Benozaxinoids in Root Exudates of Maize Attract 
*Pseudomonas putida* to the Rhizosphere

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Introduction

Plants have evolved to interact with soil-borne microbes. In addition to arbuscular mycorrhizal fungi and nodule-forming rhizobia, plants interact with a wide range of rhizosphere-colonising bacteria. These are attracted to root surfaces by chemical components in root exudates, which are rapidly assimilated into microbial biomass [1]. This so-called rhizosphere effect supports bacterial cell densities in the root vicinity up to 100-fold greater than in surrounding soil [2]. The chemical composition of root exudates differs between plant species and evidence suggests that the structure of bacterial communities in the rhizosphere differs accordingly [3]. Observations that the rhizosphere community is directly influenced by plant species have led to the hypothesis that plants may recruit specific bacteria [4]. However, it remains difficult to determine whether plants are actively recruiting specific microbes, or whether dominance of a limited number of bacterial species is simply based on a greater ‘fitness’ to exploit root exudates [5].

When rhizospheric dominance by a single micro-organism occurs, the plant-microbe interaction can range from deleterious, in the case of phytopathogens, to beneficial, where rhizobacteria can promote plant growth and resistance to plant stress. Growth promotion by rhizobacteria involves a variety of different mechanisms, including N₂-fixation by diazotrophs [6] and improved availability of poorly soluble inorganic ions, such as PO₄³⁻ and Fe[III], but can also result from modulation of plant regulatory mechanisms, such as phytohormone homeostasis [7]. In addition, rhizobacteria can promote growth indirectly by protecting the host plant against pests and diseases. This protection can be based on direct antibiosis or competition for nutrients [8], but can also result from induced systemic resistance (ISR) [9].

Evidence suggests that plant-associating bacteria have evolved the ability to metabolise plant-derived aromatic compounds [10]. For instance, plant-associating bacteria have been shown to metabolise umbelliferone, salicylic acid and 4-hydroxybenzoate [10]. As a consequence, these bacteria are often also capable of metabolising aromatic pollutants, such as naphthalene, toluene and 2,4-dichlorophenoxyacetic acid [10]. Some aromatic acids can also act as bacterial chemo-attractants [11], suggesting that plant derived aromatic compounds could serve to recruit plant-beneficial rhizobacteria to the rhizosphere.

Benzoxazinoids (BXs), such as 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), are heteroaromatic metabolites with benzoic acid moieties [12]. Since their identification as major secondary defence metabolites in *Poaceae*, investigations have predominantly focussed on their role in plant defence against above-ground pests and pathogens [13,14]. BXs are typically produced during relatively early, vulnerable plant growth stages [12]. In response to tissue damage, vacuolar reservoirs of BX-glucosides are hydrolysed by plastid-targeted β-glucosidases, causing rapid accumulation of aglucone BX biocidal metabolites.
Bacterial strains and cultivation

Two *Pseudomonas putida* strains were used. KT2440 was used for all *in vitro* experiments, including transcriptome profiling. For soil experiments, a green fluorescent protein (GFP)-tagged KT2440 derivative strain, FBC004, was used which carries a stable chromosome-inserted *Pa*1/04/05-**RBSII**-**mut3**-**T0-T1** transposon at a negligible metabolic cost [30]. Stocks of KT2440 and FBC004 were routinely stored at −80°C. For each experiment, fresh cultures were started from stocks. Depending on the experiment, cells were grown overnight at 21°C with 150 r.p.m. agitation, either in LB medium, or in M9 minimal medium supplemented with 0.1% glucose as the sole carbon source. To assess tolerance of *P. putida* to DIMBOA, the ubiquitous soil bacterium *Agrobacterium tumefaciens* was used as a comparator. *A. tumefaciens* was grown in M9 medium supplemented with 0.1 mM FeCl₃. In this case, growth of the two bacteria was followed by assessing OD₆₅₀ in five replicate 200 µL cultures at 21°C in 96-well plates with a Varioskan plate reader (Thermo Scientific, Cramlington, UK).

