PNAS RESEARCH ARTICLE | AGRICULTURAL SCIENCES OPEN ACCESS

Root-exuded specialized metabolites reduce arsenic toxicity in maize

Veronica Caggìa[a,b,1], Jan Wälchli[b], Gabriel Deslandes-Hérold[a], Pierre Mateo[a], Christelle A. M. Robert[a], Hang Guan[c], Moritz Bigalke[c,d], Sandra Spielvogel[e,f], Adrien Mestrot[c], Klaus Schlaeppi[a,b,1], and Matthias Erb[a,1]

Edited by Donald Ort, University of Illinois at Urbana Champaign, Urbana, IL; received October 7, 2023; accepted January 19, 2024

By releasing specialized metabolites, plants modify their environment. Whether and how specialized metabolites protect plants against toxic levels of trace elements is not well understood. We evaluated whether benzoxazinoids, which are released into the soil by major cereals, can confer protection against arsenic toxicity. Benzoxazinoid-producing maize plants performed better in arsenic-contaminated soils than benzoxazinoid-deficient mutants in the greenhouse and the field. Adding benzoxazinoids to the soil restored the protective effect, and the effect persisted to the next crop generation via positive plant–soil feedback. Arsenate levels in the soil and total arsenic levels in the roots were lower in the presence of benzoxazinoids. Thus, the protective effect of benzoxazinoids is likely soil-mediated and includes changes in soil arsenic speciation and root accumulation. We conclude that exuded specialized metabolites can enhance protection against toxic trace elements via soil-mediated processes and may thereby stabilize crop productivity in polluted agroecosystems.

arsenic | secondary metabolites | arsenic tolerance | W22 wild-type | *bx1* mutant

Significance

Toxic levels of arsenic in the soil can substantially reduce crop yields. Here, we reveal a mechanism to reduce plant arsenic accumulation and toxicity. Benzoxazinoids, a dominant class of specialized metabolites (also called secondary metabolites) produced by cereals such as wheat and maize, can reduce arsenic uptake and improve plant performance in the greenhouse and in the field. Furthermore, benzoxazinoid exudation into the soil enhances the performance of subsequent plant generations in soils with high levels of arsenic. This work expands the available genetically encoded resistance strategies to stabilize crop productivity in polluted agroecosystems.

Arsenic is naturally found in soil and groundwater due to natural geogenic origins (1), but also due to contaminations, from anthropogenic activities such as agriculture, mining, or industrial production (2, 3). When crops are grown in arsenic-contaminated soil or irrigated by contaminated water, their growth and yield can be severely reduced, thus leading to major challenges for food production in affected areas (4). Arsenic can also accumulate in edible plant parts and thus pose a risk to human health (5, 6). Developing crops that grow well in contaminated soils and take up little arsenic is thus important (7). Arsenic is also directly harmful to plants. It shares chemical characteristics with phosphate and is, therefore, taken up by phosphate transporters (8–10). High arsenic concentrations in plant tissues can lead to the formation of reactive oxygen species with consequent damage to proteins, nucleic acids, and cell membrane lipids (11). These damages result in cell chlorosis and necrosis and thereby suppress plant growth (12–14). The two dominant inorganic arsenic species in soils are arsenate (As^{V}) and arsenite (As^{III}). Arsenite represents only 1 to 30% of inorganic arsenic in the soil, but it is more toxic than arsenate (15).

Plants have evolved several detoxification mechanisms that allow them to cope with arsenic. For example, maize plants can reduce arsenate to arsenite (14, 16) and can thus transport it out of the plant more efficiently. In addition, it has recently been proposed that plants can tolerate arsenic contamination in soil by enhancing the exudation of phytochelatins and coumarins (17). The root microbiome may also play a role in arsenic detoxification as almost all microbes are resistant to inorganic arsenic (18) and have the potential to transform it into other species or influence its mobility (19, 20) and bioavailability (21). Thus, microbes may help plants to grow in arsenic-contaminated soils. Effects are likely dynamic, as root exudate metabolites may influence the species and activity of rhizosphere microbes, while rhizosphere microbial processes may also affect the speciation and assimilation of metal(loid)s into the plants (22). So far, we know little about the potential of exuded plant specialized metabolites in regulating arsenic toxicity, either directly or via changes in the root microbiome.

Benzoxazinoids are major specialized metabolites produced by cereals such as wheat and maize. They have been shown to be crucial in both insect herbivore and pathogen resistance (23, 24), and their production and accumulation are enhanced upon attack (25, 26). Furthermore, they can influence the performance of neighboring plants (27) and can alter the rhizosphere microbiome (28). Benzoxazinoids have also been studied for their metal-chelating properties. (29). DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) and DIMBOA-Glc (2-O-β-D-glucopyranosyl-2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one), which are secreted into the rhizosphere, have a high affinity for iron (30), can form stable iron complexes (31), and enhance iron uptake (32). Benzoxazinoids can also bind aluminum, and high benzoxazinoid production has been associated with reduced aluminum toxicity (33). To what extent benzoxazinoids

Author contributions: V.C., K.S., and M.E. designed research; V.C., G.D.-H., and H.G. performed research; P.M., C.A.M.R., M.B., S.S., A.M., and M.E. contributed new reagents/analytic tools; V.C. and J.W. analyzed data; M.B., S.S., A.M., K.S., and M.E. supervision; and V.C., J.W., and M.E. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: veronica.caggia@unibe.ch, klaus.schlaeppi@unibas.ch, or matthias.erb@unibe.ch.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2314261121/-/DCSupplemental.

Published March 21, 2024.

reduce the toxicity of trace elements such as arsenic, and whether this potential function translates into plant performance benefits in the field, is unknown.

Here, we studied the role of benzoxazinoids in protecting plants against soil arsenic. We evaluated their role by using different benzoxazinoid-deficient mutants and soil complementation with purified benzoxazinoids. We then investigated different potential mechanisms by which benzoxazinoids confer arsenic resistance, including changes in arsenic uptake, changes in rhizosphere microbial communities, and changes in rhizosphere arsenic speciation. Finally, we assessed whether benzoxazinoid-derived arsenic resistance is agriculturally relevant in the field, and whether it persists in the soil and thereby improves the performance of future plant generations via plant–soil feedbacks. By combining these results, we demonstrate that benzoxazinoids reduce arsenic accumulation in the roots and increase arsenic resistance of maize in the greenhouse and the field.

Results

Benzoxazinoid-Producing Plants Are Less Affected by Soil Arsenic than Benzoxazinoid-Deficient Mutants. To test whether benzoxazinoids reduce arsenic toxicity, we grew wild-type and benzoxazinoid-deficient *bx1* mutant maize plants in control soils and soil with added arsenic (100 mg kg^{-1}) and measured their performance. The two genotypes grew equally well in the control soil (Fig. 1 *A* and *C*), thus confirming that benzoxazinoid deficiency per se does not hamper plant performance under favorable environmental conditions (32, 34).

In soil with arsenic addition, both genotypes grew smaller ($P < 0.001$) and had less shoot biomass (dry weight, $P = 0.002$). The negative effect was significantly more pronounced in the *bx1* mutant than in wild-type plants (plant height: $P < 0.001$; shoot biomass: $P = 0.002$). Shoot chlorophyll content varied with time but was independent of genotype or arsenic addition (Fig. 1*B*). Shoot biomass (dry weight) at the end of the experiment was negatively affected by arsenic addition, with the effect being more pronounced in the *bx1* mutant ($P = 0.002$; Fig. 1*C*). No differences in shoot biomass were found between the two genotypes in the control soil. Enhanced performance of wild-type plants relative to *bx1* mutants upon arsenic addition was also observed in two other soils with different characteristics (*SI Appendix*, Tables S1 and S2 and Fig. S1 *A* and *B*), suggesting that this phenotype is conserved across soil types.

To corroborate the benzoxazinoid dependency of the above-described findings, we repeated the experiment with an

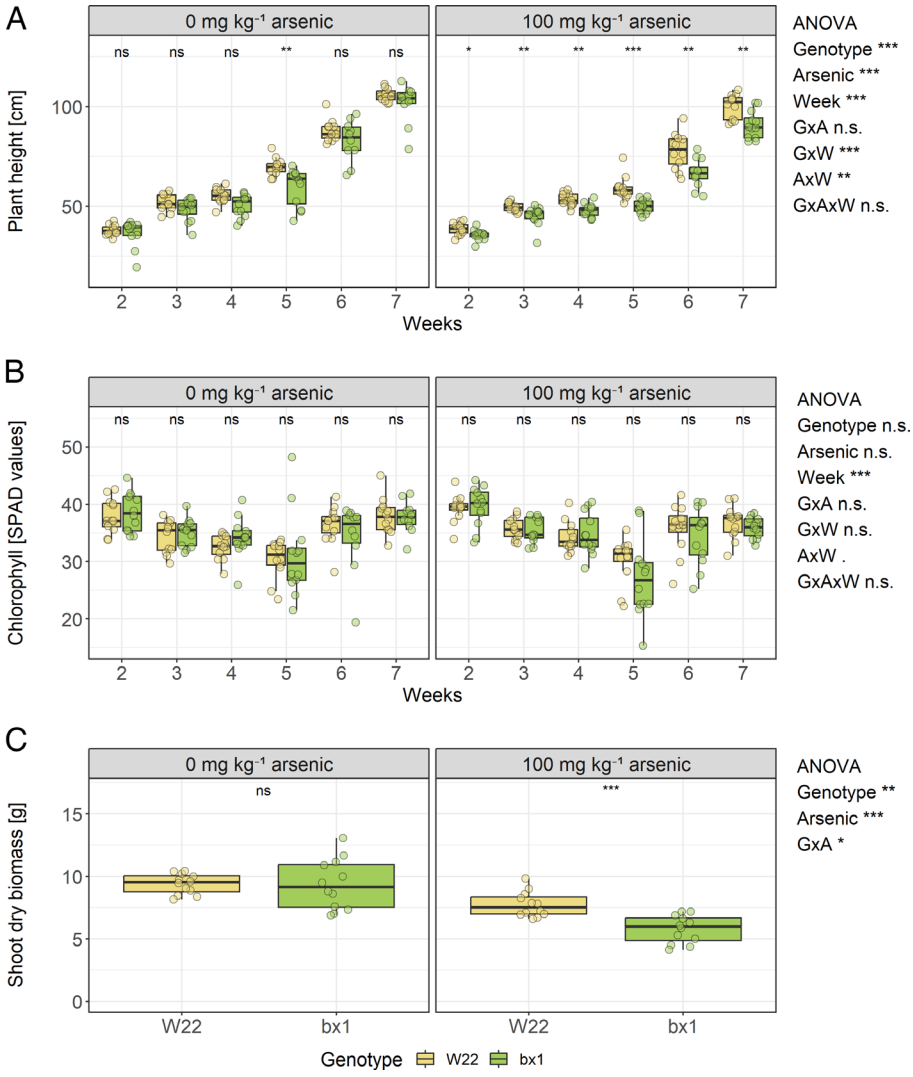

Fig. 1. Benzoxazinoid-producing maize plants are less affected by soil arsenic than benzoxazinoid-deficient mutants. (*A*) Plant height and (*B*) chlorophyll content of wild-type (W22) and benzoxazinoid-deficient *bx1* mutant plants growing in soil without (0 mg kg^{-1}) or with arsenic addition (100 mg kg^{-1}) for 7 wk. (*C*) Shoot dry biomass sampled at the end of the experiment. Asterisks indicate significant differences between genotypes (Tukey's pairwise comparisons, or Tukey's post hoc tests). Levels of significance: n.s. nonsignificant, . = marginally significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. See Dataset S1 for detailed results of Tukey HSD tests.

