The impact of benzoxazinoids on agroecological plant-soil feedbacks

Inaugural dissertation
of the Faculty of Science,
University of Bern

presented by
Valentin, Gfeller
from Röthenbach im Emmental BE

Supervisor of the doctoral thesis:
Prof. Dr. Matthias Erb
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Summary

Plants modulate their growth environment by changing their root surrounding soil, which in turn modifies the performance of the next plant growing in that soil. How such plant-soil feedbacks are affected by root exuded secondary metabolites is not well understood. In particular, we know very little about how secondary metabolite-mediated plant-soil feedbacks affect agricultural productivity and food quality in crop rotations, and how secondary metabolites could help to alleviate negative agroecological plant-soil feedbacks. In this thesis, I aim to assess the potential of benzoxazinoids, an important class of secondary metabolites that are produced by cereals, to improve crop rotations through plant-soil feedbacks. First, in a two-year field experiment, I demonstrated that maize benzoxazinoid soil conditioning improved the performance of three subsequently growing wheat varieties without compromising food quality. Cereal leaf beetle infestation was reduced in response to benzoxazinoid soil conditioning and wheat yield was increased by more than 4%, mostly caused by enhanced emergence and tillering. Second, in another two-year field experiment, I found that such benzoxazinoid-dependent plant-soil feedbacks depend on local soil parameters. Soil chemistry was closely associated with soil benzoxazinoid concentrations and rhizosphere microbial community composition. Soil chemistry also explained the magnitude and direction of the feedbacks on plant performance, resistance, and kernel quality. Further, in a climate chamber and an incubation experiment I elucidated how benzoxazinoid degradation, but not exudation, was influenced by soil chemistry. In both field experiments, benzoxazinoid soil conditioning modified soil benzoxazinoid concentrations and the community compositions of root-associated microbes. The differences in rhizosphere microbial communities were only transient, while the chemical fingerprint of benzoxazinoid degradation products persisted to the next crop. Third, in climate chamber experiments, I demonstrated that three out of five tested preceding crops suppressed growth of maize through negative plant-soil feedbacks, and that benzoxazinoid exudation reduced this growth suppression. This resistance to growth suppression was, at least partially, dependent on soil biota. Overall, the results of this thesis reveal several new facets of secondary metabolites in agroecological plant-soil feedbacks. Exuded secondary metabolites can enhance crop rotation productivity and confer resistance to negative plant-soil feedbacks, thus making them a promising breeding target to improve crop productivity in a sustainable manner.

General introduction

The increasing human population demands increasing agricultural productivity. At the same time agricultural intensification in the past decades has negatively influenced farmlands. The increased use of fertilizers and pesticides has increased agricultural productivity but at the same time also degraded farmland (Foley *et al.*, 2005). To maintain agroecosystem functioning and provide enough food in the long-term, a reduction in fertilizer and pesticide application without reduction in productivity is needed (Tilman *et al.*, 2002; Foley *et al.*, 2011). To achieve this goal, governments and intergovernmental organizations have developed strategies and recommendations (Singh *et al.*, 2020). For example, in the framework of their farm to fork strategy, the Commission of the European Union (EU) aims for a massive reduction of chemical fertilizers and pesticides and a strong increase in organic cultivation area in the next decade (European Commission, 2020). To meet such objectives without running into food shortages, sustainable intensification is necessary (Hunter *et al.*, 2017). A promising direction is to translate known ecological phenomena from natural ecosystems to agroecosystems (Altieri, 1995).

Application of ecological concepts in agriculture

Plants have evolved various traits to defend themselves directly or indirectly against pests and pathogens and some of these traits can be exploited for sustainable crop production (Stenberg, 2017). Plant defence can either be direct or indirect through multitrophic interactions (Heil, 2008). Direct defence can for example be mediated by defensive structures or secondary metabolites (Bennett & Wallsgrove, 1994; Hanley *et al.*, 2007), where secondary metabolites affect the performance and behaviour of a multitude of organisms above and belowground (Bennett & Wallsgrove, 1994). Indirect defence, in contrast, describes the process of attracting natural enemies of herbivores (Heil, 2008). Examples of indirect defence are the attraction of parasitoids wasps aboveground or entomopathogenic nematodes below ground; both being capable of reducing herbivore pressure for the plant (Turlings & Erb, 2018). Understanding the mechanisms and the genetic background of these defence strategies provides valuable information to plant breeding programmes for selection of crops with enhanced direct or indirect defence. During crop domestication some resistance traits have been lost (Tamiru *et al.*, 2015). This asks for introgression of resistance-related genes from landraces or wild relatives into modern crops. Consciously combining plant species within an agroecosystem can further protect a plant. A prominent example is the push-pull system, which combines repellent

companion plants around the crop and attractant trap plants some distance away (Pickett *et al.*, 2014). Crop diversification in space, such as intercropping and flower strips, can also protect crops, for example by increasing the density of biocontrol agents (Tschumi *et al.*, 2015). Crop diversification in time (crop rotation) represents a more widely applied strategy to reduce the density of detrimental organisms to improve crop yield (Bullock, 1992). By avoiding continuous cultivation of conspecific plants on the same field, crop rotations make use of reducing negative plant-soil feedback known from natural ecosystems.

Crop rotations

Rotation of crops in time is a long known agricultural practice to avert negative effects of continuously cultivating the same crop. The first records of crop rotations date back more than 2000 years and were optimized over time (White, 1970; van der Putten *et al.*, 2013). Crop rotations are applied to reduce yield losses typical for monocultures by suppression of weeds, pathogens, pests, and insects (Brust & King, 1994; Karlen *et al.*, 1994; Chen *et al.*, 2001; Leandro *et al.*, 2018). Increasing the diversity of cropping systems can further increase soil fertility, microbial biomass and diversity, water retention, and soil health in general (Bennett *et al.*, 2012; Tiemann *et al.*, 2015; McDaniel *et al.*, 2016; Tamburini *et al.*, 2020). The changes in the soil can increase the resilience of cropping systems to adverse growth conditions and weather events, thereby mitigating potential negative impacts of global change (Bowles *et al.*, 2020). While these long-term benefits are well understood, less is known about the effect of individual crops on their successor crop. There is growing evidence that a crop`s performance, defense, yield, and soil processes are influenced by the preceding crop identity (Sieling & Christen, 2015; McDaniel *et al.*, 2016; Benitez *et al.*, 2021), and that these effects might be mediated by altered soil microbial communities (Benitez *et al.*, 2017; Benitez *et al.*, 2021). A better understanding of these plant-soil feedbacks between two consecutively cultivated crops is needed to design superior crop rotations (Dias *et al.*, 2015).

Plant-soil feedbacks

The ecology of plant-soil feedbacks in natural environments is well understood and can, for example, explain plant diversity, vegetation succession, and plant invasion (van der Putten *et al.*, 1993; Klironomos, 2002; van der Putten *et al.*, 2013; Teste *et al.*, 2017). Different plant phenotypes were shown to be affected by plant-soil feedbacks, including biomass production, leave secondary metabolites, insect preference, and insect performance (Pineda *et al.*, 2010; Kos *et al.*, 2015b; Hu *et al.*, 2018b; Pineda *et al.*, 2020). Also, the mechanisms by which soil conditioning of the preceding plants affects the feedback on another plant vary strongly and can be summarized by the following groups: altered nutrient availability, shifts in pathogen or

mutualist communities, and soil chemistry (Bennett & Klironomos, 2019; Schandry & Becker, 2020). More recently, extracellular self-DNA has been proposed as a potential mechanism of conspecific negative plant-soil feedbacks (Mazzoleni *et al.*, 2015). The outcome of plant-soil feedbacks further depends on the environmental context, but the mechanisms behind this still need further investigation (Smith-Ramesh & Reynolds, 2017). The knowledge gained on natural plant-soil feedbacks is not yet well integrated into agroecosystems, even though it presents a promising tool towards a more sustainable agriculture (Mariotte *et al.*, 2018).

Root exudate metabolites

Plants produce an immense number of secondary metabolites in order to survive in hostile environments (Hartmann, 2007). A significant subset of them is exuded into the rhizosphere (Baetz & Martinoia, 2014). Root exudates make up to 40% of the plants carbon fixed by photosynthesis (Badri & Vivanco, 2009), where the majority of root exudates represent primary metabolites (mainly sugars, amino acids, and organic acids) and they are, at least in part, released passively (McCully & Canny, 1985; Darwent *et al.*, 2003; Jones *et al.*, 2009; Canarini *et al.*, 2019). Exudation of secondary metabolites, in contrast, is generally an active process (Sasse *et al.*, 2018). While primary metabolites are important to feed the rhizosphere inhabitants, secondary metabolites are crucial for specific host-controlled interactions, such as the establishment of symbiosis with mycorrhiza fungi or nodulating rhizobia (Abdel-Lateif *et al.*, 2012; Canarini *et al.*, 2019). Further, different groups of secondary metabolites including flavonoids, camalexins, coumarins, and benzoxazinoids have been shown to affect the composition and/or functioning of the root microbiome (Pang *et al.*, 2021; Koprivova & Kopriva, 2022), and might therefore be harnessed to increase agricultural productivity through microbiome-mediated mechanisms (Jacoby *et al.*, 2021; Trivedi *et al.*, 2021).

Benzoxazinoids as a model to study plant-soil feedbacks

Benzoxazinoids, a class of secondary metabolites present in important crops such as maize and wheat, show an intriguingly versatile bioactivity. Besides their role as defensive chemicals in leaves and as within-plant signaling molecules (Niemeyer, 2009; Ahmad *et al.*, 2011; Meihls *et al.*, 2013), they are involved in various belowground interactions. They can, for example, increase the fraction of plant available iron through chelation (Bigler *et al.*, 1996; Hu *et al.*, 2018a), suppress fungal pathogens (Martyniuk *et al.*, 2006), affect the foraging behavior and performance of root feeding herbivores (Robert *et al.*, 2017; Hu *et al.*, 2018a), shape soil nematode communities (Sikder *et al.*, 2021), and are involved in plant-nematode-insect tritrophic interactions (Robert *et al.*, 2017; Zhang *et al.*, 2019). Further, benzoxazinoids attract potentially beneficial bacteria strains (Neal *et al.*, 2012; Neal & Ton, 2013) and shape the

rhizosphere microbiome at different growth stages (Hu *et al.*, 2018b; Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019).

Recently, Hu and colleagues found that soils conditioned by benzoxazinoid producing plants affected the growth and defence of the next conspecific plant grown in that soil (Hu *et al.*, 2018b). Through chemical complementation, soil sterilization, and re-inoculation they showed that these feedbacks are mediated by benzoxazinoid exudation into the rhizosphere, and driven by shifts in the soil microbial community composition (Hu *et al.*, 2018b). Additional greenhouse experiments demonstrated that the observed phenomenon dependents on the soil origin and that maize benzoxazinoid soil conditioning can also affects wheat growth and defence (Cadot *et al.*, 2021a). It, however, remains unclear if benzoxazinoid-dependent plant-soil feedbacks affect agricultural productivity and food quality under field conditions and if benzoxazinoids reduce negative plant-soil feedbacks of preceding plants.

Thesis outline

Agricultural systems must become more productive without degrading farmlands, to feed the growing human population in the long term (Foley *et al.*, 2011; Hunter *et al.*, 2017). A promising avenue is the adaption of ecological concepts in agricultural systems (Altieri, 1995). Plant-soil feedbacks, for example, are receiving increasing attention with regard to crop rotation and represent one such promising ecological concept that could be deployed in agriculture (Dias *et al.*, 2015; Mariotte *et al.*, 2018). Recently, secondary metabolite-mediated plant-soil feedbacks were shown to affect plant growth and defence (Hu *et al.*, 2018b). This leads to the overarching question of this thesis whether plant secondary metabolites have the potential to improve the output of crop rotations through agroecological plant-soil feedbacks and thereby present a tool to make agriculture more sustainable. From this question I derived my two main hypotheses: Secondary metabolite-mediated plant-soil feedbacks enhance crop rotation productivity and food quality; Secondary metabolites mitigate negative plant-soil feedbacks of previous crops (**Fig. 1**). To test these hypotheses, two field experiments and several experiments under controlled environments were conducted. I focused on crop rotations including maize (*Zea mays*) and wheat (*Triticum aestivum*), both belonging to the most important crops to feed the world. As a model for secondary metabolites, I investigated benzoxazinoids, a class of indole-derived compounds produced by important cereals including maize, wheat, and rye (Frey *et al.*, 2009). To test for benzoxazinoid-dependent effects, benzoxazinoid-deficient *bx1* mutant plants of two maize lines, W22 and B73, were used (Tzin *et al.*, 2015; Maag *et al.*, 2016). In the three chapters of this thesis, I aimed at elucidating different aspects of benzoxazinoid-dependent plant-soil feedbacks in crop rotations.

In **chapter I**, I addressed the question if benzoxazinoids affect agricultural productivity and food quality through plant-soil feedbacks. In a two-year field experiment we tested the effect of maize benzoxazinoid soil conditioning on wheat, by first growing wild-type and benzoxazinoid-deficient *bx1* mutant maize, followed by the cultivation of three winter wheat varieties. Wheat growth and insect infestation, as well as wheat yield and kernel quality were determined. I showed that maize benzoxazinoid soil conditioning can enhance wheat productivity under agronomically relevant conditions while maintaining high food quality.

Based on the results of chapter I, I asked myself how consistent such benzoxazinoid-dependent plant-soil feedbacks are. Therefore, in **chapter II**, we conducted another two-year field experiment at a different location. The experimental field exhibited a strong special environmental gradient in soil parameters, allowing us to test how soil drives benzoxazinoid-

dependent plant-soil feedbacks. Along this soil gradient, we grew wild-type and *bx1* mutant maize in the conditioning phase, followed by wheat growth in the feedback phase. Soil benzoxazinoids, root-associated microbiomes, and chemical parameters were thoroughly investigated, to test for associations between the environmental gradient and benzoxazinoid feedbacks on wheat. Measurements of wheat growth, defence, and kernel parameters revealed that this within-field heterogeneity in soil parameters markedly affected the strength and direction of benzoxazinoid-dependent feedbacks.

In **chapter III**, to broaden our understanding of benzoxazinoids in crop rotations, I wanted to establish if benzoxazinoid exudation modify the performance of a given plant in soils conditioned by different preceding crops. To test our hypothesis that benzoxazinoids help to resist negative plant-soil feedbacks, we performed a series of experiments under controlled conditions. Fist, soils were conditioned by different precrop species, followed by wild-type and *bx1* mutant maize. To study the underlining mechanisms, we also performed chemical *bx1* mutant complementation and experiments with sterilized and re-inoculated soils. Our experiments showed that benzoxazinoids reduce growth suppressive effects of some precrops and thereby enhance crop rotation stability.

Overall, this thesis demonstrates the potential of plant secondary metabolites to enhance crop rotation productivity and highlights possible challenges, such as within-field variability of feedbacks, for employing plant secondary metabolites to improve crop rotations.

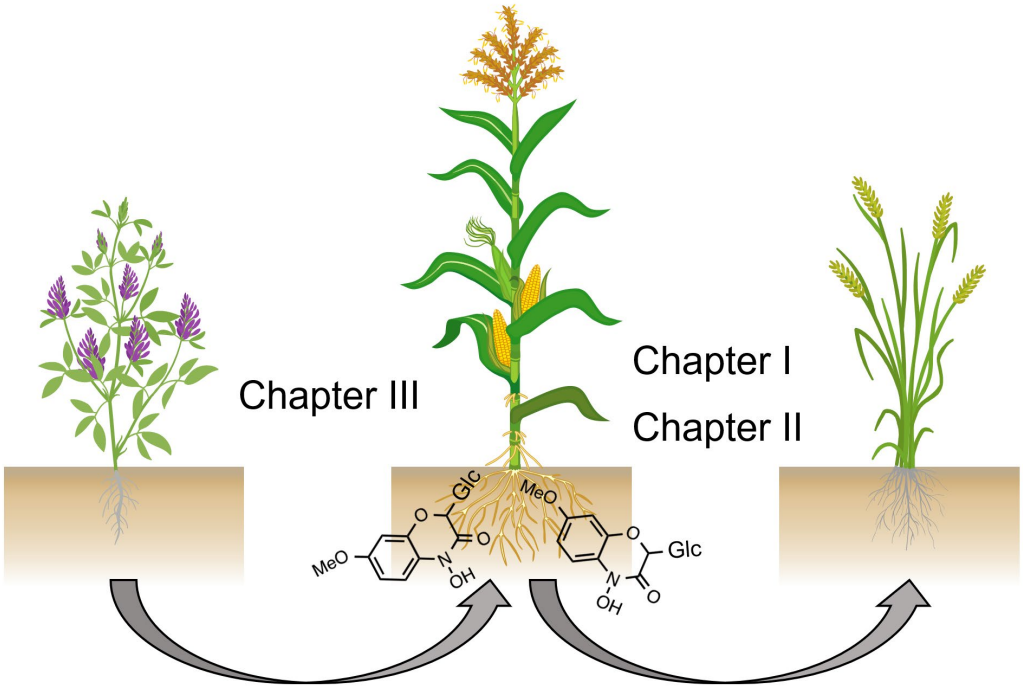

Fig. 1. Potential roles of benzoxazinoids in agroecological plant-soil feedbacks. Can benzoxazinoids reduce negative plant-soil feedbacks of preceding crops? Do benzoxazinoids affect agricultural productivity and food quality through plant-soil feedbacks? How do differences in soil mediate benzoxazinoid-dependent plant-soil feedbacks in crop rotations? These are the key questions I answer in this thesis. Pictures modified from AdobeStock.

Chapter I

Plant secondary metabolite-dependent plant-soil feedbacks can improve yield in the field

Valentin Gfeller, Jan Waelchli, Stephanie Pfister, Gabriel Deslandes-Hérold, Fabio Mascher, Gaétan Glauser, Yvo Aeby, Adrien Mestrot, Christelle A.M. Robert, Klaus Schlaeppi* and Matthias Erb*

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Abstract

Plant secondary metabolites that are released into the rhizosphere alter biotic and abiotic soil properties, which in turn affect the performance and resistance of the next plant generation. How such plant-soil feedbacks affect agricultural productivity and food quality in crop rotations is unknown. Here, we assessed the impact of maize benzoxazinoids on the performance and food quality of three winter wheat varieties in a two-year field experiment. Following maize cultivation, we detected benzoxazinoid-dependent chemical and microbial fingerprints in soil. The chemical fingerprint was still visible during wheat growth, while the microbial fingerprint was no longer detected. Benzoxazinoid soil conditioning by wild type maize led to increased wheat emergence, tillering, growth, and biomass accumulation, as well as lower insect infestation compared to conditioning by benzoxazinoid-deficient *bx1* mutant plants. Wheat yield was increased by over 4% without reduction in grain quality. Taken together, our experiments demonstrate that plant secondary metabolites can increase yield via plant-soil feedbacks under agronomically realistic conditions. Both chemical and microbial legacies are potential drivers of these feedbacks. Optimizing plant root exudation within crop rotations may be a promising strategy to enhance yields without additional agrochemical inputs.

Significance Statement

Plants release secondary metabolites into soil, which modulate microbial communities and affect plant performance. Here, we show that such plant-soil feedbacks can be harnessed for sustainable intensification of food production. In a two-year field experiment, benzoxazinoids, which are released by maize roots, promoted growth and defense of three winter wheat varieties. This resulted in an increase in wheat yields of >4% while maintaining grain quality. Our work represents a proof-of-concept that plant secondary metabolites can be harnessed to increase crop productivity without any additional input, thus paving the way to engineer crop rotations by optimizing root exudate chemistry.

Introduction

Plants alter the soil they live in, and thus modulate the growth and defense status of other plants growing in the same soil (Bever *et al.*, 1997). So-called plant-soil feedbacks can influence plant community composition and ecosystem functions (Bennett *et al.*, 2017; Teste *et al.*, 2017; Mariotte *et al.*, 2018) and have been used for centuries in crop rotation schemes to reduce pest, weed and disease pressure and improve crop yields (White, 1970; van der Putten *et al.*, 2013). So far, mechanistic work on plant-soil feedbacks has rarely been applied to improve crop rotations, thus limiting the benefit of this research for the engineering of crop rotations for ecological and sustainable agriculture (Mariotte *et al.*, 2018).

Plant-soil feedbacks can modulate a variety of plant traits. Reductions in germination for instance are common (Tawaha & Turk, 2003). Changes in plant chemistry have also been recorded, which likely influence herbivore performance and preference (Pineda *et al.*, 2010; Kos *et al.*, 2015b; Hu *et al.*, 2018b; Pineda *et al.*, 2020). Alterations in susceptibility to soil pathogens have also been observed (Ma *et al.*, 2017). Plant-soil feedbacks can also directly increase plant growth and biomass, for instance by modulating hormonal balance (van der Putten *et al.*, 2013; Pieterse *et al.*, 2014; Hu *et al.*, 2018b; Bennett & Klironomos, 2019). The response to plant-soil feedbacks is often species- and variety-specific, and thus requires detailed investigations under genetically defined conditions (Bever, 1994; Wagg *et al.*, 2015; Hu *et al.*, 2018b; Cadot *et al.*, 2021a). At the same time, the diversity of plant traits that can be affected call for broad phenotyping efforts that take into account ecologically and economically relevant parameters, including yield and yield quality measures of agricultural output.

Plant-soil feedbacks can modulate plant traits via different mechanisms, including changes in nutrient availability and chemical properties of the soil (Bennett & Klironomos, 2019; Schandry & Becker, 2020). Positive feedbacks in agriculture are often attributed to increased soil fertility, water retention, and improved pest control (Bennett *et al.*, 2012; Tamburini *et al.*, 2020). In recent years, changes in microbial communities have received substantial attention as drivers of plant-soil feedbacks (Bever *et al.*, 2012; Benitez *et al.*, 2021). Various plant health benefits have been associated to the rhizosphere microbiome (Berendsen *et al.*, 2012) and plant-soil feedbacks represent a promising way to harness these positive effects, including growth promotion and insect resistance in agricultural settings (Hu *et al.*, 2018b; Pineda *et al.*, 2020).

How do plants alter soil microbial communities? Although multiple mechanisms are likely at play, the release of small molecular weight compounds, including primary and secondary metabolites, is emerging as a major determinant of microbial community composition in the

rhizosphere (Pang *et al.*, 2021). Flavones, coumarins, triterpenes, and benzoxazinoid secondary metabolites are known to structure the rhizosphere microbiota (Hu *et al.*, 2018b; Stringlis *et al.*, 2018; Huang *et al.*, 2019; Voges *et al.*, 2019; Yu *et al.*, 2021). Flavones and benzoxazinoids have recently been shown to modulate plant-soil feedbacks via changes in microbial communities (Hu *et al.*, 2018b; Yu *et al.*, 2021). If and how secondary metabolites can alter plant performance via plant-soil feedbacks under realistic field conditions, however, remains unclear.

Benzoxazinoids, a class of indole-derived secondary metabolites produced by important food crops such as maize and wheat, are known to have multiple functions, ranging from defense to nutrient uptake (Niemeyer, 2009). Recent studies have shown that benzoxazinoids can shape rhizosphere microbial communities (Hu *et al.*, 2018b; Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019; Cadot *et al.*, 2021b). Soil conditioning by benzoxazinoids can feed back on growth and defense of maize and wheat. These effects are likely mediated by changes in rhizosphere microbial communities (Hu *et al.*, 2018b; Cadot *et al.*, 2021a). Benzoxazinoids can also chelate iron and reduce the performance of non-benzoxazinoid producing plants (Bigler *et al.*, 1996; Niemeyer, 2009; Hu *et al.*, 2018a). Based on this knowledge, we hypothesized that benzoxazinoid soil conditioning may improve the performance of subsequent crop in a crop rotation under agriculturally relevant conditions.

To test the above hypothesis, we investigated how maize benzoxazinoids affect agricultural productivity and food quality of wheat in a field experiment. Maize and wheat are among the most important crops in global food production and are commonly cultivated in sequence in rotation schemes. In a two-year field experiment involving wild-type and benzoxazinoid-deficient *bx*$_1$ mutant maize plants, we first evaluated the effects of benzoxazinoid soil conditioning on soil chemistry and microbial communities. In the following season, we planted three wheat varieties into the same field and quantified a wide variety of agronomically important traits, including yield and yield quality. This experiment allowed us to demonstrate that root exudation of secondary metabolites can improve sustainable food production under an agronomically realistic crop rotation scenario.

Results

Maize benzoxazinoid soil conditioning results in persistent chemical fingerprints in the soil

To test the hypothesis that maize benzoxazinoids improve the performance of wheat in a crop rotation scheme, we grew wild-type W22 and benzoxazinoid-deficient *bx1* mutant maize plants (in a W22 background) in the field. Compared to its wild-type counterpart, the *bx1* mutant exhibits a strong reduction in benzoxazinoid production due to a transposon insertion in the *Bx1* gene (Tzin *et al.*, 2015). We established a design where the two genotypes were alternatingly sown in 5 strips, each consisting of 12 rows of maize (**Fig. S1**). Over the course of their growth, both maize genotypes grew similarly and accumulated the same amount of biomass at the end of the growing season (**Fig. S2A**). Substantial amounts of benzoxazinoids and benzoxazinoid degradation products were detected in the soils of plots cultivated with wild-type plants (**Fig. 1A**). HDMBOA-Glc was the most abundant benzoxazinoid, followed by HMBOA, DIMBOA and DIMBOA-Glc. The breakdown products MBOA and AMPO were also detected. In contrast to soils planted with wild-type plants, most benzoxazinoids were below the limit of detection in the soils planted with *bx1* mutant plants. We only detected trace amounts of MBOA and AMPO in these soils.

To evaluate the persistence of this chemical fingerprint at the time of cultivation of the next crop, we determined benzoxazinoid profiles again 6 weeks after maize harvest, at the beginning of winter wheat cultivation. Most benzoxazinoids were only present in trace amounts, with concentrations that were 3- to 800-fold lower compared to the end of maize cultivation (**Fig. 1B**). An exception was the stable breakdown product AMPO, which had increased more than 2-fold in abundance during this time. DIMBOA, HMBOA, MBOA and AMPO were still present in higher concentrations in wild-type conditioned soils. Interestingly, the two glycosylated benzoxazinoids DIMBOA-Glc and HDMBOA-Glc were more abundant in soils previously cultivated with *bx1* mutant maize. As benzoxazinoids are released as glycosides and deglycosylated in the soil, this is indicative of a faster deglycosylation of benzoxazinoids in wild-type conditioned soils.

To test if soil conditioning by benzoxazinoids also affected other soil edaphic factors, we analyzed soil nutrient levels and pH at the end of maize cultivation. No significant differences were found between soils cultivated with wild-type or *bx1* mutant plants (**Fig. S2B**).

We then sowed 2 different wheat varieties (Claro and Fiorina) into the field, resulting in 20 plots per wheat variety with a size of 6 * 6 m where half of the plots were previously cultivated with wild-type and the other half with *bx1* mutant maize (**Fig. S1**). An additional

variety (Sailor) was sown for multiplication adjacent to the two other varieties on the same field by a third party. While Claro and Fiorina were managed without plant protection products, Sailor was treated with herbicides. As Sailor was sown within the premises of our conditioning experiment, we took the opportunity to also measure a subset of traits in Sailor.

To test if the chemical fingerprint persisted further as wheat grew in soil, we analyzed the soil benzoxazinoids again during wheat growth. As benzoxazinoids are also produced by wheat, the measurements likely represent both old maize and newly wheat produced metabolites. The previous clear differences in benzoxazinoid levels of HDMBOA-Glc, HMBOA and MBOA were not detected any more at this point (**Fig. 1C**). However, AMPO levels were still significantly and consistently higher in plots where previously wild-type maize grew, this was apparent across all three wheat varieties. Taken together, these results show that modulating benzoxazinoid production results in persistent changes in soil chemical fingerprints.

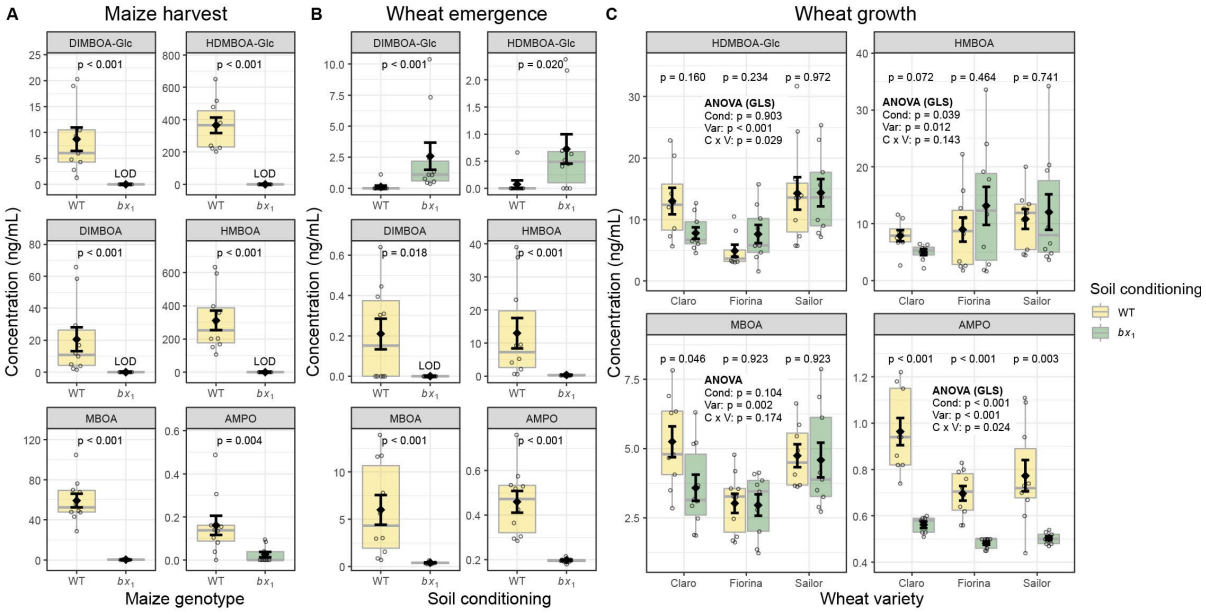
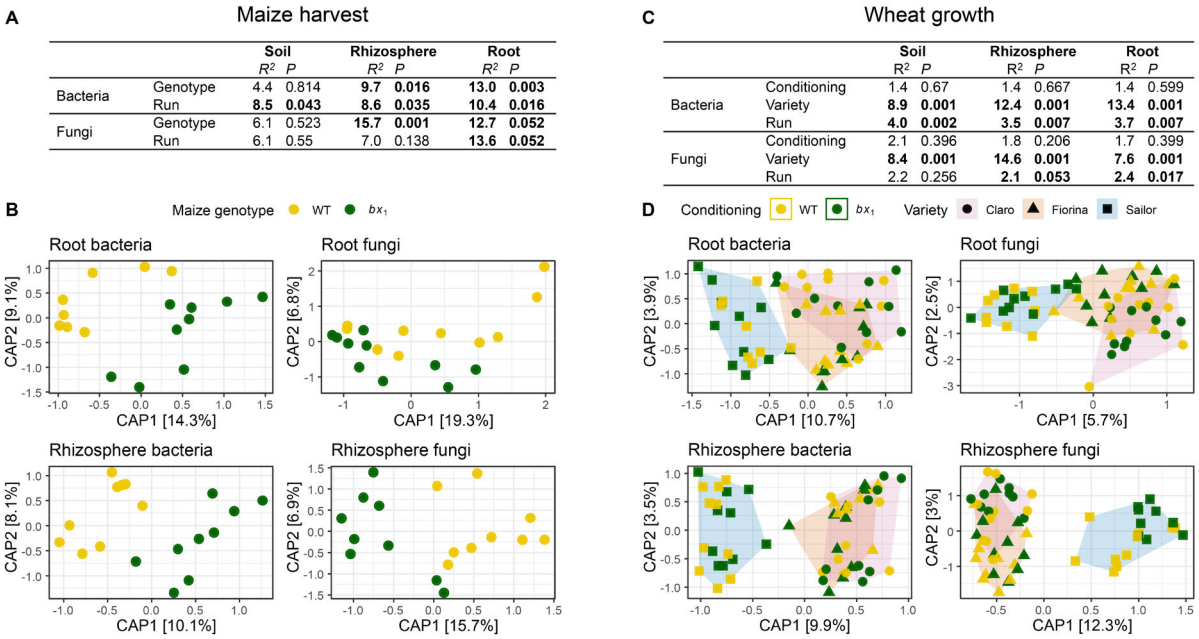


Figure 1. Root benzoxazinoid exudation results in persistent chemical fingerprints. (A) Concentrations of benzoxazinoids in soil at harvest of the wild-type (WT) or benzoxazinoid-deficient *bx₁* mutant maize plants indicated in ng per mL of soil. **(B)** Benzoxazinoids in field soil 6 weeks after maize harvest. For **(A)** and **(B)** means ± SE, boxplots, and individual datapoints are shown and Wilcoxon rank-sum tests are included (FDR-corrected *p* values, n = 10). **(C)** Benzoxazinoids were measured again during wheat growth. Means ± SE, boxplots, and individual datapoints are shown (n = 10). ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety are included (FDR-corrected *p* values). LOD: below limit of detection. Gen: maize genotype (WT and *bx₁*). Cond: soil conditioning (WT and *bx₁*). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety.

Maize benzoxazinoid soil conditioning transiently structures the microbial communities

To investigate if differences in benzoxazinoid soil conditioning affected the bacterial and fungal communities, we analyzed soil, rhizosphere, and root samples by profiling the bacterial 16S rRNA gene and the ITS1 region of the ribosomal operon for fungi. Microbiota profiling at maize harvest revealed the biggest taxonomic differences at the phylum level to be among compartments (**Fig. S3**). Permutational Multivariate Analysis of Variance (PERMANOVA) revealed significant differences between genotypes in the bacterial and fungal community composition of roots and rhizospheres after taking the effect of the sequencing run into account (**Fig. 2A**). Plant genotype accounted for 9.7 % to 15.7 % of the total variation within compartments. The benzoxazinoid effect on root bacterial and fungal community was comparable (R^2 bacteria = 13 %, R^2 fungi = 12.7 %) while in the rhizosphere we found a more pronounced effect on the fungal community relative to the bacterial community (bacteria = 9.7 %, fungi = 15.7 %). In soils, no benzoxazinoid effects were detected (**Fig. 2A**). In line with PERMANOVA, visualization of bacterial and fungal communities in roots and rhizospheres by Constrained Analysis of Principal Coordinates (CAP) showed a clear differentiation between maize genotypes in both compartments (**Fig. 2B**). Overall, these results confirm that benzoxazinoids structure root-associated microbial communities in maize.

To test if the benzoxazinoid effects on microbial community composition persisted during crop rotation, we analyzed bacteria and fungi in the root, rhizosphere, and soil compartments during wheat growth. Again, the strongest taxonomic differences at phylum level were found among compartments (**Fig. S4**). PERMANOVA on Bray-Curtis distances revealed a consistent difference in community composition between wheat varieties, with Sailor being the most dissimilar to the others (**Fig. 2C/D**). Note that these differences could also be the result of different position of Sailor in the field (**Fig. S1**). PERMANOVA did not reveal any benzoxazinoid-dependent effects on microbial community composition. Thus, there was no clear legacy effect on microbial community composition remaining at the onset of wheat maturation.