*Pseudomonas putida* transcriptome response to DIMBOA

To test the response of *P. putida* to DIMBOA, we employed a KT2440 specific cDNA microarray [31,32]. Preliminary experiments indicated that DIMBOA hydrolyses rapidly in M9 medium (half-life, 21 hours). Therefore, to test the bacterial response to DIMBOA, *P. putida* KT2440 cells were grown to mid-exponential phase in 100 mL M9 medium before DIMBOA was added to a final concentration of 5 µg mL⁻¹. After 1 hour of exposure, cells were harvested by centrifugation at 4°C. RNA was extracted from three independently performed experiments. Cell pellets were treated with RNAprotect™ (Qiagen, Valencia CA) immediately following centrifugation. Cell membrane lysis was achieved with 1 mg mL⁻¹ lysozyme in buffer containing 10 mM TRIS and 1 mM EDTA at pH 8 using Qiagen RNasea® reagent kits following the manufacturer’s instructions. Extracted RNA was purified with TURBO DNA-free™ kits (Ambion, Applied Biosystems, Foster City, CA) and quantified on a NanoDrop (Molecular Devices, Sunnyvale, CA). cDNA was synthesised and labelled using the SuperScript™ indirect cDNA labelling system (Invitrogen, Carlsbad, CA). Synthesised cDNA paired samples (control or DIMBOA) were labelled with Cy3 or HyPer5 (Amersham, Little Chalfont, UK) fluorophores. To remove dye bias, the experimental design included dye-swap normalisation procedures, as described by Dabney & Storey [33]. Dye incorporation was verified to be more than 150 pmol dye per sample. Equal amounts of Cy3-cDNA and HyPer5-cDNA, each representing a replicate comparison between control and DIMBOA-treated cells, were combined and dried in a SpeedVac before proceeding with array hybridisation.

Microarrays were pre-treated with BlockHt™ Plus blocking buffer (Arrayit Corporation, Sunnyvale, CA) in order to inactivate reactive groups on the surface. Dried cDNA was rehydrated in buffer and hybridised to arrays (Progenika Biopharma S.A., Vizcaya, Spain) for 18 hours at 42°C, according to the manufacturer’s instructions. Following hybridisation, arrays were washed, dried, and scanned with a GenePix® 4000B scanner (Molecular Devices, Sunnyvale, CA). Data were processed using TM4 microarray software [34]. Data from the three independent replicate experiments were combined and analysed together. Using Statistical Analysis for Microarrays procedures [35], only genes that were consistently induced by DIMBOA in all three independent replicates were considered significant. Functional annotation of induced genes was performed using the supporting microarray documentation and the *P. putida* KT2440 KEGG

Materials and Methods

Plant material and cultivation

Maize lines were derived from reciprocal crosses between a *bx1* single mutant and an indole-deficient *igl* mutant, as described by Ahnad et al. [17]. Since the *bx1* single mutant contains residual levels of benzoxazinoids due to a functional *Indole-3-Glycerol phosphate Lyase (IGL)* gene [17], comparisons within each progeny were made between the benzoxazinoid-producing *BX1* and benzoxazinoid-deficient *bx1* genotypes in the background of the *igl* mutant genotype (i.e. *BX1 igl* versus *bx1 igl*). For each experiment, progenies from two independent crosses (Line A and Line B) were analysed for phenotypes. Seeds were allowed to germinate at 22°C and high humidity in petri-dishes in the dark. Germinated seedlings of similar size were planted in pots containing compost and were cultivated under controlled conditions (16:8 h L:D, 22°C).

A recent study in maize revealed that *Spodoptera* larvae can detoxify DIMBOA by glycosylation and that the contribution of maize BXs to defence against these herbivores is based on an inducible conversion of DIMBOA-glc into 2-β-D-glucopyranosyl-5,7-dihydroxy-1,4-dimethoxy-1,4-benzoxazin-3-one (HDMBOA-glc) [16]. Interestingly, BXs are also active against attackers causing relatively minor tissue damage, such as aphids and pathogenic fungi. This function is based on an increased accumulation of DIMBOA in the apoplast before the onset of large-scale tissue damage, where it signals increased deposition of callose-rich papillae [17]. Thus, the above-ground defence contribution of BXs is not only limited to their biocidal properties, but also includes a within-plant signalling function in the activation of plant innate immune responses against pests and diseases.