additional benzoxazinoid-deficient line in the same genetic background. We used the *bx2* mutant with a mutation of the *Bx2* gene encoding for the second enzyme of the BXs biosynthesis that converts indole to indolin-2-one (32). Similar to the first experiments, we did not observe differences in plant height or shoot biomass in the control soil (*SI Appendix*, Fig. S2 *A* and *B*). In soil with additional arsenic (100 mg kg^{-1}), the suppression of growth ($P = 0.02$) and shoot biomass ($P = 0.07$) was more pronounced in both *bx1* and *bx2* mutants than in wild-type plants. Thus, the capacity to produce benzoxazinoids reduces the negative effects of soil arsenic on plant performance.

Soil Complementation with Benzoxazinoids Reduces Arsenic Toxicity. To test whether benzoxazinoids that are released into the soil are sufficient to reduce arsenic toxicity, we complemented soils with arsenic addition with a purified mixture of benzoxazinoids, with DIMBOA-Glc as the dominant compound (*SI Appendix*, Table S3), and measured plant performance of wild-type and benzoxazinoid-deficient mutant plants. Again, wild-type plants grew heavier than *bx1* mutant plants in the soil high in arsenic ($P = 0.0005$). Complementation of the soil with benzoxazinoids enhanced the growth of the *bx1* mutant in this soil and partially rescued the wild-type phenotype (Fig. 2*A*). The same pattern was observed for biomass accumulation (Fig. 2*B*). Thus, benzoxazinoids in the soil can reduce arsenic toxicity for maize plants.

Benzoxazinoids and Arsenic Interact to Induce Subtle Changes in Rhizosphere Microbiota. Benzoxazinoids change the microbial composition of the rhizosphere (28, 35), which may change arsenic availability for plants. To investigate whether benzoxazinoids modulate the responses of the soil microbiota to arsenic, we screened microbiota of wild-type and *bx1* mutant plants in control soils and soils with added arsenic for genotype*environment interactions. The rhizosphere microbiota was determined based on the 16S rRNA gene of bacteria and first internal transcribed spacer (ITS) region for fungi using short-read sequencing. Bacterial and fungal profiles contained between 37,055 and 77,593 (median: 49,508) and 2,172 and 5,112 (median: 3,151) high-quality sequences per sample, respectively. Rarefaction analysis confirmed that the applied sequencing depths captured the microbial diversity present in these samples (*SI Appendix*, Fig. S3). Bacterial communities were mainly composed of Verrucomicrobia, Firmicutes, Alphaproteobacteria, and Actinobacteria, all in similar abundances. Acidobacteria, Gammaproteobacteria, Planctomycetes, Deltaproteobacteria, Chloroflexi, Thaumarchaeota, Gemmatimonadetes, Rokubacteria, and others were also detected, but at lower abundance.

Fungal communities mainly consisted of Ascomycota, Mortierellomycota, Olpidiomycota, and Basidiomycota (*SI Appendix*, Fig. S4). At the coarse phylum level taxonomy, we did not find statistical support for abundance differences between benzoxazinoid exudation, arsenic levels, or their interaction (*SI Appendix*, Table S4).

We then investigated at the higher resolution individual amplicon sequence variants (ASVs) and whether the maize rhizosphere microbiota was affected by benzoxazinoids and/or the arsenic addition. Shannon diversity was unaffected by benzoxazinoid exudation, arsenic contamination, or their interaction (*SI Appendix*, Table S5). However, microbiome composition changed slightly yet significantly due to benzoxazinoid exudation (bacteria: $P = 0.034$, fungi: $P = 0.049$) and arsenic addition (bacteria: $P = 0.015$, fungi: $P = 0.002$) (Fig. 3). PERMANOVA quantified effect sizes of 3.3% (both bacteria and fungi) for benzoxazinoid exudation and for arsenic contamination effect sizes of 3.6% and 5.3% for bacteria and fungi, respectively. Interestingly, a small (4%) but significant effect was found for the interaction between

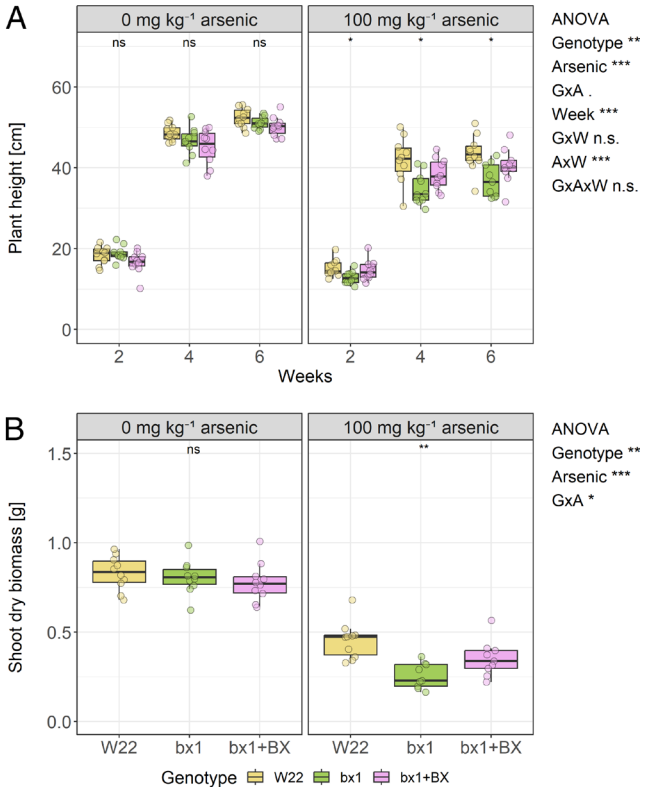

Fig. 2. Soil complementation with benzoxazinoids reduces arsenic toxicity. (*A*) Plant height and (*B*) shoot dry biomass of wild-type (W22) and benzoxazinoid-deficient *bx1* mutant plants growing without (0 mg kg^{-1}) or with arsenic addition (100 mg kg^{-1}). For a subset of *bx1* mutant plants, the soil was complemented with a mixture of purified benzoxazinoids, with DIMBOA-Glc as the dominant compound. Levels of significance (among genotypes): n.s. nonsignificant, . = marginally significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. See Dataset S1 for detailed results of Tukey HSD tests.

benzoxazinoid exudation and arsenic contamination ($P = 0.005$) (Fig. 3). CAP (Constrained Analysis of Principal) analyses visualized this finding. While bacterial communities of wild-type plants differed between control and contaminated soils, this was not seen for the mutant. In contrast to the fungi, arsenic had a stronger impact on the communities where both wild-type and mutant become similar in contaminated soil. Despite these subtle overall changes on community composition, we did not detect individual bacterial or fungal ASV which changed in abundance as a function of benzoxazinoid exudation and arsenic addition (*SI Appendix*, Table S6). Thus, we find subtle changes in the microbiome composition, but our analysis did not reveal any candidate taxa with the expected abundance pattern that could explain the observed plant phenotype and could be isolated for further functional analyses.

Benzoxazinoids Reduce Root Arsenic Accumulation. Mechanistically, benzoxazinoids may reduce arsenic uptake and/or translocation. To test for such effects, we measured total arsenic levels in the roots and leaves of wild-type and *bx1* mutant plants. As expected, only low levels of arsenic were detected in plants growing in control soils (Fig. 4). In soil with arsenic addition, we measured significantly lower arsenic concentrations in roots of wild-type plants (~70 mg kg^{-1}) compared to the *bx1* mutant (~90 mg kg^{-1}; Fig. 4*A*). Leaves contained only small amounts of arsenic (~1 mg kg^{-1}), and no differences between genotypes were found (Fig. 4*B*). Thus, benzoxazinoids reduce root arsenic accumulation. To get insights into the physiological relevance of the observed differences in root arsenic concentrations, we correlated root arsenic accumulation