		Soil		Rhizosphere		Root	
		R^2	P	R^2	P	R^2	P
Bacteria	Genotype	4.4	0.814	**9.7**	**0.016**	**13.0**	**0.003**
	Run	**8.5**	**0.043**	**8.6**	**0.035**	**10.4**	**0.016**
Fungi	Genotype	6.1	0.523	**15.7**	**0.001**	**12.7**	**0.052**
	Run	6.1	0.55	7.0	0.138	**13.6**	**0.052**

		Soil		Rhizosphere		Root	
		R^2	P	R^2	P	R^2	P
Bacteria	Conditioning	1.4	0.67	1.4	0.667	1.4	0.599
	Variety	**8.9**	**0.001**	**12.4**	**0.001**	**13.4**	**0.001**
	Run	**4.0**	**0.002**	**3.5**	**0.007**	**3.7**	**0.007**
Fungi	Conditioning	2.1	0.396	1.8	0.206	1.7	0.399
	Variety	**8.4**	**0.001**	**14.6**	**0.001**	**7.6**	**0.001**
	Run	2.2	0.256	**2.1**	**0.053**	**2.4**	**0.017**

Figure 2. Root benzoxazinoid exudation transiently affect microbial communities. Soil, rhizosphere, and root-associated microbial communities at maize harvest (**A, B**) and during wheat growth (**C, D**). **(A)** Output of PERMANOVA on Bray-Curtis dissimilarities of bacteria and fungi showing R^2 and p values for genotype and sequencing run effects in soil, rhizosphere, and root compartments. Significant effects are indicated in bold. **(B)** Constrained Analysis of Principal Coordinates (CAP) confirming the genotype effects found in the PERMANOVA, axis labels denote percentage of explained variance (n = 8-10). **(C, D)** Same as in (**A, B**) but also including the factor wheat variety (n = 6-10).

Maize benzoxazinoid soil conditioning improves subsequent wheat emergence and growth

To investigate whether benzoxazinoid soil conditioning affects wheat performance, we measured emergence shortly after seeding and chlorophyll contents, plant height and aboveground biomass during wheat growth of the three varieties. Overall, wheat seedling emergence was increased by 8 % in benzoxazinoid conditioned soils (**Fig. 3A**). Chlorophyll content in the youngest fully developed leaf (as a proxy for early plant performance) was generally increased in plants growing in benzoxazinoid conditioned soils (**Fig. 3B**). Later during wheat growth, height and biomass production per area, as well as shoot water content were increased in benzoxazinoid conditioned soils (**Fig. 3C/D, Fig. S5**). Thus, benzoxazinoid soil conditioning of the preceding crop increases wheat performance across different wheat varieties.

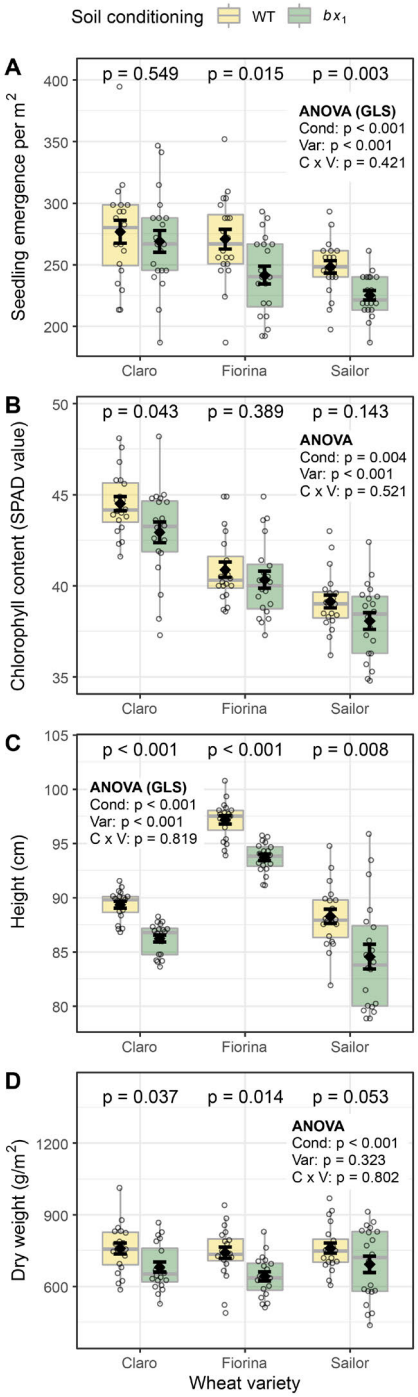


Figure 3. Benzoxazinoid soil conditioning positively affects wheat performance. (A) Seedling emergence, **(B)** chlorophyll content, **(C)** plant height, and **(D)** shoot dry weight of three wheat varieties sown in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx*$_1$ mutant maize. Means ± SE, boxplots, and individual datapoints are shown (n = 20). ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. Cond: soil conditioning (WT and *bx*$_1$). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety.

Maize benzoxazinoid soil conditioning does not change weed pressure, but reduces insect infestation

To test for possible allelopathic effects of benzoxazinoids on weeds, we surveyed the weed cover on all plots. Chickweed (*Stellaria media*), Persian speedwell (*Veronica persica*), and Shepherd's purse (*Capsella bursa-pastoris*) were the most abundant weeds on the plots. We found that weed pressure differed along the field, and therefore accounted for position effects in the analysis. If statistically significant, we included position in all further analyses. We found no effect of soil conditioning status on weed abundance for the varieties Claro and Fiorina (**Fig. 4A**). No weeds were detected with the variety Sailor, as the latter was treated with herbicides.

The main herbivore that occurred in the field was the cereal leaf beetle *Oulema melanopus*. The abundance of *Oulema* larvae was significantly reduced on wheat plants of all three varieties grown in benzoxazinoid conditioned soils, with the biggest difference in the variety Sailor (**Fig. 4B**). To investigate whether this pattern resulted in reduced damage, we quantified the consumed leaf area on the flag leaves at the end of *Oulema* development. Benzoxazinoid conditioning reduced leaf damage in the variety Sailor, but no significant differences in Claro and Fiorina were found (**Fig. 4C**). We also measured defense hormone levels, indicative for defense activation. No significant influence of benzoxazinoid soil conditioning was found (**Fig. S6**).

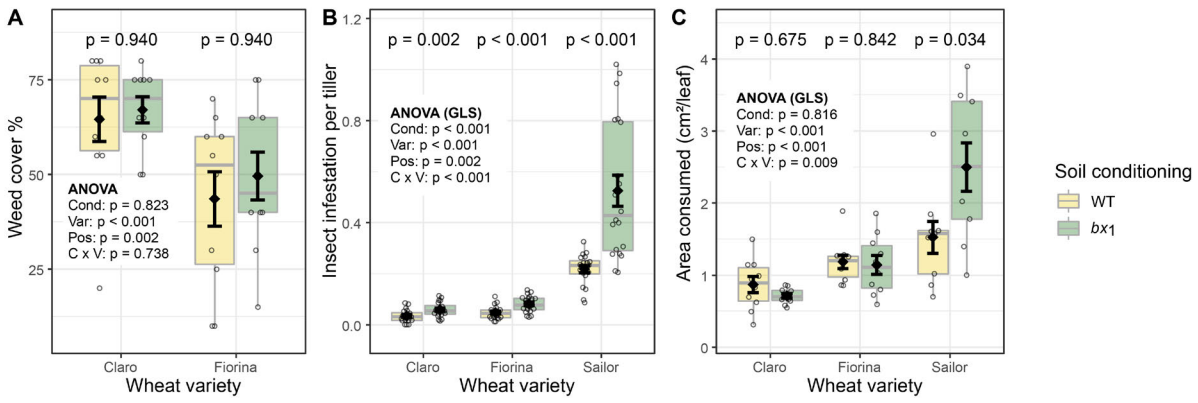


Figure 4. Benzoxazinoid soil conditioning reduces insect infestation on wheat, but does not affect weed pressure. (A) Ground cover by weed plants in plots of three wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient bx_1 mutant maize (n = 10). **(B)** Mean abundance of cereal leaf beetle (*Oulema melanopus*) per tillers (n = 20) and **(C)** Consumed flag leaf area by cereal leaf beetle (n = 9-10). Means ± SE, boxplots and individual datapoints (n = 20) are shown. ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. Cond: soil conditioning (WT and bx_1). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety. Pos: position on the field.

Maize benzoxazinoid soil conditioning increases wheat biomass at maturity

To understand how benzoxazinoid soil conditioning influences mature wheat plants, we also quantified plant performance before harvest. All wheat varieties had a higher number of tillers per area in benzoxazinoid conditioned soils (**Fig. 5A**). To test if these differences can be attributed to differences in emergence or differences in tillering, we also counted the number of tillers per plant. Overall, plants in benzoxazinoid conditioned soils produced a higher numbers of tillers per plant. This pattern was consistent across all varieties, with the most pronounced difference in the variety Sailor (**Fig. 5B**). Next, we measured if the higher tiller density resulted in a higher aboveground biomass per area. Consistent with the results during wheat growth, benzoxazinoid soil conditioning also increased biomass at plant maturity (**Fig. 5C**). The weight of individual tillers was similar (**Fig. 5D**), demonstrating that benzoxazinoid soil conditioning increased biomass by promoting tiller density, both through enhanced germination and tillering.

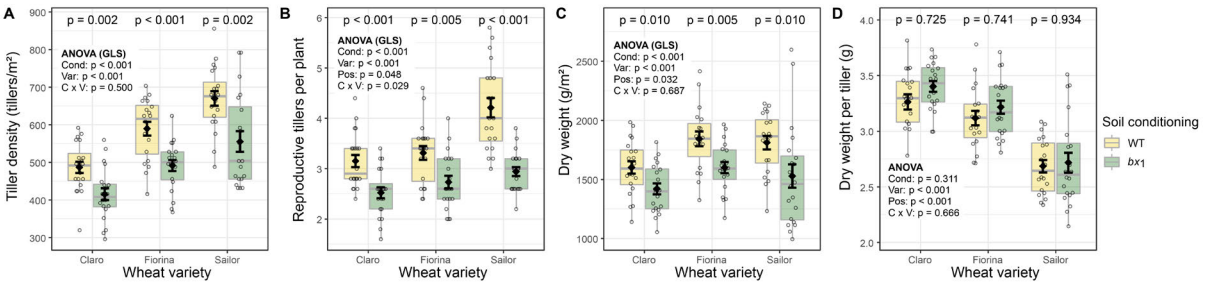


Figure 5. Benzoxazinoid soil conditioning increases wheat biomass in a density-dependent manner. (A) Tiller density, **(B)** reproductive tillers per plant, **(C)** shoot dry weight, and **(D)** dry weight per tiller of three wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx*$_1$ mutant maize. Means ± SE, boxplots, and individual datapoints (n = 20) are shown. ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. Cond: soil conditioning (WT and *bx*$_1$). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety. Pos: position on the field.

Maize benzoxazinoid soil conditioning improves wheat yield

We evaluated whether benzoxazinoid soil conditioning improved wheat yield and quantified the kernel weight per plot at harvest. For each Claro and Fiorina plot, 9 m^2 were harvested. Yield could not be determined for Sailor, as this field was harvested in bulk for seed multiplication. Yield in both Claro and Fiorina was increased by 4-5 % on benzoxazinoid conditioned soils (**Fig. 6A**). The number of kernels per tiller, the kernel weight per tiller and the thousand kernel weight did not differ between soil conditioning treatments (**Fig. 6B, Fig. S7A/B**), showing that the increase in yield is primarily the result of more kernels per area being produced.

To investigate whether the increased wheat yield comes with a penalty in terms of grain quality, we first determined a number of physical kernel properties. Volume per weight, kernel surface area, kernel length and kernel width were not affected by soil conditioning (**Fig. S7C-F**). We further assessed various agronomically important parameters that are indicative of kernel quality and suitability for baking. We measured protein content, Zeleny index, falling number, as well as dough water absorption, stability, and softening. Kernel quality and baking quality were high and showed no differences between soil conditioning treatments (**Fig. 6C-E, Fig. S7G-I**). To test if micronutrient content is affected by soil conditioning, we also quantified 21 elements in the harvested wheat kernels. No benzoxazinoid conditioning effects were found (**Fig. 6F, Fig. S8**). Taken together, these results demonstrate that maize benzoxazinoid soil conditioning increases wheat yield without affecting kernel quality.

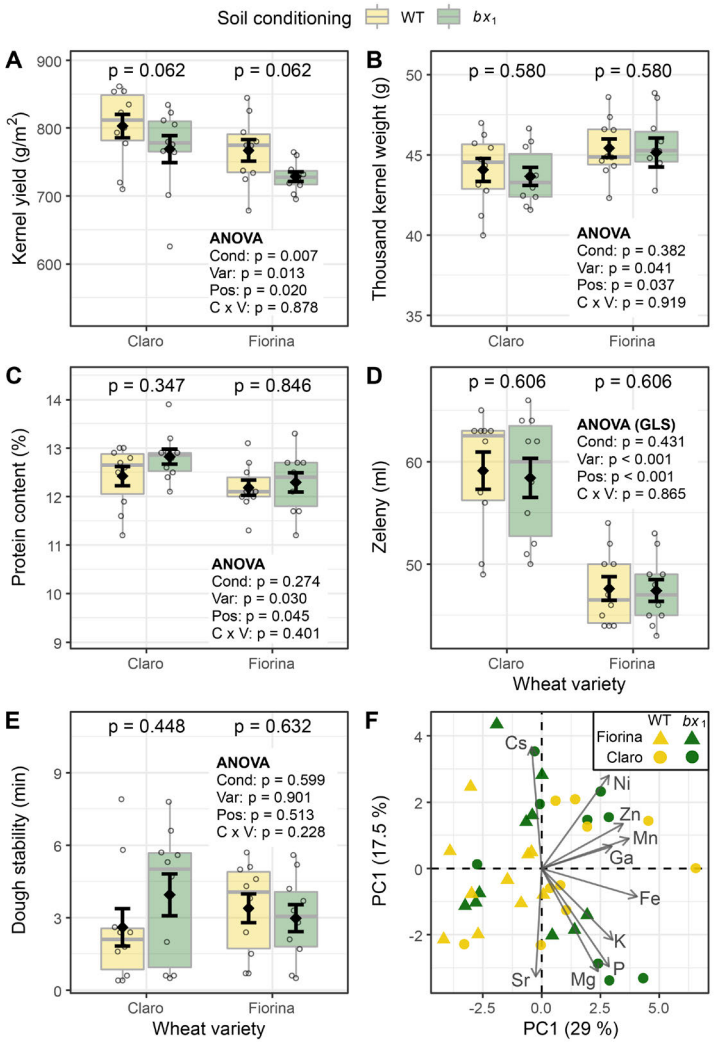


Figure 6. Benzoxazinoid soil conditioning enhances crop yield but not kernel quality. (A) Yield of two wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx*$_1$ mutant maize. Kernel quality measures included **(B)** thousand kernel weight, **(C)** kernel protein content, **(D)** Zeleny index (flour quality), **(E)** dough stability, and **(F)** PCA of kernel micronutrient composition. For **(A)-(E)** means ± SE, boxplots, and individual datapoints are shown (n = 10). ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. **(F)** reports the first two axes of the micronutrient PCA, including individual samples and the contribution of the 10 elements explaining most of the variation in the dataset (arrow length denotes relative contribution). Cond: soil conditioning (WT and *bx*$_1$). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety. Pos: position on the field.

Discussion

Plants exude secondary metabolites into the rhizosphere and thereby influence the growth and defense of subsequently growing plants (Hu *et al.*, 2018b; Cadot *et al.*, 2021a). Whether this phenomenon is also relevant in the field, and whether it can be exploited to improve crop productivity, is unknown. Here, we demonstrate that root secondary metabolites can improve plant growth and crop yield via plant-soil feedbacks under agronomically realistic conditions in an experimental field. Below, we discuss mechanisms underlying this phenomenon as well as its potential to improve sustainable food production.

Translating plant-soil feedback mechanisms to crop resistance and productivity has been proposed as a promising approach in sustainable agriculture (Mariotte *et al.*, 2018). Plant secondary metabolites and their degradation products are known to suppress the growth of other plants (Schandry & Becker, 2020) and improve herbivore and pathogen resistance (Niemeyer, 2009). Less is known about their potential to influence seedling establishment (Lamichhane *et al.*, 2018) and the agronomically most relevant parameters in the field, yield quality and quantity (Cadot *et al.*, 2021a; Pang *et al.*, 2021). We found that benzoxazinoid soil conditioning by the preceding crop increased subsequent wheat emergence, tillering and plant performance in the field, resulting in higher plant biomass and kernel yield. Because weed cover was unaffected, and increased insect infestation only resulted in increased leave damage for a subset of varieties, we conclude that the positive effects on yield were the result of directly improved growth rather than changes in plant competition or pest damage. Interestingly, the observed increase in biomass is different from what was observed in an earlier greenhouse study with maize and wheat (2021a). This discrepancy is explained by the fact that the greenhouse study investigated individual plant performance and did thus not take into account germination effects. Taken together, our work illustrates the power of agriculturally relevant field experiments to detect benefits of plant-soil feedbacks for sustainable agriculture.

In crop rotations the identity of the preceding crop is known to affect growth, tiller density, yield, and kernel protein content of wheat (Anderson, 2008; Rieger *et al.*, 2008; Sieling & Christen, 2015). Our work expands this knowledge by demonstrating that the release of chemicals by in the preceding crop is sufficient to enhance overall crop yield through enhanced emergence and tillering. Although higher plant densities are often associated with lower grain quality (Bastos *et al.*, 2020), we found that the yield increase did not affect physical parameters, grain micronutrient composition, grain quality, and baking quality. The considerable increase in yield of 4 – 5 % is equivalent to more than two years of breeding (Le Gouis *et al.*, 2020), and

represents a true advantage because quality remained constant without additional agricultural inputs. This work thus demonstrates that the release of a specific class of secondary metabolites into the rhizosphere can directly improve agricultural productivity. Benzoxazinoid exudation and responsiveness to benzoxazinoid soil conditioning are thus promising targets for future breeding efforts. Future crop rotations could be designed using varieties that are optimized for such traits. One can for instance envisage a scenario where high benzoxazinoid maize hybrids are selected specifically to precede highly responsive wheat cultivars. Future field experiments will have to evaluate how other crops respond to benzoxazinoid conditioning in the field and how generalizable the obtained results are across different years and locations. Such work could help to further unlock the potential of plant-soil feedbacks for the much needed sustainable intensification of agriculture (Hunter *et al.*, 2017).

From a mechanistic perspective, plant-soil feedbacks can be triggered by different mechanisms (Bennett & Klironomos, 2019): the first plant generation changes soil chemistry (Schandry & Becker, 2020), root-associated microbiota (Bever *et al.*, 2012) or their interaction, with changes in chemistry mediating changes in microbiota (Hu *et al.*, 2018b; Yu *et al.*, 2021). The persistence of chemical and microbiological changes is seen as a key factor in this context. It has been proposed that chemical changes may be more short lived than microbial changes, as plant secondary metabolites often degrade rapidly (Bennett & Klironomos, 2019). In line with previous studies, we found benzoxazinoids alter the composition of root-associated microbes (Hu *et al.*, 2018b; Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019; Cadot *et al.*, 2021b). However, these effects disappeared by the end of the vegetative growth of the next crop. By contrast, the benzoxazinoid chemical fingerprint persisted across both cultivation periods. AMPO, a microbial degradation product with a half live of months (Macías *et al.*, 2004; Niemeyer, 2009), was found in higher concentrations in benzoxazinoid conditioned soils of all three wheat varieties. Thus, we concluded that the chemical fingerprint was more long-lived that the microbial fingerprint in our study. However, microbial community changes may still have contributed to plant-soil feedback effects observed at the late timepoints, as many of the late phenotypes (**Fig. 5, Fig. 6**) were caused by the differential wheat emergence. With emergence and tillering, early microbial legacies may explain most of the late and agronomically important yield effects. More research is needed to disentangle the relative importance of chemical and microbial fingerprints which will aid to optimize the design of agroecologically smart crop rotations.

Conclusions

Taken together this study presents a proof-of-concept for the utilization of plant root exuded metabolites to increase agricultural yield without additional external inputs. This opens a new avenue to optimize plant traits in crop rotations for a more sustainable agriculture. Future studies with different varieties and crop species and in a wider range of soils and under various farming regimes will help to unravel the generalizability and applicability of using exudate-mediated plant-soil feedbacks in sustainable agriculture.

Materials and Methods

Plant material

The field experiment was conducted in two phases, the conditioning phase with maize (*Zea mays*) and the feedback phase with wheat (*Triticum aestivum*, **Fig. S1**). The wild-type maize inbred line W22 (referred to as WT) and the benzoxazinoid-deficient *bx1* transposon knockout mutant (in W22 background, (Tzin *et al.*, 2015) were grown in the conditioning phase. During conditioning, the inbred lines were surrounded by a buffer zone of the hybrid maize variety Gottardo. In the feedback phase the wheat varieties CH Claro (referred to as Claro), Fiorina, and Sailor were grown. All three wheat varieties are commonly cultivated in Switzerland (recommended varieties by Agroscope). Claro is an obligate winter wheat, Fiorina can be cultivated as winter or spring wheat, and Sailor is a common forage winter wheat variety.

Experimental setup

The conditioning phase indicates the first season where the field was cultivated with WT and *bx1* mutant maize to condition the soil with or without benzoxazinoids. 'Benzoxazinoid soil conditioning' refers to the process of benzoxazinoid exudation into the surrounding soil and the resulting changes in the soil (e.g. microbial community composition). In the second season, i.e. the feedback phase, wheat was grown to survey the effects of previous benzoxazinoid soil conditioning on wheat performance. To test for genotype-specific responses, we investigated two wheat varieties Claro and Fiorina. In addition, the seed company Saatzucht Düdingen has grown a third wheat variety (Sailor) adjacent to our two wheat varieties, and we were kindly allowed to phenotype that variety as well. Therefore, we had three wheat varieties to survey during growth, but could not obtain data on yield and kernel quality for Sailor (**Fig. S1**). At the end of the conditioning phase maize biomass, belowground microbiota and soil parameters, including benzoxazinoids, were measured. In the feedback phase we determined wheat emergence, growth, and weed and insect infestation. Soil benzoxazinoids and microbiota were analyzed again during wheat growth. At the end of the feedback phase kernel quantity and quality were evaluated (**Fig. S1**). For detailed methods see below.

Field experiment

Field specifications

The experiment was carried out in 2019 and 2020 on a field at the Agroscope research station in Posieux, Switzerland (parcel 2.3, 46°46′23.09″N 7°06′22.95″E). The soil was classified as a sandy loam. The cropping history of this field was a fodder meadow (mixture of red clover and Italian ryegrass; 2018), winter barley (2017), triticale and alfalfa (field divided,

2016), maize and alfalfa (field divided, 2015), alfalfa and maize (field divided, 2014), and alfalfa (2012-2013). The crops were managed according to Swiss conventional agricultural practices by the field team of Agroscope and the education farm of the Agricultural competence center in Grangeneuve, nearby Posieux. There was a long-lasting drought period in spring 2020 (feedback phase).

Maize conditioning phase

WT and *bx1* inbred lines were alternately sown in 5 strips of 12 rows each (**Fig. S1**). Distance between maize rows was 75 cm, distance between plants within a row was 15 cm. The inbred lines were surrounded by a minimum of 18 rows of hybrid maize. Before sowing, the soil was fertilized with manure (40 m^3/ha), ploughed, and harrowed. Weeds were once treated with herbicide (Equip Power 1.5 l/ha). During plant growth, maize was fertilized twice, firstly with ammonium nitrate supplemented with sulfur 100 kg/ha (25% N, 5% Mg, 8.5% S) and secondly with urea 180 kg/ha (46% N). Maize was harvested and silaged after 22 weeks. One week before harvest, 4 plants per maize strip were randomly selected for phenotyping resulting in 20 replicates per genotype (WT and *bx1*). The aboveground biomass was harvested, dried at 80 °C and weighed. For half of the samples (n = 10) soil cores of 20 x 20 x 20 cm containing the root system were excavated and used for analysis of benzoxazinoid concentrations, microbiomes, and further soil parameters as described below.

Wheat feedback phase

The wheat varieties were sown one week after maize harvest. Claro and Fiorina were sown in two alternating strips, each perpendicular to the orientation of the maize rows (**Fig. S1**). Sailor was sown in the same orientation as the maize. Distance between wheat rows was 12.5 cm. Prior to sowing the soil was harrowed. During plant growth, wheat was fertilized twice, first with 50 kg N/ha of urea-ammonium nitrate solution (UAN; 39 % N) combined with 120 kg/ha Kiserite (15% Mg, 20% S) and second with 55 kg N/ha of UAN solution (39 % N). No plant protection products were applied to Claro and Fiorina, whereas the field of Sailor was treated with a herbicide against weeds. 4 weeks after sowing, at wheat emergence, soil samples were taken for benzoxazinoid analysis. With a soil sampler 10 soil cores per plot (17 mm diameter, 20 cm deep) were taken and combined to one sample (n = 10 per soil conditioning). Germination, plant growth, and insect infestation were phenotyped as described below. During wheat growth, at the end of the vegetative phase soil cores (7 x 7 cm wide, 12 cm deep) were taken below 3 randomly selected wheat plants per plot and pooled for benzoxazinoid and microbiome analysis (n = 10 per treatment combination). After 41 weeks of growth, the wheat was harvested (see below).

Phenotyping wheat in feedback phase

To survey benzoxazinoid-dependent plant-soil feedbacks on wheat growth, we measured various parameters. Phenotyping was carried out on all subplots (**Fig. S1**), resulting in 20 replicates for each combination of soil conditioning status (WT, *bx1*) and wheat variety (Claro, Fiorina, Sailor). Weed cover estimation, determination of insect damage, and harvesting was done on plot level, resulting in 10 replicates for each treatment combination.

Vegetative growth

Emerged seedlings were counted on 1.5 m of a randomly selected wheat row within a subplot one month after the wheat was sown. Seedling emergence per m^2 was calculated. At the end of tillering, we measured chlorophyll content with a SPADE-502 chlorophyll meter (Konica Minolta, Japan). Chlorophyll was determined in the middle of the youngest fully expanded leaf of 20 randomly selected plants per subplot and the mean value was recorded. During stem elongation, weed abundance was surveyed by estimating percentage weed cover per plot. At the end of the vegetative growth stage plant height of 10 randomly selected plants per subplot was measured and averaged for analysis. In addition, biomass accumulation was measured, by harvesting wheat plants along 1 m of a randomly selected row per subplot. Fresh biomass was weighed before plant material was dried at 80 °C until constant weight, dry biomass was determined, and plant water content was calculated.

Insect infestation, leave damage, and sampling for phytohormone analysis

Infestation by the cereal leaf beetle (*Oulema melanopus*) was surveyed at the end of stem elongation. Along 9 m of a row within a subplot all larvae were counted and infestation per m^2 was calculated. To determine the total larval damage on the leaves, 10 flag leaves were sampled per plot before the leaves started to wilt. Leaves were transported to the laboratory in a wettened plastic bag stored in cooled container. Leaves were then scanned and the consumed area per leaf was determined using the R packages *EBImage* and *pliman* (Pau *et al.*, 2010; Tiago Olivoto, 2021). In addition, at the end of the vegetative phase five flag leaves of five plants per plot were randomly selected, wrapped in aluminum foil and snap frozen in liquid nitrogen for later determination of phytohormone levels (see below).

Biomass, tiller density, and harvesting

Once the kernels were ripe, total biomass accumulation was determined by harvesting wheat plants along 1 m of a randomly selected row per subplot. Plant material was dried at 80 °C before measuring biomass. To calculate tiller density and weight per tiller, the number of tillers in the dried material were counted. A subsample of five randomly selected heads were

threshed with a laboratory thresher (LT-15, Haldrup GmbH), and kernels were counted and weighed. Next, we randomly selected five plant per subplot and counted the number of tillers per plant, mean tiller number per plant was taken for statistical analysis.

At the end of the feedback phase, we harvested the experiment plots with a compact plot combine harvester (Zürn 110, Zürn GmbH). Yield was determined based on a 9 m^2 area in the center of the plots (**Fig. S1**) and kernel weight per plot was determined. A subset of these kernels was taken for analyzing kernel quality and micronutrient composition (see below).

Analyses

Benzoxazinoid analysis

At the end of maize growth, at wheat emergence and during wheat growth soils were sampled as described above and benzoxazinoids and break down products were analyzed. Soil samples were processed with a test sieve (5 mm mesh size), 25 mL of soil were transferred into a 50 mL centrifuge tube and homogenized in 25 mL acidified MeOH/H2O (70:30 v/v; 0.1% formic acid). For extraction, the suspension was incubated for 30 minutes at room temperature on a rotary shaker, followed by a centrifugation step (5 min, 2000 g) to sediment the soil. The supernatant was passed through a filter paper (Grade 1; Size: 185 mm; Whatman, GE Healthcare Live Sciences), 1 mL of the flow through was transferred into a 1.5 mL centrifuge tube, centrifuged (10 min, 19000 g, 4 °C), and the supernatant was sterile filtered (Target2TM, Regenerated Cellulose Syringe Filters. Pore size: 0.45 µm; Thermo Scientific) into a HPLC glass tube for further analysis.

To obtain detectable concentrations at wheat emergence and wheat growth, the samples needed to be concentrated before the second centrifugation step 20 and 10 times, respectively. To obtain that, 20 mL or 10 mL of each sample was completely evaporated (45 °C; CentriVap, Labconco) and the pellet was resuspended in 1 mL of acidified MeOH/ H2O (70:30 v/v; 0.1% formic acid).

The analysis was performed as previously described (Robert *et al.*, 2017). Briefly, an Acquity UHPLC system coupled to a G2-XS QTOF mass spectrometer equipped with an electrospray source and piloted by the software MassLynx 4.1 (Waters AG, Baden-Dättwil, Switzerland) was used. Gradient elution was performed on an Acquity BEH C18 column (2.1 x 50 mm i.d., 1.7 mm particle size) at 90-70% A over 3 min, 70-60% A over 1 min, 40-100% B over 1 min, holding at 100% B for 2.5 min, holding at 90% A for 1.5 minutes where A = 0.1% formic acid/water and B = 0.1% formic acid/acetonitrile. The flow rate was 0.4 mL/min. The temperature of the column was maintained at 40 °C, and the injection volume was 1 µL.

The QTOF MS was operated in positive mode. The data were acquired over an m/z range of 50-1200 with scans of 0.15 seconds at a collision energy of 4 V and 0.2 seconds with a collision energy ramp from 10 to 40 V. The capillary and cone voltages were set to 2 kV and 20 V, respectively. The source temperature was maintained at 140 °C, the desolvation was 400 °C at 1000 L/hr and cone gas flow was 50 L/hr. Accurate mass measurements (< 2 ppm) were obtained by infusing a solution of leucin encephalin at 200 ng/mL and a flow rate of 10 mL/min through the Lock Spray probe. Absolute quantities were determined through standard curves of pure compounds. For that MBOA (6-methoxy-benzoxazolin-2(3H)-one) was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). DIMBOA-Glc (2-O-β-D-glucopyranosyl-2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) and HDMBOA-Glc (2-O-β-D-glucopyranosyl-2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one) were isolated from maize plants in our laboratory. 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 (9-methoxy-2-amino-3H-phenoxazin-3-one) were synthesized in our laboratory.

Soil parameters

A subsample of the soil of each root system excavated at the end of maize growth (see above), was taken and pooled to obtain 4 representative samples of the field per genotype. Soil parameters were then analyzed by LBU Laboratories (Eric Schweizer AG, Thun, Switzerland). Water (H_2O), ammonium acetate EDTA (AAE), and carbon dioxide saturated water (CO_2) extractions were performed for different nutrients. H_2O extracts serve as a proxy for plant available nutrients, AAE extracts for nutrients available through plant chelation mechanisms and CO_2 extracts are a common extraction procedure for magnesium, phosphorus, and potassium (similar to H_2O extracts).

Phytohormones analysis

Concentrations of salicylic acid (SA), oxophytodienoic acid (OPDA), jasmonic acid (JA), jasmonic acid-isoleucine (JA-Ile) and abscicic acid (ABA) were determined by UHPLC-MS/MS. First, wheat leave samples were ground to a fine powder under constant cooling with liquid nitrogen. An aliquot of 100 mg (± 20%) was taken and the exact weight was noted for the final determination of hormone concentration. Next, phytohormones were extracted as described in Glauser, Vallat, & Balmer (2014) with minor adjustments: 10 µL of labelled internal standards (d5-JA, d6-ABA, d6-SA, and 13C6-JA-Ile, 100 ng/mL in water) were added to the samples and hormones were extracted in ethylacetate/formic acid (99.5:0.5, v/v), the samples were centrifuged and evaporated to dryness, and finally resuspended in 200 µL of MeOH 50% for analysis. Two µL of extract were injected in an Acquity UPLC (Waters, USA)

coupled to a QTRAP 6500, (Sciex, USA). Analyst v.1.7.1 was used to control the instrument and for data processing. Each phytohormone peak was normalized to that of its corresponding labelled form except that of OPDA which was normalized to that of 13C6-JA-Ile.

Kernel analysis

For morphological analysis of kernels, a subsample of 25 mL kernels was taken. Volume weight, thousand kernel weight (TKW), kernel surface area, kernel length, and kernel width were determined by means of a microbalance and a MARVIN kernel analyzer (GTA Sensorik GmbH, Germany). A subset of kernels was milled for further analysis. To test flour quality, we determined the falling number (according to ICC standard method 107/1), Zeleny index (according to ICC standard method 116/1) and protein content, which was evaluated by near-infrared reflectance spectroscopy (NIRS) using a NIRFlex N-500 (Büchi Labortechnik AG, Switzerland). We further tested dough quality using a micro-doughLAB farinograph (model 1800, Perten Instruments, PerkinElmer United States). Dough stability (min), dough softening (Farinograph Units, FU), and water absorption capacity of the flour (%) during kneading were analyzed according to the manufacturer's protocol.

Kernel micronutrient analysis

We analyzed total element concentrations for 21 elements as grain micronutrients. 40 g of kernels per plot were ground to fine powder using a cutting mill (Pulverisette, Fritsch). Element extraction and analysis was performed as previously described (Cadot *et al.*, 2021b), with small adjustments: An aliquot of 250 mg grain powder was extracted in 4 ml of concentrated HNO_3 (35%) overnight and 2 mL of H_2O_2 (30%) was added. Samples were vortexed for 5 seconds before microwave extraction at 95 °C for 30 min. Before analysis, tubes were filled to 50 mL with HNO_3 (1%) and centrifuged (5 minutes at 2500 rpm) to remove remaining particles. Elements in the extracts were quantified with inductively coupled plasma mass spectrometry (ICP-MS, 7700x, Agilent, USA).

Microbiota profiling

The sampling of the soil cores on the field was describe above. To prepare the soil samples, the root system was removed from the soil core, subsequently the soil was sieved through a test sieve (mesh size 5 mm). Root and rhizosphere samples were prepared as previously reported (Hu *et al.*, 2018b), with minor modifications: Root segments corresponding to -5 to -10 cm below soil level were harvested and large soil particles were removed, before washing the roots twice in a 50 mL centrifuge tube with 25 mL of sterile ddH_20, by vigorously shaking the tube 10 times. The wash fractions were combined, centrifuged (5 minutes at 3000 g) and the resulting

pellet was frozen at -80 °C for further processing (rhizosphere sample). The washed roots were freeze-dried for 72 h and subsequently milled to fine powder using a Ball Mill (Retsch GmBH; 30 seconds at 30 Hz using one 1 cm steel ball).

For DNA extraction, a subsample of 200 mg soil and rhizosphere, and 20 mg of root powder was taken. DNA from all compartments were extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals LLC, Solon, OH, USA) following the manufacturer instruction. In brief, after adding 978 µL of sodium phosphate buffer and 122 µL of MT buffer to each aliquot, the samples were homogenized with a Retsch Mixer Mill during 40 seconds at 25 Hz. Following 10 minutes of centrifugation, 250 µL of PPS was added to the supernatant. After mixing ten times by inversion, samples were centrifuged for 5 minutes. The supernatant was mixed by inversion with 1 mL of binding matrix suspension, transferred to a SPIN™ filter and then centrifuged for 1 minute. The binding matrix was washed with 500 µL of SEWS-M and a total of 3 minutes of centrifugation was performed. The matrix was air-dried for 5 minutes, and the binding matrix was resuspended with 100 µL of DNAse/Pyrogen-Free water. After incubating 5 minutes, DNA was eluted by centrifuging for 1 minute. Extraction was performed at room temperature and all centrifugation steps were done with 14000 g. After that step, the DNA was distributed into 96-well plates in a random and equal manner. The DNA concentrations were quantified with the AccuClear® Ultra High Sensitivity dsDNA quantification kit (Biotium, Fremont, CA, USA) and diluted to 2 ng μL^{-1} using a Myra Liquid Handler (Bio Molecular Systems, Upper Coomera, Australia).

