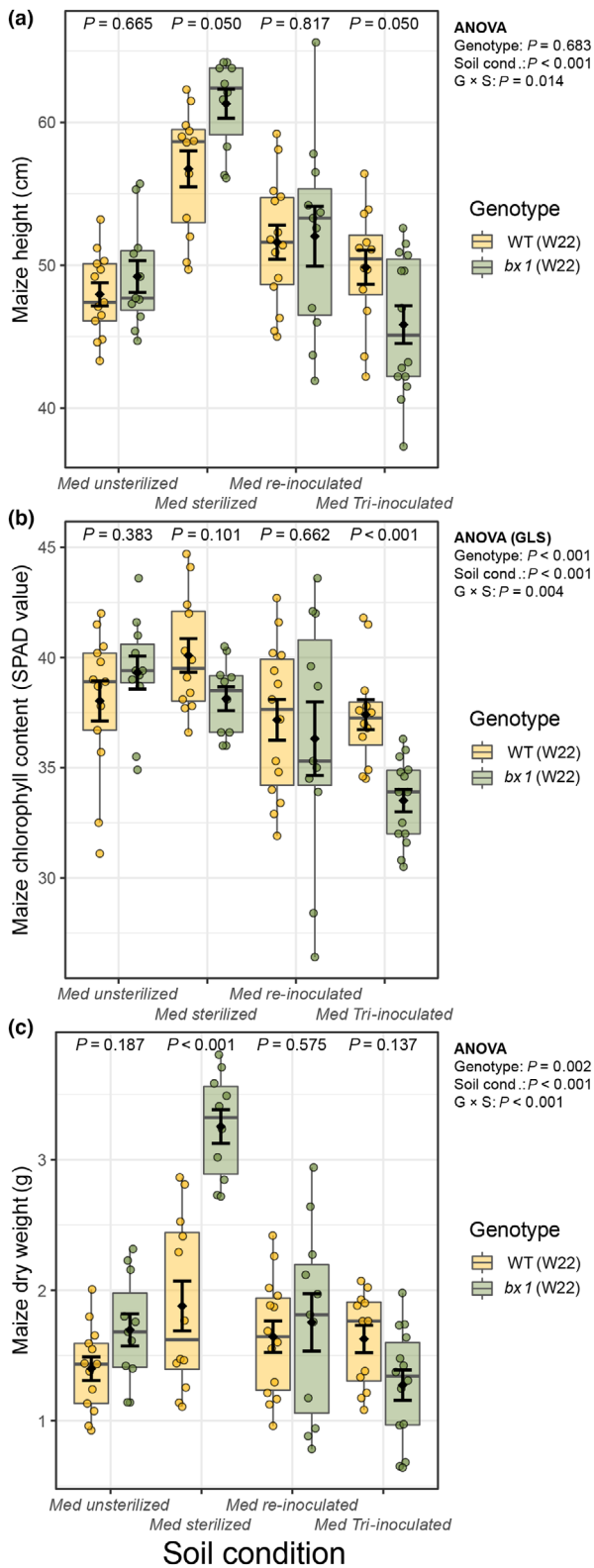


Fig. 6 Benzoxazinoid-dependent resistance to negative plant–soil feedbacks depends on precrop-specific soil biota. (a) Height, (b) chlorophyll content, and (c) dry weight of wild-type (WT) or benzoxazinoid-deficient *bx1* mutant plants grown for 4 wk in *Medicago sativa* conditioned soil that was either unsterilized, sterilized, sterilized and reinoculated with unsterilized *M. sativa* soil (Med-inoculated), or sterilized and reinoculated with unsterilized *Triticum aestivum* soil (Tri-inoculated). Means \pm SE, boxplots, and individual datapoints are shown ($n = 10–14$). ANOVA table and pairwise comparisons of estimated marginal means from all three treatments (FDR-corrected P values) are provided. ‘ $G \times S$ ’, interaction between genotype and soil condition; GLS, generalized least squares (linear model).

benzoxazinoid effects on the exuding plants differ from their effects on the next plant generation, most likely due to differences in their abundance and identity.

A recent study found that overall, conspecific and heterospecific plant–soil feedbacks across different plant species are correlated (Wilschut *et al.*, 2023). Our experiments do not allow for a direct comparison of con- and heterospecific effects, as we did not include conspecific soil conditioning in our comparison, and we currently only have one plant species available to manipulate benzoxazinoid production. Nevertheless, such comparisons would be an interesting avenue for future work.

Plant–soil feedbacks can be driven by specialist and generalist plant–microbial interactions (Semchenko *et al.*, 2022). While resistance to specialists might be important in repeated cultivation of conspecifics (monocultures), resistance to generalists might be more important in heterospecific crop sequences (crop rotations). This may indicate that benzoxazinoid exudation can suppress generalist pathogens that accumulated during the soil conditioning of the different precrops. Indeed, in this study we show an overall protective effect of benzoxazinoids to heterospecific negative feedbacks in the exuder plant. Benzoxazinoids can also promote the accumulation of specialist pests and pathogens (Glenn *et al.*, 2001; Saunders & Kohn, 2008, 2009), which may explain why neutral to negative feedback effects of benzoxazinoids were found on conspecific successors (Hu *et al.*, 2018b). Continuous cropping of benzoxazinoid producing plants (e.g. conspecifics) might therefore diminish or even reverse the positive effect of benzoxazinoids on plant performance. Diverse crop rotations may take advantage of the positive protective effect of benzoxazinoids by reducing the abundance of negative generalists without the build-up of specialist pests and pathogens. Future experiments with longer-term conspecific and heterospecific cropping sequences coupled with in-depth microbiome analysis are needed to test for the involvement of specialist and generalist pathogens.