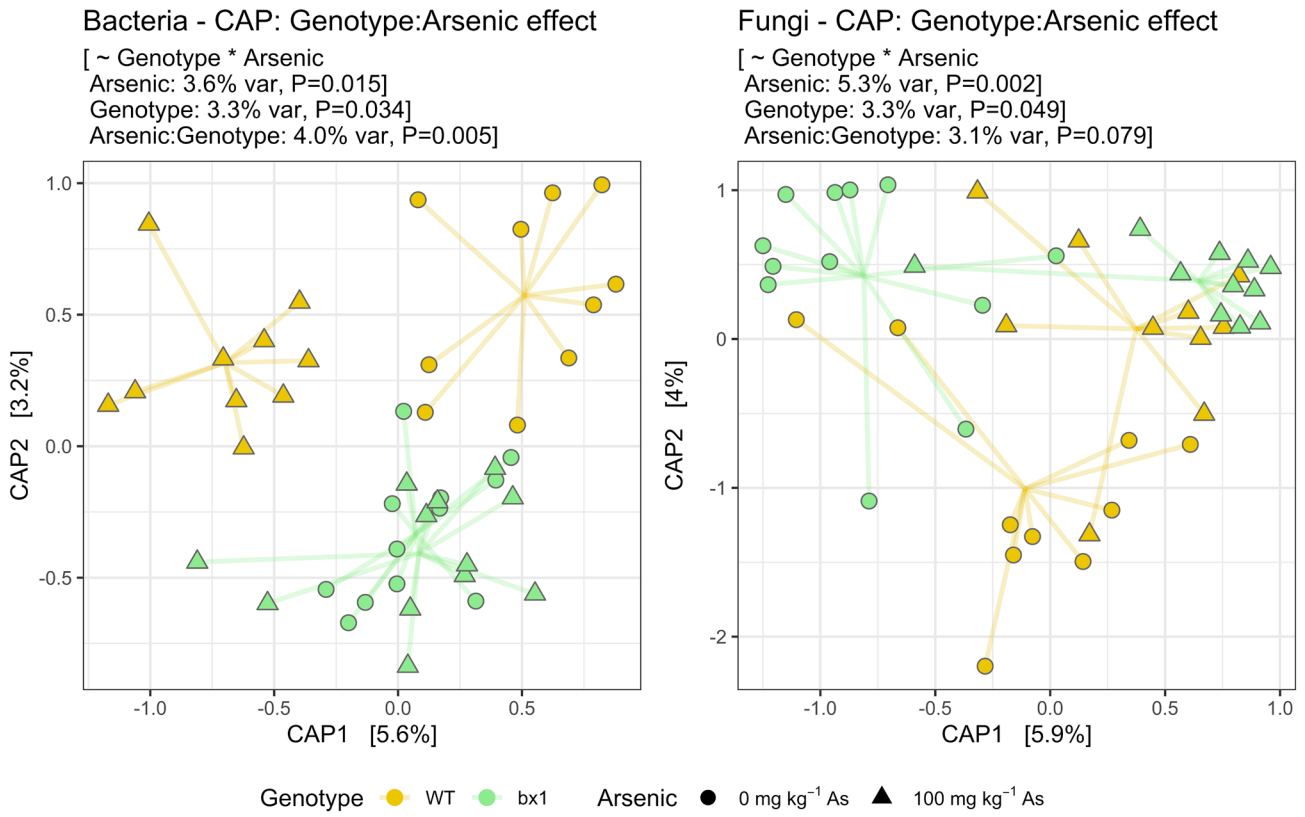

Fig. 3. Benzoxazinoids and arsenic interact to induce subtle changes in rhizosphere microbiota. The results of a CAP coordinates for bacteria and fungi computing dissimilarities with the model *Bray–Curtis distance ~ arsenic * genotype* is shown. Rhizosphere samples of wild-type (W22) and benzoxazinoid-deficient *bx1* mutant plants growing without (0 mg kg^{-1}) or with arsenic addition (100 mg kg^{-1}) were analyzed. Variances and *P*-values were computed by a PERMANOVA with 999 permutations. Levels of significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

with growth across three levels of soil arsenic addition. We found that root arsenic concentration differences of 50 mg kg^{-1} were associated with a reduction in biomass accumulation of more than 50% (Fig. 4*C*). These data suggest that the concentration difference in total arsenic between wild-type and *bx1* mutant roots of 20 mg/kg is sufficient to explain the growth differences between these two genotypes when growing in arsenic-amended soils.

Benzoxazinoids Change Arsenic Speciation in the Soil. To test whether the reduced arsenic uptake and/or the enhanced plant growth is associated with differential arsenic speciation, we quantified the different arsenic species in the rhizosphere. Most of the arsenic was present as As^{V}, the arsenic species that we spiked into the soil (*SI Appendix*, Fig. S5). We found a significantly higher concentration of remaining As^{V} in the rhizosphere of the *bx1* mutant (~12.5 mg kg^{-1}) compared to the rhizosphere of the wild-type plants (~7.5 mg kg^{-1}). We detected small amounts of As^{III} and two unknown arsenic species, without any significant differences between genotypes (*SI Appendix*, Fig. S5). Thus, benzoxazinoids change arsenic speciation in the soil and decrease the concentration of the initially added As^{V}. The fate of the disappearing As^{V} is currently unclear.

Field-Grown Plants Benefit More from Benzoxazinoids in a Field with High Arsenic Levels. To test whether benzoxazinoids benefit maize plants growing in an arsenic-contaminated field under realistic agricultural conditions, we grew wild-type and *bx1* mutant plants in a maize field with high biogenic arsenic (430 mg kg^{-1}, "As+ field"). We also included a field nearby that contains 10 times lower concentrations of arsenic (43 mg kg^{-1}; termed "As– field"). In both fields, wild-type plants grew significantly better than the *bx1* mutant. While this effect was subtle in the As– field, it was stronger in the As+ field, with wild-type plants grew 22 to 40% taller than *bx1* mutant plants (Fig. 5*A*), compared to 15 to 22% in As– field. Shoot biomass showed the same significant pattern, albeit less pronounced (Fig. 5*B*). Thus, benzoxazinoids promote plant performance in the field, and this effect is stronger when arsenic is present at high levels.

The Reduction of Arsenic Toxicity by Benzoxazinoids Persists via Plant–Soil Feedbacks. To further investigate the importance of benzoxazinoids that are released into the soil for arsenic toxicity of future plant generations, we performed a plant–soil feedback experiment. As benzoxazinoids can have feedback effects independently of arsenic in certain soils (28), we chose a soil (Q-matte) that does not show feedback effects in the absence of additional stressors (36). We grew wild-type plants in control soils and soils with arsenic addition in which wild-type or *bx1* mutant plants had been growing previously. As expected, we did not observe any differences in height or shoot biomass of wild-type plants growing in control soils (Fig. 6 *A* and *B*) In arsenic-amended soil, plants grew taller and accumulated more biomass when benzoxazinoid-producing wild-type plants had been growing before compared to when *bx1* mutants had been growing in this soil. Thus, benzoxazinoid-mediated arsenic tolerance can be transmitted as a soil legacy to the next generation of plants.

Discussion

Exuded plant specialized metabolites may help plants to deal with contamination by toxic levels of trace elements. Despite the substantial potential for agriculture, few studies have investigated this phenomenon and its underlying mechanisms (33, 37). Here, we

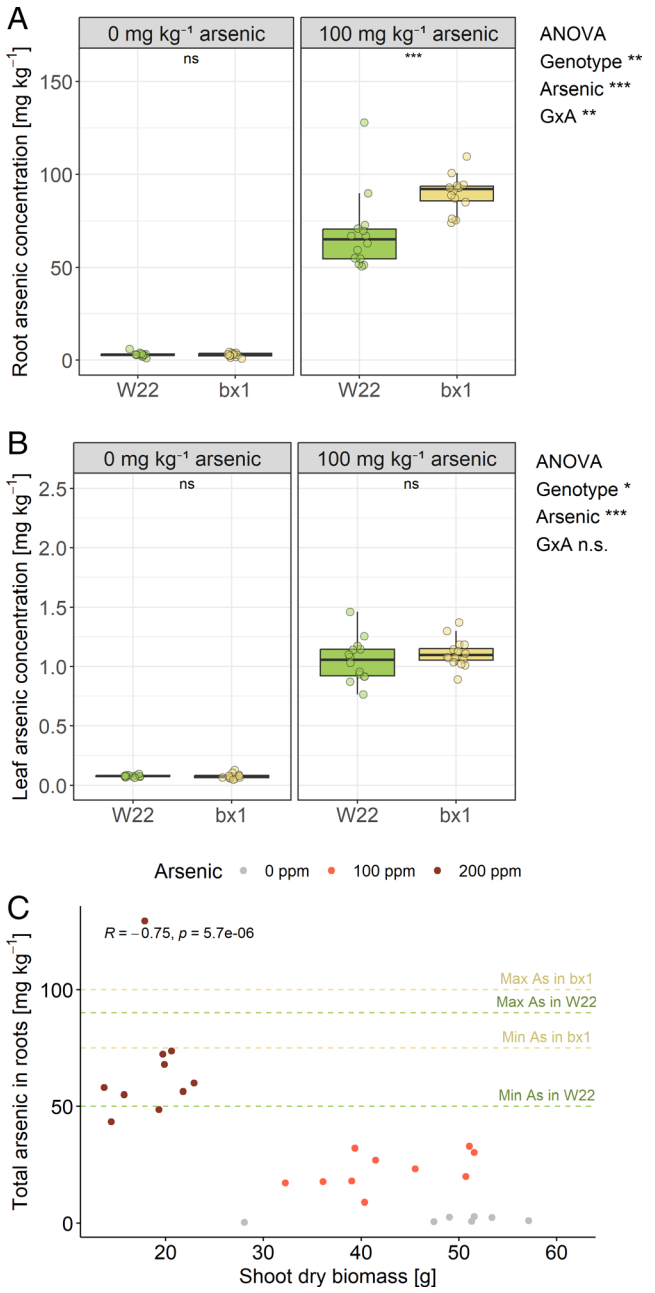

Fig. 4. Benzoxazinoids reduce root arsenic accumulation. (*A*) Root and (*B*) leaf arsenic concentrations of wild-type (W22) and benzoxazinoid-deficient *bx1* mutant plants growing without (0 mg kg^{-1}) or with arsenic addition (100 mg kg^{-1}). (*C*) Correlation between root arsenic concentration and plant dry biomass of wild-type plants growing in soils with different levels of arsenic addition. For comparison, the yellow and green lines correspond to minimal and maximal arsenic concentrations in W22 and *bx1* mutant plants growing in soil with arsenic addition from *A*. Levels of significance (between genotypes): n.s. nonsignificant, . = marginally significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. See Dataset S1 for detailed results of Tukey HSD tests.

demonstrate that benzoxazinoids reduce arsenic toxicity in the greenhouse and in agriculturally relevant conditions in the field. We show that the improved plant performance is associated with direct interactions between benzoxazinoids and the soil environment. Below, we discuss the underlying mechanisms and agricultural implications of this finding.

Specialized metabolites exuded from plant roots can improve plant growth by mobilizing micronutrients, suppressing pathogens and pests, attracting beneficial microbes, and attracting natural enemies of herbivores (38–42). Several studies also suggest that they may help plants to cope with trace elements toxicity (17). A maize cultivar that exudes benzoxazinoids from the root tips in response to aluminum for instance was more resistant to aluminum than another cultivar, suggesting a link between benzoxazinoid exudation and metal tolerance (37). Here, we performed genetically and environmentally controlled experiments to investigate whether and how benzoxazinoids enhance maize tolerance to arsenic, an important metalloid whose toxicity affects at least 140 million people worldwide (43). We show that benzoxazinoid deficiency, conferred by mutations in two different benzoxazinoid biosynthesis genes, reduced growth, and biomass accumulation in different soils, both in the greenhouse and the field in the presence of toxic arsenic levels in the soil. The link between benzoxazinoids and arsenic tolerance was corroborated by the fact that soil spiking with purified benzoxazinoids enhanced arsenic tolerance of the mutants. By demonstrating their involvement in arsenic tolerance, our study expands our view on the multifunctionality of benzoxazinoids and specialized metabolites in general (44).