For the bacterial library, a first PCR reaction was performed with the non-barcoded 16S rRNA gene primers 799-F (AACMGGATTAGATACCCKG, Chelius & Triplett, 2001) and 1193-R (ACGTCATCCCCACCTTCC, Bodenhausen *et al.*, 2013). A second PCR tagged the PCR product with custom barcodes. The first PCR program consisted of an initial denaturation step of 2 minutes at 94 °C, 25 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 30 seconds, elongation at 65 °C for 30 seconds, and a final elongation at 65 °C for 10 minutes. The second PCR program was similar, with the difference that the number of cycles was reduced to 10.

For the fungal library, a first PCR reaction was performed with the non-barcoded internal transcribed spacer (ITS) region primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA, Gardes & Bruns, 1993) and ITS2 (GCTGCGTTCTTCATCGATGC, White *et al.*, 1990). A second PCR tagged the PCR product with custom barcodes. The first PCR program consisted of an initial denaturation step of 2 minutes at 94 °C, 23 cycles of denaturation at 94 °C for 45 seconds,

annealing at 50 °C for 60 seconds, elongation at 72 °C for 90 seconds, and a final elongation at 72 °C for 10 minutes. The second PCR program was similar, with the difference that the number of cycles was reduced to 7.

All PCR reactions were performed with the 5-Prime Hot Master Mix (Quantabio, QIAGEN, Beverly, MA, U.S.A.). All PCR products and pooled library were purified with CleanNGS beads (CleanNA, Waddinxveen, The Netherlands) according to manufacturer protocol with a ratio of 1:1.

All the PCR products were quantified with the AccuClear® Ultra High Sensitivity dsDNA quantification kit (Biotium, Fremont, CA, USA) and subpooled by sample type, library type and sequencing run (Table S1). Subpools were assembled using a Myra Liquid Handler by adding an equal mass of each PCR product. For the bacterial library, the rhizosphere and root subpools were purified on an agarose gel (amplicon ~450 bp) using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), whereas all other subpools were purified with CleanNGS beads. Subpools were quantified with the Qubit™ dsDNA BR kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and equally divided into two sequencing libraries (BE09 & BE10). All samples were paired-end sequenced (v3 chemistry, 300 bp paired end) on an Illumina MiSeq instrument at the NGS platform of the University of Bern.

Statistical analysis and bioinformatics

Bioinformatics

The raw sequencing data is available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena) with the study accession PRJEB53704 and the sample IDs SAMEA110170660 (BE09) and SAMEA110170661 (BE10). Raw reads were first quality inspected with *FastQC* and demultiplexed using *cutadapt* (Andrews, 2010; Martin, 2011). The barcode-to-sample assignments are documented in the supporting Dataset S1. With *cutadapt* we also removed primer and barcode sequences from the reads (error 0.1, no indels). We utilized the *DADA2* pipeline of Callahan et al. (Callahan *et al.*, 2016; R package *DADA2* v.3.10) to infer exact amplicon sequences variants (ASVs) from the sequencing reads. The raw reads were quality filtered (max. expected errors: 0; max. N's allowed: 0), truncated to the minimal lengths (250 bp, forward read; 170 bp, reverse) and shorter and low quality reads (truncQ=2) or reads matching PhiX were discarded. The error rates were learned for the separate sequencing runs using the *DADA2* algorithm to denoise the reads and infer true sequence variants. Next, the paired forward and reverse sequences were merged by a minimal overlap of

twelve identical bases, a count table was created, and chimeras were removed using the *DADA2* scripts. Finally, the taxonomy was assigned using a *DADA2* formatted versions of the *SILVA* v.132 database (Quast *et al.*, 2013; Callahan, 2018) for bacteria and the FASTA general release from *UNITE* v8.3 (Abarenkov *et al.*, 2021) for fungi. The bioinformatic code is available on GitHub (https://github.com/PMI-Basel/Gfeller_et_al_Posieux_field_experiment).

Statistical analysis

All statistical analyses were conducted in R (R Core Team, 2021). Data management and visualization was performed using the *tidyverse* package collection (Wickham *et al.*, 2019). Microbiota of root, rhizosphere and soil compartments were analyzed separately for maize samples (conditioning phase) and wheat samples (feedback phase). The variation between sequencing runs was taken into account in all models. We rarefied the data (100x; depth: bacteria: 8000, maize, fungi: 1'200), because this normalization technique efficiently mitigates artifacts of different sampling depths between sample groups (Weiss *et al.*, 2017). Effects on community composition were tested by Permutational Multivariate Analysis of Variance (PERMANOVA, 999 permutations) on Bray-Curtis distances in the R package *vegan* (Oksanen *et al.*, 2020). For maize, we tested for differences between genotypes (model: beta diversity ~ genotype + run), and for wheat, we tested for effects of soil conditioning and wheat variety (model: beta diversity ~ genotype + variety + run). We visualized the beta diversity by plotting the Canonical Analysis of Principal coordinates (CAP) using the R package *phyloseq* (McMurdie & Holmes, 2013).

Plant phenotyping data was analyzed by analysis of variance (ANOVA). Statistical assumptions such as normal distribution and homoscedasticity of error variance were inspected visually from diagnostic quantile-quantile and residual plots. If unequal variance among treatment groups was observed, a model using generalized least squares (GLS, *nlme* package) was fitted, taking into account different variances for each grouping factor (Pinheiro *et al.*, 2021). Possible correlations of the response variables with the position on the field were tested, and, if significant, the position on the field was factored into the model to account for otherwise unexplained variation. For linear models in the feedback phase, we tested for soil conditioning effects within each wheat variety by calculating estimated marginal means (EMMs; *emmeans* package) and reporting false discovery rate (FDR) corrected *p* values (Benjamini & Hochberg, 1995; Lenth, 2022). Wilcoxon rank-sum tests were performed to test for differences in benzoxazinoid concentrations between WT and bx_1 conditioned soil in the conditioning phase and at wheat emergence; *p* values were also FDR adjusted. Maize genotype-dependent differences on soil parameters were tested by Welch's two-sample *t*-test and *p* values were FDR

adjusted. Possible differences in element profile of wheat kernels were visualized through principal component analysis (*FactoMineR* package; Lê *et al.*, 2008). The 10 elements explaining most of the variance in PCA-axes 1 and 2 were visualized as arrows. All R code and data (microbiome and plant phenotyping) is available on GitHub (https://github.com/PMI-Basel/Gfeller_et_al_Posieux_field_experiment).

Acknowledgement

We thank Jean-François Rauber, Wolfram Schuwey and Raphaël Grandgirard (Agricultural competence center canton Freiburg, Grangeneuve, Switzerland) for their field assistance and Lilia Levy, Lydia Michaud and Noemi Schaad (Agroscope, Changins, Switzerland) for their help during wheat harvest. Further, we are grateful to Florian Enz and Sophie Gulliver for field and laboratory assistance. This work was supported by the Interfaculty Research Collaboration “One Health” of the University of Bern.

Supplementary Information

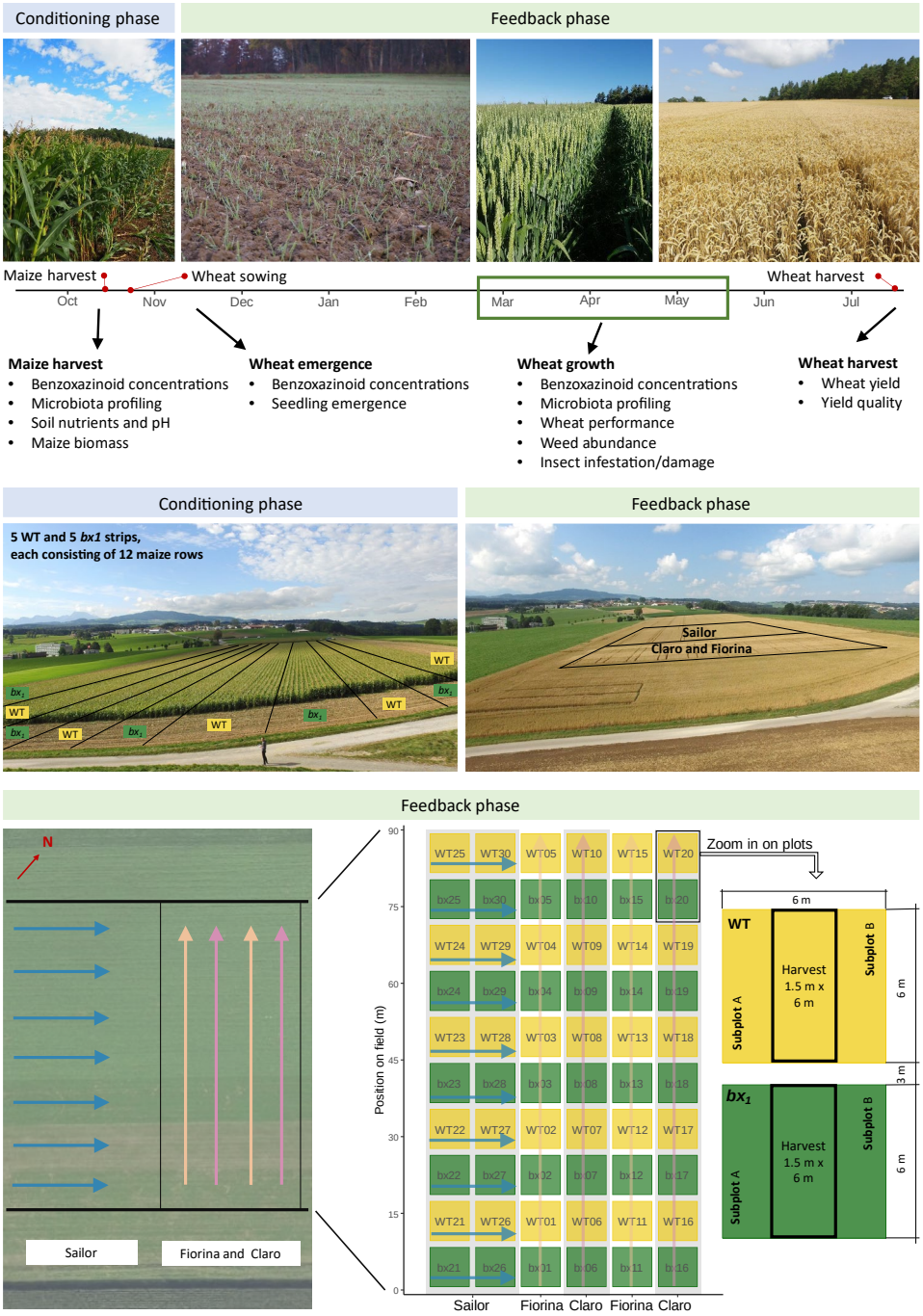


Figure S1. Experimental set-up. A two-year field experiment was conducted in Posieux, Switzerland. In the first season (conditioning phase) the field was cultivated with 10 strips of maize where wild-type (WT, n = 5) and the benzoxazinoid-deficient *bx*$_1$ mutant (n=5) maize were sown alternatingly. Each strip consisted of 12 rows of maize plants. Soil conditioning refers to the process of root benzoxazinoid exudation and the resulting changes in the soil. The impact of soil conditioning was evaluated by analysis of microbiomes, soil benzoxazinoid concentrations, soil nutrients, and pH. In the second season (feedback phase), after the maize was harvested, three wheat varieties (Claro, Fiorina, and Sailor) were sown. Most wheat phenotypes were measured in all subplots, indicated in the zoomed plots. Feedbacks of benzoxazinoid soil conditioning on wheat seedling emergence, growth, and defense were surveyed. Microbial and chemical (benzoxazinoid) soil legacies were again analyzed during wheat growth. At wheat harvest, wheat yield and yield quality were determined. For more details see the method description.

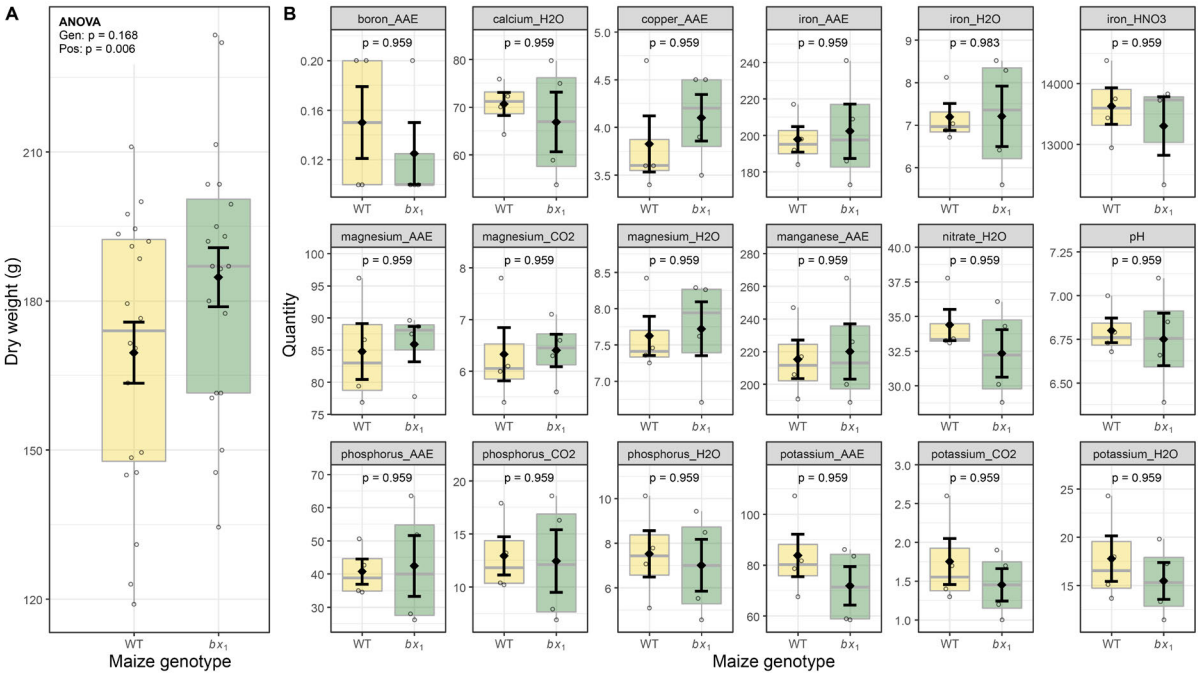


Figure S2. Additional parameters maize harvest. (**A**) Shoot dry weight of individual wild-type (WT) and *bx₁* maize plants at harvest. Means ± SE, boxplots, and individual datapoints are shown (n = 20). ANOVA table is included. (**B**) Soil nutrient levels at the end of the maize conditioning phase. Except for pH all values are concentrations in mg/kg soil. Means ± SE, boxplots, and individual datapoints are shown (n = 4). Welch's two-sample *t*-tests are included (FDR-corrected *p* values). Gen: maize genotype (WT and *bx₁*). Var: wheat variety. Pos: position on the field. AAE: ammonium acetate EDTA extraction; H_2O: water extraction; CO_2: carbon dioxide saturated H_2O extraction.

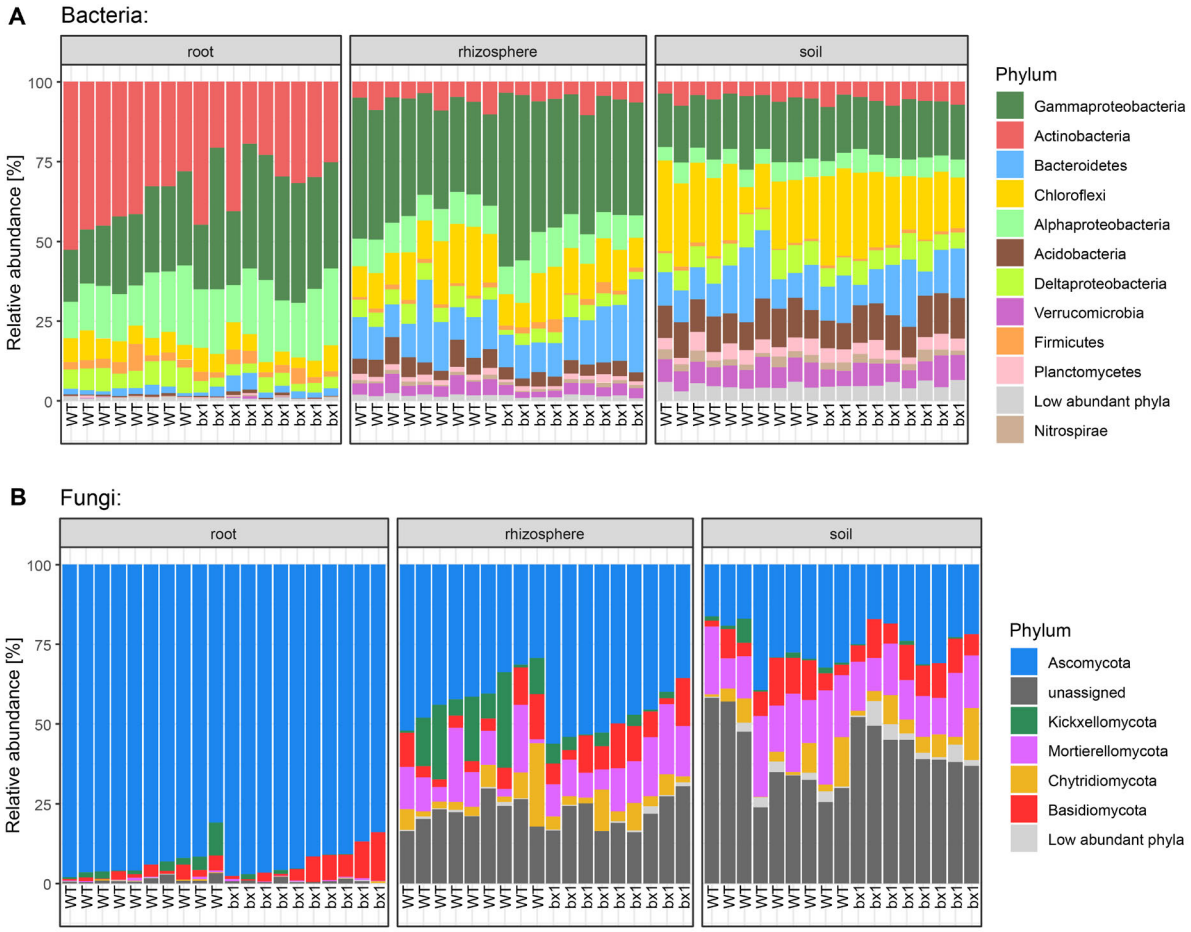


Figure S3. Relative abundance of microbial phyla at maize harvest. (**A**) Taxonomy of bacteria and (**B**) fungi in roots, rhizospheres, and soil of wild-type or benzoxazinoid-deficient *bx*$_1$ mutant maize plants. All samples are shown.

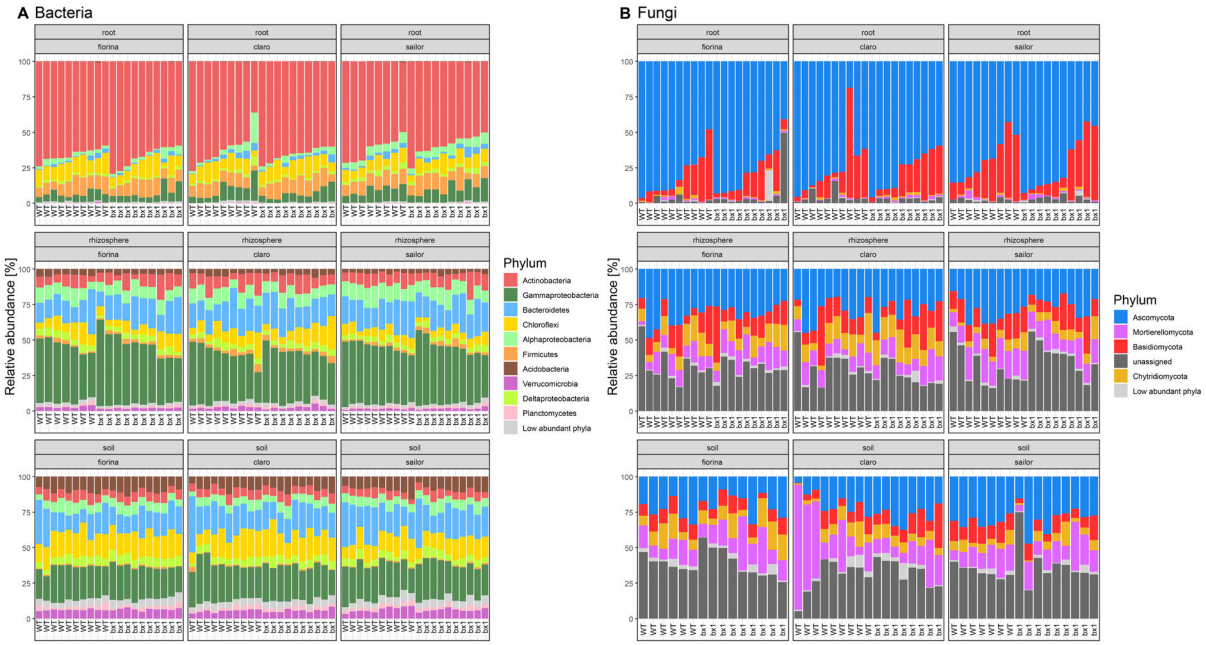


Figure S4. Relative abundance of microbial phyla in the wheat feedback phase. (**A**) Taxonomy of bacteria and (**B**) fungi in roots, rhizospheres, and soils in three wheat varieties grown on wild-type or benzoxazinoid-deficient *bx*$_1$ mutant conditioned soil. All samples are shown.

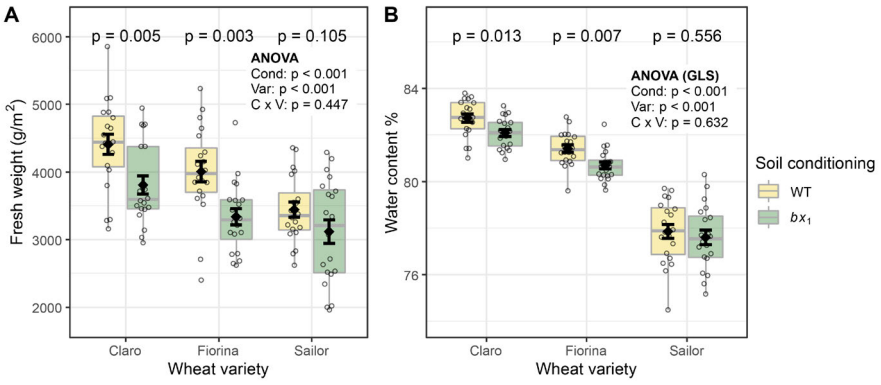


Figure S5. Additional benzoxazinoid soil conditioning effects on wheat growth. (**A**) Shoot fresh weight of three wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx*$_1$ mutant maize. **(B)** Shoot water content. Means ± SE, boxplots, and individual datapoints are shown (n = 20). ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. Cond: soil conditioning (WT and *bx*$_1$). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety.

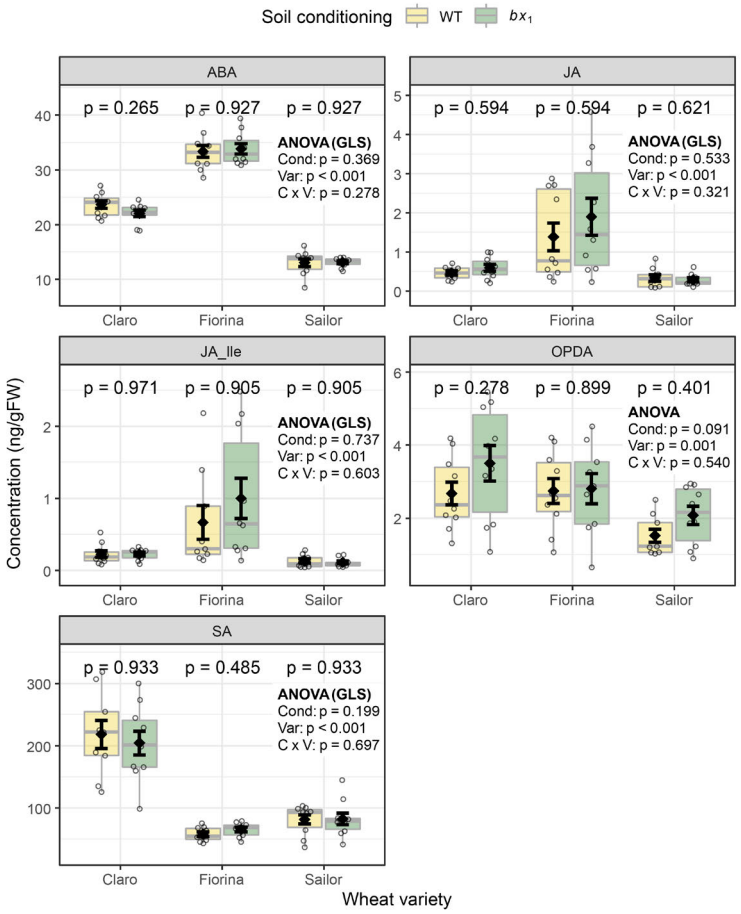


Figure S6. Benzoxazinoid soil conditioning does not affect leaf phytohormone levels of wheat. Phytohormone levels of three wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx*$_1$ mutant maize were measured (n = 9-10). Means ± SE, boxplots, and individual datapoints are shown. ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. FW: fresh weight. Cond: soil conditioning (WT and *bx*$_1$). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety. Pos: position on the field. ABA: abscicic acid. JA: jasmonic acid. JA-Ile: jasmonic acid-isoleucine. OPDA: oxophytodienoic acid. SA: salicylic acid.

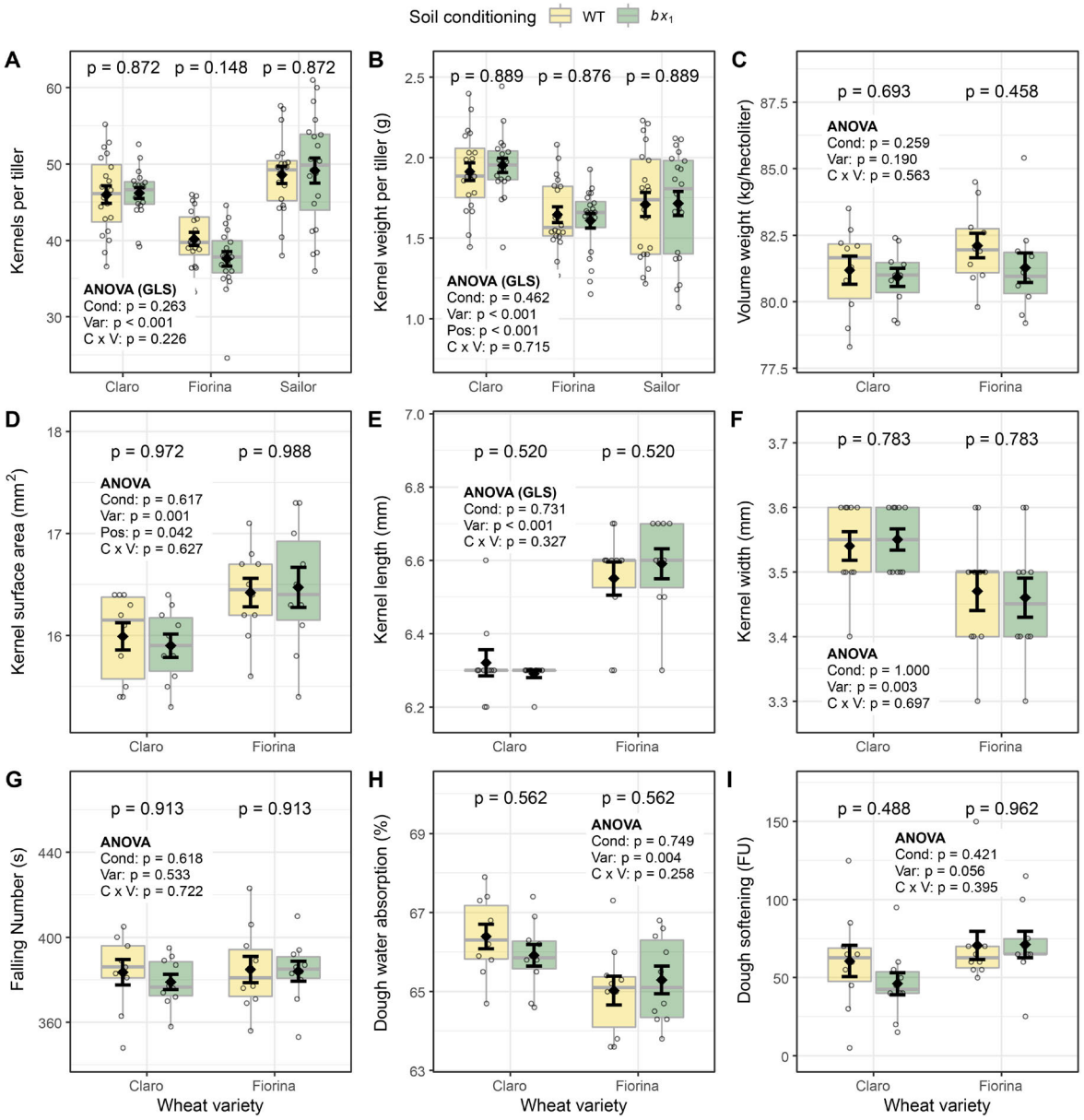


Figure S7. Benzoxazinoid soil conditioning does not affect wheat kernel measurements and baking quality. (**A**) Kernels per tiller of three wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx₁* mutant maize (n = 10). (**B**) Kernel weight per tiller (n = 10), (**C**) kernel volume per weight (n = 10), **(D)** kernel surface area (n = 10), **(E)** kernel length (n = 10), **(F)** kernel width (n = 10), **(G)** falling number (flour quality, n = 10), **(H)** flour water absorption (n = 10), and (**I**) dough softening (n = 9-10). Means ± SE, boxplots, and individual datapoints are shown. ANOVA tables (if unequal variance, on generalized least squares model GLS) and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. Cond: soil conditioning (WT and *bx₁*). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety. Pos: position on the field.

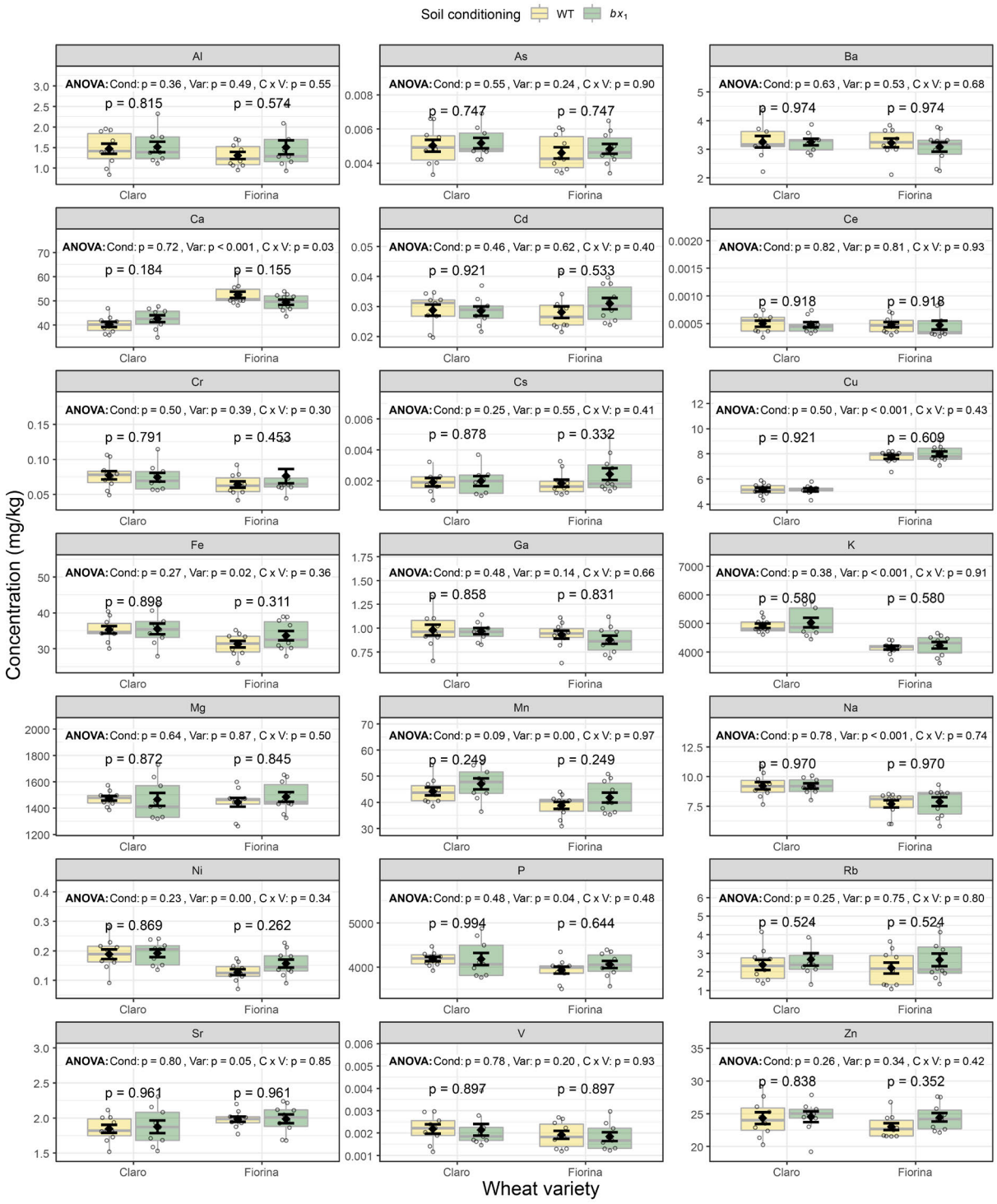


Figure S8. Benzoxazinoid soil conditioning does not affect micronutrient concentrations in wheat kernels. Concentration of individual element in kernels of two wheat varieties growing in soils previously conditioned with wild-type (WT) or benzoxazinoid-deficient *bx₁* mutant maize (n = 8-10). Means ± SE, boxplots, and individual datapoints are shown. ANOVA tables and pairwise comparisons within each wheat variety (FDR-corrected *p* values) are included. Same data as shown in PCA Fig. 6F. Cond: soil conditioning (WT and *bx₁*). Var: wheat variety. 'C x V': interaction between conditioning and wheat variety.