Plant–soil feedback effects are known to be highly context dependent, rendering them variable to a point where seemingly stochastic patterns are observed. Plant–soil feedbacks are for instance known to depend on the growth environment, soil origin, aboveground herbivores, soil microbes, and temperature and soil moisture (Smith-Ramesh & Reynolds, 2017). Small variations in abiotic and biotic parameters may have contributed to the variation within and between experiments that we observed

Finally, while substantial effects of benzoxazinoids on the microbiome can be seen in the rhizosphere of the exuding plant, these changes disappear in the surrounding soil and during the feedback phase (Hu *et al.*, 2018b; Gfeller *et al.*, 2023a,b). Thus,

in our study, even under controlled conditions (Wei *et al.*, 2019). Despite this variation, we observed a remarkable consistency in the directionality of our effects, suggesting that, while quantitatively variable, the net protective effect of benzoxazinoids toward negative plant–soil feedbacks is relevant for plant performance. Nevertheless, the benefits of benzoxazinoid exudation can depend on the soil environment, even within the same field (Cadot *et al.*, 2021a; Gfeller *et al.*, 2023a). While negative plant–soil feedbacks are observed in one soil, they are absent in another soil, and thus, no protection is afforded by benzoxazinoids in this second situation. Interestingly, this second soil, Q-matte, has previously been shown to be incapable of provoking benzoxazinoid-dependent plant–soil feedbacks on successor plants (Cadot *et al.*, 2021a). This coincidence may be explained by similar underlying mechanisms by which benzoxazinoids trigger and cope with feedbacks. A potential mechanism could be the benzoxazinoid-driven shaping of the root-associated microbiota that relies on the microbial reservoir in a particular soil. Experiments with additional soils that differ in microbial composition, pathogen pressure, and chemical and physical characteristics will show how important benzoxazinoid-mediated resistance is across different soils.

Crop rotations have been incorporated into agricultural practices for centuries to lower negative effects of crops, such as accumulation of species-specific soil-borne pathogens or nutrient depletion (van der Putten *et al.*, 2013). Only recently, cultivar-specific feedbacks within agricultural plant–soil feedbacks have been demonstrated (Wagg *et al.*, 2015; Carrillo *et al.*, 2019; Cadot *et al.*, 2021a; Awodele & Bennett, 2022). The mechanisms responsible for tolerating a given precrop were largely unexplored. In our work, we find that one single group of secondary metabolites whose production is largely dependent on the *Bx1* gene, responsible for the first dedicated step of benzoxazinoid biosynthesis, determines the resistance of maize against negative plant–soil feedbacks. Given that the same metabolites can increase agricultural productivity of the following crop (Gfeller *et al.*, 2023b), this makes the genes involved in biosynthesis and exudation of such metabolites a potential breeding target for superior crop rotations. Many maize lines already produce substantial amounts of benzoxazinoids in their roots, but substantial genetic variation is commonly observed (Handrick *et al.*, 2016). It should thus be possible to develop cultivars that are particularly suited to crop rotations or that may deliver better performance following specific preceding crops. Broader field experiments will be needed to quantify the potential of optimized benzoxazinoid release to promote sustainable crop production by improving yields and food quality while reducing inputs.

Conclusion

Plants strongly interact with the soil, where the release of secondary metabolites has a strong effect on soil biota (Sasse *et al.*, 2018). Our study shows that such exudation may increase crop rotation stability by reducing negative plant–soil feedbacks. The use of agroecological plant–soil feedbacks has been proposed as a possible way toward more sustainable systems (Mariotte *et al.*, 2018), and

with our work, we provide an additional mechanism to apply this concept. As the release of diverse secondary metabolites into the rhizosphere is a common plant trait (Baetz & Martinoia, 2014), studying their effect on crop rotations offers a big reservoir of possible mechanisms to make agriculture more sustainable through plant–soil feedbacks (Mariotte *et al.*, 2018).

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Competing interests

None declared.

Author contributions

VG and ME designed research. VG and LT performed research. VG and ME analyzed data and wrote the first draft of the paper. All authors revised the paper.

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Data availability

All data generated for this study can be downloaded from the DRYAD repository (doi: [10.5061/dryad.j6q573nmf](https://doi.org/10.5061/dryad.j6q573nmf)). R code to reproduce statistical analysis and visualizations is available under https://github.com/ValentinGfeller/Precrop_paper.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Schematic representation of the different feedback experiments.

Fig. S2 Characterization of benzoxazinoid complementation.

Fig. S3 Precrop weight and maize height of initial precrop screening experiment.

Fig. S4 Precrop weight and maize height of experiment comparing different soil types and genetic backgrounds.

Fig. S5 Dry weight of precrop species grown to condition the soil for replicated experiments 1–3.

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