Specialized metabolites may protect plants from toxic levels of trace elements by acting inside the affected tissues, and/or by acting outside of the plant, i.e., in the rhizosphere and soil (17, 33). Benzoxazinoids are released in substantial quantities by maize roots (45), and we provide several lines of evidence that suggest that they unfold their protective role in the surrounding soil. First, our complementation experiments demonstrated that external addition of benzoxazinoids to the soil partially rescued the wild-type phenotype. Second, we found that arsenic speciation in the soil was changed, and root uptake was reduced in wild-type

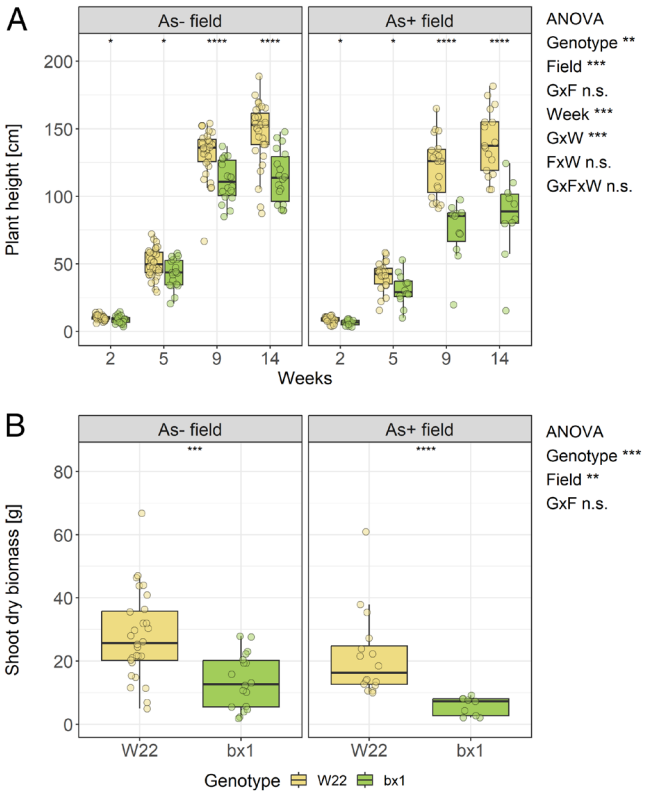

Fig. 5. Field-grown plants benefit more from benzoxazinoids in a field with high arsenic levels. (*A*) Plant height was recorded during maize growth season by 4 time points (2, 5, 9, and 14 wk after sowing) and (*B*) shoot dry biomass was measured after harvesting in wild-type (W22) and benzoxazinoid-deficient *bx1* mutant plants. *Left-hand* panels are from a field with low arsenic (As−) (43 mg kg^{-1}) and the *Right-hand* panels are from a field with high arsenic contamination (As+) (430 mg kg^{-1}). Levels of significance (between genotypes): n.s. nonsignificant, . = marginally significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. See Dataset S1 for detailed results of Tukey HSD tests.

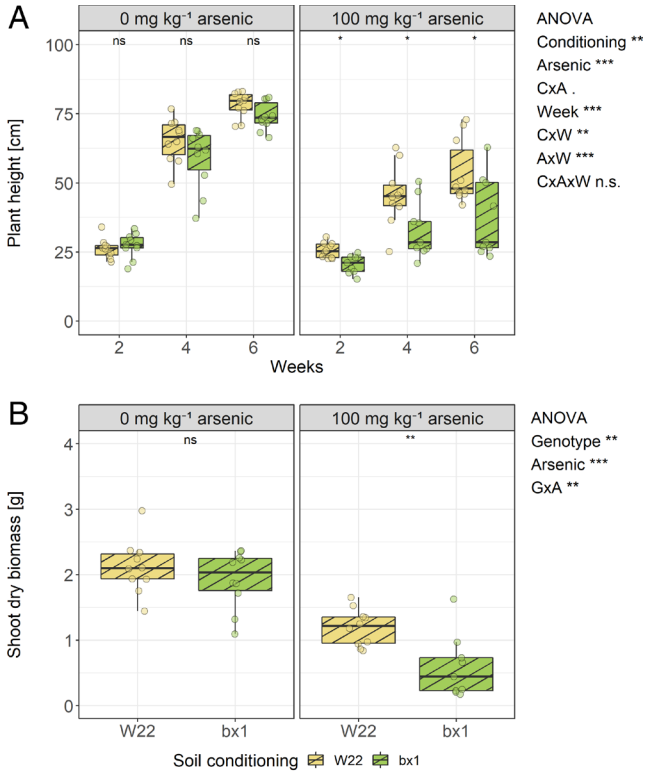

Fig. 6. The reduction of arsenic toxicity by benzoxazinoids persists via plant–soil feedbacks. (*A*) Plant height and (*B*) shoot dry biomass of wild-type (W22) plants growing in soils without (0 mg kg^{-1}) or with arsenic addition (100 mg kg^{-1}) that were conditioned by either wild-type (W22) or benzoxazinoid-deficient *bx1* mutant plants. Levels of significance (between genotypes): n.s. nonsignificant, . = marginally significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001. See Dataset S1 for detailed results of Tukey HSD tests.

plants, both pointing at effects that occur outside of the roots. Third, the protective effect of benzoxazinoids was transferred via the soil to the next plant generation, independently of their own capacity to produce benzoxazinoids, illustrating that changes in the soil are sufficient to modulate arsenic toxicity for maize. Given that many different specialized metabolites are released by plants into the soil (46, 47), and that several of them can interact with trace elements in the soil (48, 49), such interactions are likely widespread in the plant kingdom and may determine interactions between soil chemistry, plants, and plant consumers (50).

Benzoxazinoids can modify the speciation and bioavailability of trace elements such as iron (32, 50) and aluminum (33). Here, we demonstrate that benzoxazinoids also modify arsenic speciation and uptake. First, we observed that they enhance the conversion of arsenate (As^V), most likely to yet unknown forms of arsenic. As^V and its conversion product As^{III} are directly taken up by plant through different transporters and are toxic to plants (51), the decrease in As^V may reduce the availability of inorganic arsenic to plants and thus mitigate toxic effects. Second, either as a consequence or independently, maize roots of wild-type plants accumulate ~30% less total arsenic, a difference which is physiologically relevant, as shown by our spiking experiments. As the shoot concentration was only 1 to 2% of what was found in the roots, major toxic effects of arsenic can be expected to occur mainly in the root tissues. The reduced root accumulation in benzoxazinoid-producing plants may thus confer a true advantage for plant performance. Whether and how benzoxazinoids interact with inorganic arsenic, either directly or through interactions with soil microbes or other soil chemicals, including chelators, remains to be determined. In particular, more experiments are needed to determine the fate of As^V in benzoxazinoid-containing soils and to investigate the mechanisms leading to the reduced levels of arsenic in maize roots. Another open question in this context is which benzoxazinoids are the active compounds that reduce arsenic toxicity. Benzoxazinoid glycosides are rapidly converted to aglucones after being released into the rhizosphere, and the latter are then further metabolized to more stable breakdown products (reviewed by ref. 45). Detailed in vitro studies and complementation experiments with individual benzoxazinoids will be required to determine their relative importance in protecting plants from toxic levels of arsenic.

Benzoxazinoids may change the microbial community composition of the soil, which again may modify arsenic uptake dynamics (52). We profiled changes in rhizosphere bacteria and fungi to gain insights into this possibility. We detected only subtle interactive effects between benzoxazinoids and arsenic on the rhizosphere microbiota and found no candidate microorganisms whose changes in abundance would fit the observed plant performance patterns. Furthermore, we found that benzoxazinoids reduce arsenic toxicity across four different soils (three laboratory and one field soil) with widely different soil characteristics and likely very different microbial communities. Interactions between benzoxazinoids and rhizosphere microbiota are known to be highly context and soil-type specific (35, 36). Thus, these findings are not in support of a major role of the soil microbiota in the observed phenotype. Further experiments could focus on testing this hypothesis and investigating the role of soil microbial communities and dynamics in more detail, for instance though sterilization and complementation approaches (28, 53).

Arsenic toxicity is a major challenge for sustainable food production in many regions of the world, and modifying plants to cope better with arsenic is seen as a promising strategy in this context (54). Our work reveals two directly applicable benefits of benzoxazinoids, a genetically resolved trait of cereals. First, benzoxazinoids reduce the plant performance penalty of maize plants growing in arsenic-contaminated fields. Second, benzoxazinoids enhance the performance of subsequent crops growing in the same soil—thus adding a benefit to crop rotations. Most maize genotypes produce high amounts of benzoxazinoids, with notable variation in terms of structure and distribution (29). In the future, boosting benzoxazinoid production, especially of older maize plants, and boosting benzoxazinoid exudation into the rhizosphere may be desirable traits for traditional and modern breeding efforts. Producing such plants would not only enhance arsenic resistance and uptake but would also allow farmers to benefit from the other positive effects of benzoxazinoids, including iron uptake (32) and resistance to generalist insect pests (55). However, it has to be kept in mind that benzoxazinoids also favor the occurrence of specialized insect herbivores (56–58). Appropriate management of herbivore and pathogen communities would thus need to be part of an effort to harness the potential of benzoxazinoids for sustainable agriculture.

In conclusion, we demonstrate that benzoxazinoids, that are released into the soil, benefit plant performance in arsenic-contaminated environments. From an ecological point of view, this finding underscores how a single class of bioactive specialized metabolites can provide a wide range of benefits to plants, whose relative importance varies with environmental conditions. From an applied point of view, our work suggests that employing cultivars with enhanced benzoxazinoid exudation may be a useful strategy to maintain agricultural yields in arsenic-contaminated areas. The biosynthesis of benzoxazinoids is well understood, and proof-of-concept experiments show that benzoxazinoid production can be enhanced through genetic engineering (59). As soon as the mechanisms of benzoxazinoid exudation are elucidated, hyper-releasing maize plants could be employed

to support areas of the world where arsenic contamination constrains food production.

Methods

Performance of Benzoxazinoid-Deficient Mutant Plants in Arsenic-Amended Soil.