Table S1. Specifications to library preparation for sequencing

Sample type	Library type	Sequencing Run	Number of sample	Purification method
Soil + Control	16S (Bacteria)	BE09	43	Beads
rhizosphere	16S (Bacteria)	BE09	38	Agarose gel
Root	16S (Bacteria)	BE09	40	Agarose gel
Soil + Control	16S (Bacteria)	BE10	47	Beads
rhizosphere	16S (Bacteria)	BE10	42	Agarose gel
Root	16S (Bacteria)	BE10	40	Agarose gel
Soil + Control	ITS (Fungi)	BE09	43	Beads
rhizosphere	ITS (Fungi)	BE09	38	Beads
Root	ITS (Fungi)	BE09	40	Beads
Soil + Control	ITS (Fungi)	BE10	47	Beads
rhizosphere	ITS (Fungi)	BE10	42	Beads
Root	ITS (Fungi)	BE10	40	Beads

Chapter II

Soil environmental gradients determine local variation in root exudate-mediated plant-soil feedbacks

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[1] These authors contributed equally to this work

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Abstract

Introduction:

Harnessing positive plant-soil feedbacks via crop rotations is a promising strategy for sustainable agriculture. Plants can influence soil properties, including microbial composition, by exuding specialized metabolites. While these effects are well established in the laboratory, little is known about their prevalence in the field. In particular, the impact of within-field environmental variation on plant-soil feedbacks is not well understood. Benzoxazinoids are specialized metabolites that are released in high quantities by cereals such as wheat and maize. They have been shown to alter rhizosphere microbiota and the performance of plants growing in the same soils and are thus an excellent model to study agriculturally relevant plant-soil feedback mechanisms.

Materials & methods:

To assess within-field patterns of benzoxazinoid-mediated plant-soil feedbacks, we conditioned soils with wild-type and benzoxazinoid-deficient *bx1* mutant maize in a grid pattern in an arable field, and then grew winter wheat across the entire field in the following season. We determined benzoxazinoid degradation, root-associated microbial communities, abiotic soil properties and wheat performance in each plot, and then assessed associations between chemical environmental variation and benzoxazinoid-mediated plant-soil feedbacks.

Results:

Benzoxazinoid concentrations and microbial communities at the end of the conditioning phase varied strongly along a spatial environmental gradient in the soil. Benzoxazinoid conditioning affected microbial richness and community composition of maize and while the microbial legacy was lost during wheat growth, fingerprints of a benzoxazinoid degradation product were still observed. Phenotyping efforts in the feedback phase showed that vegetative biomass accumulation was negatively affected by benzoxazinoid soil conditioning. Further, wheat performance, insect performance and kernel characteristics exhibited distinct local benzoxazinoid plant-soil feedbacks, depending on the soil parameters.

Conclusion:

Overall, this study revealed that plant-soil feedbacks differ in strength and direction depending on soil parameters. Understanding this context-dependency of agricultural plant-soil feedbacks is crucial to make them exploitable to promote sustainable agriculture.

Introduction

Plants influence the soil they grow in, which again influences the performance of other plants. In crop production, designing a suitable crop rotation makes use of positive plant-soil feedbacks (van der Putten *et al.*, 2013). Identifying and exploiting the mechanisms of plant-soil feedbacks in crop rotations has been proposed as a promising tool to promote sustainable agriculture by leveraging agroecological effects (Mariotte *et al.*, 2018). A key challenge in this context is the spatial and temporal variability of plant-soil feedbacks in the field (Smith-Ramesh & Reynolds, 2017). Thus, newly identified mechanisms need to be evaluated in the light of environmental heterogeneity.

Plant-soil feedbacks are attributed to a number of different mechanisms, including changes in mutualist and pathogen abundance, including microbiome composition, nutrient availability, and other soil chemical properties (Bever *et al.*, 2012; Bennett & Klironomos, 2019; Pineda *et al.*, 2020). These drivers can affect germination, plant performance (Tawaha & Turk, 2003; van der Putten *et al.*, 2013), as well as pathogen and herbivore resistance (Kos *et al.*, 2015b; Ma *et al.*, 2017; Pineda *et al.*, 2020). Common to these mechanisms is that they are indirect, i.e. that they involve soil abiotic and biotic factors. Given that all these factors are highly heterogeneous, one can expect strong context-dependency and spatiotemporal variation in the resulting feedback effects.

Plant-associated microbial communities have gained attention as drivers of plant-soil feedbacks in the past (Bever *et al.*, 2012). An important mechanism in how plants shape their microbiome is through root exudates, i.e. the secretion of primary and secondary metabolites to the surrounding soil (Pang *et al.*, 2021). Prominent examples of secondary metabolites involved in influencing the root or rhizosphere microbiome are benzoxazinoids, coumarins, flavones, and triterpenes (Hu *et al.*, 2018b; Huang *et al.*, 2019; Stringlis *et al.*, 2019; Voges *et al.*, 2019; Yu *et al.*, 2021). Changes in the root-associated microbiome can in turn boost plant growth and defence (Berendsen *et al.*, 2012; Pieterse *et al.*, 2014). The dynamic interplay between secondary metabolite exudation, degradation, metabolization, and soil microbial communities can be expected to add to the variation in plant-soil feedbacks.

Spatial and temporal variation in plant-soil feedbacks has been studied mostly for soil nutrients, temperature (Smith-Ramesh & Reynolds, 2017; Long *et al.*, 2019), drought (Fry *et al.*, 2018), and the interaction of abiotic factors with soil biota (Kaisermann *et al.*, 2017; Long *et al.*, 2019). Further, soil biotic communities represent key determinants of plant-soil feedbacks across time and space (Revillini *et al.*, 2016; Bennett *et al.*, 2017). How environmental

heterogeneity affects plant-soil feedbacks driven by exuded root metabolites is largely unknown, and local (i.e. within-field variation) in the underlying dynamics has not been studied so far.

Benzoxazinoids, a class of secondary metabolites common in grasses including maize and wheat, have been shown to be bioactive in many ways (Niemeyer, 2009). They are long known to be involved in allelopathy and defence against insects and pathogens (Niemeyer, 2009; Schandry & Becker, 2020). They are also involved in chelating iron for more efficient uptake and chelating aluminium for plant tolerance (Zhou *et al.*, 2018; Zhao *et al.*, 2019). In the past few years benzoxazinoids have several times been shown to shape root-associated microbiomes (Hu *et al.*, 2018b; Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019; Cadot *et al.*, 2021b), in addition benzoxazinoids can function as an attractant for single bacteria as shown for *Pseudomonas putida* to locate the maize roots (Neal *et al.*, 2012). Maize roots predominantly excrete DIMBOA-Glc, HDMBOA-Glc, and DIMBOA and these benzoxazinoid compounds are rapidly converted into MBOA (Hu *et al.*, 2018b). Ultimately, soil microorganisms can further degrade MBOA to AMPO and metabolize AMPO with a half-life of days or several months, respectively (Macías *et al.*, 2004; Etzerodt *et al.*, 2008). So far, benzoxazinoid-dependent plant-soil feedbacks have been shown for maize-maize and maize-wheat cropping sequences in the greenhouse and maize-wheat in the field (Hu *et al.*, 2018b; Cadot *et al.*, 2021b; Gfeller *et al.*, 2022b). In a relatively homogenous experimental field, benzoxazinoid exudation by maize resulted in an increase in wheat yield. If and how such feedbacks act under more heterogeneous conditions is largely unknown.

In this study, we investigated how environmental soil heterogeneity within a single agricultural field influences benzoxazinoid-mediated plant-soil feedbacks in a maize-wheat crop rotation. We set up a two-year field experiment where we first grew maize to condition the soil followed by winter wheat (**Fig. 1**). The soil was conditioned either by benzoxazinoid-producing wild-type or benzoxazinoid-deficient *bx1* mutant maize plants, and we then assessed feedbacks in 20 distinct plots within the field. Feedback effects were then analyzed taking into account the innate gradient in soil chemistry present in the field. Detailed measurements of benzoxazinoid accumulation and changes in soil microbiota were used to determine to what extent these factors interact with soil heterogeneity to explain the observed variation in plant-soil feedbacks. Overall, our results show a hight context-dependency of secondary metabolite-mediated plant-soil feedbacks in crop rotations. Understanding such context-dependencies is crucial to successfully employ the concept of plant-soil feedbacks in sustainable agriculture.

Material and Methods

Plant material

To test benzoxazinoid-dependent soil conditioning effects, wild-type maize (Zea mays) and the corresponding benzoxazinoid-deficient *bx1* transposon insertion mutant (referred to as *bx1*) of the inbred line W22 (Tzin *et al.*, 2015) were planted in the field. To subsequently test benzoxazinoid feedbacks on wheat, we grew the winter wheat variety CH Claro (referred to as Claro), a top variety commonly cultivated in Switzerland.

Field experiment

The field experiment was conducted in Changins (Nyon, Switzerland) on a field at Agroscope (Parcel 29, 46°23''58'N, 6°14''25'E, **Fig. 1**) during the growing seasons of 2018 and 2019 and consisted of a maize-wheat crop rotation, the maize being the conditioning crop and the wheat being the feedback crop. The field was first ploughed (20 cm depth) on April 24th, and then harrowed (10 cm depth) on the 25th. On April 26th the maize seeds were sown (200-220 seeds for a 20 m^2 plot surface) on 20 plots consisting of 10 wild-type plots alternating with 10 *bx1* mutant plots, each measuring 6 m long and 3 m wide and separated from each other by 3 m of buffer (**Fig. 1**). The six maize rows sown per plot were separated by 50 cm, and plants along the rows were separated by 20 cm. A net (14 g/m^2) was put on the field during germination to protect seeds from being eaten. On the 26th of May, alfalfa (*Medicago sativa*) was sown as a buffer plant between and around the maize plots. The same day, N fertilizer as ammonium nitrate (27.5%, 100 kg/ha) was applied and the soil was superficially worked to weed and incorporate the fertilizer. On the 24th of July, 35 mm water was applied on the field for irrigation. No herbicide was applied but manual weeding was performed on the 12th of June. The preceding crops were alfalfa (*Medicago sativa*, 2016-2017), spring wheat (2015), and maize (2014). On the 6th of September, plants were harvested at maturity.

From autumn 2018 to summer 2019, wheat was grown on the same field. The field was treated with a goose foot cultivator (15 cm depth) and then harrowed (10 cm depth) to prepare the seedbed on the 9th of October. On the following day, wheat seeds of the variety Claro were sown (416 g seeds/m^2) with a row distance of 20 cm. On the 20th of February, 50 kg/ha of N fertilizer (39% N in the form of a liquid mixture of urea (50%), ammoniac nitrate (25%) and nitrate (25%)) was applied, and the same day Mg fertilizer was applied as 120 kg/ha of Kiserite (15% Mg and 20% S). One month later, on the 21st of March, 55 kg/ha of the same N fertilizer (39%) was again applied. On the 25th and the 27th of February, the field was treated with a weeding harrow. Finally, wheat was harvested on the 19th of July at maturity (14% humidity).

Sample collection

To check benzoxazinoid exudation into the soil and to test for effects on the soil microbes, at the end of the maize growth we sampled root, rhizosphere, and soil samples. For that, from one randomly selected plant per plot the root system (20 x 20 x 20 cm) was excavated and used for chemical and microbial analysis (n = 10 see below). To investigate soil benzoxazinoids and microbes at the onset of wheat germination, we again sampled soil one day after wheat sowing. At 10 randomly selected positions on each plot soil cores of the top 20 cm were taken with a 17 mm diameter soil sampler. Samples of each plot were combined and further processed for chemical and microbial analysis (n = 10). To study the benzoxazinoid concentrations and the microbial community in the wheat feedback phase, we again sampled root, rhizosphere, and soil samples during wheat growth. From 3 randomly selected plants per plot, the root system (7 x 7 cm wide, 12 cm deep) was excavated and pooled for chemical and microbial analysis (n = 10, see below). To study within-field variation of soil parameters, soil was sampled after the experiment was finished. On each plot soil was taken from 5 randomly selected positions at a soil depth of 5-20 cm, resulting in total of 2 kg pooled soil per plot (n = 20).

Phenotyping wheat emergence and vegetative growth

One month after sowing we counted all emerged seedlings along 1 m of three randomly selected wheat rows per plot, to determine possible benzoxazinoid soil conditioning effects on wheat emergence. The sum of all counted seedlings per plot was taken and wheat emergence per area was calculated.

To determine the effect of benzoxazinoid soil conditioning on wheat vegetative growth, we measured plant chlorophyll content, height, and biomass accumulation during wheat growth. Chlorophyll content of 15 randomly selected flag leaves per plot was measured by means of a SPAD-502 chlorophyll meter (Konica Minolta, Japan) and the average value was taken for statistical analysis. Height of 5 randomly selected wheat plants per plot was determined and averaged for statistical analysis. Aboveground biomass accumulation was evaluated by harvesting two times 1 m of wheat row per plot at ground level. Dry weight was determined after the plant material was dried at 80 °C until constant weight. The obtained data was used to calculate biomass accumulation per area.

Phenotyping insect infestation and performance

Insect infestation was evaluated by counting the number of *Oulema melanompus* larvae at wheat growth. The number of larvae was evaluated by 5 times randomly selecting 0.5 meter of

wheat row on each plot and counting all larvae present on the flag leaves. In parallel, the number of tillers was recorded to calculate the number of *O. melanompus* larvae per plant.

To further evaluate benzoxazinoid conditioning effects on plant defence, we assessed insect performance on detached wheat leaves. For that, we collected 2 randomly selected wheat plants per plot and stored them in a zip-lock plastic bag moistened with a wet cotton pad at 4 °C, to draw upon as required throughout the insect performance assay. Two transparent solo cups (4 cm height and 3.5 cm diameter) per plot were equipped with a wet filter paper, and the top 6 cm of the youngest fully developed leaf was placed inside. *Spodoptera littoralis* larvae were reared on an artificial diet until used in the bioassay. One healthy 3rd instar larva per solo cup was pre-weighed on a microbalance and placed on the wheat leaf before closing the cup with an air permeable lid. Leaves were moistened daily and renewed on day 2 and 4 of the assay to assure excess food for all larvae. To evaluate larvae performance, larvae were weighed after one week of feeding. Larvae weight gain per day was calculated (weight end - weight start / number of days feeding * 100) and the mean of the two replicates per plots was used for statistical analysis.

Phenotyping at wheat harvest

To estimate final plant biomass accumulation, we again collected two times 1 m of a wheat row on each plot 12 days before harvest. The plant material was dried at 80 °C until constant weight and dry biomass was determined. The number of tillers was counted and plant density and aboveground biomass per tiller was calculated.

The wheat was harvested once the kernels were ripe (14 % humidity). Nine m^2 per plot were harvested with a compact plot combine harvester (Quantum, Wintersteiger), and kernel weight per plot was determined. A subset of these kernels was taken for analysing agronomic kernel quality and food quality related parameters (see below).

Kernel analysis

To determine morphological kernels traits, an aliquot of 25 mL was taken. By means of a MARVIN kernel analyzer (GTA Sensorik GmbH, Germany) and a microbalance, volume weight, thousand kernel weight (TKW), kernel surface area, kernel length, and kernel width were determined. To test flour quality, an aliquot of kernels was milled and falling number (according to ICC standard method 107/1), Zeleny index (according to ICC standard method 116/1), and protein content were analyzed, where protein content was evaluated by near-infrared reflectance spectroscopy (NIRS) using a NIRFlex N-500 (Büchi Labortechnik AG, Switzerland). Further, dough quality was determined using the micro-doughLAB farinograph

(model 1800, Perten Instruments, PerkinElmer United States), by measuring dough stability (min), dough softening (Farinograph Units, FU), and water absorption capacity of the flour (%) during the kneading process according to the manufacturer's protocol.

To test for possible benzoxazinoid conditioning effects on food quality related parameters, we send 750 g of kernels per plot to Eurofins Scientific AG (Schönenwerd, Switzerland) to analyse the most important nutritional values and mycotoxins.

Soil analysis

To test for gradients of soil parameters on the field, we went back to the experimental site after the experiment and sampled soil on every plot. The freshly collected soil was sent to LBU Laboratories (Eric Schweizer AG, Thun, Switzerland) and analyzed with different extraction methods: water (H_2O), ammonium acetate EDTA (AAE), and carbon dioxide saturated water (CO_2). H_2O extracts are a proxy for plant available nutrients, AAE extracts represent nutrients available through plant chelation mechanisms and CO_2 extracts are a common extraction procedure for magnesium, phosphorus, and potassium (similar to H_2O extracts). In addition, total iron was extracted in nitric acid (HNO_3) and quantified with inductively coupled plasma mass spectrometry (ICP-MS) as previously described (Cadot *et al.*, 2021b).

Benzoxazinoid analysis

To determine the dynamics of benzoxazinoids and their degradation products in the soil over the course of the experiment, we analyzed soil benzoxazinoid concentrations at the end of maize soil conditioning, after wheat sowing, and at the end of wheat vegetative growth. Samples were collected as described above, soils were passed through a test sieve (5 mm mesh size), 25 mL of soil was transferred into a 50 mL centrifuge tube and completely suspended in 25 mL acidified MeOH/ H_2O (70:30 v/v; 0.1% formic acid) by vigorously shaking and vortexing the tube. After 30 minutes of shaking at room temperature in a rotary shaker, samples were centrifuged (5 min, 2000 g) to sediment the soil. The supernatant was passed through a filter paper (Grade 1; Size: 185 mm; Whatman, GE Healthcare Live Sciences), 1 mL of the flow through was transferred into a 1.5 mL centrifuge tube, centrifuged (10 min, 19000 g, 4 °C), and the supernatant was sterile filtered (Target2TM, Regenerated Cellulose Syringe Filters. pore size: 0.45 µm; Thermo Scientific) into a glass tube for further analysis.

All samples collected at wheat sowing and wheat vegetative growth were concentrated 20 times before the second centrifugation step. For that, 20 mL of soil extract per sample was dried (45 °C; CentriVap, Labconco) and resuspended in 1 mL of acidified MeOH/ H_2O (70:30 v/v; 0.1% formic acid).

Benzoxazinoids and degradation products were analyzed with an Acquity UHPLC system coupled to a G2-XS QTOF mass spectrometer (Waters AG, Bade-Dättwil, Switzerland) as previously described (Gfeller *et al.*, 2022b). Absolute quantification was done through standard curves of pure compounds. For that, MBOA (6-methoxy-benzoxazolin-2(3H)-one) was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). DIMBOA-Glc (2-O-β-D-glucopyranosyl-2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) and HDMBOA-Glc (2-O-β-D-glucopyranosyl-2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one) were isolated from maize plants in our laboratory. DIMBOA (2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), DIMBOA-d_3 (2,4-dihydroxy-7-(methoxy- d_3)-2H-1,4-benzoxazin-3(4H)-one), HMBOA (2-hydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), and AMPO (9-methoxy-2-amino-3H-phenoxazin-3-one) were synthesized in our laboratory.

Benzoxazinoid exudation and degradation

To evaluate if maize benzoxazinoid exudation or degradation depends on soil nutrients or other soil parameters, we performed a climate chamber experiment comparing soils from both sides of the field. For that, we collected soil in the north and south (N: plots WT07/bx04; S: plots WT10/bx07), sieved the soil (10 mm mesh size), and filled the soil into 130 mL pots before a wild-type (W22) maize seed was sown (n = 10). Plants were grown in walk-in climate chambers under controlled conditions (day/night: 14/10h; temperature: 22 °C/18 °C; light 550 µmol $m^{-2}s^{-1}$; humidity: 60%) and fertilized with 10 mL nutrient solution (0.4% (w/v); Plantactive Typ K, Hauert) supplemented with iron (1 ‰ (W/V); Sequestrene rapid, Maag) twice a week. All plants were randomized weekly and watered as needed. After 3 weeks the maize plants were harvested. First, benzoxazinoid exudation was measured by taking a given plant out of the pot, gently removing the soil of the root system at the very bottom from 4 randomly selected root tips and rinsing 2 cm of the root tips 4 times with 100 µL of sterile water. Immediately after, 60 µL of this suspension was added to 140 µL pure acidified MeOH resulting in MeOH/H_2O (70:30 v/v; 0.1% formic acid). After centrifugation for 10 minutes at 19000 g the supernatant was stored at -20 °C prior to analysis of benzoxazinoid (as described above). Second, roots were cut at soil level, cleaned off adhering soil with water, dried at 80 °C until a constant weight, and dry biomass was determined on a microbalance. Third, the remaining soil in the pot was homogenized, passed through a 5 mm test sieve, 25 mL were put in a 50 mL centrifuge tube, and stored at -80 °C. Benzoxazinoid extraction and measurement of this soil was done as described above. For statistical analysis the benzoxazinoid concentration in the soil was corrected for differences in root dry weight.

Benzoxazinoid degradation experiment

To evaluate possible differences in the benzoxazinoid degradation in soils at both ends of the field, we performed a degradation experiment with labelled deuterated DIMBOA-d_3 under controlled conditions in the lab. Soils were collected at the northern and southern end of the field (N: plots WT07/bx04; S: plots WT10/bx07/WT03). A 10 mL (≈10 mg) aliquot of this soil was mixed with 10 mL of sterile water in a 50 mL centrifuge tube and blended with a Polytron (30 s at 15000 rpm), to obtain a suspension (n = 4 per soil). The soil acidity was between pH 6.96 and pH 7.13, therefore we used a phosphate buffer at pH 7 for the negative and no soil controls. Six mL of soil suspension or buffer were transferred into a 14 mL culture tubes and incubated at 22 °C in a thermoshaker (at 150 rpm) under oxic conditions. We let the soil acclimate for 3 days before the DIMBOA-d_3 was added. DIMBOA-d_3 was dissolved in autoclaved ddH_2O and added to each culture tube (except negative controls) to obtain a final concentration of 30 µg/mL (≈140 µmol/L). To elucidate the kinetics of benzoxazinoid degradation, we sampled from each reaction mix after 1 min, 7.5 min, 15 min, 1 hour, 4 hours, 1 day, and 4 days. At every sampling, 300 µL reaction mix was pipetted into a 1.5 mL centrifuge tube containing 700 mL acidified MeOH to result in MeOH/H_2O (70:30 v/v; 0.1% formic acid). The suspension was vigorously vortexed and stored at -80 °C. Once all samples were collected the tubes were thawed, soil particles were removed by centrifugation (20 min, 19000 g, 4 °C), the supernatant was filtered (Target2TM, Regenerated Cellulose Syringe Filters. pore size: 0.45 µm; Thermo Scientific) and stored in a glass vial at -20 °C until analysis. Benzoxazinoids were analyzed as described above.

Microbiota library preparation

For the microbiota profiling the samples were collected as described above followed by sample preparation as previously described (Gfeller *et al.*, 2022b). In short, soil samples were obtained by gently removing soil from the root systems and passing them through a 5 mm test sieve. Roots were cut at a soil depth of 5 cm to 15 cm, placed in a 50 mL centrifuge tube, and washed 4 times with 25 mL of sterile ddH_2O by vigorously shaking them 10 times. Roots samples, containing endophytes and epiphytes, were then freeze-dried, and milled to fine powder using a Ball Mill (Retsch GmBH; 30 s at 30 Hz using one 1 cm steel ball). To prepare the rhizosphere samples, the first two washes of the root cleaning were combined, centrifuged (5 min at 3000 g), and the resulting pellet was frozen at -80 °C before further processing.

DNA was extracted using the Spin Kit for Soil (MP Biomedical, USA), following the instructions of the manufacturer. For that, 20 mg of roots powder and 200 mg for rhizosphere

and soil were taken. DNA concentrations were evaluated by means of a AccuClear Ultra High Sensitivity dsDNA Quantitation Kit (Biotium, USA).

Bacterial and fungal community profiling were performed following a two-step PCR profiling protocol described in (Gfeller *et al.*, 2022b) with a few changes. Briefly, bacterial profiles are based on PCR primers 799-F (Chelius & Triplett, 2001) and 1193-R (Bodenhausen *et al.*, 2013) that span the hypervariable regions V5 to V7 of the 16S rRNA gene. Fungal profiles are derived from the internal transcribed spacer region 1 (ITS1) and were amplified with the PCR primer pair ITS1-F (Gardes & Bruns, 1993) and ITS2 (White *et al.*, 1990). The PCR reactions mix included 5-prime HotMastermix (1x, QuantaBio, USA), bovine serum albumin BSA (0.3%), forward primer (300 nM), reverse primer (300 nM), with 1 ng input DNA in the case of soil and rhizosphere bacterial samples, 10 ng for root bacterial samples, 2 ng for soil and rhizosphere fungal samples and 20 ng for root fungal samples; H_2O was added to the solution to obtain 20 uL. One negative control with no DNA and one validated positive control were added to each PCR plate. PCR settings for bacteria included a first 3-min step at 94 °C for denaturation, and 25 cycles of 45 s at 94 °C, 1 min at 50 °C, 1 min 30 s at 72 °C, followed by a final step of 10 min at 72 °C. The PCRs for fungal samples were done similarly, except that the number of cycles was increased from 25 to 35 to increase the amplification. Amplification success and contamination events were evaluated by migrating PCR product aliquots on a 1.5% agarose gel. Next, PCR products were purified using self-made Solid Phase Reversible Immobilisation (SPRI) magnetic beads (https://openwetware.org/wiki/SPRI_bead_mix) with a 0.8:1 beads (16 µl) to PCR products (20 µl) ratio in 10 mM Tris-HCl pH 8 buffer solution. After binding the beads with the adhering DNA to a magnet, the supernatant was removed, the beads were washed twice with 80% ethanol, briefly air-dried, and eluted in 22.5 µl Tris-HCl buffer (10 mM pH 8). Twenty µl of cleaned amplicon DNA was then transferred to new 96-well plates. These clean PCR products were then quantified with NanoDrop (Thermo Fisher, USA), equimolarly pooled, purified, and concentrated with beads in a 1:1 beads-to-library ratio, and eluted in 20 µl of buffer. Finally, the library was quantified with Qubit (Thermo Fisher, USA). Library preparation was completed by ligation of the Illumina adapters by the Next Generation Sequencing (NGS) Platform at University of Bern, where they were subsequently sequenced on a MiSeq (v3) instrument in paired-end 2 × 300 bp mode (Illumina, USA). The raw sequencing data is available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena).

Microbiota bioinformatics

The sequencing data was processed as previously described (Gfeller *et al.*, 2022b). In short, raw reads were quality checked and demultiplexed using *FastQC* and *cutadapt*, respectively

(Andrews, 2010; Martin, 2011). Information on barcode-to-sample assignments can be found in the supporting Dataset S1. Exact amplicon sequences variants (ASVs) were generated using the *DADA2* pipeline (Callahan *et al.*, 2016; R package *DADA2* v.3.10). Taxonomic assignment of ASVs was performed using a *DADA2* formatted version of the *SILVA* v.132 database (Quast *et al.*, 2013; Callahan, 2018) for bacteria and the FASTA general release from *UNITE* v8.3 (Abarenkov *et al.*, 2021) for fungi. The bioinformatic code is available on GitHub (https://github.com/PMI-Basel/Gfeller_et_al_Changins_field_experiment).

Statistical analysis

Analyses were conducted using the open-source software R (R Core Team, 2021). Data management and visualisation was facilitated with the *tidyverse* packages (Wickham *et al.*, 2019). Root, rhizosphere, and soil microbiomes were analyzed at maize harvest, soil microbiomes at wheat sowing, and again all three compartments during wheat growth. ASVs were first filtered to exclude sequences assigned to eukaryotes, cyanobacteria, mitochondria, or chloroplasts. Based on the inspection of the sequencing depth of all samples and testing if sequencing depth was significantly different among variable groups by a Kruskal-Wallis test, microbial community data was rarefied for all downstream analysis using the v*egan* package (Weiss *et al.*, 2017; Oksanen *et al.*, 2020). Thresholds for rarefaction were 6701 for bacteria and 186 for fungi, resulting in the loss of four bacterial samples with sequence numbers below the threshold. First, we examined the microbial community composition across the field after maize growth. Unconstrained Principal Coordinate Analysis (PCoA) using Bray-Curtis distances were performed for bacterial and fungal communities and the effect of compartment and field position on community composition were tested by Permutational Multivariate Analysis of Variance (PERMANOVA, 999 permutations) on Bray-Curtis distances using the R package *vegan* (Oksanen *et al.*, 2020). The correlation between field position (width and length) and microbial communities (PCoA axes) were further investigated by Pearson's correlation test. Next, we investigated the effect of benzoxazinoid exudation on alpha and beta diversity by comparing samples from wild-type and *bx1* plots within each compartment (root, rhizosphere, soil). Alpha diversity was analyzed by calculating the Shannon index in each sample and performing an ANOVA, where we included a soil chemistry variable (PC1, see below) to account for otherwise unexplained variance (model: diversity ~ genotype * soil chemistry PC1). The same model was used for beata diversity by applying a PERMANOVA. We visualized the beta diversity by plotting the Canonical Analysis of Principal coordinates (CAP) using the R package *phyloseq* (McMurdie & Holmes, 2013). To test if the differences in microbial communities found at the end of maize growth persisted, we again analyzed the soil microbiota

at wheat sowing and soil, rhizosphere, and root microbiota during wheat growth. This was done by compartment-wise PERMANOVA for bacteria and fungi at each sampling time and unconstrained PCoA visualization during wheat growth.

Differences in concentrations of soil benzoxazinoid and their degradation products between the two maize genotypes at the end of the conditioning phase, at wheat sowing, and during wheat growth were tested by Wilcoxon rank-sum tests and false discovery rate (FDR) corrected *p* values were reported (Benjamini & Hochberg, 1995), followed by correlation analysis to test for associations between benzoxazinoid concentrations and field position and/or soil chemistry. To get an overview of the variation of soil chemistry, we performed a principal component analysis (PCA, *FactoMineR*; Lê *et al.*, 2008). PC axes were extracted for further analysis. First, the PC axes were used to check for correlations of soil parameters with the field position. Second, in further analysis the first PC axis, referred to as soil chemistry PC1, was factored in the linear models to account for variation explained by soil parameters.

Wheat growth and defence related data were analyzed by Analysis of Variance (ANOVA). Homoscedasticity and normal distribution of error variance was checked visually. For plant phenotypes two different statistical analysis were applied to get an overview: (i) Possible overall soil benzoxazinoid conditioning effects, effects of the chemical gradient, and the interaction between the two were tested with a linear mode: lm(phenotype ~ soil conditioning * soil chemistry PC1); (ii) to test for local benzoxazinoid-dependent plant-soil feedbacks we calculated the log-response ratio (LRR) for every plot. This was calculated with the following formula: On wild-type conditioned plots log(local value/surrounding mean) and on *bx1* conditioned plots log(surrounding mean/local value), where log() is the natural logarithm, the local value is the realised value of a certain phenotype on the plot of interest, and the surrounding mean is the mean of all adjusting plots of the opposite treatment (**Fig. S1**). The LRRs were then used to test associations between soil parameters and the direction and strength of the feedback, where positive LRRs indicate positive benzoxazinoid plant-soil feedbacks. In the greenhouse experiment and lab degradation experiments, differences between the two soil origins (S, N) were tested by means of Welch's two-sample t-tests and false discovery rate (FDR) corrected *p* values were reported (Benjamini & Hochberg, 1995). All code for statistical analysis and visualization and the corresponding data can be downloaded from GitHub (https://github.com/PMI-Basel/Gfeller_et_al_Changins_field_experiment).

Results

Benzoxazinoid accumulation shows a distinct spatial pattern

To characterize the conditioning phase (**Fig. 1**), we collected soil samples at maize harvest for benzoxazinoid analysis. We confirmed the presence of benzoxazinoids in soils of wild-type plots while we did not detect them in plots where mutant *bx1* plants were grown (**Fig. 2A**). We measured high amounts of HMBOA and HDMBOA-Glc followed by MBOA, DIMBOA-Glc and DIMBOA, and low amounts of AMPO in soils of wild-type plots. The benzoxazinoid measurements varied strongly across replicates. Soil levels of several benzoxazinoids, in particular DIMBOA-Glc and DIMBOA, gradually increased on plots along the length of the field (**Fig. 2A, Fig. S2**).

Microbial community composition shows a distinct spatial pattern

We also performed a microbiota profiling to describe the microbial communities of maize roots, its rhizospheres, and the soil at maize harvest. Again, we noticed that the variation in microbiota composition coincided with the position of the plot in the field (**Fig. 2B**). Permutational Multivariate Analysis of Variance (PERMANOVA) revealed significant positional effects for the bacteria (both width and length of the field) and for the fungi (length). Taking R^2 values as indicators for effect size, positional effects on bacteria were stronger. The strong positional effects in bacterial communities were apparent in Principal Coordinate Analysis (PCoA), where the second axes largely separated the replicates following their position along the length of the field (**Fig. 2B, Fig. S3**).

Characterization of natural variation in soil chemistry

To assess potential variation in soil chemical properties that may explain the strong gradient in benzoxazinoid accumulation and microbial community composition, we measured pH and nutrients in water (H_2O), CO_2, and ammonium acetate EDTA (AAE) extracts on the 20 plots following a grid pattern (**Fig. 1**). Principal Component Analysis (PCA) revealed a strong chemical gradient. Axis 1, associated with the northeast-southwest axis of the field (field position length), explained 60% of the chemical variation (**Fig. 2C**, **Fig. S4**), while the axis 4, associated with the southeast-northwest axis of the field (field position width), explained 7% of the variation. Overall, we observed a chemical gradient, best described by PC axis 1, running roughly in a diagonal across the field (**Fig. 2D**). This gradient was also apparent when looking at individual soil nutrients (**Fig. S5**). It was characterized by elevated levels of water-soluble iron and magnesium (all extracts) towards the southern corner and elevated levels of Ca (H_2O), K (CO_2) and Mn, P and Bo (all AAE extract) towards the northern corner of the field. Thus, the

observed gradient in benzoxazinoid accumulation and microbial community composition is associated with a pronounced innate soil chemical gradient. To account for this environmental gradient, we included the PCA axis 1, referred to as soil chemistry PC1, as covariable in all downstream analyses.

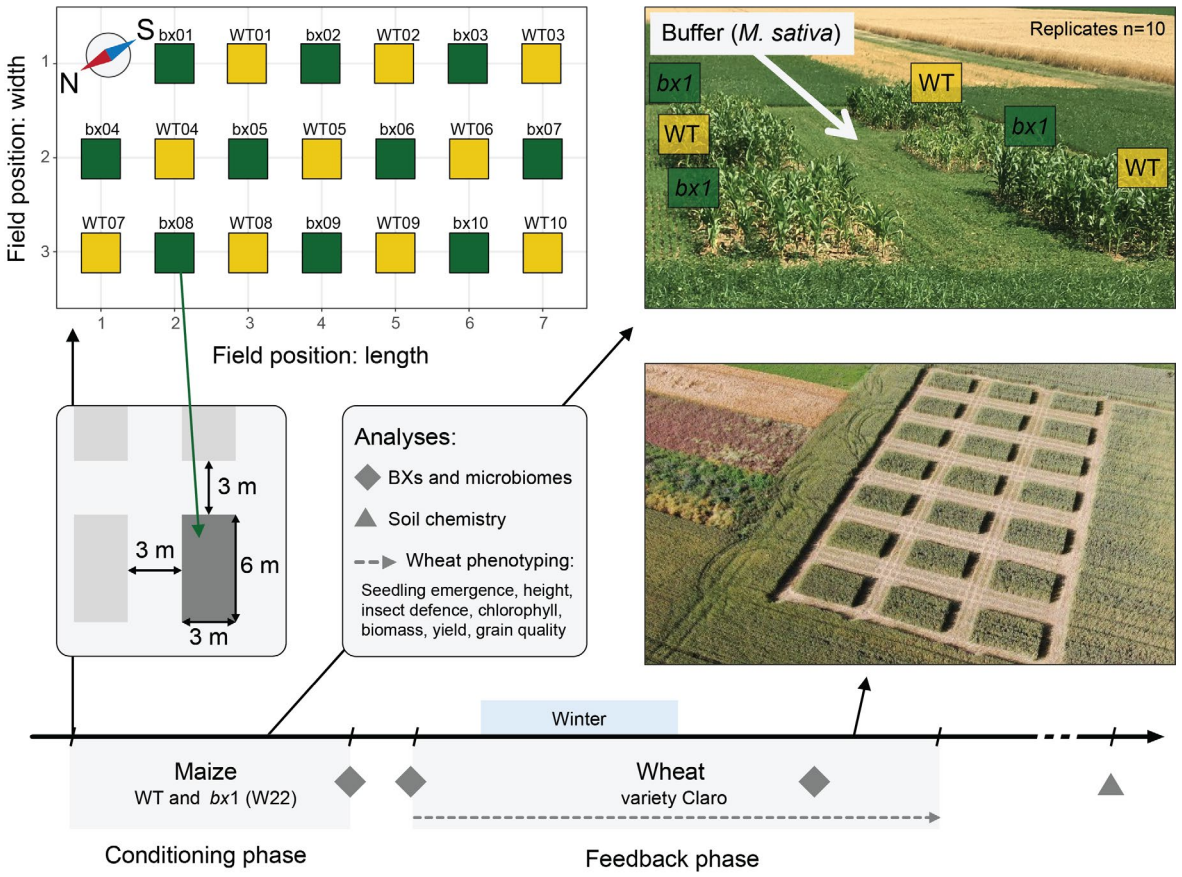


Figure 1. Experimental setup. To examine the effect of maize benzoxazinoid soil conditioning on subsequent wheat growth, defence, yield, and grain quality we conducted a two-year field experiment in Changins, Switzerland. First, wild-type (WT) and benzoxazinoid-deficient *bx1* mutant plants of the maize line W22 were grown on 10 plots each (plot dimensions 3 m x 6 m). As a buffer, *Medicago sativa* was grown between maize plots. After maize harvest, the winter wheat variety Claro was sown. Wheat growth and defence were intensively phenotyped. At harvest, yield was determined, and grain quality was analyzed. Soil benzoxazinoid concentrations and microbiomes were analyzed at maize harvest, wheat sowing, and wheat growth. For microbiome analysis roots, rhizospheres, and the soils surrounding the plants were sampled, except for the time point at wheat sowing, where only soil was present. After wheat harvest, soils for various analysis and for degradation experiments were collected on each plot. For more details, please refer to the method section.