BXs have also been implicated in plant defence below-ground. BXs are exuded in relatively large quantities from cereal roots, where they can act as allelochemicals against microbes, insects or competing plants [13,18]. Once released, BXs degrade relatively quickly in aqueous environments with a half-life of less than 24 hours [19]. Upon hydrolysis, DIMBOA is converted into 6-methoxy-benzoxazolin-2-one (MBOA), a compound considerably more stable in sterile soil, but with significantly less toxicity than DIMBOA [20]. Biodegradation of MBOA leads to accumulation of phenoxazinones [21], and requires activity by microbes, such as *Acinetobacter calcoaceticus* [22], soil-borne fungi [23], or unidentified members in the rhizosphere community of oat [24]. Phenoxazinone products are typically more biocidal than benzoxazolinones and have antifungal [25], antibacterial [26], and plant allelopathic properties [27]. Hence, BX exudation by plants can have a major impact on rhizosphere communities in the soil.

Plant-derived aromatic metabolites can act as chemo-attractants for *Pseudomonas putida* [10,11]. We therefore hypothesised that BXs from root exudates of maize may attract and support *P. putida* cells. To address this hypothesis, we studied the influence of BXs on *P. putida* KT2440, a competitive coloniser of the maize rhizosphere with plant-beneficial traits [28,29]. We identified DIMBOA as the *KT2440* specific cDNA microarray [31,32]. Preliminary experiments indicated that DIMBOA hydrolyses rapidly in M9 medium (half-life, 21 hours). Therefore, to test the bacterial response to DIMBOA, *P. putida* KT2440 cells were grown to mid-exponential phase in 100 mL M9 medium before DIMBOA was added to a final concentration of 5 µg mL⁻¹. After 1 hour of exposure, cells were harvested by centrifugation at 4°C. RNA was extracted from three independently performed experiments. Cell pellets were treated with RNAprotect™ (Qiagen, Valencia CA) immediately following centrifugation. Cell membrane lysis was achieved with 1 mg mL⁻¹ lysozyme in buffer containing 10 mM TRIS and 1 mM EDTA at pH 8 using Qiagen RNasea® reagent kits following the manufacturer’s instructions. Extracted RNA was purified with TURBO DNA-free™ kits (Ambion, Applied Biosystems, Foster City, CA) and quantified on a NanoDrop (Molecular Devices, Sunnyvale, CA). Synthesised cDNA paired samples (control or DIMBOA) were labelled with Cy3 or HyPer5 (Amersham, Little Chalfont, UK) fluorophores. To remove dye bias, the experimental design included dye-swap normalisation procedures, as described by Dabney & Storey [33]. Dye incorporation was verified to be more than 150 pmol dye per sample. Equal amounts of Cy3-cDNA and HyPer5-cDNA, each representing a replicate comparison between control and DIMBOA-treated cells, were combined and dried in a SpeedVac before proceeding with array hybridisation. Microarrays were pre-treated with BlockHt™ Plus blocking buffer (Arrayit Corporation, Sunnyvale, CA) in order to inactivate reactive groups on the surface. Dried cDNA was rehydrated in buffer and hybridised to arrays (Progenika Biopharma S.A., Vizcaya, Spain) for 18 hours at 42°C, according to the manufacturer’s instructions. Following hybridisation, arrays were washed, dried, and scanned with a GenePix® 4000B scanner (Molecular Devices, Sunnyvale, CA). Data were processed using TM4 microarray software [34]. Data from the three independent replicate experiments were combined and analysed together. Using Statistical Analysis for Microarrays procedures [35], only genes that were consistently induced by DIMBOA in all three independent replicates were considered significant. Functional annotation of induced genes was performed using the supporting microarray documentation and the *P. putida* KT2440 KEGG
In vitro Pseudomonas putida KT2440 chemotaxis assay

Chemo-attractiveness of DIMBOA was quantified using a modified capillary-based chemotaxis assay [11], which relies on accumulation of bacterial cells in microcapillary tubes (1 µL volume, Drummond Scientific Company, Broomall, PA). Tubes containing glucose-free M9 medium (control), M9 medium with 5 µg g⁻¹ DIMBOA, or M9 medium with 0.1% casamino acids (positive control) [11] were incubated in individual wells of a 96-well plate. Each well contained 200 µL glucose-free M9 suspension with P. putida KT2440 bacteria (OD₅₆₀ = 0.06). After 30 minutes, capillary contents were carefully collected and plated onto LB agar for cell enumeration.