Soil selection. To evaluate the interaction between benzoxazinoids and arsenic, we first identified a suitable test soil. For this purpose, we screened numerous agricultural soils from Switzerland. Based on its low level of arsenic (2.9 ± 0.5 mg/kg), we chose to work with a silty loam soil (*SI Appendix*, Table S7) from "Q-Matte" in Frauenkappelen, Switzerland (46°57′20.5″N, 7°19′58.3″E). The A-horizon of this soil was collected and stored en bloc (24 m^3) next to our greenhouse facility (Bern, Switzerland). To vary arsenic levels in this soil, we collected bulk soil from storage and sieved it to 1 cm. We measured the soil water content by weighing a precise amount of soil (n = 3) and then drying it. Twenty kg of moist soil was decanted in 5 transparent plastic boxes (REGALUX Clear Box L, 54.8 × 38.4 × 28.3 cm, Bauhaus, Switzerland) and kept semiclosed with the respective plastic lid (REGALUX Clear Box-Deckel, 54.8 × 38.4 cm, Bauhaus, Switzerland). The soil was then watered with arsenic salt (sodium arsenate dibasic heptahydrate, ≥98%, Sigma-Aldrich, Switzerland) dissolved in MilliQ water to reach 100 mg kg^{-1} of arsenic and 60% water content. The soil was watered with arsenic solution in 4 steps and mixed thoroughly in between to spread the solution homogeneously. For the control treatment without arsenic, we applied the same procedure without adding sodium arsenate to the MilliQ water. The soil was incubated at room temperature (*SI Appendix*, Fig. S6*A*) for 6 wk at 60% water holding capacity (WHC), allowing for As equilibration between soil water and solid phases and simulating aging (60). We then merged, homogenized, and decanted the soils of the different boxes. For plant growth experiments, we added 1.8 kg of As spiked or control soil (n = 12) at 60% WHC in each pot (Rosentopf Soparco 2.0 L, Hortima AG, Switzerland). Each pot had an underpot (Untersetzer mit flachem Rand 16 cm, Hortima AG, Switzerland) to avoid cross-contamination during watering.

Plant germination. To evaluate the influence of benzoxazinoids on arsenic toxicity, we used the maize (*Zea mays L.*) inbred line W22 and the benzoxazinoid-deficient *bx1* and *bx2* mutants in a W22 background (61). Maize seeds were surface sterilized by soaking them for 6 min in commercial bleach containing 5% active hypochlorite (Potz Javel-Wasser Natur, Migros, Switzerland). Afterward, the bleach was removed, and the seeds were washed 5 to 6 times with autoclaved MilliQ water. For pregermination, the seeds were soaked in autoclaved MilliQ water in the dark for 8 h. After 8 h, the seeds were placed on a moist filter paper (Rundfilter Sorte 1 Whatman, 90 mm, Huberlab, Switzerland) in plastic petri dishes (petri dish 94 × 16 mm, without vents, sterile, Greiner Bio-One, Switzerland) overnight, before sowing them. All the work was performed at a clean bench.

Plant growth and performance. We sowed three surface-sterilized seeds per pot to ensure germination. After germination, we removed the extra seedlings and left only one seedling per pot. Maize plants (n = 12) were grown for 6 to 7 wk under greenhouse conditions (ranges: light/dark 14 h:10 h, daily temperature 14 °C to 22 °C, night temperature 10 °C to 14 °C, humidity 50 to 70%), and the position of the pots was randomized weekly (*SI Appendix*, Fig. S6*B*). We fertilized the maize plants from week 2 with Plantaktiv Typ K (concentration 2 g/L, Hauert, Switzerland) by dissolving 50 g of nutrient salt in 1 L of water (stock solution). We transferred 80 mL of the stock solution in a 2-L container and diluted it with tap water. Two and three weeks after sowing, plants were watered with 100 mL nutrient solution. Four weeks after sowing, plants were watered with 200 mL nutrient solution. We measured plant height and chlorophyll content weekly or every 2 wk. Plant height was determined by straightening the leaves manually and measuring the highest point; chlorophyll content was determined with a SPAD meter (Chlorophyll meter SPAD-502, Minolta Camera CO., LTD., Japan), taking an average of nine measurements on the youngest fully open leaf (three on the tip, three in the middle, and three at the base of the leaf). At harvest, leaves were cut and dried in an oven (UF 1060 Plus, Memmert Experts in Thermostatics, Hettich Laborapparate) at 70 °C for 72 h, and dry mass was determined.

Impact of Soil Type on Arsenic Toxicity. To assess whether the observed effect of benzoxazinoids on arsenic toxicity depends on the soil type, we repeated the experiment as described above using two additional agricultural soils, Changins and Posieux (n = 10). These soils come from different areas of Switzerland (*SI Appendix*, Fig. S7) and have different soil characteristics (*SI Appendix*, Table S2). Arsenic incubation, plant growth and fertilization, and plant performance measurements were done as described in the section above.

Soil Complementation with Purified Benzoxazinoids.

Benzoxazinoid purification. To determine whether the mode of action of benzoxazinoids on arsenic resistance is taking place in or outside the plant, we complemented the soil of *bx1* mutant plants with purified benzoxazinoids. We purified benzoxazinoids from germinated wild-type kernels. For that we prepared four batches of 40 g each of W22 seeds previously sterilized in containers and soaked them in autoclaved water for 14 h. Then the water was removed and every morning and evening the seeds were rinsed with autoclaved water several times. We left the seeds germinate for 4 d at 26 °C in the dark. Before harvesting we did not water the seeds for 12 h. After 4 d, we immediately put the germinated kernels (*SI Appendix*, Fig. S8*A*) into a blender (Mio Star Blend 600s, Migros, Switzerland) with 600 mL of methanol (methanol ≥ 99.8%, HPLC grade, Fisher Chemical) and blended them at full power for 5 min (*SI Appendix*, Fig. S8*B*). We filtered the mixture through a sintered glass filter (Filternutschen, 500 mL, Por. 3, Huberlab) and two filter papers (Qualitative Filter Paper, DP 595 090, 90 mm, ALBET LabScience, Germany) (*SI Appendix*, Fig. S8*C*). We evaporated the solvent on a rotary evaporator (RC900, KNF Neuberger AG, Balterswil, CHE) (*SI Appendix*, Fig. S8*D*). We freeze-dried this residue with a lyophilizator (LyoQuest -55, Telstar, Terrassa, ESP) (*SI Appendix*, Fig. S8*E*) until only crystallized residues were left in the flask. We added methanol to the extract and dissolved it by means of a sonicator (Ultrasonic bath, XUBA1, Grant Instruments). Afterward, we added 2 g of silica (silica 60, 0.062 to 0.2 mm, Macherey-Nagel, Macherey-Nagel GmbH & Co. KG, Germany), mixed it thoroughly and evaporated the methanol on the rotary evaporator. We scraped the silica and the extract from the walls and loaded them onto a solid loading cartridge (Solid Sample Cartridge, Teledyne). The compounds were separated on an automated flash column chromatography apparatus (CombiFlash Rf+, Teledyne ISCO Inc., Lincoln NE, USA). (*SI Appendix*, Fig. S8*F*) by using a 120 g silica cartridge, chloroform (suitable for HPLC, ≥ 99.8%, 0.5 to 1% ethanol as stabilizer, Sigma-Aldrich, Switzerland) and an increasing proportion of methanol as eluent. The elution gradient was as follows: 0 to 13% MeOH over 7 min, 13 to 16% MeOH over 9 min, and 16 to 35% MeOH over 9 min. After separation, we collected the fractions of interest (*SI Appendix*, Fig. S8*G*), poured them into a round-bottom flask, and evaporated the solvent on the rotary evaporator. When only liquid was left, we added the silica again and repeated the separation with the CombiFlash with a smaller silica column (40 g, normal phase, disposable columns for flash chromatography, RediSepRF) and collected fractions of interest again. We evaporated the solvent and collected the extract with a 2-mL syringe (Thermo Scientific™ National Target All-Plastic Disposable Syringes, Fisher Scientific, Switzerland) with a needle (hypodermic needle, 0.8 × 70 mm, 21G, NIPRO Europe N.V., Belgium), exchanged the needle with a PTFE 0.20 filter (chromafilXtra, Macherey-Nagel GmbH & Co. KG, Germany), and filtered the extract in a preweighed small round-bottom flask. We evaporated the solvent and dried the extract, dissolved it in distilled water, and freeze-dried it to obtain crystallized compounds (*SI Appendix*, Fig. S8*H*).

Ultra-high performance liquid chromatography mass spectrometry analysis. To determine the purity of the extracted benzoxazinoids, we analyzed them by UHPLC-MS and adapting a previous protocol (62). 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, Bade-Dättwil, Switzerland) was used. Gradient elution was performed on an Acquity BEH C18 column (2.1 × 50 mm i.d., 1.7 mm particle size) at 90 to 70% A over 3 min, 70 to 60% A over 1 min, 40 to 100% B over 1 min, holding at 100% B for 2.5 min, holding at 90% A for 1.5 min where A = 0.1% formic acid (FA)/water and B = 0.1% FA/acetonitrile (ACN). 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 to 1,200 with scans of 0.15 s at a collision energy of 4 V and 0.2 s 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 1,000 L/h and cone gas flow was 50 L/h. Accurate mass measurements (<2 mg kg^{-1}) 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.

Chemicals. Optima LC–MS FA and ACN, as well as HPLC grade methanol were purchased from Fisher Scientific AG. BOA (benzoxazolin-2(3*H*)-one) and MBOA

(6-methoxy-BOA) were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). *N*-(3-methoxy-2-hydroxyphenyl)malonamic acid was received as a gift from Francisco A. Macías (University of Cádiz, Spain). DIMBOA-Glc and 2-O-β-D-glucopyranosyl-2-hydroxy-4,7-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)-one were isolated from maize plants in our laboratory. DIMBOA, 2,4-dihydroxy-7-(methoxy-d_3)-2*H*-1,4-benzoxazin-3(4*H*)-one, MBOA-Glc, 2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one, APO (2-amino-3*H*-phenoxazin-3-one), AMPO (9-methoxy-APO), 9-methoxy-AMPO, were synthesized in our laboratory.

Soil complementation. The greenhouse experiment was performed in the same conditions as described before and lasted 6 wk (n = 10). The crystallized benzoxazinoids were dissolved in MilliQ water and complemented to the mutant every 3 d, with a total amount of 1.6 mg of benzoxazinoids per pot per week. This amount corresponds to physiologically relevant doses used in previously published work (28, 32), as determined by Gfeller et al. 2023 (53).

Impact of Arsenic on the Rhizosphere Microbiota.