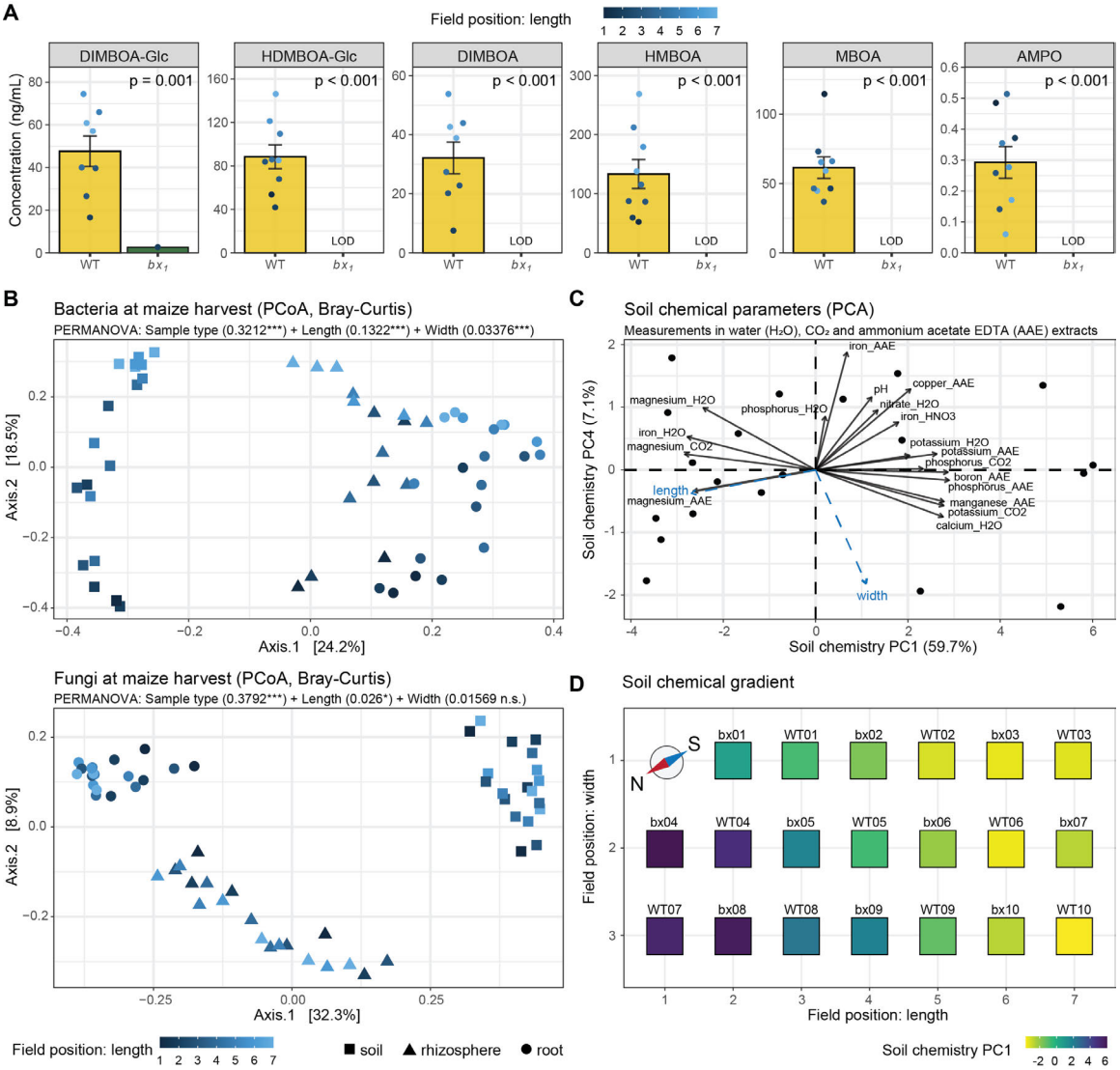


Figure 2. Within-field variation of benzoxazinoids, microbiota, and soil chemistry. (A) Soil benzoxazinoid concentrations collected on plots conditioned by wild-type (WT) or benzoxazinoid-deficient *bx1* mutant plants in ng/mL soil (Means ± SE). Statistical significance was calculated by Wilcoxon rank-sum tests and *p* values were corrected for multiple testing (FDR). (B) Unconstrained Principal Coordinate Analysis (PCoA) using Bray-Curtis distances of bacterial (top) and fungal (bottom) communities in soil, rhizosphere, and root samples. R^2 and significance level of PERMANOVA on Bray-Curtis distances for bacteria and fungi are shown. (C) Principal Component (PC) axes 1 (PC1) and 4 (PC4) of soil chemistry PCA are show. Individual samples (circles), soil parameters (arrows), and direction of field width and length (blue arrows) are included. (D) Field map showing values of soil chemistry PC1 across the field. Levels of significance: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *, $p > 0.05$ ^{ns}.

Benzoxazinoid exudation shapes root microbiota

To determine whether benzoxazinoids shape microbial communities in maize roots and rhizospheres, as observed before (Cadot *et al.*, 2021b), we first analyzed the impact of benzoxazinoids on microbial alpha diversity in maize roots and rhizospheres. Alpha diversity of root and rhizosphere bacterial as well as rhizosphere fungal communities were enhanced in wild-type samples relative to *bx1* samples (**Fig. S6**). We then measured changes in beta diversity using PERMANOVA to validate benzoxazinoid conditioning and compare the effect size (R^2 values) relative to PC1 (**Table S1**). Benzoxazinoid conditioning shaped microbial communities in the roots and rhizospheres, with stronger effects for fungi than bacteria. Effects of PC1 were generally stronger than benzoxazinoid effects. Constrained Analysis of Principal Coordinates (CAP) visually confirmed these findings (**Fig. 3**). Thus, benzoxazinoid exudation led to a microbial conditioning.

Chemical legacy of benzoxazinoid exudation

To test the persistence of benzoxazinoid-dependent effects, we measured them again at wheat sowing and during wheat growth in the feedback phase (**Fig. 1**). At wheat sowing we found 10-100 fold reduced levels of benzoxazinoids compared to our first measurements (**Fig. 4A**). We thus performed analyses on concentrated samples, which also resulted in the detection of low benzoxazinoid levels in *bx1* conditioned soils. Most benzoxazinoids were still significantly more abundant in soils of wild-type plots (**Fig. 4B**). These quantitative differences were lost during wheat vegetative growth and some benzoxazinoid compounds (HDMBOA-Glc, DIMBOA, and HMBOA) became more abundant compared to wheat sowing, as wheat also releases benzoxazinoids (**Fig. 4B**). AMPO, the microbial metabolization product of MBOA, behaved differently than the other benzoxazinoids (**Fig. 4A**): Its concentration decreased only marginally across time points, and it remained significantly higher in wild-type conditioned plots during the entire experiment (**Fig. 4B**). We observed a significant co-variation of AMPO with PC1. Interestingly, the concentration gradient of AMPO was opposite to other benzoxazinoids such as HDMBOA-Glc, DIMBOA and HMBOA (**Fig. S7**), suggesting that the gradient of AMPO may be the result of differential conversion of benzoxazinoids to their breakdown products.

To test the persistence of the microbial legacy found at maize harvest (**Fig. 3**), we profiled the soil microbiomes at wheat sowing, and during wheat growth the soil, rhizosphere, and root microbiomes again. PERMANOVA revealed significant effect sizes for the soil chemical gradient, at wheat sowing and during wheat growth (**Table S2**). Unconstrained PCoA visualized the structuring of the bacterial communities and of rhizosphere fungi by the soil chemical gradient (**Fig. 4C**). However, no significant impact of benzoxazinoid conditioning on the soil and wheat microbial community composition was detected (**Table S2**). Thus, while chemical legacies of benzoxazinoid exudation remained present during wheat growth, microbial legacies disappeared in the feedback phase.

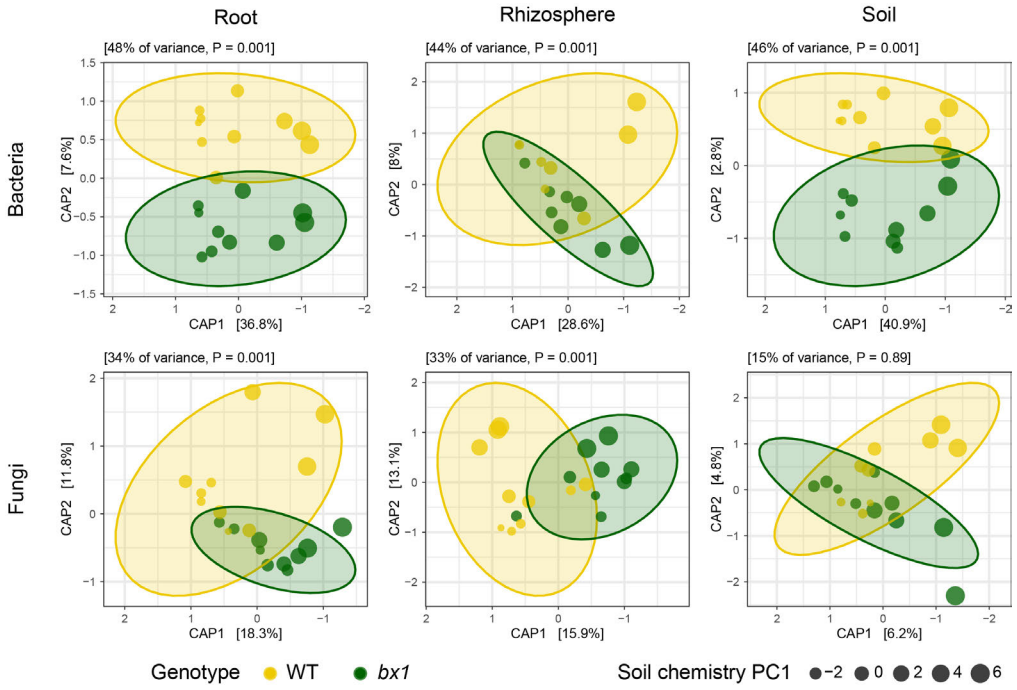


Figure 3. Benzoxazinoid exudation modulates rhizosphere and root microbiota. Compartment-wise Constrained Analysis of Principal Coordinates (CAP) using Bray-Curtis distances of community profiles from bacteria (top) and fungi (bottom). CAPs were performed using the model '~genotype * soil chemistry PC1'. Wild-type (WT) and *bx1* mutant samples are shown for roots, rhizospheres, and soils. The size of the datapoints represent the value of soil chemistry PC1. Total variance explained by the model and model significance are shown at the top of each panel. Axis labels indicate percentage of variance explained.

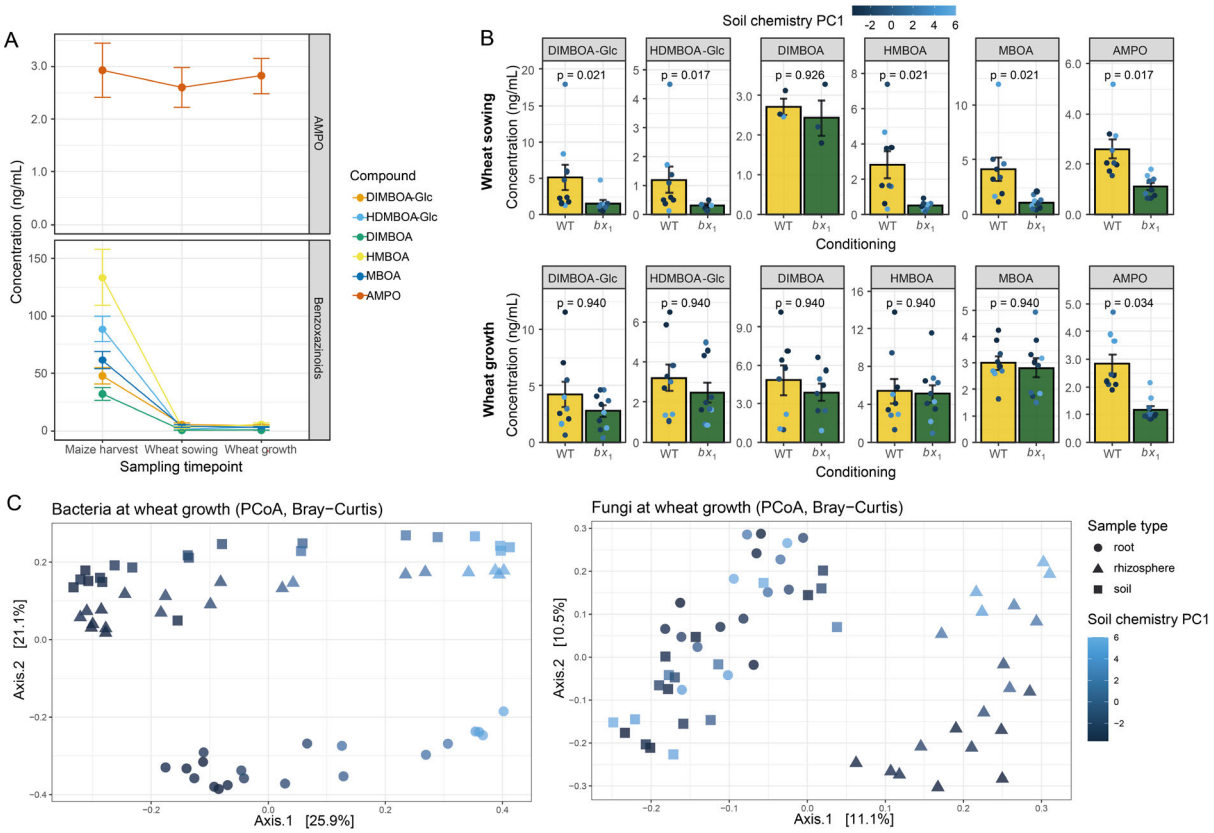


Figure 4. Persistence of benzoxazinoid-mediated chemical legacy. (A) Progression of concentrations of benzoxazinoids and their degradation product (AMPO) in wild-type (WT) plots over time (Means ± SE). (B) Concentrations of benzoxazinoids in soils collected on plots conditioned by wild-type or benzoxazinoid-deficient *bx1* mutant plants in ng/mL of soil (Means ± SE) at wheat sowing (top) and during wheat growth (bottom). *P* values were calculated by Wilcoxon rank-sum tests and corrected for multiple testing (FDR). (C) Unconstrained Principal Coordinate Analysis (PCoA) using Bray-Curtis distances of bacterial (left) and fungal (right) communities in root, rhizosphere, and soil samples. Axis labels indicate percentage of variance explained.

Soil chemistry directly determines benzoxazinoid degradation

Differences in soil chemistry may change benzoxazinoid exudation and degradation, thus accounting for the marked gradient in the directionality of the observed benzoxazinoid accumulation and microbial community composition. To test whether differences in soil properties can account for differential benzoxazinoid accumulation, we sampled soil from the opposite ends of the soil chemical gradient, i.e. south (S) and north (N, **Fig. 5A**). We then grew wildtype maize plants in these soils for 3 weeks and measured benzoxazinoid accumulation in the soil and benzoxazinoid exudation from freshly harvested roots. We did not detect significant differences in benzoxazinoid exudation from roots (**Fig. 5B**). However, we found significantly higher benzoxazinoid levels in S soil compared to N soil (**Fig. 5C**). Benzoxazinoid glucosides and their conversion products were more abundant in soil of the S compared to the N corner; a finding consistent with the field measurements (**Fig. S7, Fig. S8**).

To further investigate benzoxazinoid metabolization, we performed an incubation experiment with labeled DIMBOA-d_3 directly spiked in S and N soils and quantified the benzoxazinoid degradation over time. Most of the DIMBOA was rapidly metabolized in the field soil (**Fig. S9**). In N soil, DIMBOA was metabolized to MBOA more rapidly, resulting in a faster and stronger accumulation of AMPO compared to S soil (**Fig. 5D**). In the S soil, almost no AMPO was formed despite complete metabolization of DIMBOA and MBOA, suggesting that other degradation pathways operate in this corner of the field. Overall, these experiments revealed that benzoxazinoid metabolization is strongly dependent on soil properties, which explains the strong gradient observed across the different plots of the field experiment.

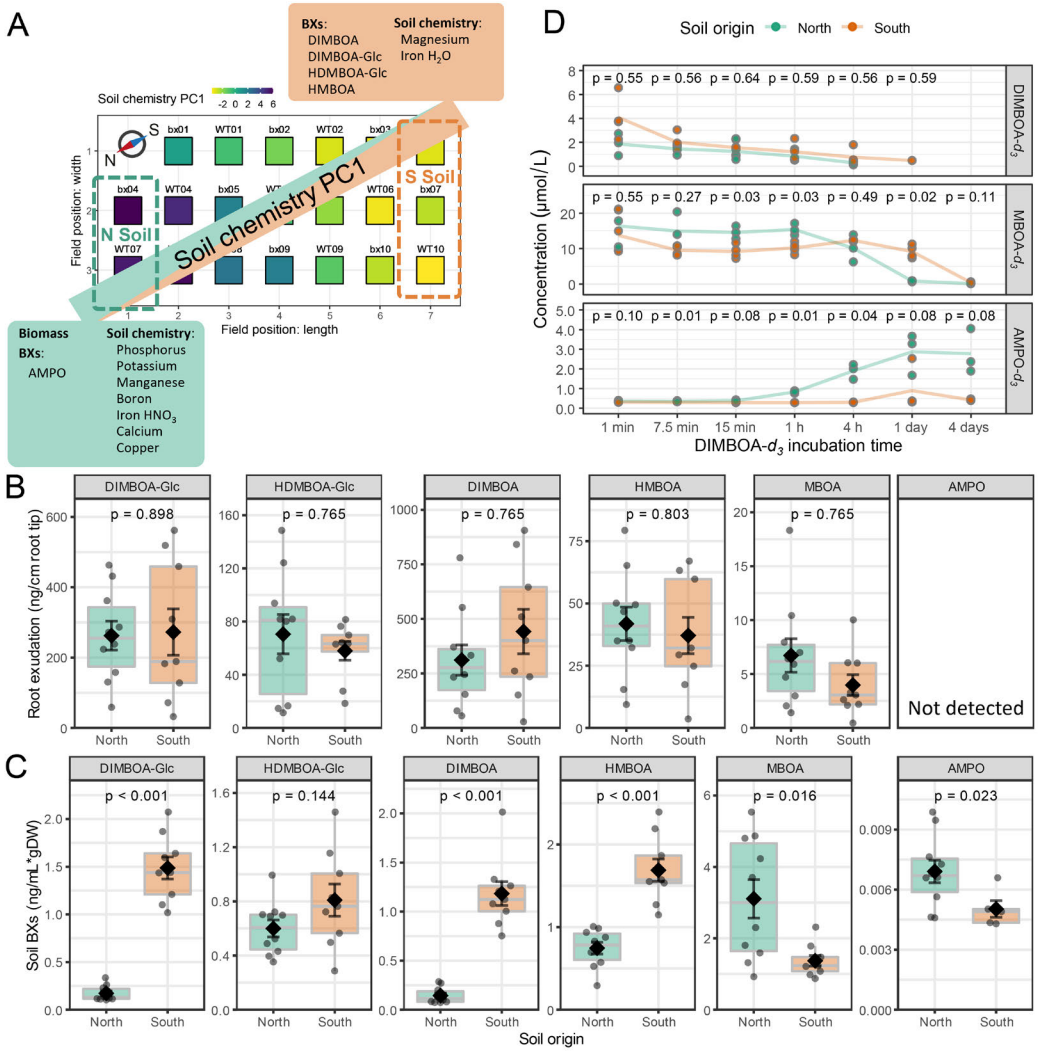


Figure 5. Degradation of benzoxazinoids depend on local soil environment. (A) Filed soil at both extremes of the soil chemistry gradient were collected for benzoxazinoid exudation and degradation experiments under controlled conditions. (B) Root benzoxazinoid exudation of 3-week-old maize plants (W22). (C) Benzoxazinoid concentration in soils of 3-week-old maize plants (W22) measured in ng/mL of soil and corrected for root dry weight. (D) Degradation of deuterated DIMBOA-d_3 in a plant free system monitored for 4 days. For (B) and (C) boxplots, means ± SE, and individual datapoints are shown and for (B)-(D) outputs of Welch's two-sample t-tests are included (FDR-corrected *p* values). N: north, S: south.

Chemical soil gradients are associated with benzoxazinoid-dependent plant-soil feedbacks

To determine benzoxazinoid-dependent feedback effects along our soil chemical gradient, we measured wheat performance and resistance in the different plots. For each phenotype, we tested for benzoxazinoid conditioning effects, effects of the chemical environmental gradient (PC1), and their interaction. We also quantified the feedback for each plot individually as log-response ratio of wild-type relative to *bx1* soil conditioning at a given location (see **Fig. S1**). This approach allowed us to compute local benzoxazinoid effects.

Overall, seedling emergence was not significantly affected by soil conditioning or PC1 (**Fig. 6A**). Analysis of local effects however revealed a negative effect of benzoxazinoids in plots to the north, and a positive effect in the plots to the south.

During wheat growth, overall chlorophyll content and height were not significantly affected by benzoxazinoid soil conditioning, but local effects were again detected (**Fig. 6B-C**). Positive effects of benzoxazinoids on chlorophyll and height were observed in plots to the north, while negative effects were observed in plots to the south. Plant biomass was negatively affected by benzoxazinoid soil conditioning, with effects that were more pronounced towards the northern end of the gradient in the field (**Fig. 6D)**.

As defence-related phenotypes, we counted the number of *Oulema melanopus* larvae on the plants in the field and we tested the performance of *Spodoptera littoralis* feeding on leaf material collected in the field. Both defence phenotypes were not affected by benzoxazinoid soil conditioning (**Fig. 6E-F**). For *S. littoralis* performance, analysis of local effects revealed a positive effect of benzoxazinoids on larval growth in plots to the north, and a negative effect in the plots to the south.

At wheat harvest, no significant benzoxazinoid effects on shoot biomass, biomass per tiller, and tiller density were found (**Fig. 7A-C**). Yield was also not affected by benzoxazinoid conditioning overall (**Fig. 7D**). However, a weak effect was observed along the gradient, with positive effects in plots to the north and slightly negative effects in plots to the south.

Agronomically important kernel quality parameters including grain characteristics, protein content, and bakeability were also not affected by overall benzoxazinoid soil conditioning (**Fig. S10**). Gradients of feedback effects on grain width, volume weight and dough stability were detected. Nutritional and food quality properties were not changed by benzoxazinoid conditioning or along the soil chemical gradient (**Fig. S11**).

Thus, benzoxazinoid soil conditioning influences wheat growth, defence, yield, and grain quality, but the directionality of the effect follows environmental gradients associated with differences in soil chemistry.

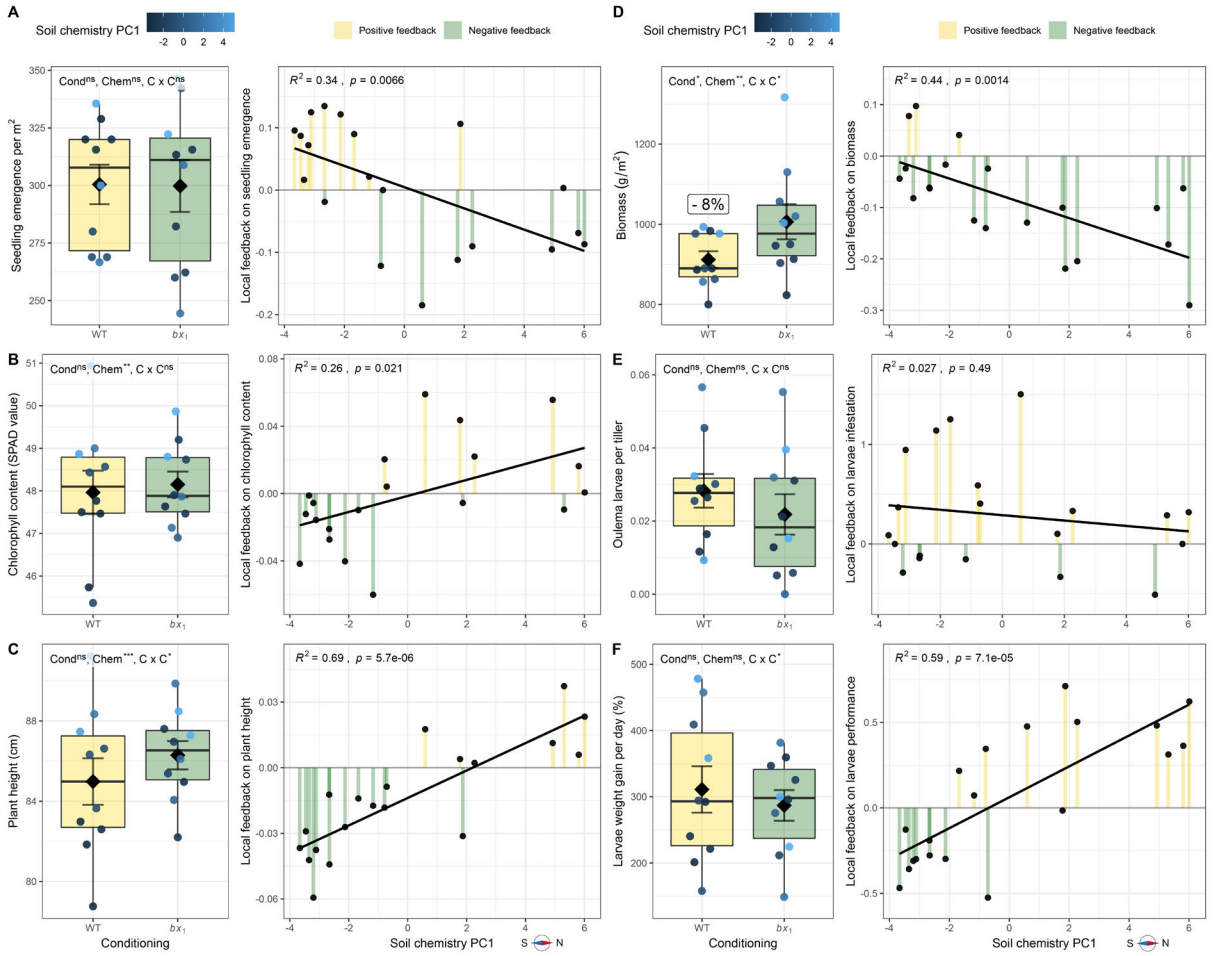


Figure 6. Benzoxazinoid-dependent feedbacks during wheat emergence and growth are associated with soil chemistry. (A) Seedling emergence, (B) chlorophyll content, (C) plant height, (D) dry biomass, (E) *Oulema* infestation, and (F) *Spodoptera* performance during wheat growth. For each phenotype boxplots (left) and local feedbacks of individual plots along the soil chemistry PC1 (right) are shown. For boxplots, phenotypes measured on plots conditioned by wild-type (WT) or benzoxazinoid-deficient *bx1* mutant maize are shown. Means ± SE and individual datapoints are included. Further, significance of ANOVA output is shown, where benzoxazinoid soil conditioning (Cond), the soil chemistry PC1 (Chem), and their interaction (C x C) were modelled. For the local feedbacks, values of individual plots are shown and R^2 and *p* value of linear regression are indicated in the top. For more details on the local feedback refer to method section. Levels of significance: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *, $p > 0.05$ ^{ns}.

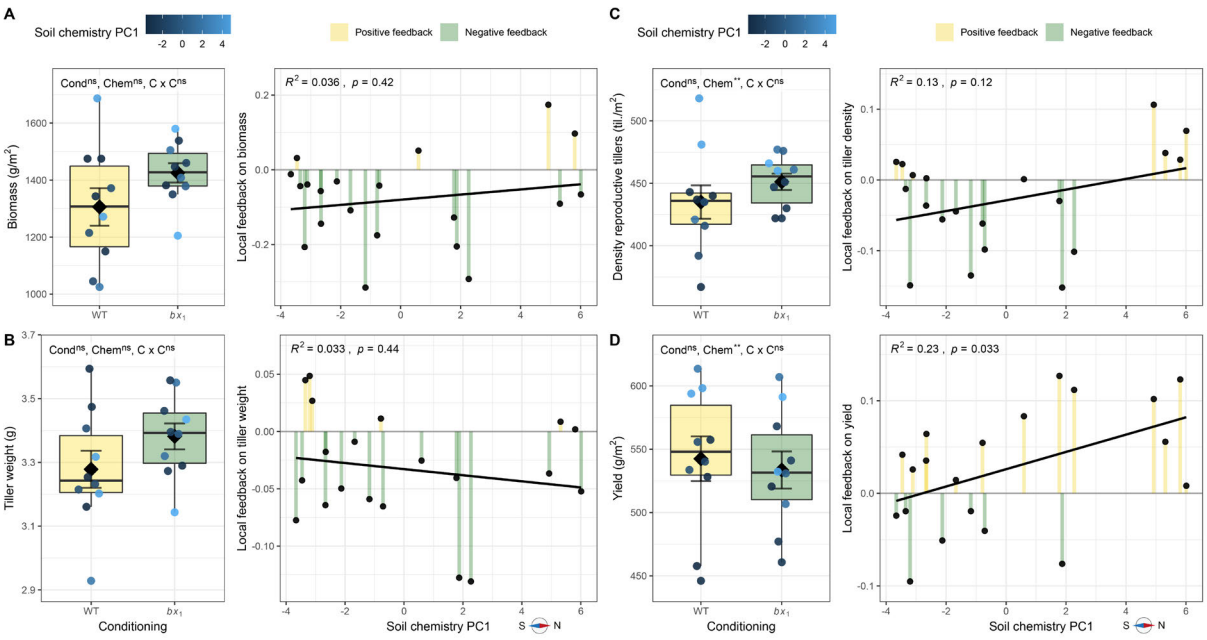


Figure 7. Local benzoxazinoid-dependent feedbacks on grain yield at wheat harvest. (A) Biomass, (B) tiller weight, (C) tiller density, and (D) yield at wheat harvest. For each phenotype, boxplots (left) and local feedbacks of individual plots along the soil chemistry PC1 (right) are shown. For boxplots, phenotypes measured on plots conditioned by wild-type (WT) or benzoxazinoid-deficient *bx1* mutant maize are shown. Means ± SE and individual datapoints are included. Further, significance of ANOVA output is shown, where benzoxazinoid soil conditioning (Cond), the soil chemistry PC1 (Chem), and their interaction (C x C) were modelled. For the local feedbacks, values of individual plots are shown and R^2 and p value of linear regression are indicated in the top. For more details on the local feedback refer to method section. Levels of significance: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *, $p > 0.05$ ^{ns}.

Discussion

Soil conditioning by plant secondary metabolites can affect the growth and defence of the following crop through plant-soil feedbacks. If and how such feedbacks depend on the soil type, and how soil heterogeneity may influence their spatial patterning, remains unknown. Here, we show that the effect of maize benzoxazinoids on wheat performance is entirely dependent on soil properties, leading to a distinct effect gradient within a single field. Correlation analysis revealed strong associations between soil parameters (chemistry, microbiome), soil benzoxazinoid concentrations, and the magnitude and direction of the benzoxazinoid-dependent feedback effects on wheat growth, defence, and grain quality. Below, we discuss these findings from a mechanistic perspective and derive implications for the use of secondary metabolite-driven plant-soil feedbacks in agriculture.

Impact of soil chemistry on benzoxazinoid accumulation

We find that innate differences in soil chemistry are associated with marked changes in benzoxazinoid accumulation during maize growth. Benzoxazinoid exudation can, for example, be altered in response to soil iron (Zhou *et al.*, 2018) and aluminium (Zhao *et al.*, 2019). Soil parameters may also influence the metabolization of secondary metabolites (Nannipieri *et al.*, 2002). The degradation to MBOA for instance is pH dependent (Maresh *et al.*, 2006), and the conversion of MBOA to AMPO as well as AMPO metabolization are mediated by soil microbes (Etzerodt *et al.*, 2008; Niemeyer, 2009). Our climate chamber experiments suggest that the differential accumulation in the field is the result of differences in metabolization rather than exudation by maize roots. As benzoxazinoids in the rhizosphere are directly responsible for changes in microbial composition and feedback effects on other plants (Hu *et al.*, 2018b), differences in metabolization may influence plant-soil feedback effects directly. The pronounced differences in metabolization we observe within the same field point to substantial potential for fine-scale variation in secondary metabolite-mediated feedback effects.

Interactions between benzoxazinoids and soil microbiota

Root-associated bacterial and fungal community compositions are well documented to be affected by benzoxazinoid exudation (Hu *et al.*, 2018b; Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019; Cadot *et al.*, 2021b; Gfeller *et al.*, 2022b). Here, we confirm this result and show that benzoxazinoid effects on microbiome composition and alpha diversity are significant, even in heterogeneous soils. The community structure of fungi, compared to bacteria, was more strongly affected by benzoxazinoids, which is in line with previous findings (Cadot *et al.*, 2021b). Bacterial communities showed a strong association with soil chemistry, possibly

because they respond more dynamically to local changes in environmental conditions. Previous work showed that bacteria are, for example, more strongly affected by soil acidification compared to fungi (Rousk *et al.*, 2010; Choma *et al.*, 2020). Given that benzoxazinoid accumulation is dependent on variation in soil properties and the root and rhizosphere microbiota are shaped by benzoxazinoids, one would expect the benzoxazinoid effect on microbiomes to vary across the field. In our study we did not observe this behaviour. A possible explanation for this is that root and rhizosphere microbiota are shaped directly by root internal and root-exuded benzoxazinoids, which were shown to be unaffected by soil parameters. In line with our previous field study (Gfeller *et al.*, 2022b), chemical, but not microbiota patterns persisted to the next crop generation. This is in contrast to previous pot and container experiments, where microbial fingerprints form the soil conditioning phase were still present during the feedback plant's growth (Hu *et al.*, 2018b; Hannula *et al.*, 2021). A likely explanation for this discrepancy is that in our experiments the process of seedbed preparation for wheat, with a complete soil homogenization at a depth of 10 cm, the microbial fingerprints were diluted in the surrounding soil. In summary, both soil chemistry and benzoxazinoid exudation shapes root microbiota, which likely adds to the variation and dynamics of plant-soil feedback effects.