Rhizosphere sampling, DNA extraction, and library preparation. To assess whether arsenic contamination influences the rhizosphere microbiome of wild-type and benzoxazinoid-deficient *bx1* mutant plants, we profiled bacterial and fungal community composition by next-generation sequencing (n = 12). Rhizosphere was collected at 6 wk by cutting the root section from −2 to −7 cm, placing it in a 50 mL tube (Sigma Corning® 50 mL centrifuge tubes, polypropylene, conical bottom w/ CentriStar cap, sterile, Sigma-Aldrich, Switzerland) with 25 mL MilliQ water. We shook the tubes 10 times and then added another 25 mL MilliQ to the same tube and shook it again 10 times before removing the root material. We centrifuged the tubes at 3,220 × g for 5 min (Sorvall Legend XTR centrifuge, Unity Lab Services, Thermo Scientific, Switzerland). We poured out the water from the tube and stored the sample pellets at −80 °C until DNA extraction. DNA extraction was based on the EMP DNA Extraction Protocol (63, 64). DNA was prepared using the DNeasy PowerSoil Pro Kit (QIAGEN, Beverly, MA, USA) following the manufacturer's instructions. The mix of 200 mg of rhizosphere material and 800 μL of Solution CD1 was homogenized with a Retsch Mixer Mill at 25 Hz for 10 min. DNA was eluted from the MB Spin Column with 100 μL of Solution C6. The DNA concentrations were quantified with the AccuClear® Ultra High Sensitivity dsDNA quantification kit (Biotium, Fremont, CA) and diluted to 2 ng μL^{-1} using a Myra Liquid Handler (Bio Molecular Systems). During that step, the DNA was distributed into two 96-well plates in a random and equal manner.

For the bacterial library, a first PCR reaction was performed with the 16S rRNA gene primers CS1-515-F [ACACTGACGACATGGTTCTACA*GTGYCAGCMGCCGCGGTAA – CS1 tagged version of Parada et al. (65)] and CS2-806-R [TACGGTAGCAGAGAC TTGGTCT*GGACTACNVGGGTWTCTAAT – CS2 tagged version of Apprill et al. (66)]. A second PCR tagged the PCR product with Access Array™ Barcode Library for Illumina® Sequencers–384, Single Direction (Fluidigm, South San Francisco, CA, USA). The first PCR program consisted of an initial denaturation step of 3 min at 94 °C, 25 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, elongation at 72 °C for 90 s, and a final elongation at 72 °C for 10 min. The second PCR program was similar, with the difference that the annealing temperature was increased to 60 °C and that the number of cycles was reduced to 10. For the fungal library, first a PCR reaction was performed with the ITS region primers CS1-ITS1f-F [ACACTGACGACATGGTTCTACA*CTTGGTCATTTAGAGGAAGTAA–CS1tagged version of Gardes and Bruns 1993 (67)] and CS2-ITS2-R [TACGGTAG CAGAGACTTGGTCT*GCTGCGTTCTTCATCGATGC–CS2tagged version of White et al. (68)]. A second PCR tagged the PCR product with Access Array™ Barcodes. The first PCR program consisted of an initial denaturation step of 3 min at 94 °C, 25 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, elongation at 72 °C for 90 s, and a final elongation at 72 °C for 10 min. The second PCR program was similar, with the difference that the annealing temperature was increased to 60 °C and that the number of cycles was reduced to 10. The sample-to-barcode assignment is available in Dataset S2.

All PCR reactions were performed with NGS grade Oligos (Eurofins Genomics, Ebersberg, Germany) and the Platinum Hot Start PCR MM (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). All PCR products and pooled libraries were purified with SPRIselect beads (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol with a ratio of 1:1; and were quantified with the AccuClear® Ultra High Sensitivity dsDNA quantification kit (Biotium, Fremont, CA). Subpools of bacteria and fungi samples were assembled by library type using a Myra Liquid Handler by adding an equal mass of each PCR product. All samples were sequenced (v3 chemistry, 300 bp paired end) on an Illumina MiSeq instrument at the NGS platform of the University of Bern.

Bioinformatics. First, we performed a quality control using FastQC [V0.11.8, (69)]. Barcodes were previously removed and written to the sequence headers by the NGS platform. We removed primers with cutadapt [V3.4, (70)] and used sequence headers information to demultiplex the data. Nine samples were discarded due to a very low number of sequences or because they were detected as outliers. With the same methods as used in Gfeller et al. (71) we used dada2 [V1.16.0, (72)] in R [V4.0.0, (73)] to infer exact sequences variants and assign taxonomies. The sequencing data and bioinformatic code are publicly available (see section *Data, Materials, and Software Availability*).

Impact of Benzoxazinoids on Arsenic Uptake and Speciation.

Total arsenic in roots and leaves. To test whether the capacity to produce benzoxazinoids influences arsenic accumulation, we measured total arsenic in roots and leaves (n = 12). After sampling the fresh samples at 6 wk, we freeze-dried them for 72 h in a lyophilizer (Swiss Vacuum Technologies SA, Telstar LyoQuest, Switzerland) and ground them with a mill (Retsch MM400 Mixer Mill, Fisherbrand™, Waltham, MA) at 30 Hz for 2 min. We weighed, 0.25 g of freeze-dried material directly into centrifuge tubes (Corning® 50 mL centrifuge tubes, sterile, Sigma-Aldrich, Switzerland). As a control, we included triplicates for blanks and a certified reference material (CRM; NIST 1573a, tomato leaves, National Institute of Standards and Technology, U.S. Department of Commerce, Gaithersburg, MD) (certified arsenic concentration of 0.112 ± 0.004 mg kg^{-1}; measured concentration 0.13 ± 0.01) with the following number of samples for each microwave run: 44, 47, 16, and 24. We added 4 mL of concentrated HNO_3 (65%; sub-boiled) to each sample and let it react overnight and 2 mL of 30% (w/w) peroxide (Suprapur H_2O_2; Sigma-Aldrich®, Switzerland) to each tube in three 5 min intervals and let it stand for 30 min to prevent over frothing. We vortexed each sample and digested it in a microwave oven (Microwave Digestion System MARS™ 6; CEM GmbH) (74) at 55 °C for 10 min, 75 °C for 10 min, and 95 °C for 30 min. After digestion, the samples were diluted to 50 mL with MilliQ water, weighed, and stored at 4 °C until analysis. Before analysis, we centrifuged them at 2,500 rpm for 5 min (Multifuge™ X1 Centrifuge Series, Thermo Scientific™, Reinach, Switzerland) and diluted them with a dilution factor of 5 (roots and leaves) or 2 (CRM) with 1% HNO3. The samples were finally analyzed by inductively coupled plasma mass spectrometer (ICP-MS; 7700× Agilent Technologies).

Arsenic speciation in the rhizosphere. To determine whether benzoxazinoids exudation has an impact on arsenic speciation in the rhizosphere, we performed additional analyses (n = 6) using a previously published method (75). At harvesting, a root section (2 to 7 cm below the soil surface) was put into a 50 mL centrifuge tube (Sigma Corning® 50 mL centrifuge tubes, polypropylene, conical bottom w/CentriStar cap, sterile, Sigma-Aldrich, Switzerland), with 25 mL of autoclaved Q-MilliQ water. The tube was shaken manually upside down 10 times, 25 mL of autoclaved MilliQ water was added, and the shaking was repeated. We removed the roots with tweezers, centrifuged the tubes at 3,220 × g for 5 min (Sorvall Legend XTR centrifuge, Unity Lab Services, Thermo Scientific, Switzerland) and removed the supernatant. The material was freeze-dried for 72 h in a lyophilizer (Swiss Vacuum Technologies SA, Telstar LyoQuest, Switzerland). After drying, the rhizosphere was homogenized with mortar and pestle and stored at room temperature before analysis. The eluent for the speciation analysis was also used as extractant and was composed of 5 mM tetrabutylammonium hydroxide (Sigma-Aldrich, Switzerland), 5 % (v/v) methanol and 3 mM malonic acid (Sigma-Aldrich, Switzerland). It was then pH-adjusted to 5.9. The buffer was deoxygenated by bubbling it with nitrogen for 30 min. We weighed 2-mL test tubes (Safe-Lock Eppenforf tubes, Eppendorf AG, Germany) containing 5 acid-washed zirconium oxide beads before transferring 60 mg of material (dry weight). We added 2 mL of buffer, closed the tubes, weighed them again (tube and beads + sample + buffer), and extracted the samples at 30 rpm for 10 min (Mixer Miller RETSCH MM 400, Fisherbrand™, Waltham, MA). After extraction, we centrifuged the samples at 2,500 rpm for 5 min (Multifuge™ X1 Centrifuge Series, Thermo Scientific™, Reinach, Switzerland), filtered with a 0.22 μm hydrophilic polytetrafluoroethylene filter (13 mm syringe filter, BGB®, Switzerland) and transferred 500 to 700 μL of the supernatant to a 0.7 mL PP HPLC vials (BGB®, Switzerland). The As speciation analysis was done by high-performance liquid chromatography-ICP-MS (HPLC-ICP-MS) with a 1260 Infinity HPLC coupled to a 7700× ICP-MS (both Agilent Technologies). The As species were separated by ion-pairing chromatography using a Zorbax SB-C18 column (150 mm × 4.6 mm, 5 μm particle size, Agilent, Switzerland) and the previously described eluent. The column was maintained at 50 °C and the flow

rate was set to 1.2 mL min^{-1}. The usual QA/QC procedures of calibration, spiking, chloride interference check, and determination of column recovery were applied. To calibrate the HPLC-ICP-MS, we used dimethylarsinic acid standards, freshly prepared in the eluent, at concentrations ranging from 0.1 to 100 µg kg^{-1}. The column recovery for the method was 105.6 ± 25.8%. The species were identified by retention time and spiking. We used two distinct As species mix to check for optimal separation. However, As^{III} and trimethylarsenic oxide can coelute with this method. Thus, we also used a combination of spiking and oxidizing of samples with hydrogen peroxide on one sample from each treatment. After oxidation, As^{III} was converted to As^{V}, and no peak remained, thus showing that only As^{III} was present. We also checked for polyatomic interference with chloride.

Protective Role of Benzoxazinoids in the Field. To test for the importance of benzoxazinoids in arsenic tolerance in the field, we chose an agricultural area in Switzerland with high geogenic arsenic (76). The contaminated region is situated in the Canton Basel-Landschaft, Switzerland, around the village of Liesberg. In this area, arsenic concentrations in soils have been found to exceed the remediation value. The Canton of Basel-Landschaft has measured the arsenic concentration of food crops (between 0.01 and 0.46 mg $kg^{-1}$of wet weight) growing on the contaminated soil (between 28 and 300 mg kg^{-1}) (76) and grass from permanent grassland, which is used as feed (77). We grew plants in two closely neighboring fields, one a heavily contaminated field (430 mg kg^{-1}, 47°24′19.6″N 7°25′38.2″E) and the other a weakly contaminated field (43 mg kg^{-1}, 47°24′33.4″N 7°25′02.8″E) (*SI Appendix*, Fig. S9). In each field, we had six subplots where we planted both genotypes (W22 and *bx1*, n = 6 per subplot) in a line (in total n = 36 per genotype per field). The other lines were planted with a commercial maize hybrid (*SI Appendix*, Fig. S10 *A* and *B*). Seeds were planted on the 1st of June 2021, and the plants were harvested on the 21st of September 2021 before the harvest of the rest of the field by the farmer (*SI Appendix*, Fig. S10*C*). The fields were managed following the details in *SI Appendix*, Table S8. Plant height and chlorophyll content were measured 4 times during the period of growth (2, 5, 9, and 14 wk after sowing). Plant height was determined by straightening the leaves and measuring the highest point. Chlorophyll content was determined with the SPAD meter (Chlorophyll meter SPAD-502, Minolta Camera CO., LTD., Japan) by taking the average among nine measurements (three at the base, three in the middle, and three on the tip of the leaf). After 14 wk of growth, plants were harvested by cutting the aboveground part 1 cm from the crown roots to separate it from the roots that were sampled and washed with tap water. Both aboveground and belowground parts were dried in an oven (UF 1060 Plus, Memmert Experts in Thermostatics, Hettich Laborapparate, Switzerland) at 70 °C for 72 h.

Protective Role of Benzoxazinoids for Subsequent Crops. To test whether benzoxazinoid exudation protects the next plant generation growing in the same soil, we performed a plant–soil feedback experiment (n = 10). Following the same methods for spiking the soil with sodium arsenate and growing the plants in the same conditions as explained before, the feedback experiment consisted of two phases. During the first phase (conditioning phase) we grew W22 and *bx1* mutant in both arsenic-free and arsenic-contaminated soils for 6 wk. After the conditioning phase, we harvested the plants including the root system; we sieved the soil of each pot through a 1-cm sieve and decanted it again in the same pot. In the second phase (feedback phase), we planted wild-type W22 seeds in all pots. The feedback phase also lasted 6 wk, during which plant performance was measured. At the end, plants were harvested, and the leaves were dried for 72 h at 70 °C in a dry oven to get the dry biomass.

Correlation between Root Arsenic Accumulation and Plant Performance. To correlate root arsenic accumulation and plant performance, we grew W22 maize plants in soils spiked with 0, 100, and 200 mg kg^{-1} of arsenic (calculated and spiked as explained above) in soil for 5 mo (n = 10). Plants grew in the greenhouse, with the fertilization and soil WHC as described before. We measured plant height regularly and we dried aboveground biomass at the end of the experiment, after 72 h drying at 70 °C. We also harvested plant roots, lyophilized them, and determined total arsenic levels following the procedure described above.

Statistical Analyses. All statistical analyses were performed in R (version 4.1.2). All datasets were checked for normality with Shapiro–Wilk tests ($P < 0.05$) and transformed as necessary (square-root, log, or rank) to meet assumptions for subsequent analyses. Eventual data transformations are indicated in the statistics tables for each figure. Such a general approach in treating the data permitted us to assess all the data with the same statistical test (ANOVA and pairwise test), using the following model: data ~ *Genotype* (G) * *Arsenic* (A) * (*Week* (W)). The factor "Week" was only considered with parameters taken through time (plant height and chlorophyll content), while ignored when a parameter was taken at a single time point. ANOVA tables and details on data transformations can be found in Supplementary Information (*SI Appendix*, Tables S9 and S10). Differences between treatments were tested with pairwise Tukey HSD tests (Dataset S1). The boxplots represent by default the first (Q1) and third quartile (Q3), known as the interquartile range (IQR), with the median of each group. The whiskers display the minimal and maximal values, calculated with the following equation: min(max, Q3+1.5*(Q3-Q1)) and max(min, Q1-1.5*(Q3-Q1)).

For the microbiota analysis, one fungal sample was removed because of low sequencing yield. Four bacterial and four fungal samples were not considered for analysis based on the result of a CLOUD outlier analysis. Data were normalized by total sum scaling. Bray–Curtis (BC) distances were calculated, and a permutation ANOVA (PERMANOVA, ~ genotype * arsenic, 999 permutations) was performed to conduct for differences in phylum-abundances. The alpha diversity of each sample was determined by calculating Shannon diversity. An ANOVA with the factors *genotype* and *arsenic* (~ genotype * arsenic) was performed to test for alpha diversity differences. BC distances were calculated as an index for beta-diversity and a PERMANOVA (~ genotype * arsenic; 999 permutations) was used to test for differences. Beta diversity was visualized using ordinations. Differential abundance analyses (DAA) were performed to find ASVs differing in abundance between the maize genotypes in arsenic-amended soil. Four DAA tools [ALDEx2 (78), ANCOMBC (79), Maaslin2 (80), and metagenomeSeq (81)] were used and predicted ASVs to be different in abundance if they were detected by at least two tools.

Data, Materials, and Software Availability. The raw sequencing data are available from the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under the study accession PRJEB55585 and the sample ID ERS12841720 (82). The bioinformatic code of sequence processing and the R codes of the statistical analyses are available on GitHub (https://github.com/PMI-Basel/Caggia_et_al_As-BX_interaction) (83).

ACKNOWLEDGMENTS. We thank Antoine Baud for his precious help in setting up and harvesting the greenhouse and field experiments and Mirco Hecht, Stephanie Pfister, and Anika Sidler for their help with the analysis in the lab. This work was supported by the Interfaculty Research Cooperation "One Health" of the University of Bern. Portions of the paper were developed from the thesis of V.C. (2022) entitled "Effects of environmental chemicals on soil and plant health".

Author affiliations: [a]Institute of Plant Sciences, University of Bern, Bern CH-3013, Switzerland; [b]Department of Environmental Sciences, University of Basel, Basel 4056, Switzerland; [c]Institute of Geography, University of Bern, Bern CH-3012, Switzerland; [d]Institute of Applied Geoscience, Technical University Darmstadt, Darmstadt D-64287, Germany; [e]Institute of Plant Nutrition and Soil Science, Christian-Albrechts-Universität, Kiel 24118, Germany; and [f]Institute of Bio- and Geosciences, Forschungszentrum Jülich GmbH, Jülich 52425, Germany

1. M. Amini *et al.*, Statistical modeling of global geogenic fluoride contamination in groundwaters. *Environ. Sci. Technol.* **42**, 3662–3668 (2008).
2. J. Matschullat, Arsenic in the geosphere {\textemdash} a review. *Sci. Total Environ.* **249**, 297–312 (2000).
3. F. X. Han *et al.*, Assessment of global industrial-age anthropogenic arsenic contamination. *Naturwissenschaften* **90**, 395–401 (2003).
4. FAO/WHO Expert Commettee on Food Additives, Nutrition and Food Safety (NFS), Standard & Scientific Advice on Food Nutrition (SSA), "Evaluation of certain food additives and contaminants. Thirty-third report of the Joint FAO/WHO Expert Committee on Food Additive" WHO Technical Report series;1036 (1989).
5. D. X. Guan, Z. H. Dai, H. J. Sun, L. Q. Ma, Arsenic and selenium in the plant-soil-human ecosystem: CREST publications during 2018–2021. *Crit. Rev. Environ. Sci. Technol.* **52**, 3567–3572 (2021).
6. F. J. Zhao, S. P. McGrath, A. A. Meharg, Arsenic as a food chain contaminant: Mechanisms of plant uptake and metabolism and mitigation strategies. *Annu. Rev. Plant Biol.* **61**, 535–559 (2010).
7. S. Prabpai, L. Charerntanyarak, B. Siri, M. R. Moore, B. N. Noller, Effects of residues from municipal solid waste landfill on corn yield and heavy metal content. *Waste Manag.* **29**, 2316–2320 (2009).