Within-field variation in plant-soil feedbacks

Plant-soil feedbacks are well known to depend on the growth environment, the responsible mechanisms are however only partly understood (van der Putten *et al.*, 2013; Smith-Ramesh & Reynolds, 2017). Plant nutrient supply, for example, can influence the outcome of plant-soil feedbacks in crops and wild plants (Kos *et al.*, 2015a; Kuerban *et al.*, 2022). Generally, it is assumed that increasing soil fertility will weaken the strength of plant-soil feedbacks by lowering soil nutrient feedbacks, reducing the plant's dependency on mutualists, and decreasing the role of pathogens if plants have more resources to allocate in defence and immunity (Smith-Ramesh & Reynolds, 2017). Here, we found that depending on soil chemistry, the effect of benzoxazinoid-dependent plant-soil feedbacks on growth, defence, and food quality differ in strength and/or direction. During vegetative growth we found that under more fertile conditions, as indicated by higher wheat yield, benzoxazinoid conditioning led to faster plant growth, but less biomass accumulation and lower plant defence; While at harvest yield, kernel width, and dough stability were increased at the expense of kernel volume per weight, showing that the influence of soil chemistry on benzoxazinoid-dependent plant-soil feedbacks is plant growth stage-dependent. Because soil fertility positively correlated with benzoxazinoid degradation and affected microbial community composition, the exact underling mechanism remains to be investigated. The observed context-dependency of plant-soil feedbacks within one field could

explain why greenhouse experiments often cannot be reproduced under natural conditions (Schittko *et al.*, 2016; Forero *et al.*, 2019). We further found context-dependencies of benzoxazinoid-dependent plant-soil feedbacks between studies: In this field experiment, at the end of wheat vegetive growth, we found an overall negative effect of benzoxazinoid conditioning on wheat biomass accumulation. This finding is in line with what was found in a previous greenhouse experiment (Cadot *et al.*, 2021a), but the opposite of what was found in a previous field experiment (Gfeller *et al.*, 2022b). Given that the two field experiments were conducted in different soils at different locations, the observed variation could be explained by benzoxazinoid-dependent plant-soil feedbacks being soil specific, as it was shown in this study and in a maize-maize experiment before (Cadot *et al.*, 2021a). Taken together, our findings show the importance to take into account local and regional variation of plant-soil feedbacks to understand them in diverse environments and further examine their potential in sustainable agriculture.

Conclusion

Plants closely interact with their belowground environment. Root exuded secondary metabolites can directly or indirectly, mediated through changes in the microbiome, affect the next plant grown in that soil. Our work shows that such secondary metabolite-mediated plant-soil feedbacks occur within crop rotations under agronomically relevant conditions and that they are highly context-dependent. Together with previous work showing that direct effects of benzoxazinoids on aboveground insects depend on soil chemistry (Hu *et al.*, 2021), this study highlights how local environmental variation influences the effect of secondary metabolites. Understanding the context-dependency of plant-soil feedbacks within crop rotations is necessary to make them applicable to sustainable agriculture.

Acknowledgement

We thank Nicolas Widmer and the team of Agroscope Changins (Switzerland) for their field assistance during the two-year field experiment. Further, we thank Florian Enz for field and laboratory assistance. This work was supported by the Interfaculty Research Collaboration “One Health” of the University of Bern.

Supplementary information

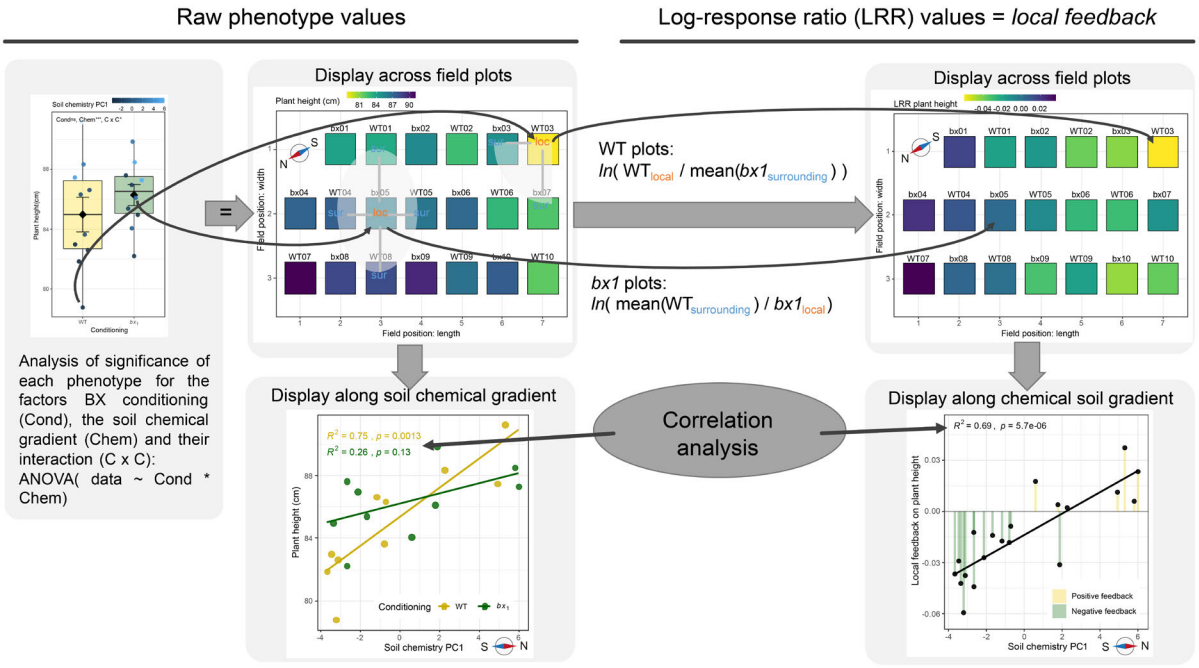


Figure S1. Explanation of the local feedback. Two different statistical analysis were performed on wheat phenotypes. First, the raw data was inspected for overall differences depending on benzoxazinoid soil conditioning and correlations with the soil chemical gradient. Second, the log-response ratio (LRR) was calculated for each plot, to estimate the local feedback at a given position on the field. To do so, on wild-type (WT) conditioned plots, the phenotype measurement was divided by the mean of measurements on the surrounding *bx1* mutant conditioned plots and log transformed (natural logarithm). On *bx1* conditioned plots, the mean phenotype measurement of the surrounding wild-type plots was divided by the measurement on the focal *bx1* plot and log transformed. These local feedbacks were further used to test for associations between soil parameters and the direction and strength of the feedback, where positive LRRs denote positive benzoxazinoid plant-soil feedbacks and negative LRRs denote negative benzoxazinoid-dependent plant-soil feedbacks.

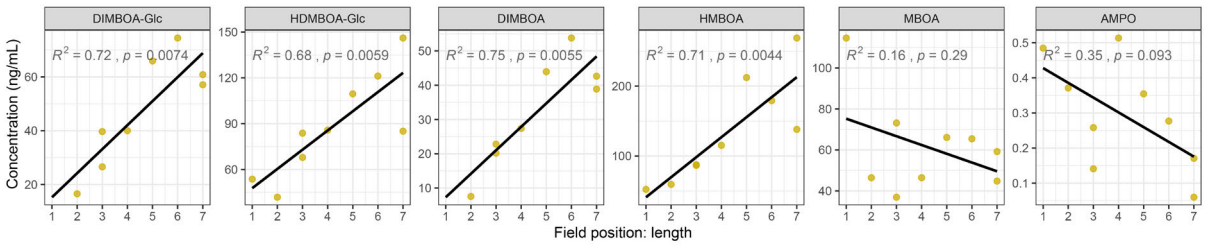


Figure S2. Soil benzoxazinoid concentrations at maize harvest are correlated with the position along the field length. The correlation between the field position and concentrations of benzoxazinoids in soils surrounding wild-type (WT) roots at the end of the condition phase in ng/ mL of soil are shown. R^2 and p value of linear regression are shown in the top.

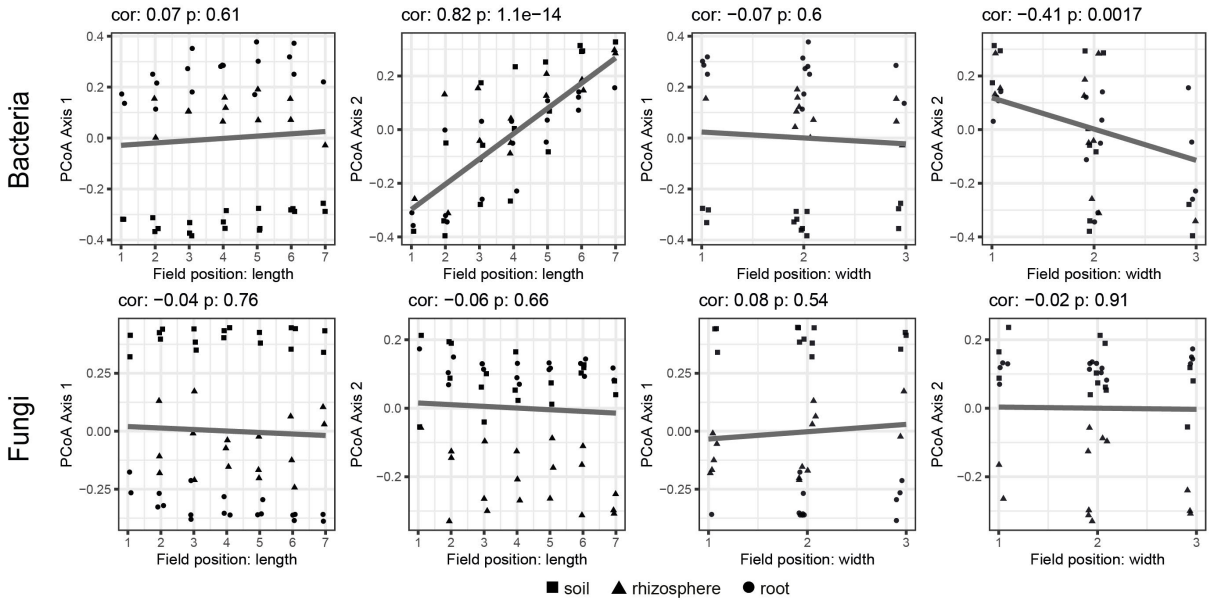


Figure S3. Microbial community composition (PCoA Axes) varies with field positions. Axis form Principal Coordinate Analysis (PCoA) of bacterial (top) and fungal (bottom) communities in roots (red), rhizospheres (green) and soils (brown) samples. Pearson correlation and corresponding significance is denoted on top of each panel.

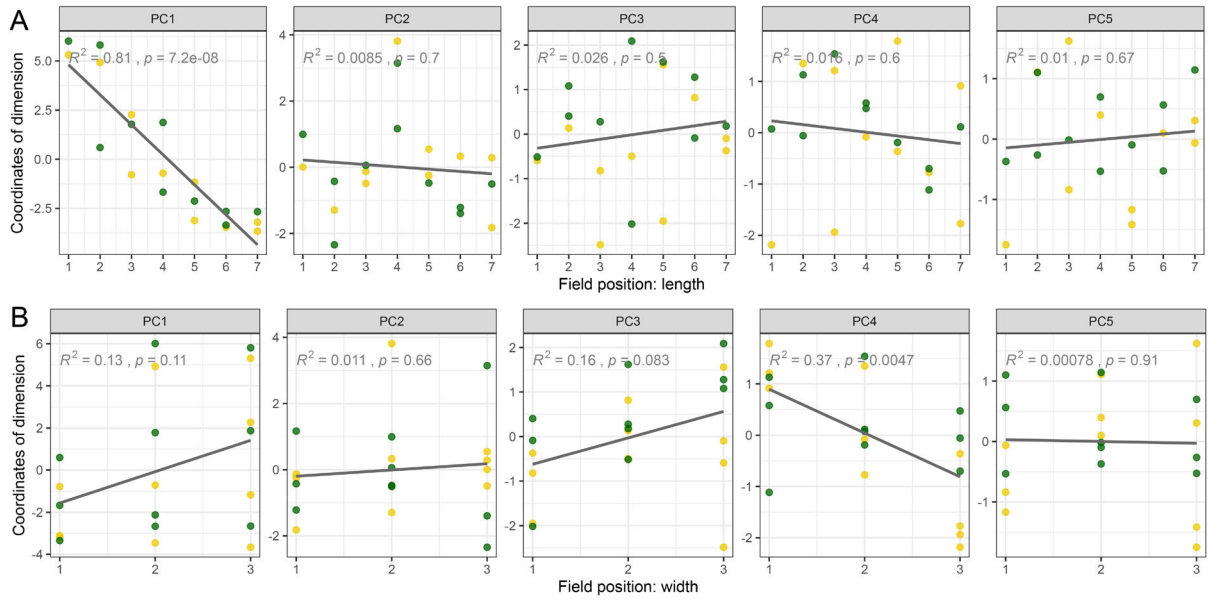
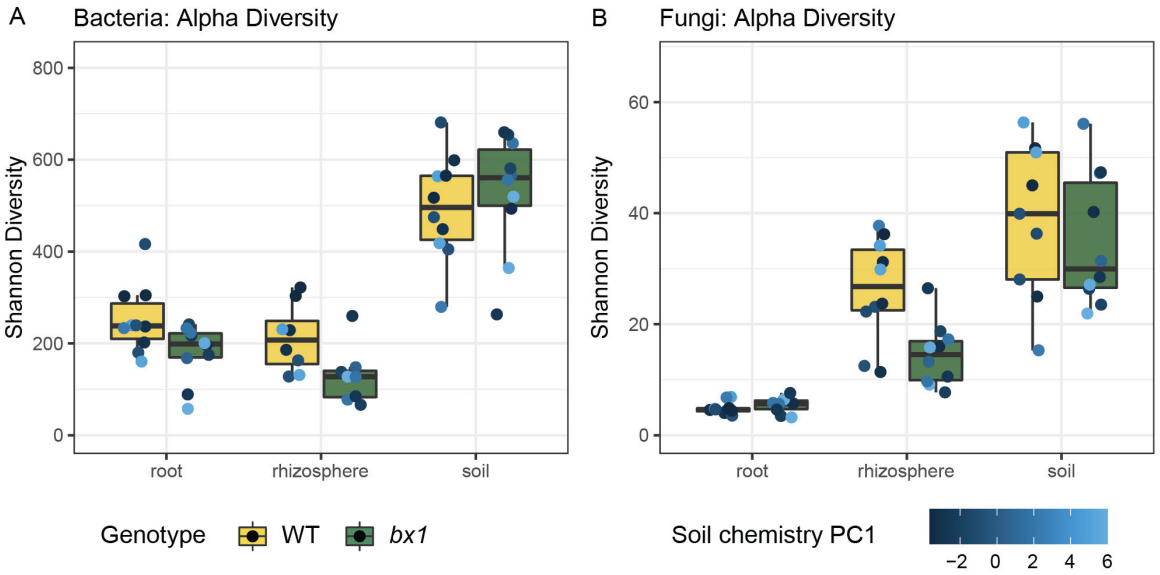


Figure S4. Composition of soil chemistry (PCA dimensions) varies with field positions. The correlation between soil chemistry Principal Component (PC) axes 1-5 and the position along field length (top) or filed width (bottom) are shown. R^2 and p value of linear regression are indicated in the top. Yellow circles: wild-type (WT) plots; Green circles: *bx1* mutant plots.

Figure S5. Chemical gradient along the field. Field map showing the soil chemical gradient along field plots for all individual soil parameters.



ANOVA statistics by sample type

Bacteria roots

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotype	1	25310	25310	5.449	0.03294
Soil chemistry PC1	1	8401	8401	1.809	0.1974
Genotype : Soil chemistry PC1	1	1015	1015	0.2185	0.6465
Residuals	16	74319	4645	NA	NA

Bacteria rhizosphere

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotype	1	27435	27435	6.079	0.02973
Soil chemistry PC1	1	8087	8087	1.792	0.2055
Genotype : Soil chemistry PC1	1	1607	1607	0.3561	0.5618
Residuals	12	54157	4513	NA	NA

Bacteria soil

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotype	1	5931	5931	0.3805	0.546
Soil chemistry PC1	1	15535	15535	0.9966	0.333
Genotype : Soil chemistry PC1	1	1639	1639	0.1051	0.7499
Residuals	16	249424	15589	NA	NA

ANOVA statistics by sample type

Fungi roots

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotype	1	1.226	1.226	0.8449	0.3716
Soil chemistry PC1	1	1.199	1.199	0.8264	0.3768
Genotype : Soil chemistry PC1	1	3.207	3.207	2.21	0.1566
Residuals	16	23.22	1.451	NA	NA

Fungi rhizosphere

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotype	1	685.5	685.5	11.41	0.003837
Soil chemistry PC1	1	13.58	13.58	0.226	0.6409
Genotype : Soil chemistry PC1	1	107.9	107.9	1.797	0.1988
Residuals	16	961.3	60.08	NA	NA

Fungi soil

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Genotype	1	62.4	62.4	0.3468	0.5647
Soil chemistry PC1	1	3.426	3.426	0.01904	0.8921
Genotype : Soil chemistry PC1	1	114.3	114.3	0.6353	0.4379
Residuals	15	2698	179.9	NA	NA

Figure S6. Microbial alpha diversity at maize harvest. (A) Bacterial and (B) fungal alpha diversity calculated as Shannon index are shown in boxplots (top) and compartment wise ANOVA outputs are included (bottom; model '~ genotype * soil chemistry PC1').

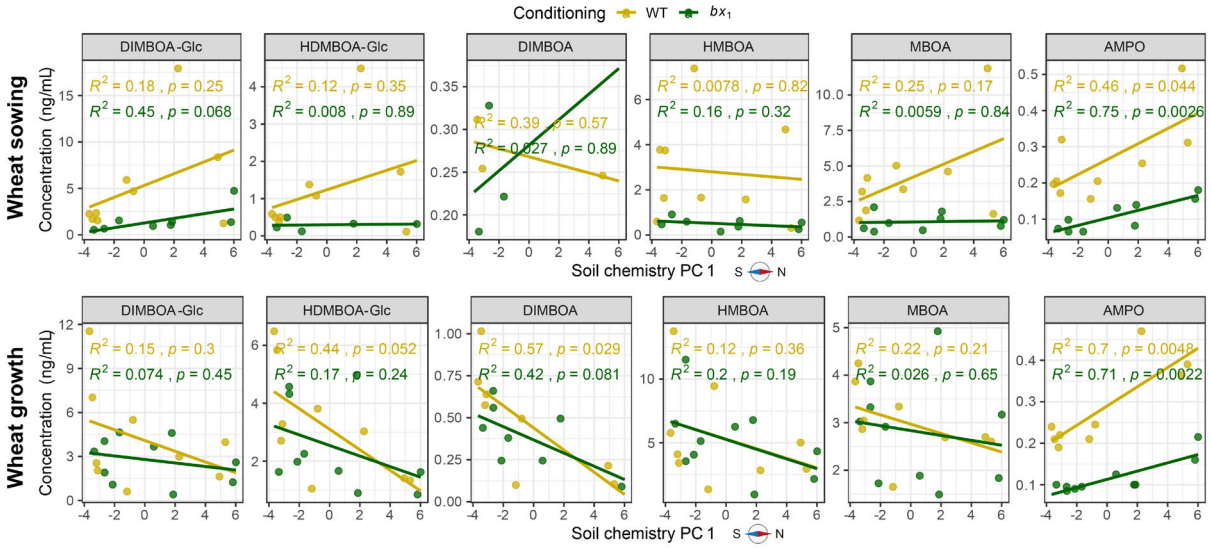


Figure S7. Soil benzoxazinoid measurements at wheat sowing and during wheat growth. The correlation between soil benzoxazinoid concentration and soil chemistry PC1 are shown at wheat sowing (A) and during wheat growth (B). R^2 and p values of linear regressions for plots conditioned by wild-type (WT) or benzoxazinoid-deficient *bx1* mutant plants are indicated in the top.

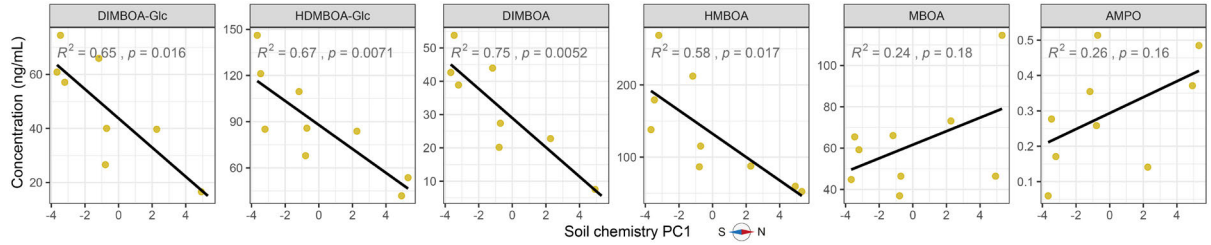


Figure S8. Soil benzoxazinoid concentrations at maize harvest are correlated with soil chemistry. The correlation between soil chemistry PC1 and the concentrations of benzoxazinoids in soils surrounding wild-type (WT) roots at the end of the condition phase in ng/mL of soil are shown. R^2 and p value of linear regression are in the top.

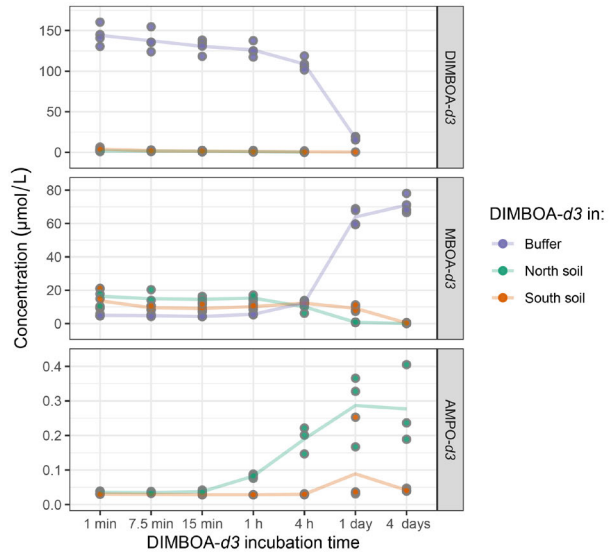


Figure S9. Degradation of benzoxazinoids in soil suspensions and buffer. Degradation of deuterated DIMBOA-d_3 in soil suspensions originating from both extremes of the soil chemistry gradient and in buffer was monitored for 4 days.

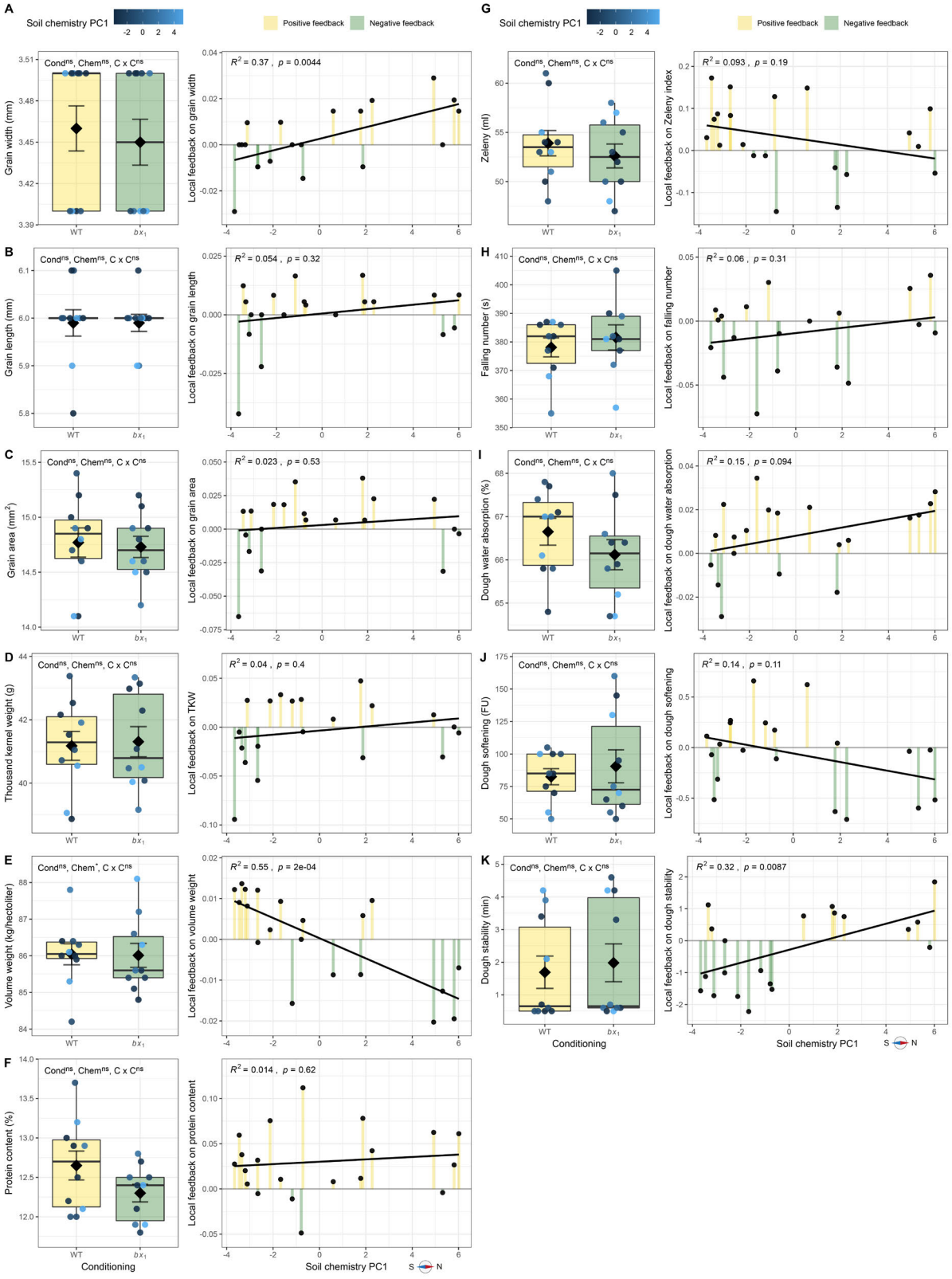


Figure S10. Benzoxazinoid-dependent plant-soil feedbacks on agronomic kernel parameters. For each agronomic kernel parameter boxplots (left) and local feedbacks of individual plots along the soil chemistry PC1 (right) are shown. For boxplots, phenotypes measured on plots conditioned by wild-type (WT) or benzoxazinoid-deficient *bx1* mutant maize are shown. Means ± SE and individual datapoints are included. Further, significance of ANOVA output is shown, where benzoxazinoid soil conditioning (Cond), the soil chemistry PC1 (Chem) and their interaction (C x C) were modelled. For the local feedbacks, values of individual plots are shown and R^2 and *p* value of linear regression are indicated in the top. For more details on the local feedback refer to method section. Levels of significance: p < 0.001 ***, p < 0.01 **, p < 0.05 *, p > 0.05 ^{ns}.

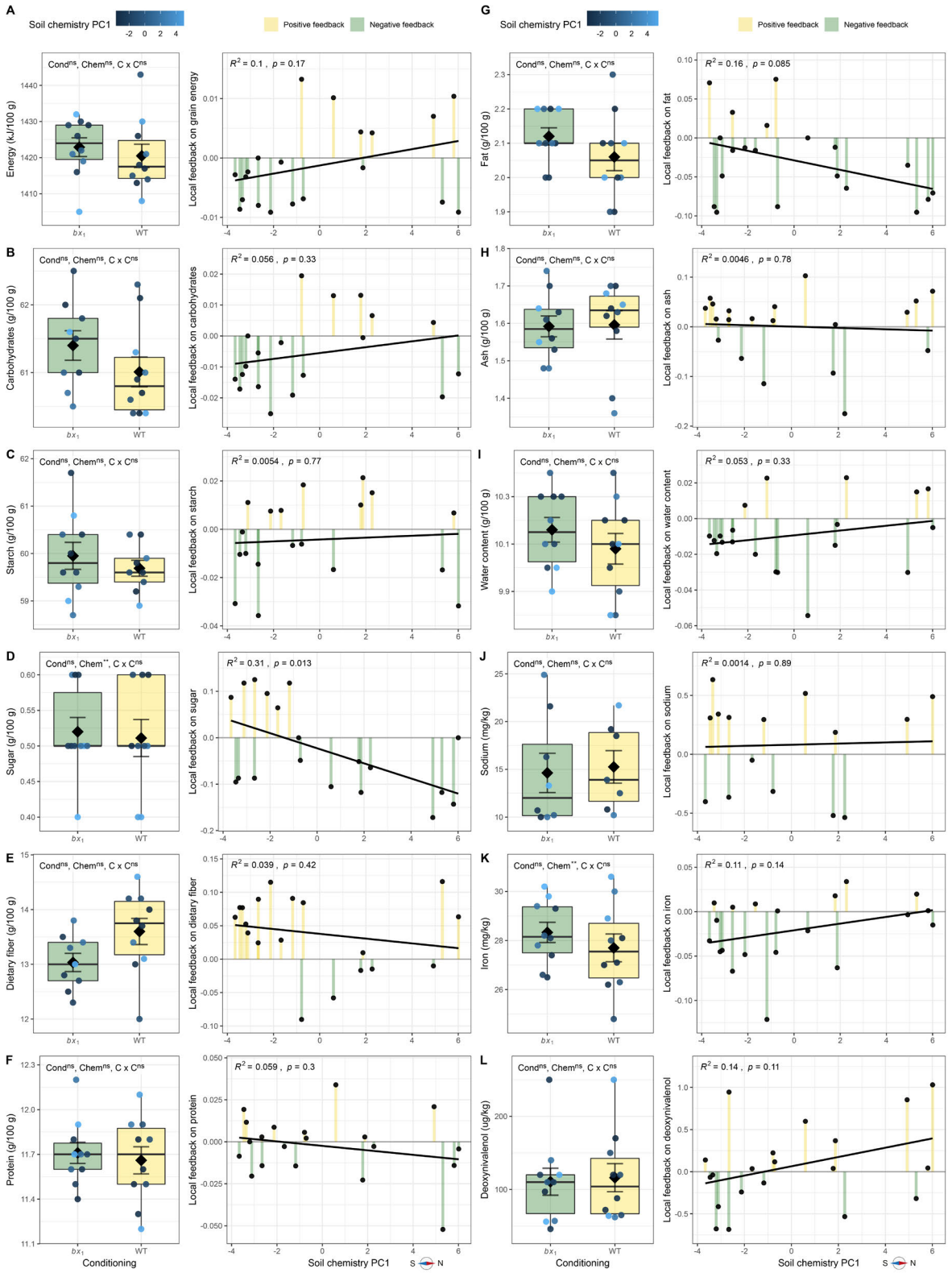


Figure S11. Benzoxazinoid-dependent plant-soil feedbacks on food quality parameters. Various food quality parameters were determined in the wheat kernels. For each parameter boxplots (left) and local feedbacks of individual plots along the soil chemistry (right) are shown. For boxplot, phenotypes measured on plots conditioned by wild-type (WT) or benzoxazinoid-deficient *bx1* mutant maize are shown; means ± SE and individual datapoints are included (n = 10). Further, significance of ANOVA output is shown, where benzoxazinoid soil conditioning (Cond), the soil chemistry PC1 (Chem), and their interaction (C x C) were modelled. For the local feedbacks, values of individual plots are shown and R^2 and p value of linear regression are indicated in the top. For more details on the local feedback refer to method section. Levels of significance: $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *, $p > 0.05$ ^{ns}.

Supplementary Table 1. PERMANOVA of Microbiomes at maize harvest. Compartment-wise analysis of maize genotype (WT, *bx1*), soil chemistry (PC axis 1), and their interaction for bacteria (top) and fungi(bottom). Significant *p* values are shown in bold.

Bacteria		Df	SumOfSqs	R2	F	Pr(>F)	
Root	Genotype	1	0.2582	0.07514	2.2878	**0.049**	*
	Soil chemistry PC1	1	1.2611	0.36695	11.1727	**0.001**	***
	Genotype:SoilChemPC1	1	0.1114	0.03241	0.9867	0.381	
	Residual	16	1.8060	0.52550			
	Total	19	3.4367	1.00000			
Rhizo.	Genotype	1	0.22447	0.07741	1.6452	0.100	.
	Soil chemistry PC1	1	0.82186	0.28342	6.0236	**0.001**	***
	Genotype:SoilChemPC1	1	0.21615	0.07454	1.5842	0.117	
	Residual	12	1.63728	0.56463			
	Total	15	2.89976	1.00000			
Soil	Genotype	1	0.1264	0.03169	0.9343	0.404	
	Soil chemistry PC1	1	1.6104	0.40374	11.9031	**0.001**	***
	Genotype:SoilChemPC1	1	0.0872	0.02186	0.6445	0.699	
	Residual	16	2.1646	0.54271			
	Total	19	3.9886	1.00000			
Fungi		Df	SumOfSqs	R2	F	Pr(>F)	
Root	Genotype	1	0.32678	0.13110	3.1550	**0.002**	**
	Soil chemistry PC1	1	0.40106	0.16090	3.8721	**0.001**	***
	Genotype:SoilChemPC1	1	0.10750	0.04313	1.0379	0.411	
	Residual	16	1.65722	0.66487			
	Total	19	2.49255	1.00000			
Rhizo.	Genotype	1	0.40395	0.15156	3.6085	**0.003**	**
	Soil chemistry PC1	1	0.33356	0.12515	2.9797	**0.005**	**
	Genotype:SoilChemPC1	1	0.13664	0.05127	1.2207	0.259	
	Residual	16	1.79110	0.67202			
	Total	19	2.66526	1.00000			
Soil	Genotype	1	0.2436	0.04152	0.7295	0.944	
	Soil chemistry PC1	1	0.3574	0.06092	1.0704	0.291	
	Genotype:SoilChemPC1	1	0.2573	0.04386	0.7706	0.903	
	Residual	15	5.0089	0.85371			
	Total	18	5.8672	1.00000			

Signif. codes: *** 0.001
** 0.01
* 0.05
. 0.1

Supplementary Table 2. PERMANOVA of Microbiomes at wheat sowing and during wheat growth. Compartment-wise analysis of soil conditioning (WT, *bx1*), soil chemistry (PC axis 1), and their interaction for bacteria (top) and fungi(bottom) at wheat sowing and during wheat growth. Significant *p* values are shown in bold.