8. X. Cao, L. Q. Ma, Effects of compost and phosphate on plant arsenic accumulation from soils near pressure-treated wood. *Environ. Pollut.* **132**, 435–442 (2004).
9. A. A. Meharg, M. R. Macnair, An altered phosphate uptake system in arsenate-tolerant Holcus lanatus L. *New Phytol.* **116**, 29–35 (1990).
10. Z. Wu, H. Ren, S. P. Mcgrath, P. Wu, F. J. Zhao, Investigating the contribution of the phosphate transport pathway to arsenic accumulation in rice. *Plant Physiol.* **157**, 498–508 (2011).
11. I. M. Møller, P. E. Jensen, A. Hansson, Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol.* **58**, 459–481 (2007).
12. N. Stoeva, M. Berova, Z. Zlatev, Stoeva 2004.Pdf. *Biol. Plant.* **3**, 449–452 (2004).
13. N. Singh, L. Q. Ma, M. Srivastava, B. Rathinasabapathi, Metabolic adaptations to arsenic-induced oxidative stress in Pteris vittata L and Pteris ensiformis L. *Plant Sci.* **170**, 274–282 (2006).
14. X. ke Ci, *et al.*, Arsenic distribution, species, and its effect on maize growth treated with arsenate. *J. Integr. Agric.* **11**, 416–423 (2012).
15. Y. Hong, K. Song, J. Chung, Health effects of chronic arsenic exposure. *J. Prev. Med. Public. Health* 245–252 (2014).
16. X. Y. Xu, S. P. McGrath, F. J. Zhao, Rapid reduction of arsenate in the medium mediated by plant roots. *New Phytol.* **176**, 590–599 (2007).
17. A. Frémont *et al.*, Phytochelatin and coumarin enrichment in root exudates of arsenic-treated white lupin. *Plant Cell Environ.* **45**, 936–954 (2022).
18. D. Paez-Espino, J. Tamames, V. de Lorenzo, D. C´novas, Microbial responses to environmental arsenic. *Biometals* **22**, 117–130 (2009).
19. A. Sessitsch *et al.*, The role of plant-associated bacteria in the mobilization and phytoextraction of trace elements in contaminated soils. *Soil Biol. Biochem.* **60**, 182–194 (2013).
20. X. Di *et al.*, Microbial arsenic methylation in soil and uptake and metabolism of methylated arsenic in plants: A review. *Int. J. Environ. Res. Public Health* **16**, 1–13 (2019).
21. Y. Ma, R. S. Oliveira, H. Freitas, C. Zhang, Biochemical and molecular mechanisms of plant-microbe-metal interactions: Relevance for phytoremediation. *Front. Plant Sci.* **7**, 1–19 (2016).
22. D. V. Badri, T. L. Weir, D. van der Lelie, J. M. Vivanco, Rhizosphere chemical dialogues: Plant-microbe interactions. *Curr. Opin. Biotechnol.* **20**, 642–650 (2009).
23. D. Maag *et al.*, Highly localized and persistent induction of Bx1-dependent herbivore resistance factors in maize. *Plant J.* **88**, 976–991 (2016).
24. W. Oa, A. Huffaker, N. J. Dafoe, E. A. Schmelz, Regulates maize innate immunity and enhances. *Plant Physiol.* **155**, 1325–1338 (2011).
25. A. Köhler *et al.*, Within-plant distribution of 1, 4-benzoxazin-3-ones contributes to herbivore niche differentiation in maize. *Plant Cell Environ.* **38**, 1081–1093 (2015).
26. V. Tzin *et al.*, Rapid defense responses in maize leaves induced by Spodoptera exigua caterpillar feeding. *J. Exp. Bot.* **68**, 4709–4723 (2017).
27. M. I. Hussain *et al.*, Benzoxazinoids in wheat allelopathy–From discovery to application for sustainable weed management. *Environ. Exp. Bot.* **202**, 104997 (2022).
28. L. Hu *et al.*, Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nat. Commun.* **9**, 1–13 (2018).
29. S. Zhou, A. Richter, G. Jander, Beyond defense: Multiple functions of benzoxazinoids in maize metabolism. *Plant Cell Physiol.* **59**, 1528–1533 (2018).
30. L. Tipton, E. L. Buell, Ferric iron complexes of hydroxamic acid from maize. *Phytochemistry* **9**, 1964–1966 (1970).
31. C. Niculaes, A. Abramov, L. Hannemann, M. Frey, Plant protection by benzoxazinoids–recent insights into biosynthesis and function. *Agronomy* **8**, 143 (2018).
32. L. Hu *et al.*, Plant iron acquisition strategy exploited by an insect herbivore. *Science* **361**, 694–697 (2018).
33. C. Poschenrieder, R. P. Tolrà, J. Barceló, A role for cyclic hydroxamates in aluminium resistance in maize? *J. Inorg. Biochem.* **99**, 1830–1836 (2005).
34. V. Gfeller *et al.*, Plant secondary metabolite-dependent plant-soil feedbacks can improve crop yield in the field. *Elife* **12**, 1–20 (2023).
35. S. Cadot *et al.*, Specific and conserved patterns of microbiota-structuring by maize benzoxazinoids in the field. *Microbiome* **9**, 1–19 (2021).
36. S. Cadot *et al.*, Soil composition and plant genotype determine benzoxazinoid-mediated plant–soil feedbacks in cereals. *Plant Cell Environ.* **44**, 3502–3514 (2021).
37. Z. Zhao *et al.*, A unique aluminum resistance mechanism conferred by aluminum and salicylic-acid-activated root efflux of benzoxazinoids in maize. *Plant Soil* **437**, 273–289 (2019).
38. F. D. Dakora, D. A. Phillips, Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant Soil* **245**, 35–47 (2002).
39. E. N. Kudjordjie, R. Sapkota, S. K. Steffensen, I. S. Fomsgaard, M. Nicolaisen, Maize synthesized benzoxazinoids affect the host associated microbiome. *Microbiome* **7**, 59 (2019).
40. S. Mikic, S. Ahmad, Benzoxazinoids–Protective secondary metabolites in cereals: The role and application. *Ratar. povrt.* **55**, 49–57 (2018).
41. A. L. Neal, S. Ahmad, R. Gordon-Weeks, J. Ton, Benzoxazinoids in root exudates of maize attract pseudomonas putida to the rhizosphere. *PLoS One* **7**, e35498 (2012).
42. X. Zhang *et al.*, Plant defense resistance in natural enemies of a specialist insect herbivore. *Proc. Natl. Acad. Sci. U.S.A.* **116**, 23174–23181 (2019).
43. WHO, Chemical fact sheets: Arsenic Arsenic. Technical document (2022).
44. M. Erb, D. J. Kliebenstein, Plant secondary metabolites as defenses, regulators, and primary metabolites: The blurred functional trichotomy1[OPEN]. *Plant Physiol.* **184**, 39–52 (2020).
45. C. A. M. Robert, P. Mateo, The chemical ecology of benzoxazinoids. *Chimia (Aarau).* **76**, 928–938 (2022).
46. C. A. M. Robert *et al.*, A specialist root herbivore exploits defensive metabolites to locate nutritious tissues. *Ecol. Lett.* **15**, 55–64 (2012).
47. P. Pétriacq *et al.*, Metabolite profiling of non-sterile rhizosphere soil. *Plant J.* **92**, 147–162 (2017).
48. W. Ma *et al.*, Root exudates contribute to belowground ecosystem hotspots: A review. *Front. Microbiol.* **13**, 1–19 (2022).
49. L. Sun *et al.*, Root exudation as a major competitive fine-root functional trait of 18 coexisting species in a subtropical forest. *New Phytol.* **229**, 259–271 (2021).
50. L. Hu *et al.*, Soil chemistry determines whether defensive plant secondary metabolites promote or suppress herbivore growth. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2109602118 (2021).
51. P. M. Finnegan, W. Chen, Arsenic toxicity: The effects on plant metabolism. *Front. Physiol.* **3**, 182 (2012).
52. M. J. Abedin, J. Feldmann, A. A. Meharg, Uptake kinetics of arsenic species in rice plants. *Plant Physiol.* **128**, 1120–1128 (2002).
53. V. Gfeller, L. Thönen, M. Erb, Root-exuded secondary metabolites can alleviate negative plant-soil feedbacks. *New Phytol.*, 10.1111/nph.19401 (2023).
54. D. Sinha *et al.*, Negative impacts of arsenic on plants and mitigation strategies. *Plants* **12**, 1–44 (2023).
55. F. C. Wouters, B. Blanchette, J. Gershenzon, D. G. Vassão, Plant defense and herbivore counter-defense: Benzoxazinoids and insect herbivores. *Phytochem. Rev.* **15**, 1127–1151 (2016).
56. E. Stahl, O. Hilfiker, P. Reymond, Plant–arthropod interactions: Who is the winner? *Plant J.* **93**, 703–728 (2018).
57. R. Karban, A. A. Agrawal, Herbivore offense. *Annu. Rev. Ecol. Syst.* **33**, 641–664 (2002).
58. D. G. Heckel, "Insect detoxification and sequestration strategies". Annual Plant reviews: Insect-Plant Interactions, Volume 47 (2014).
59. L. Zheng *et al.*, Prolonged expression of the BX1 signature enzyme is associated with a recombination hotspot in the benzoxazinoid gene cluster in Zea mays. *J. Exp. Bot.* **66**, 3917–3930 (2015).
60. J. Song, F. Zhao, S. McGrath, Y. Luo, Influence of soil properties and aging on arsenic phytotoxicity. *Environ. Toxicol. Chem.* **25**, 1663–1670 (2009).
61. V. Tzin *et al.*, Dynamic maize responses to aphid feeding are revealed by a time series of transcriptomic and metabolomic assays. *Plant Physiol.* **169**, 1727–1743 (2015).
62. C. A. M. Robert *et al.*, Sequestration and activation of plant toxins protect the western corn rootworm from enemies at multiple trophic levels. *Elife* **6**, e29307 (2017).
63. C. Marotz *et al.*, DNA extraction for streamlined metagenomics of diverse environmental samples. *Biotechniques* **62**, 290–293 (2017).
64. D. Berg-Lyons *et al.*, EMP DNA extraction protocol (2018).
65. A. E. Parada, D. M. Needham, J. A. Fuhrman, Every base matters: Assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ. Microbiol.* **18**, 1403–1414 (2016).
66. A. Apprill, S. Mcnally, R. Parsons, L. Weber, Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* **75**, 129–137 (2015).
67. M. Gardes, T. D. Bruns, ITS primers with enhanced specificity for basidiomycetes–Application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**, 113–118 (1993).
68. T. J. White, T. Bruns, S. Lee, J. Taylor, *Amplification and Direct Sequencing of Fungal Ribosomal Rna Genes for Phylogenetics* (Academic Press Inc, 1990).
69. S. Andrews, FastQC: A quality control tool for high throughput sequence data. *Babraham Bioinform.* (2010).
70. M. Martin, Cutadapt removes adapter sequences from high-throughput sequencing reads (Tech. notes, 2011).
71. V. Gfeller *et al.*, Plant secondary metabolite-dependent plant-soil feedbacks can improve 1 crop yield in the field. *Elife* **12**, e84988 (2022).
72. B. J. Callahan *et al.*, DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **13**, 581–583 (2016).
73. R Core Team, R: A Language and Environment for Statistical Computing (R Found. Stat. Comput., 2022).
74. G. Norton *et al.*, Arsenic speciation and localization in horticultural produce grown in a historically impacted mining region. *Environ. Sci. Technol.* **47**, 6164–6172 (2013).
75. V. Müller, T. Chavez-Capilla, J. Feldmann, A. Mestrot, Increasing temperature and flooding enhance arsenic release and biotransformations in Swiss soils. *Sci. Total Environ.* **838**, 156049 (2022).
76. S. Daniel, "Gemeinde Liesberg: Arsen in Gartenböden und in angebautem Gemüse, Bau- und Umweltschutzdirektion" (2018).
77. S. Daniel, "Gemeinde Liesberg: Arsen in Landwirtschaftsböden und Futterpflanzen, Bau- und Umweltschutzdirektion" (2018).
78. A. D. Fernandes, J. M. Macklaim, T. G. Linn, G. Reid, G. B. Gloor, ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-seq. *PLoS One* **8**, e67019 (2013).
79. H. Lin, S. Das Peddada, Analysis of compositions of microbiomes with bias correction. *Nat. Commun.* **11**, 3514 (2020).
80. H. Mallick *et al.*, MaAsLin 2: Multivariable association in population-scale meta-omics studies. *PLoS Comput. Biol.* **17**, e1009442 (2021).
81. J. Paulson *et al.*, metagenomeSeq: Statistical analysis for sparse high-throughput sequncing (2013).
82. J. Wälchli, Caggia et al 2024; As-BX interaction. European Nucleotide Archive. https://www.ebi.ac.uk/ena/submit/webin/report/studies/ERP140498. Deposited 25 August 2022.
83. J. Wälchli, Caggia_et_al_As-BX_interaction. GitHub. https://github.com/PMI-Basel/Caggia_et_al_As-BX_interaction. Deposited 13 July 2023.