Bacteria	**Wheat sowing**	Df	SumOfSqs	R2	F	Pr(>F)	
Soil	Conditioning	1	0.1003	0.02729	0.8888	0.403	
	Soil chemistry PC1	1	1.7012	0.46288	15.0735	**0.001**	***
	Conditioning:SoilChemPC1	1	0.0679	0.01849	0.6020	0.702	
	Residual	16	1.8058	0.49134			
	Total	19	3.6752	1.00000			

Fungi	**Wheat sowing**	Df	SumOfSqs	R2	F	Pr(>F)	
Soil	Conditioning	1	0.3173	0.04511	0.8016	0.873	
	Soil chemistry PC1	1	0.4594	0.06531	1.1605	0.163	
	Conditioning:SoilChemPC1	1	0.3197	0.04544	0.8075	0.868	
	Residual	15	5.9381	0.84414			
	Total	18	7.0345	1.00000			

Bacteria	**Wheat growth**	Df	SumOfSqs	R2	F	Pr(>F)	
Root	Conditioning	1	0.0710	0.02041	0.6123	0.765	
	Soil chemistry PC1	1	1.4612	0.42022	12.6027	**0.001**	***
	Conditioning:SoilChemPC1	1	0.0900	0.02588	0.7761	0.535	
	Residual	16	1.8551	0.53349			
	Total	19	3.4773	1.00000			
Rhizo.	Conditioning	1	0.0929	0.02615	0.8268	0.455	
	Soil chemistry PC1	1	1.5780	0.44399	14.0371	**0.001**	***
	Conditioning:SoilChemPC1	1	0.0845	0.02377	0.7516	0.551	
	Residual	16	1.7987	0.50608			
	Total	19	3.5541	1.00000			
Soil	Conditioning	1	0.1085	0.02560	0.7443	0.609	
	Soil chemistry PC1	1	1.6969	0.40044	11.6416	**0.001**	***
	Conditioning:SoilChemPC1	1	0.1000	0.02360	0.6862	0.664	
	Residual	16	2.3322	0.55035			
	Total	19	4.2377	1.00000			

Fungi	**Wheat growth**	Df	SumOfSqs	R2	F	Pr(>F)	
Root	Conditioning	1	0.2785	0.07517	1.3154	0.064	.
	Soil chemistry PC1	1	0.2041	0.05509	0.9640	0.569	
	Conditioning:SoilChemPC1	1	0.2581	0.06966	1.2189	0.146	
	Residual	14	2.9645	0.80008			
	Total	17	3.7052	1.00000			
Rhizo.	Conditioning	1	0.1415	0.03350	0.7605	0.760	
	Soil chemistry PC1	1	0.9289	0.21990	4.9917	**0.001**	***
	Conditioning:SoilChemPC1	1	0.1763	0.04174	0.9476	0.507	
	Residual	16	2.9775	0.70485			
	Total	19	4.2243	1.00000			
Soil	Conditioning	1	0.2055	0.04254	0.7826	0.860	
	Soil chemistry PC1	1	0.2256	0.04670	0.8591	0.705	
	Conditioning:SoilChemPC1	1	0.1981	0.04100	0.7543	0.893	
	Residual	16	4.2023	0.86975			
	Total	19	4.8316	1.00000			

Signif. codes:
*** 0.001
** 0.01
* 0.05
. 0.1

Chapter III

Benzoxazinoids enhance maize performance by mitigating negative plant-soil feedbacks

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Summary

- Plants can suppress the growth of other plants by modifying soil properties. These effects are often species-specific, suggesting that some plants are resistant to negative plant-soil feedbacks. However, the underlying resistance mechanisms remain unknown.
- Benzoxazinoids exuded by maize roots shape root microbiota and subsequent plant performance. We thus hypothesized that these secondary metabolites may help the plants to reprogram growth suppressive soils and thus to resist negative plant-soil feedbacks. Using benzoxazinoid-deficient mutants, chemical complementation, sterilization and re-inoculation experiments, we determined how benzoxazinoids influence maize growth responses to different preceding crop species.
- We find that maize growth is suppressed following soil conditioning by 3 out of 5 tested crop species. The capacity to produce benzoxazinoids reduced these negative effects.
- Our study demonstrates that plant secondary metabolites can confer resistance to negative plant-soil feedbacks. These findings expand our understanding of the role of plant secondary metabolites in mediating plant-soil interactions and represent a promising avenue to improve plant performance in crop rotations.

Introduction

Plants constantly interact with their soil environment. They change the soil`s biotic and abiotic attributes, which then, in turn, alter the performance of proceeding plants. These so-called plant-soil feedbacks can either enhance or lower the performance of the following plant, leading to positive or negative feedbacks (Bever *et al.*, 1997; van der Putten *et al.*, 2013). 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; Klironomos, 2002; van der Putten *et al.*, 2013; Teste *et al.*, 2017). In agriculture, this concept has been applied for centuries to mitigate negative impacts of monocropping. Up to now the ecological knowledge generated in the field of plant-soil feedback has not translated into improved crop rotations (Mariotte *et al.*, 2018). Evidence based crop rotation design represents a promising avenue towards more sustainable agriculture (Dias *et al.*, 2015; Mariotte *et al.*, 2018).

Crop rotations are best studied for their long-term benefits. 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), this will be of importance to alleviate adverse impacts of global change. Soil conditioning by a given crop species can alter the growth, defence, 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 seedings 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 maize seedling performance are driven by precrop-dependent microbiomes. More work is needed to understand the mechanisms determining suitable pairs of crops in a sequence to ultimately improve crop rotations.

Root exudates shape the rhizosphere microbiome (Sasse *et al.*, 2018). For benzoxazinoids and flavones these changes were linked to the performance of succeeding conspecific plants (Hu *et al.*, 2018b; Yu *et al.*, 2021). Through plant-soil feedbacks, the trait of benzoxazinoid exudation in maize also affects wheat performance under controlled conditions and improves wheat yield under field conditions (Cadot *et al.*, 2021a; Gfeller *et al.*, 2022b). Considering that benzoxazinoids can structure the rhizosphere microbiome of maize already at the seedling stage (Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019), that benzoxazinoids can act fungistatic against

soil pathogens (Wilkes *et al.*, 1999; Martyniuk *et al.*, 2006), and that benzoxazinoids can attract a *Pseudomonas putida* strain that potentially induces plant resistance (Neal *et al.*, 2012; Neal & Ton, 2013), we hypothesize that they could also increase crop rotation stability by alleviating negative plant-soil feedbacks.

Benzoxazinoids, a class of indole-derived plant secondary metabolites, are well known for their multifaceted bioactivities (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 defence metabolites against insects and pathogens (Niemeyer, 2009), and they can act as signalling molecules (Ahmad *et al.*, 2011). Further, it is known that benzoxazinoids have chelating properties leading to improved iron acquisition (Hu *et al.*, 2018a). Given the various positive functions of benzoxazinoids, it seems likely that they improve crop rotation stability.

Here, we investigate the role of benzoxazinoids in tolerating crop rotation legacies. We hypothesize that benzoxazinoid exudation into the rhizosphere reduces negative plant-soil feedbacks caused by the precrops. By growing wild-type and benzoxazinoid-deficient *bx1* mutant maize, we examined how benzoxazinoids alter soil legacy effects of different precrops. In several plant-soil feedback experiments (**Fig. S1**), we tested if direction 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 underlying mechanism. We found that benzoxazinoids increase crop rotation stability through root exudation and soil biota dependent mechanisms, while also unmeasured factors contributed to the outcome of some experiments.

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 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* cv. green shell (soybean), *Medicago sativa* (alfalfa), *Brassica napus* (rapeseed), *Phacelia tanacetifolia* (lacy phacelia), and *Triticum aestivum* cv. Claro (winter wheat). *G. max*, *M. sativa*, and *P. tanacetifolia* seeds were obtained from Sativa Rheinau AG (Switzerland), *B. napus* seeds were purchased online (www.saemereien.ch), and *T. aestivum* seeds were kindly provided by Saatzucht Düdingen (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. For all the other experiments, soil was sampled in two batches on another filed, parcel 30. 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. All soils were characterized in previous publications (Hu *et al.*, 2018b; Cadot *et al.*, 2021a; Gfeller *et al.*, 2022a).

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: ~ 550 µmol $m^{-2}s^{-1}$). In the conditioning phase the precrops were grown in 2 L pots (Rosentopf Soparco 2.0 l; Hortima, Switzerland) for 6 weeks, followed by the maize feedback phase in either 2L or 1L pots (Rosentopf Soparco 1.0 l; Hortima, Switzerland) for 6 weeks or 4 weeks, respectively. To avoid the roots from growing out of the pot, fleece (Geotex; Windhager, 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 one day before sowing. For each precrop an excess of seeds was sown

and thinned out to 2 plants per plot after 1 week, except for the fast-growing soybean, where we only kept 1 plant. Plants were watered as needed, and once a week, 100 mL of a nutrient solution (0.2% [w/v]; Plantaaktiv Typ K, Hauert, 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). Pots were randomly arranged in the climate chamber and re-randomized on a weekly basis. At precrop harvest shoot biomass was collected and dried until constant weight at 80 °C, before dry weight was determined on a microbalance. After removing the root system, the remaining soil was sieved (10 mm mesh size), homogenized within each precrop (in all but one experiment), and used for the feedback phase. In the feedback phase, pot preparation was performed identical to the conditioning phase and maize seeds were sown. At harvest, plant height was measured and in some experiments chlorophyll content was determined by averaging 9 measurements equally distributed along the youngest fully opened leaf by means of a SPADE-502 chlorophyll meter (Konica Minolta, Japan), and maize biomass was weighed after drying at 80 °C until constant weight. See **Fig. S1** for specific information on pot size, length of feedback period, and soil treatments between conditioning and feedback phase.

Screening benzoxazinoid-mediated resistance to plant-soil feedbacks of different precrops

In our initial experiment we examined the role of benzoxazinoids in resisting plant-soil feedbacks of 5 selected precrops. In the response phase wild-type B73 maize and its *bx1* mutant were grown for 6 weeks in 2 L pots. To analyse benzoxazinoid exudation during the course of the experiments, after harvesting, we selected a random subset of 3 wild-type pots and 1 *bx1* pot per precrop species. 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. The tubes were then stored at -80 °C until further processing (see below).

Examination of soil type, maize line, and plant age dependency

To test if the observed effects depend on soil type, maize line, and/or plant age, we performed a feedback experiment comparing B73 and W22 maize in soil from Changins, and for W22 we compared soil form Changins with an additional soil, Q-Matte. Based on our initial experiment, we decided to grow *M. sativa* and *T. aestivum* as precrop. In the response phase, maize was grown for 6 weeks in 2 L pots. In comparison to all the other experiments, (i) experimental units were kept separate from conditioning to feedback phase without mixing, and (ii) fertilization was maintained low during precrop conditioning and maize response: 100 mL of a nutrient solution (0.2% [w/v]; Plantaaktiv Typ K, Hauert, Grossaffoltern, Switzerland) supplemented with iron (1 ‰ [w/v]; Sequestrene rapid, Maag) was supplied on a weekly base.

Testing the underlying mechanisms

To get a deeper understanding of the phenomenon, we performed complementation experiments, sterilization and re-inoculation experiments. To test for repeatability, we performed these experiments three times. Experiment 1 and 2 were performed in the same soil batch as the previous experiment, whereas for experiment 3 we freshy collected soil from the same location (Changins, **Fig. S1**). In all three experiments, feedbacks were conducted with W22 maize in 1 L pots for 4 weeks.

To test if the observed differences in performance of wild-type and *bx1* mutant plants are triggered by benzoxazinoids exuded in the soil matrix, we externally applied a mixture of benzoxazinoids to *bx1* mutant plants growing in *T. aestivum* conditioned soil. In the three experiments, benzoxazinoids were applied in three different concentrations (**Fig. S5a,b**). Benzoxazinoid levels in the soil were determined at the end of each experiment, to estimate the effectiveness of our treatment. AMPO, a stable degradation product of benzoxazinoids, 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. S5c-e**). Compared to levels in the soil of wild-type plants, AMPO concentrations in complemented *bx1* mutant soil were very low in the first experiment, where we applied 50 ug 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 wild-type plants. For the third experiment, we thus used an intermediate dose, 1.6 mg, resulting in wild-type levels (**Fig. S5c-e**). For complementation, benzoxazinoids were purified form 4-day old seedlings (see below), dissolved in $_{dd}H_2O$, and 5 mL of this solution was pipetted to the *bx1* plants every 3 days, starting two days after sowing (at germination). Control *bx1* plants and wild-type plants were supplied with the same amount of $_{dd}H_2O$. To investigate benzoxazinoid accumulation in the pots, soil was sampled as described above for a random subset of plants.

Next, we evaluated if soil biota is driving the positive effects of benzoxazinoids on plant growth. For that, part of the *T. aestivum* conditioned soils were X-ray sterilized (20-60 kGy; Steris, Däniken, Switzerland). In the feedback phase wild-type and *bx1* mutant plants were grown in unsterilized, sterilized, and re-inoculated soil. Re-inoculation was achieved by complementing 95 % of sterilized soil with 5 % of unsterilized (living) soil and homogenizing thoroughly. All soils were acclimatized for 1 week in the climate chamber before sowing. All plants across the entire experiment were watered with autoclaved tab H_2O.

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 4 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.

Purification of benzoxazinoids for complementation

To purify benzoxazinoids, 40 g of maize seeds (var. Akku) were placed in a 1 L glass beaker and soaked in autoclaved $_{dd}H_2O$ for 14 h. Kernels were washed twice a day and harvested after 4 days. Soaking and growth took place in the dark at 26 °C. During harvest, kernels were immediately put into a blender (MioStar Beld 600s; Migros, Switzerland) prefilled with 600 mL methanol (MeOH), blended at maximum speed for 5 minutes, and passed through a filter paper (Grade 1; Whatman, GE Healthcare Live Sciences, USA). Next, we removed MeOH and H_2O 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), evaporated to dryness, and compounds were separated on a flash chromatography purification system (CombiFlash RF+, Teledyne ISCO, 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, 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 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; MN, Germany) filter, and evaporated to dryness. To crystallize the benzoxazinoid mixture, the compounds were dissolved in $_{dd}$H2O and lyophilized. The resulting white powder was used for complementation and an aliquot was characterized on a UHPLC-MS system (see below).

Analysis of benzoxazinoids

To analyse benzoxazinoids and break down products, soil was sampled as described above. 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 minutes 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), a 1 mL aliquot of the filtrate was transferred into a 1.5 mL centrifuge tube, centrifuged (10 min, 19000 g, 4 °C), and the supernatant was

sterile filtered (Target2TM, Regenerated Cellulose Syringe Filters. Pore size: 0.45 µm; Thermo Scientific) into a glass tube for analysis.

Benzoxazinoids extracted from soils and the purified benzoxazinoid mixture from germinated maize kernels were analysis as described before (Gfeller *et al.*, 2022b). In short, an Acquity UHPLC system coupled to a G2-XS QTOF mass spectrometer equipped with an electrospray source and piloted by the software MassLynx 4.1 (Waters AG, Baden-Dättwil, Switzerland) was used. Absolute quantities were determined through standard curves of pure compounds. For that, MBOA (6-methoxy-benzoxazolin-2(3H)-one) was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). DIMBOA-Glc (2-O-β-D-glucopyranosyl-2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one) and HDMBOA-Glc (2-O-β-D-glucopyranosyl-2-hydroxy-4,7-dimethoxy-2H-1,4-benzoxazin-3(4H)-one) were isolated from maize plants in our laboratory. 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 (9-methoxy-2-amino-3H-phenoxazin-3-one) were synthesized in our laboratory.

Statistical analysis

All statistical analysis were conducted in R version 4.1.2. (R Core Team, 2021). Data management and visualisation was facilitated with the *tidyverse* package collection (Wickham *et al.*, 2019). Phenotypic data was analysed 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 analysed 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 test for differences in benzoxazinoid production between wild-type and *bx1* mutant maize as well as validation of complementation success, Wilcoxon rank-sum tests were performed. Differences in weight gain 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 of wild-type 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 endpoint analysis of the time series experiment was also analysed by Welch's two-sample *t*-test.

Results

Benzoxazinoids enhance resistance to negative plant-soil feedbacks

To test whether benzoxazinoids can assist the plant to cope with negative plant-soil feedbacks, we grew five crop species for 6 weeks under controlled conditions, followed by a feedback phase with wild-type (B73) and benzoxazinoid-deficient *bx1* mutant maize. Biomass accumulation after 6 weeks ranged from 3.4 g to 10.5 g dry weight (**Fig. S2a**). After harvesting the conditioning plants, conditioned soils were separately sieved, and wild-type and *bx1* mutant maize was sown (**Fig. S1**). After 6 weeks of growth, we observed differences in biomass accumulation of maize depending on the precrop. Maize plants accumulated significantly less biomass on soils conditioned by *T. aestivum*, *P. tanacetifolia*, and *B. napus* than *G. max* and *M. sativa*. The growth suppression of *T. aestivum*, *P. tanacetifolia*, and *B. napus* was less pronounced in wild-type plants compared to *bx1* mutant plants (**Fig. 1a**). A similar pattern was observed for plant height (**Fig. S2b**). Analysing soil benzoxazinoid concentrations and their degradation products in the soil after harvest confirmed that wild-type plants produced significantly more benzoxazinoids than *bx1* mutant plants (**Fig. 1b**). Thus, benzoxazinoid production in maize can convey resistance against negative plant-soil feedbacks.

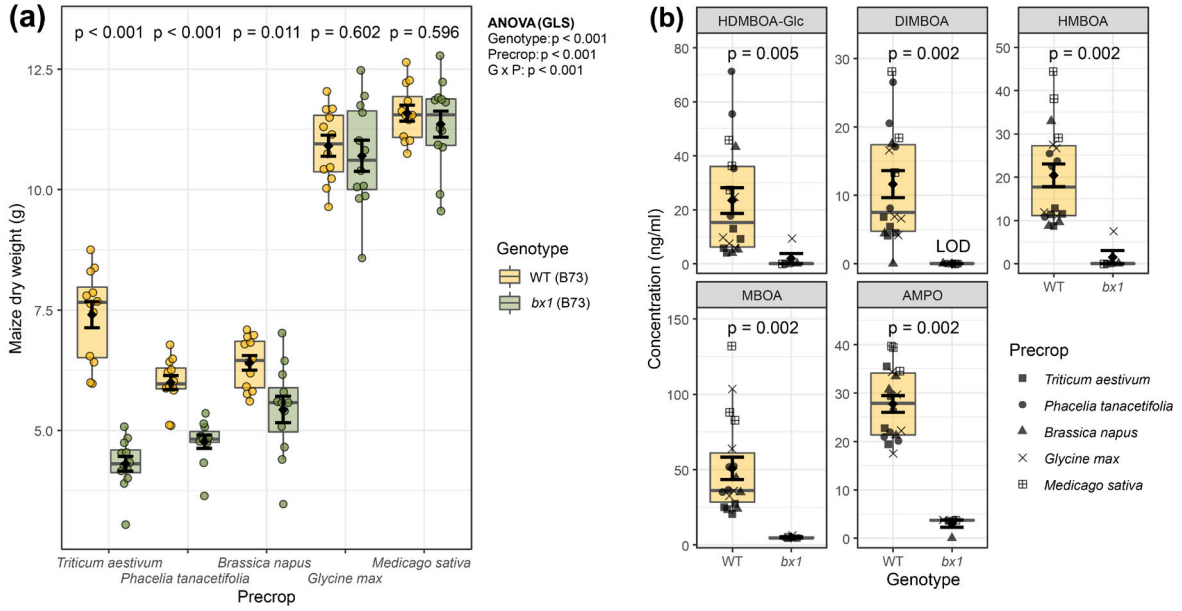

Fig. 1. **Benzoxazinoid production is associated with resistance to negative plant-soil feedbacks**. (a) Dry weight of wild-type or benzoxazinoid-deficient bx1 mutant maize grown in soils conditioned by five precrop species. Means ± SE, boxplots, and individual datapoints are shown (n = 11-12). ANOVA table and pairwise comparisons of estimated marginal means within each precrop (FDR-corrected p values) are provided. (b) Soil benzoxazinoid concentration after maize growth of wild-type (WT) or benzoxazinoid-deficient bx1 mutant plants indicated in ng per mL of soil. Means ± SE, boxplots, and individual datapoints are shown (WT: n = 18, bx1: n = 5). Symbols indicate the precrop species that conditioned the soil before maize growth. LOD: below limit of detection. GLS: generalized least squares (linear model). 'G x P': interaction between genotype and precrop.

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) and grew wild-type and mutant plants in two different soils (Changins and Q-Matte). We also included B73 plants grown in Changins soil as a positive control. Two plant species with different feedback effects, *T. aestivum* and *M. sativa*, were used to condition the soils (**Fig. S3a**). Three weeks after sowing, the height of wild-type plants of both genetic backgrounds was increased compared to *bx1* mutants on *T. aestivum* conditioned Changins soil. Genotypes grew similarly on *M. sativa* conditioned Changins soil, thus confirming our earlier results that benzoxazinoids increase resistance to negative plant-soil feedbacks (**Fig. 2a**). In contrast to the Changins soil, no difference between wild-type and *bx1* mutants in the W22 background was found in Q-Matte soil, illustrating that the effect depends on soil type (**Fig. 2a**).

Six weeks after sowing, the differences in height between wild-type and mutant plants were less pronounced, and even reversed in the B73 background in the Changins soil (**Fig. S3b**). Dry weight patterns for W22 were as expected from the early height data, with the *bx1* mutant accumulating less biomass than the wild-type in *T. aestivum* conditioned Changins soil, and no difference in *M. sativa* conditioned Changins 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 soil from Changins (**Fig. S1, Fig. S4**). First, we conducted 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 days of growth, wild-type plants grew significantly taller than *bx1* mutant plants in *T. aestivum* conditioned soil. The effect was most pronounced 27 days after sowing. Chlorophyll contents and dry weight were increased in wild-type compared to *bx1* mutant plants at day 27 (**Fig. 3b/c**). Thus, feedback effects appear two weeks after sowing maize and are clearly visible 4 weeks after sowing. Based on these results, we set the feedback phase to 4 weeks in all further experiments.

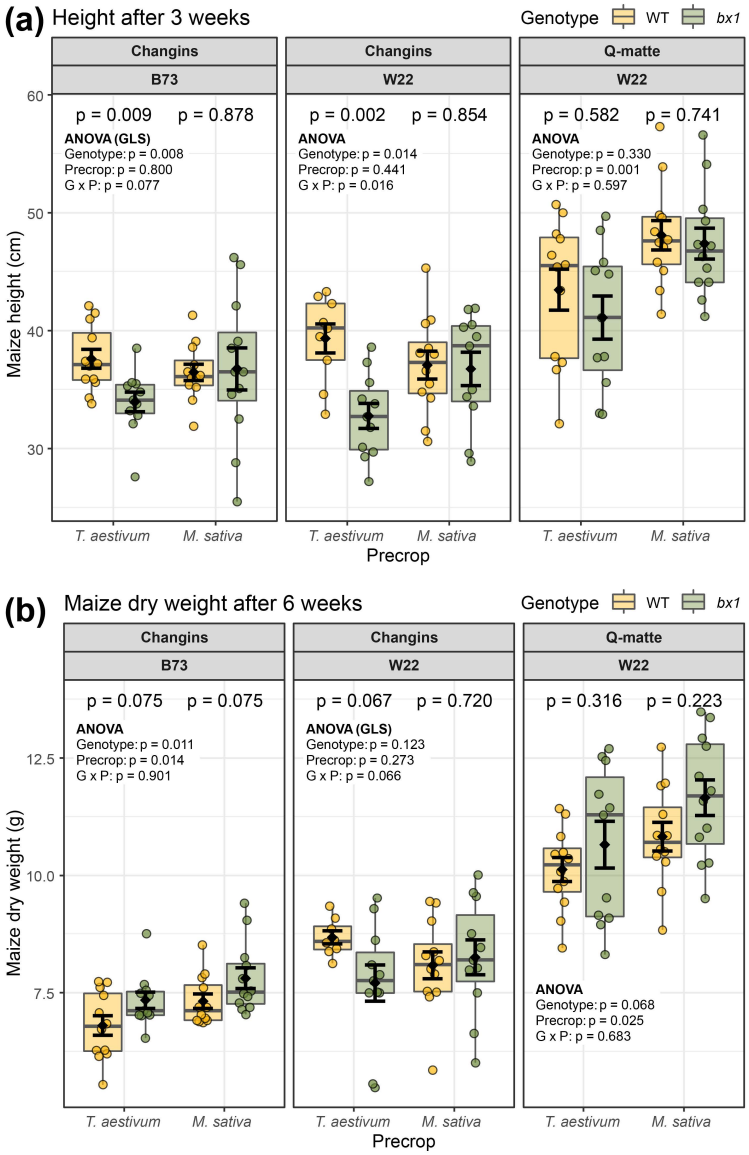

Fig. 2. **Benzoxazinoid-dependent resistance to negative plant-soil feedbacks is soil-specific and transient**. **(a)** Height after 3 weeks of growth and **(b)** dry weight at harvest of wild-type or benzoxazinoid-deficient *bx1* mutant plants of the maize lines B73 or W22 growing in different soils (Changins, Q-matte) that were previously conditioned by *Triticum aestivum* or *Medicago sativa*. Means ± 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. GLS: generalized least squares (linear model). 'G x P': interaction between genotype and precrop.

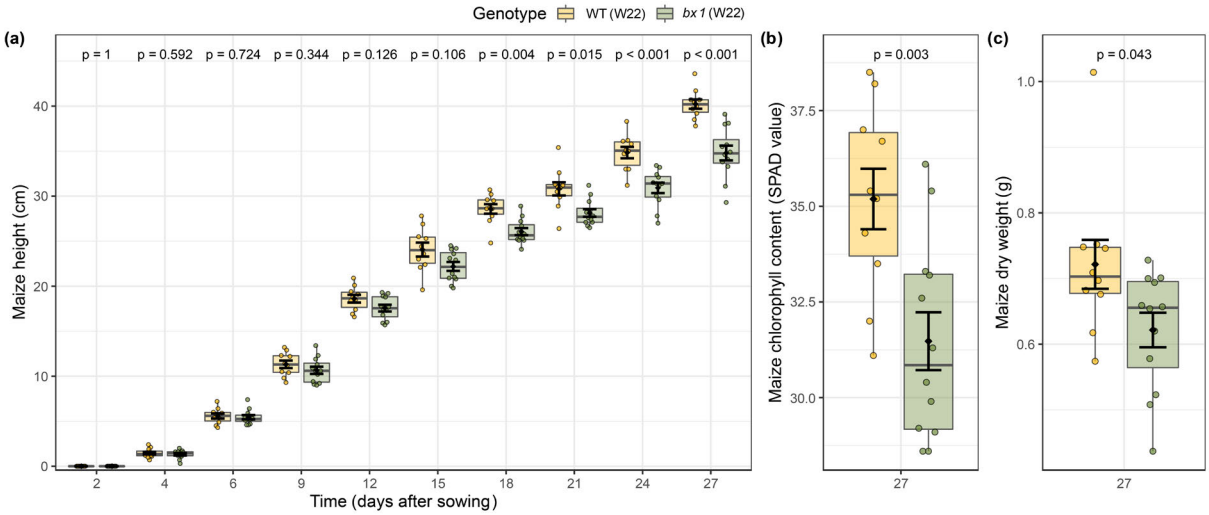

Fig. 3. **Benzoxazinoid-dependent resistance to negative plant-soil feedbacks appears in early seedling growth**. **(a)** Time series of plant height, **(b)** end point chlorophyll content, and **(c)** dry weight at harvest of wild-type or benzoxazinoid-deficient *bx1* mutant plants grown in soils that were conditioned by *Triticum aestivum*. Means ± 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).**

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

To test if benzoxazinoids increase resistance by acting in the rhizosphere, we complemented the soil of *bx1* mutant plants with a benzoxazinoid mixture typical for young maize seedlings (**Fig. S5a/b**). We then compared their performance to wild-type and *bx1* mutants grown in *T. aestivum* conditioned soil without benzoxazinoid supplementation. Three different complementation concentrations were applied in three consecutive experiments.

There was considerable variation in the observed phenotypic effects, with at least one out of three measured plant performance parameters being enhanced in wild-type over *bx1* mutant plants (**Fig. 4**). In each case, benzoxazinoid supplementation partially rescued the lower resistance of *bx1* mutants. The clearest effect was observed for chlorophyll contents (**Fig. 4**). Interestingly, even though the concentrations applied 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 partially be explained by benzoxazinoids in the rhizosphere.

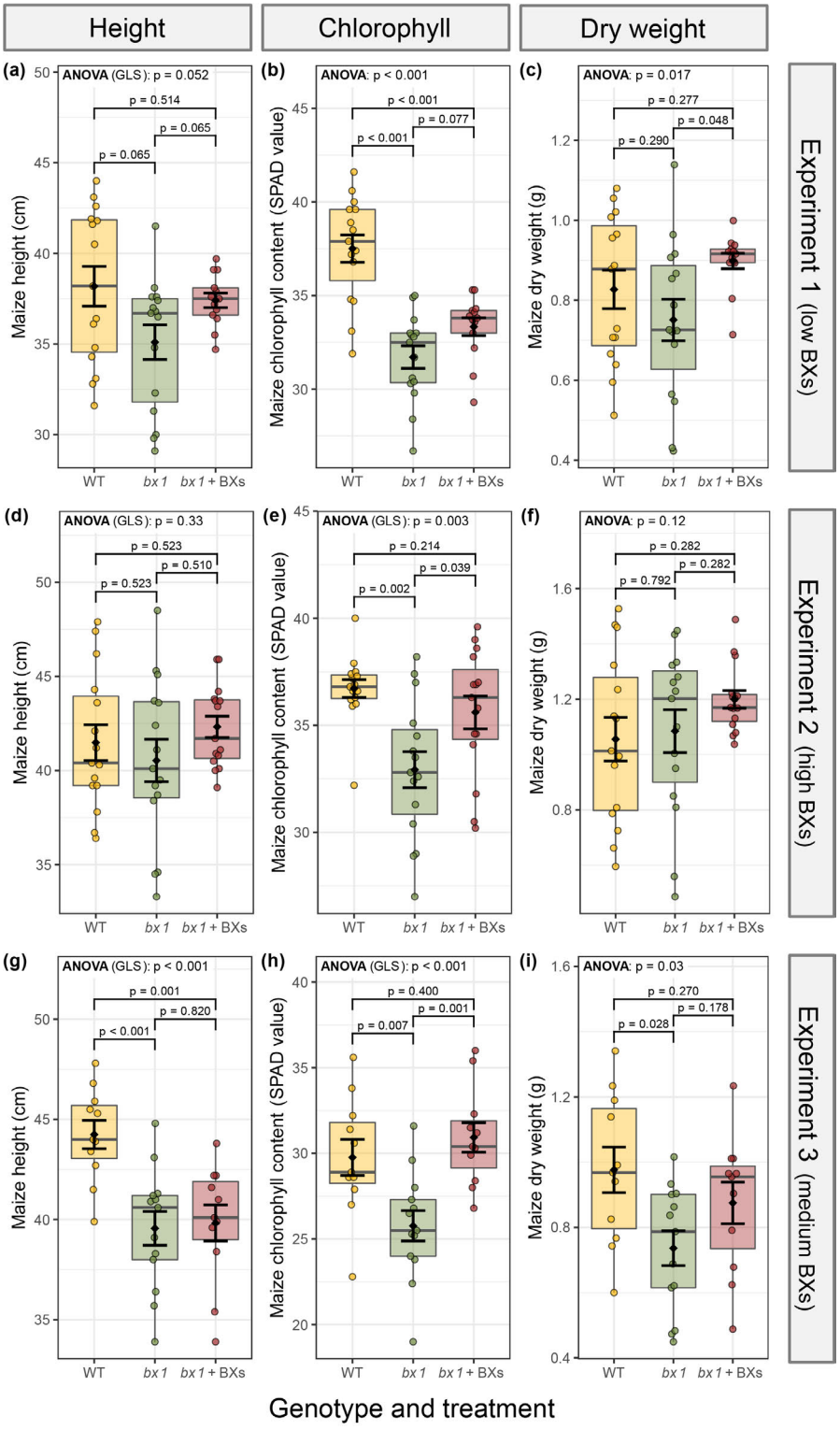

Fig. 4. **Benzoxazinoid-dependent resistance to negative plant-soil feedbacks is partially associated with benzoxazinoids in the rhizosphere**. For all three replications of this experiment height, chlorophyll content, and dry weight of wild-type, benzoxazinoid-deficient *bx1* mutant plants, or *bx1* plants complemented with benzoxazinoids grown in soils that were previously conditioned by *Triticum aestivum*. Means ± 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, 2 and 3 were complemented with low, high, and medium amounts of benzoxazinoids. Experiment 1: n = 13-15, experiment 2: n = 15, experiment 3: n= 11-13. GLS: generalized least squares (linear model). 'G x P': interaction between genotype and precrop. BXs: benzoxazinoids.

Benzoxazinoid-dependent resistance to negative plant-soil feedbacks is partially explained by soil microbiota

To investigate the role of soil microbiota in benzoxazinoid-mediated resistance against negative plant-soil feedbacks, we X-ray sterilized part of the conditioned soil and grew wild-type and *bx1* mutant plants in the soils. A microbial re-inoculation control was included to control for changes in soil chemical and physical properties that may result from the sterilization treatment (Berns *et al.*, 2008). Again, wild-type plants outperformed *bx1* mutants across experiments in one or several performance parameters (**Fig. 5**). All resistance effects were lost in sterilized soil. Re-inoculation restored all resistance effects in experiment 2. In experiments 1 and 3, only tendencies for restored resistance effects were found in re-inoculated soil. Thus, there is clear evidence that the resistance is mediated by elements that are labile to sterilization. At least in some cases, soil biota can account for these labile elements, as re-inoculation 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, wild-type and *bx1* mutant plants grew similarly well, as observed before (**Fig. 6**). In sterilized soils, the *bx1* mutant outperformed wild-type plant growth. This effect disappeared when the soil was re-inoculated with *M. sativa* soil (**Fig. 6**). When the soil was inoculated with *T. aestivum* biota, wild-type plants outperformed *bx1* mutant plants. This reciprocal transplant experiment demonstrates that the negative effects of *T. aestivum* soil biota can be overcome by benzoxazinoids.

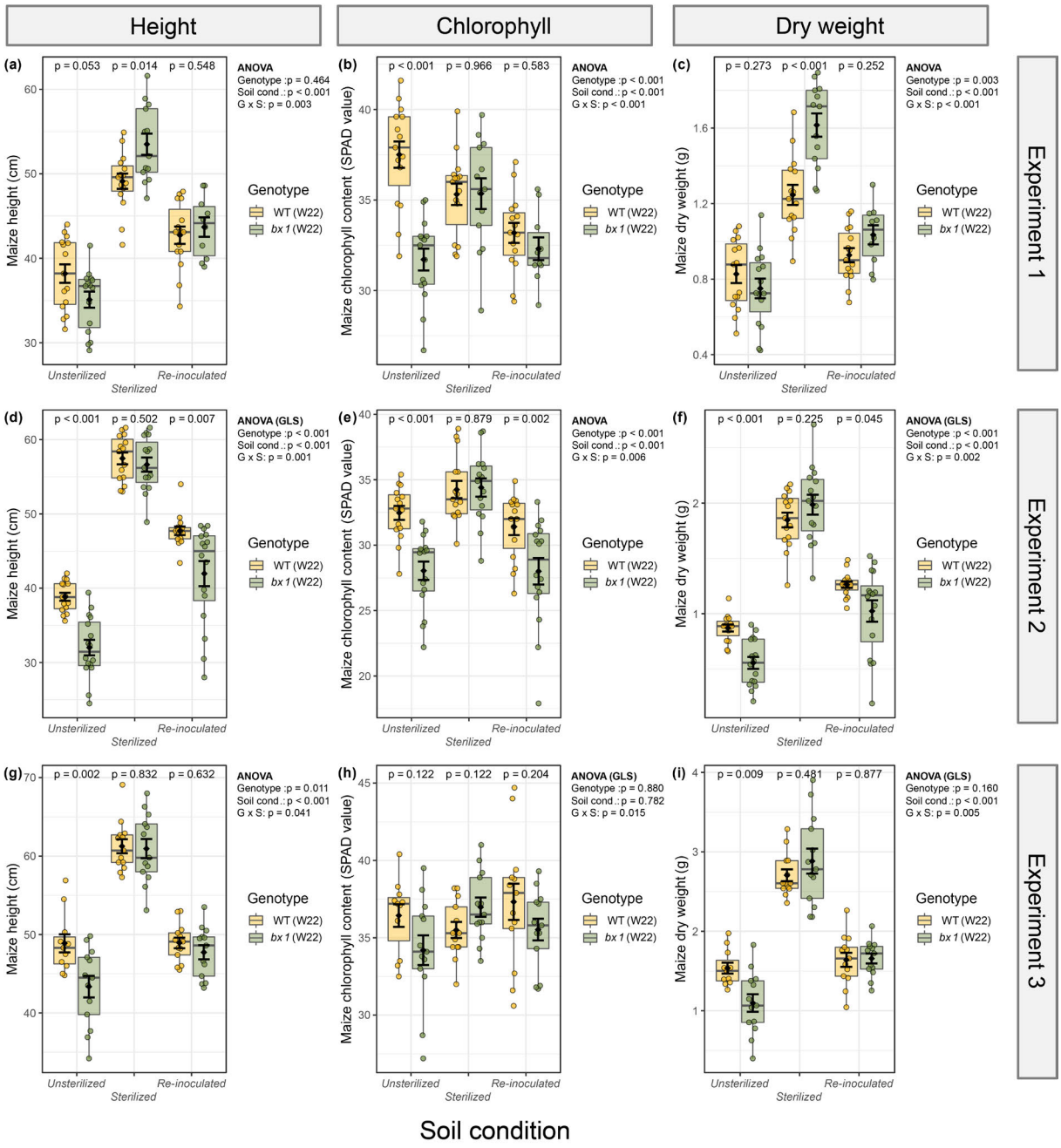

Fig. 5. **Benzoxazinoid-dependent resistance to negative plant-soil feedbacks can depend on soil biota**. For all three replications of this experiment height, chlorophyll content, and dry weight of wild-type or benzoxazinoid-deficient *bx1* mutant plants grown in *Triticum aestivum* conditioned soil that was either unsterilized, sterilized, or sterilized and re-inoculated with unsterilized soil. Means ± 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: n = 10-15, Experiment 2: n = 15-16, Experiment 3: n= 11-13. GLS: generalized least squares (linear model). 'G x S': interaction between genotype and soil condition.

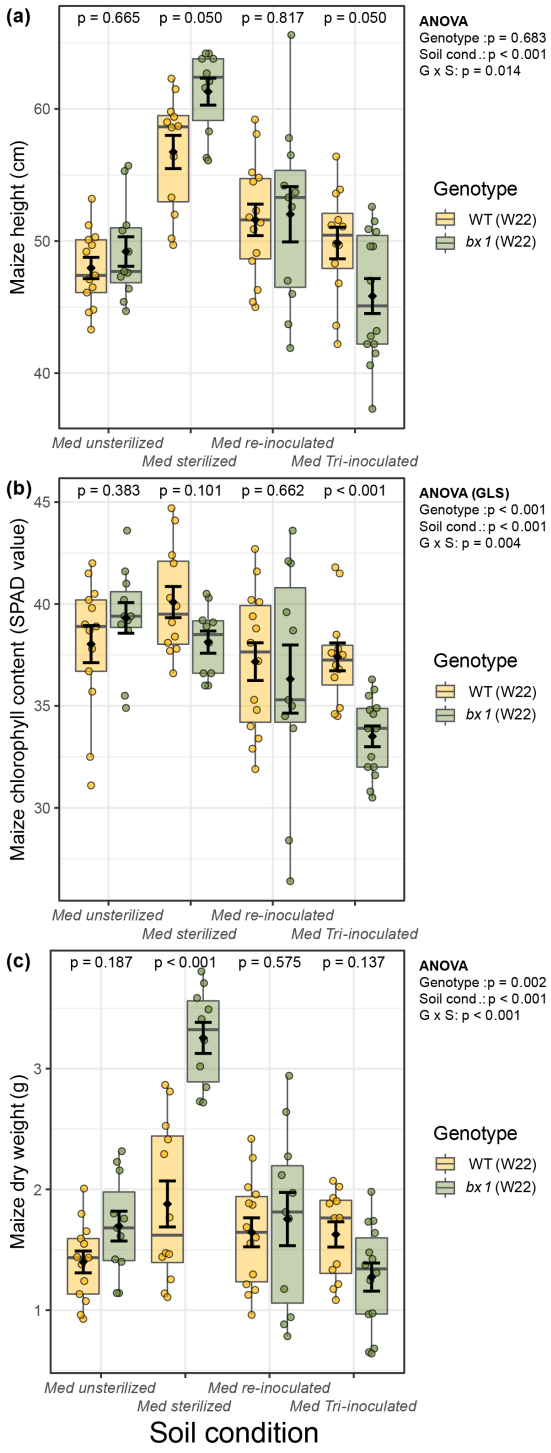

Fig. 6. **Benzoxazinoid-dependent resistance to negative plant-soil feedbacks depends on precrop-specific soil biota. (a)** Height, **(c)** chlorophyll content, and **(c)** dry weight of wild-type or benzoxazinoid-deficient *bx1* mutant plants grown in *Medicago sativa* conditioned soil that was either unsterilized, sterilized, sterilized and re-inoculated with unsterilized *Medicago sativa* soil (Med-inoculated), or sterilized and re-inoculated with unsterilized *Triticum aestivum* soil (Tri-inoculated). Means ± SE, boxplots, and individual datapoints are shown (n = 10-14). ANOVA table and pairwise comparisons of estimated marginal means between all three treatments (FDR-corrected *p* values) are provided. GLS: generalized least squares (linear model). 'G x S': interaction between genotype and soil condition.

Discussion

Plant-soil feedbacks have a major impact on the performance of plants and their successor plants. How plants resist negative feedback effects is not well known. In this study we demonstrate that benzoxazinoids can help plants to cope with negative plant-soil feedbacks. This effect is, at least partially, mediated by the interaction between benzoxazinoids and microbiota in the soil. Substantial spatial, temporal, and stochastic variation in these patterns is observed. 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 (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 excretion into the rhizosphere can mitigate negative plant-soil feedbacks. This effect was found in two maize lines and was most pronounced for early performance; an important trait in crop cultivation (Ellis, 1992; Steege *et al.*, 2005; Shi *et al.*, 2020). Benzoxazinoid 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 the mitigation of adverse impacts of soil born plant pathogens, which are known to be capable of massively reducing seedling performance (Packer & Clay, 2000). Indeed, sterilization resulted in the disappearance of the negative effect and the capacity of benzoxazinoids to improve plant performance, and (re)-inoculation with soil biota partially re-established the 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.

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 known to depend on the growth environment (Schittko *et al.*, 2016), soil origin, above-ground herbivores, soil microbes, as well as temperature and soil moisture (Long *et al.*, 2019; Cadot *et al.*, 2021a). Small variations in abiotic and biotic parameters may have contributed to the variation within and between experiments that we observed 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 towards negative plant-soil feedbacks is relevant for plant performance. Nevertheless, the benefits of benzoxazinoid exudation is likely to depend on the soil environment (van der Putten *et al.*, 2013; Smith-Ramesh & Reynolds, 2017; Cadot *et al.*, 2021a). 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). Experiments with additional soils will show how widespread negative plant-soil feedbacks are and how important benzoxazinoid-mediated resistance is in an agroecological context. Such experiments could also help to narrow down the microbes that drive the observed patterns.

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 is largely unexplored. In our work, we find that one single group of secondary metabolites controlled by one single gene (*Bx1*) 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.*, 2022b), 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 stochastic patterns make our system an excellent tool to unravel why plant-soil feedbacks tend to be conditional and thereby make their application more predictable. The use of agroecological plant-soil feedbacks has been proposed as a possible way towards 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).

Acknowledgement

We thank Florian Enz, Sophie Gulliver, and Pascal Wyss for their assistance with the growth and maintenance of plants. Further, we are grateful to Pierre Mateo for helpful advice on benzoxazinoid purification and analytics. This work was supported by the Swiss National Science Foundation (Grant. Nr. 192564) and the Interfaculty Research Collaboration “One Health” of the University of Bern.

Supplementary information

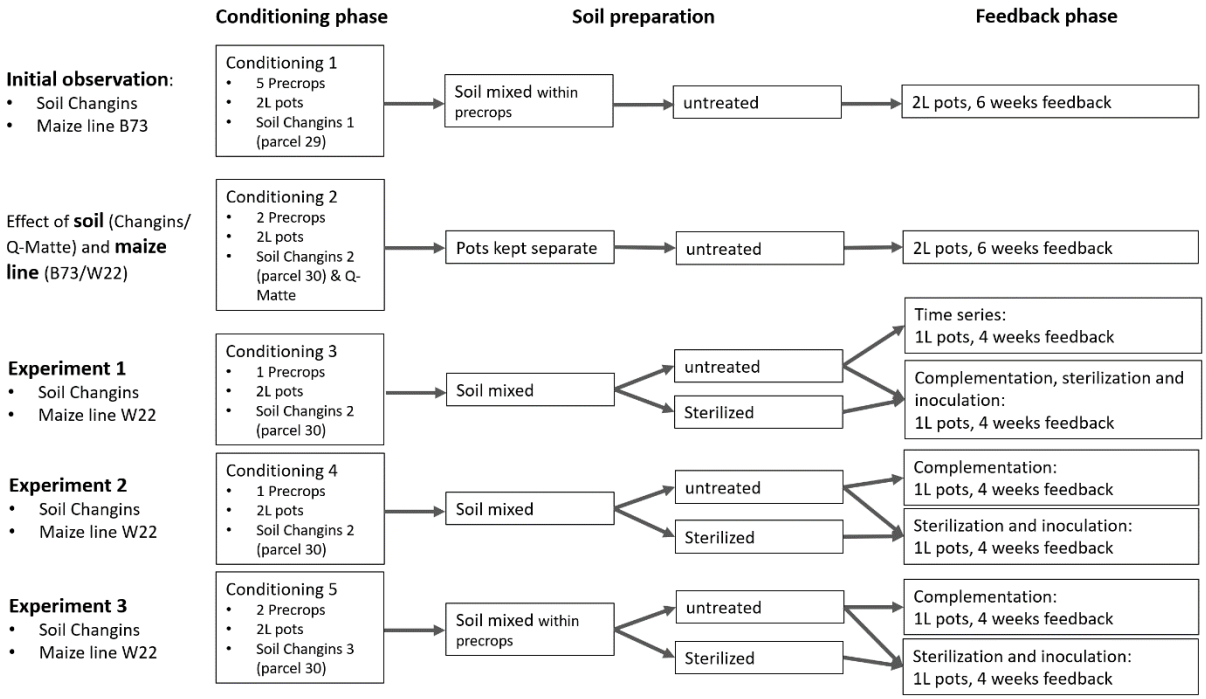

Fig. S1. **Experimental setup**. All experiments are listed and specification concerning the conditioning and the feedback phase are indicated. Further, soil preparation between conditioning and feedback is shown.

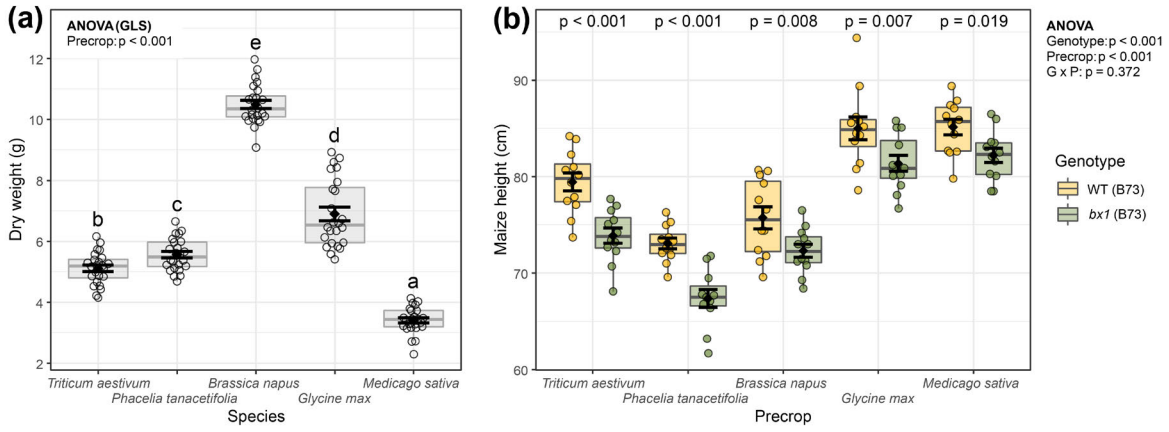

Fig. S2. **Precrop weight and maize height of initial precrop screening experiment**. **(a)** Dry weight of precrops grown to condition the soil for the initial experiment and **(b)** height of wild-type or benzoxazinoid-deficient *bx1* mutant maize grown in soils conditioned by five precrop species. **(a)** Means ± SE, boxplots, and individual datapoints are shown (n = 24). ANOVA table and compact letter display of all pair-wise comparisons (Significance-level: FDR-corrected $p < 0.05$) of estimated marginal means are provided. **(b)** Means ± 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. 'G x P': interaction between genotype and precrop.

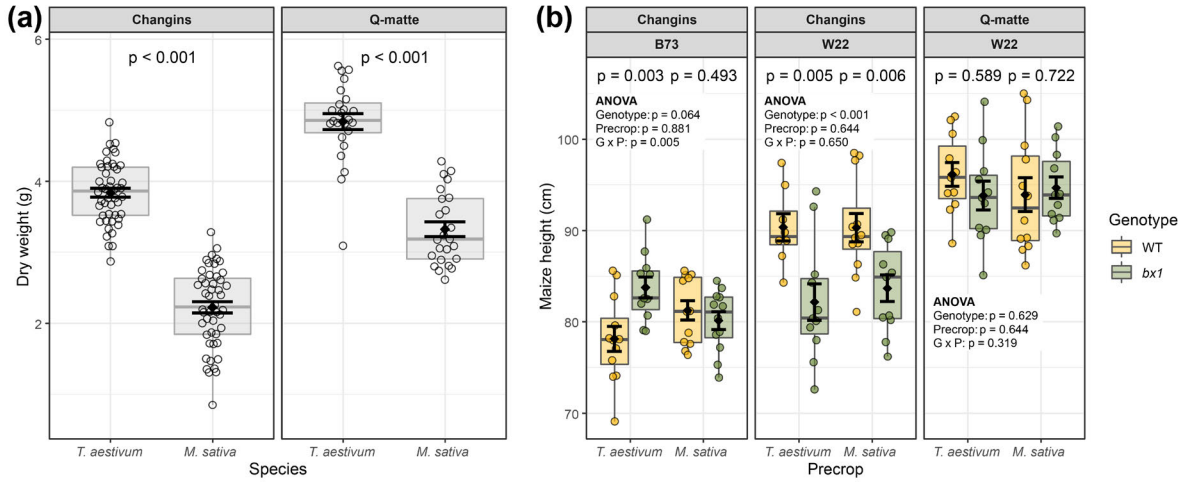

Fig. S3. **Precrop weight and maize height of experiment comparing different soil origins and maize lines**. **(a)** Dry weight of precrop species grown in two different soils (Changins and Q-matte) and **(b)** height at harvest of wild-type or benzoxazinoid-deficient *bx1* mutant plants of the maize lines B73 or W22 growing in different soils that were conditioned by *Triticum aestivum* or *Medicago sativa*. **(a)** Means ± SE, boxplots, and individual datapoints are shown (Changings: n = 48, Q-matte: n= 24). Statistical significance is indicated as *p* values computed by Welch's two-sample *t*-test. **(b)** Means ± 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. GLS: generalized least squares (linear model). 'G x P': interaction between genotype and precrop.

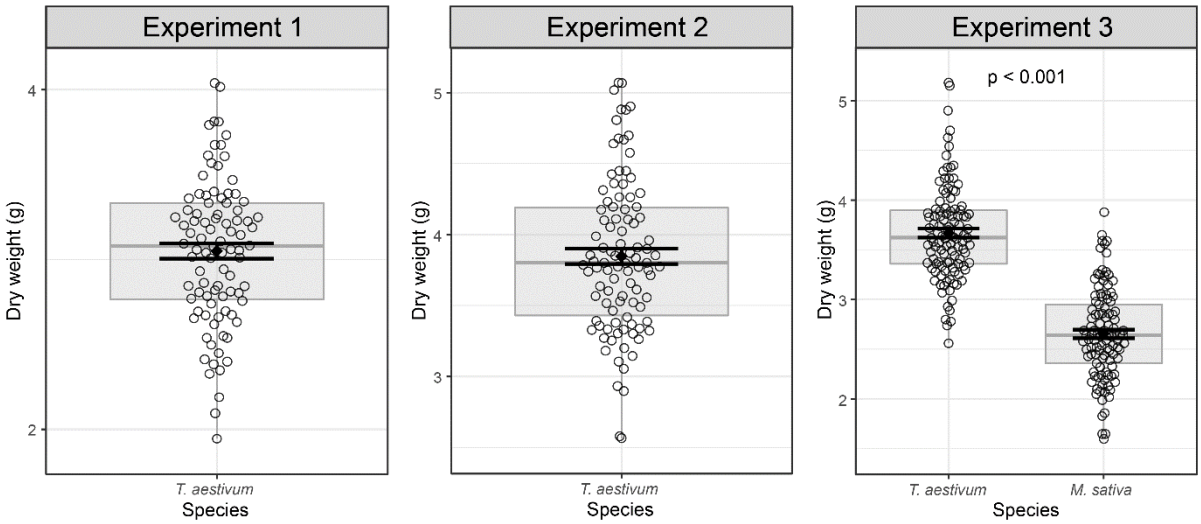

Fig. S4. **Dry weight of precrop species grown to condition the soil for replicated experiments 1-3**. Means ± SE, boxplots, and individual datapoints are shown. For experiment 3 statistical significance is indicated as *p* values computed by Welch's two-sample *t*-test. Experiment 1: *T. aestivum* n = 92; experiment 2: *T. aestivum* n = 98; experiment 3: *T. aestivum* n = 110, *M. sativa* = 109.

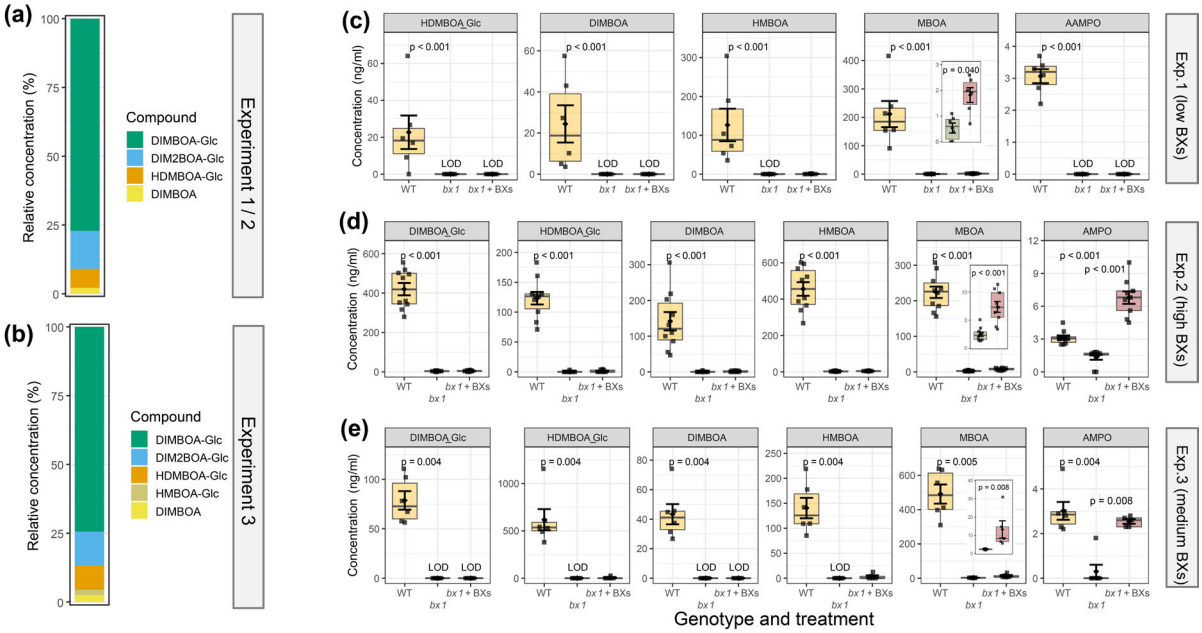

Fig. S5. **Characterization of benzoxazinoid complementation**. **(a, b)** relative abundance of single benzoxazinoids in mixture purified from 4-days germinated maize kernels and applied for complementation. **(c-e)** Soil benzoxazinoid concentration after maize growth of wild-type, benzoxazinoid-deficient *bx1* mutant plants, or *bx1* plants complemented with benzoxazinoids indicated in ng per mL of soil. Means ± SE, boxplots, and individual datapoints are shown. Provided *p* values for the comparison between wild-type and *bx1* plants were computed by Wilcoxon rank-sum tests and corrected for multiple testing (FDR). If significant, p values for the comparison between *bx1* plants and complement *bx1* pants are also provided. Experiment 1, 2 and 3 were complemented with low, high, and medium amounts of benzoxazinoids. Experiment 1: n = 6, experiment 2: n = 9-10, experiment 3: n= 5-6. LOD: below limit of detection. BXs: benzoxazinoids.

General discussion

In this thesis, I explored the role of root exudate metabolites on agricultural plant-soil feedbacks. In two field experiments, I found that maize benzoxazinoids can increase wheat performance through plant-soil feedbacks, and that these effects depend on soil parameters. Further experiments under controlled conditions revealed that the trait of benzoxazinoid production in maize increases crop rotation stability through the mitigation of negative plant-soil feedbacks. Taken together, these results present a proof-of-concept that secondary metabolite-mediated plant-soil feedbacks can be applied as effective agroecological practice under certain environmental conditions. Below, I will discuss how these results expand our understanding of plant secondary metabolites in agroecosystems, their potential to improve crop rotations in a sustainable way, and possible challenges in their implementation.

Secondary metabolites in agroecosystems

Secondary metabolites are crucial for plants to survive in hostile environments. They play an important role in defence against insect herbivores and plant pathogens (War *et al.*, 2012), and could therefore be harnessed to increase crop defence and productivity as an alternative strategy to agrochemical inputs. While some secondary metabolites reduce the performance of insects or pathogens directly, others defend the plant though tritrophic interactions by attracting the natural enemies of plant attackers (Heil, 2008). For example, leaf emitted volatile organic compounds can attract parasitoids of leaf feeding insects, or root emitted volatile compounds can attract entomopathogenic nematodes that in turn reduce the performance of root feeding insects (Turlings & Erb, 2018). Many secondary metabolites show multiple functions depending on plant organ, age, and environmental context (Erb & Kliebenstein, 2020). A class of secondary metabolites that exhibits a high multifunctionality are the benzoxazinoids (Niemeyer, 2009; Zhou *et al.*, 2018). Their bioactivity against insect herbivores, fungal pathogens, and competing weed species are well studied (Niemeyer, 2009). More recently, they have been shown to interact with individual soil microbes and modify entire rhizosphere microbiomes (Neal *et al.*, 2012; Cadot *et al.*, 2021b). Under controlled conditions benzoxazinoid-dependent changes in the soil microbiome have been shown to affect the performance of the following conspecific crops though plant-soil feedbacks (Hu *et al.*, 2018b). In this thesis, I expand this knowledge by showing that benzoxazinoid-dependent plant-soil feedbacks affect crop productivity under agronomically relevant conditions (**Chapter I**, **Fig. 1A**). Further, I show that benzoxazinoids confer resistance to growth suppressing plant-soil

feedbacks caused by preceding crop (**Chapter III**). This is of high relevance as this shows that one class of secondary metabolites can both improve the resistance to soil cultivation legacies and increase the performance of the succeeding crops This expands the multifunctionality of benzoxazinoids by two additional layers of bioactivity. The same group of secondary metabolites could therefore be applied to tackle multiple agricultural challenges. To make use of this, the effects of metabolite quantity and quality (composition) on overall plant-environment interactions must be further investigated. A better and more comprehensive understanding of all these roles of secondary metabolites will clarify their full potential in sustainable agriculture.

Crop selection for superior crop rotations

Translating the concept of plant-soil feedback in crop rotation design, in order to increase crop resistance and productivity, has been proposed as a promising tool for sustainable agriculture (Dias *et al.*, 2015; Mariotte *et al.*, 2018). To achieve this, a suitable precrop identity has to be selected to create a positive plant-soil feedback, and/or a suitable response plant identity has to be selected to improving the feedback to a given soil conditioning. Given that the outcome of plant-soil feedbacks can be species- and variety-specific, it is important to find the right crop species or crop cultivar for a given crop rotation sequence (Wagg *et al.*, 2015; Hu *et al.*, 2018b; Pineda *et al.*, 2020; Cadot *et al.*, 2021a). In **chapter III**, I show that benzoxazinoids can alleviate species-specific negative plant-soil feedbacks on maize, indicating that not only specific crop cultivars, but the activity of one single gene responsible to produce a class of secondary metabolites is sufficient to change the response to a given soil legacy. Results from **chapter I** and **chapter II** further indicate that the soil conditioning undertaken with a particular group of secondary metabolites can affect agroecological plant-soil feedbacks under field conditions. Taken together, the results of this thesis show that traits related to root secondary metabolites are promising candidates to engineer crop rotations. To evaluate the potential of benzoxazinoids in different crop rotations, more research on various response plants is now needed. The observed diversity of root exudates in the plant kingdom represents a promising reservoir for additional candidate compounds to improve crop rotations (Baetz & Martinoia, 2014) and testing their effects on crop rotations will shed light on how common this phenomenon is. While the need for improving crop rotation sequences through evidence-based crop selection has been suggested before (Dias *et al.*, 2015; Koyama *et al.*, 2022), our results call for the evaluation of individual varieties and individual crop traits in crop rotation design.

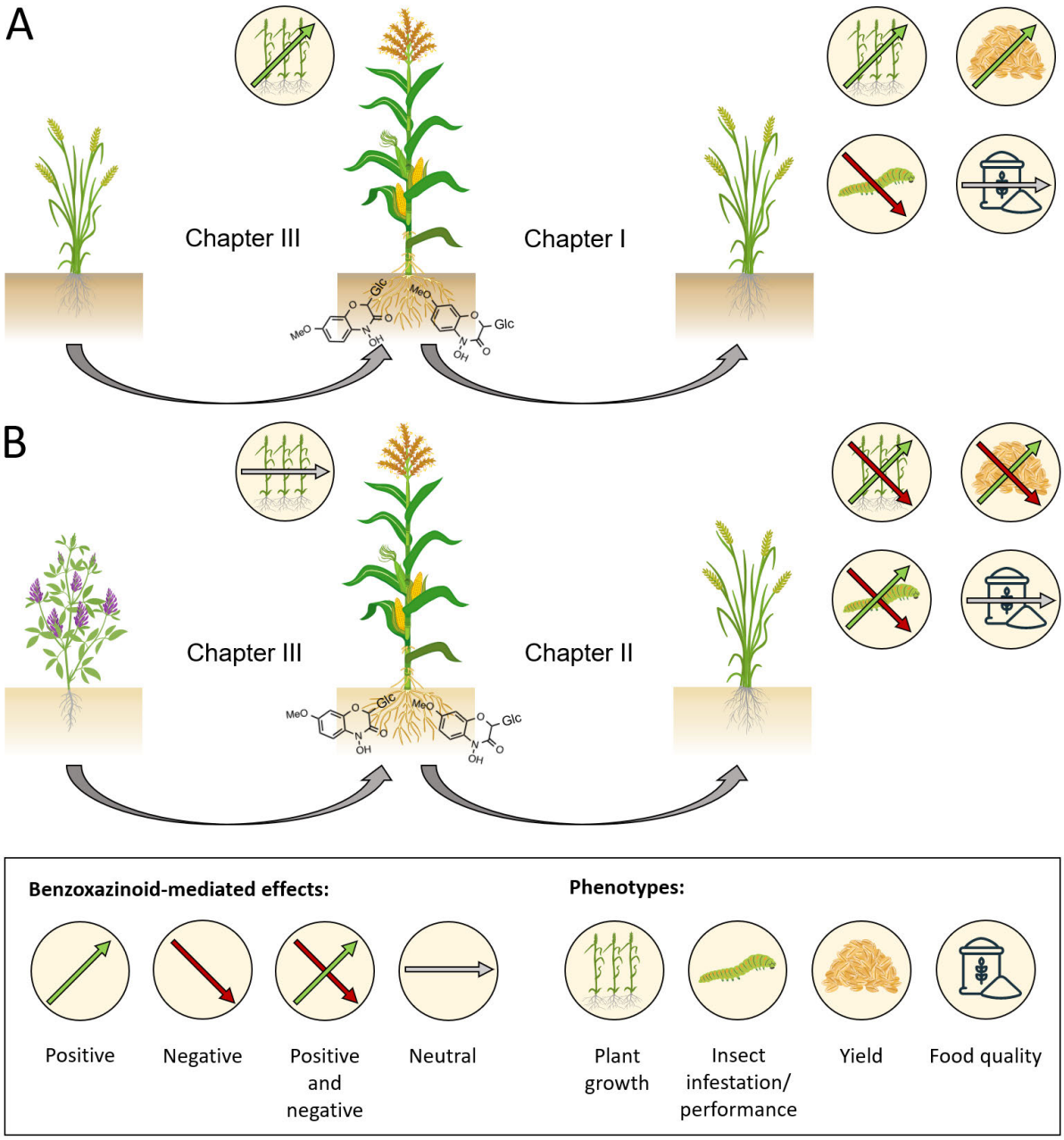

Fig. 1. The role of benzoxazinoids in agroecological plant-soil feedbacks. (A) In this thesis I demonstrate that benzoxazinoid exudation can reduce negative plant-soil feedbacks of preceding crops (**Chapter III**), and that benzoxazinoid-dependent plant-soil feedbacks on wheat can increase growth, defence, and yield without reducing food quality and thereby boost agricultural productivity (**Chapter I**). **(B)** These benzoxazinoid-dependent effects are highly context-dependent: The positive effect of benzoxazinoids on resistance to growth suppressive soils depends on soil origin and precrop species (**Chapter III**), and the strength and direction of benzoxazinoid-dependent plant-soil feedbacks can change within one field depending on local soil parameters (**Chapter II**). Next, investigations of these context-dependencies are necessary to assess the full potential of secondary metabolite-dependent plant-soil feedbacks to sustainably enhance productivity in crop rotations. Pictures modified from AdobeStock.

Shaping plant-soil feedbacks

Plant-soil feedbacks affect plant performance though different mechanisms. Soil conditioning can modify nutrient availability, soil community composition of mutualistic and pathogenic soil biota, and the chemical properties of the soils (Bennett & Klironomos, 2019; Schandry & Becker, 2020), where the focus on microbiome-mediated plant-soil feedbacks has increased in recent years (Bever *et al.*, 2012). For example, feedbacks of different precrops on chrysanthemum leaf defence have been attributed to soil microbiomes (Pineda *et al.*, 2020) and microbial legacies have been shown to persist for months during the feedback phase (Hannula *et al.*, 2021). Plants can shape their root-associated microbiomes through the exudation of secondary metabolites (Pang *et al.*, 2021). For example, flavones, coumarins, triterpenes, and benzoxazinoids have all been shown to modulate rhizosphere microbial communities (Hu *et al.*, 2018b; Stringlis *et al.*, 2018; Huang *et al.*, 2019; Voges *et al.*, 2019; Yu *et al.*, 2021), and for benzoxazinoids and flavones such changes in the microbial communities have been demonstrated to drive plant-soil feedbacks on the next conspecific plant (Hu *et al.*, 2018b; Yu *et al.*, 2021). In **chapter I** and **chapter II** benzoxazinoid-dependent plant-soil feedbacks were found under field conditions, and in line with previous studies these effects coincided with changes in the rhizosphere microbial community at the end of the conditioning phase (Hu *et al.*, 2018b; Cotton *et al.*, 2019; Kudjordjie *et al.*, 2019; Cadot *et al.*, 2021b). In both field experiments differences in the microbial composition vanished at the onset of wheat cultivation. In contrast, the benzoxazinoid chemical fingerprints were still present after several months of wheat growth, showing that microbial fingerprints were transient and chemical fingerprints are more long-lasting. Nevertheless, because plant-soil feedback effects were generally strongest during germination and early growth, changes in microbial community are still a likely cause of the observed effects. In **chapter III**, I demonstrate that soil microbes are, at least partially, responsible for negative plant-soil feedback effects. This growth suppression was mitigated through benzoxazinoids, which are known to suppress soil pathogens (Wilkes *et al.*, 1999; Martyniuk *et al.*, 2006). Integrating the results of this thesis into the current literature makes benzoxazinoids a promising class of secondary metabolites to tolerate and shape soil microbial communities and thereby potentially enhance the performance of the focal and the following crop. Harnessing microbiomes has been proposed as one of the most promising ways towards a more sustainable agriculture (Singh *et al.*, 2020). Until recently, microbiologists largely focused on individual beneficial or pathogenic microbes when studying plant-microbe interactions, but there is growing evidence for the importance to incorporate the entire microbiomes (Ray *et al.*, 2020; Trivedi *et al.*, 2020). One way to steer the microbiome is through

soil conditioning by plants (Pineda *et al.*, 2020). Selecting the right plants or varieties in crop rotations could increase crop performance through microbiome-mediated effects (Benitez *et al.*, 2021). Future breeding efforts to optimize plant-microbiome interactions in crop rotations may consider root exudates as important plant traits.

Context-dependency of plant-soil feedbacks

Plant-soil feedbacks can be highly context-dependent (van der Putten *et al.*, 2013; Smith-Ramesh & Reynolds, 2017). Many factors, including growth environment, biotic and abiotic soil properties, temperature, and above-ground herbivory have been associated with different outcomes of plant-soil feedbacks (Kos *et al.*, 2015a; Schittko *et al.*, 2016; Long *et al.*, 2019; Cadot *et al.*, 2021a), but a large fraction of context-dependency in plant-soil feedbacks is still not well understood (Smith-Ramesh & Reynolds, 2017). A recent greenhouse study on several crop species demonstrated that water and nutrient availability can influence the outcome of microbial plant-soil feedbacks (Kuerban *et al.*, 2022). Soil chemistry and water availability are also known to strongly influence the composition of microbial communities (Fierer, 2017), which can in turn affect the plant-soil feedback outcome (Bever *et al.*, 2012). In **chapter II**, rhizosphere microbial communities were shown to be strongly associated with soil chemistry and covaried with benzoxazinoid degradation, as well as the strength and direction of plant-soil feedback effects (**Fig. 1B**). It is striking that plant-soil feedbacks on growth, defence, and kernel quality related traits change in direction within one field. In **chapter III**, we observed that benzoxazinoid exudation promotes plant growth in one soil, while no effect was observed in another soil (**Fig. 1B**). Further, we observed variation within and between experiments. The variation between soils as well as experiments could both be explained by small differences in the microbial communities at the onset of the experiments (Wei *et al.*, 2019). Recently, soil chemistry dependent effects of benzoxazinoids on aboveground insect resistance were shown (Hu *et al.*, 2021), therefore direct interactions between benzoxazinoids and soil chemistry cannot be excluded. Future experiments will reveal the source of the observed context-dependency. Besides soil manipulation experiments (e.g. sterilization and re-inoculation), other soil biota such as nematodes and protists should also be analysed in parallel to fungi and bacteria, as they could contribute to soil biota-mediated effects directly or indirectly through the modification of members of another kingdom (Durán *et al.*, 2018; Gao *et al.*, 2019; Sikder *et al.*, 2021; Wilschut & Geisen, 2021). A better understanding of the context-dependency of agroecological plant-soil feedbacks will provide much needed insights to promote plant-soil feedbacks for sustainable agriculture.

Conclusion

This thesis establishes root exudate metabolites as tools to enhance agricultural productivity. In two field experiments, I demonstrated that root exudate-mediated plant-soil feedbacks affect plant performance and yield, and that these effects depend on soil parameters (**Fig. 1**). Comprehensive experiments under controlled conditions allowed us to further show that root exudate metabolites increase crop rotation stability by resisting growth suppression triggered by some preceding crops. These findings pave the way to optimize crop rotations through plant secondary metabolites. In a next step, more classes of secondary metabolites and their interactions should be tested to determine how widespread this phenomenon is. Further field experiments across a range of soils and under different farming regimes will show if exudate-dependent plant-soil feedbacks can be implemented in agroecosystems, to securely feed the growing population on planet earth while maintaining healthy farmlands.

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