Maize - Pseudomonas putida FBC004 colonisation assays

Seeds germinating after 2 days of imbibition were planted in 100 mL-pots (3 seeds per pot; 4 pots per genotype), containing autoclaved (120°C; 20 min) or non-autoclaved soil that had been supplemented with washed cells from overnight FBC004 cultures at an approximate density of 5 x 10⁷ colony forming units (CFU) g⁻¹ soil. At 7, 14, and 21 days of growth, root systems were gently removed from the soil, rinsed in water, weighed and gently shaken for 20 minutes in 30 mL phosphate-buffered saline (mmol L⁻¹; NaCl 137, KCl 2.7, Na₂HPO₄·2H₂O 10, KH₂PO₄ 1.76; pH 7.4). Serial dilutions of rhizosphere bacteria were plated onto LB agar, containing 200 µg L⁻¹ cyclohexamide to inhibit fungal growth. Plates were incubated at room temperature for 48 hours. GFP-expressing colonies were counted using a DR5000X Dark Reader® transilluminator (Clare Chemical Research Inc., Dolores, CO); the total numbers of non-agonist, other culturable cells, assessed as CFU g⁻¹ root fresh weight, was analysed for each maize line and time point by two-factor analysis of variance (ANOVA), using the maize BX genotype (BX1, bx1) and bacterial cell type (P. putida, other culturable cells) as factors. All data were log₁₀-transformed before analysis to stabilise variances. Post-test comparisons were made using Holm–Šidák step-down pairwise comparisons. All statistical analyses were performed using SigmaPlot version 12.

Extraction and chromatographic analysis of benzoazinoids in root exudates and bacterial cultures

Seeds germinating after 2 days imbibition were planted in soil (3 seeds per pot). At days 7, 14, and 21 after planting, root systems were gently removed from the soil, rinsed in water, and placed in 50 mL tubes containing 30 mL water for seven hours to collect root exudates. Root exudates were lyophilised, re-suspended in 1 mL extraction buffer (2% acetic acid in methanol), sonicated for 5 min and centrifuged (12,600 x g, 10 min). Supernatants were analysed by high performance liquid chromatography coupled to diode array detection (HPLC-DAD), as described by Ahmad et al. [17]. Root exudates from BX1 genotypes contained three main peaks, absent in samples from bx1 genotypes. Spiking experiments with previously confirmed standards [16,17] revealed that exudates from BX1 wild-type roots consistently contain three main BX species: DIMBOA and to a lesser extent DIMBOA-glc and HDMBOA-glc. For analysis of DIMBOA breakdown by P. putida KT2440 in M9 growth medium, 0.5 mL samples were periodically removed and filtered (<0.2 µm) to remove cells. The samples were then stored in an equal volume of extraction buffer until analysis. Detection of DIMBOA and MBOA was based on a modified HPLC protocol, using a mobile phase of 0.05% trifluoroacetic acid in water (solution A) and 0.03% trifluoroacetic acid in methanol (solution B) at a flow rate of 1 mL min⁻¹. The gradient consisted of 0–1 minute 3–20% solution B, 1–20 minutes, 20–100% solution B, and 20–35 minutes isocratic conditions of 100% solution B. Chromatograms were recorded at 254 nm and retention times of DIMBOA and MBOA were established from standards.

Results

Exudation of benzoazinones from maize roots

Roots of BX1 wild-type and bx1 mutant lines were incubated for 7 hours in water, after which the collected exudates were subjected to HPLC-DAD analysis of BXs. Root exudates from BX1 wild-type plants consistently contained three BX compounds, all of which were absent from exudates of bx1 mutant lines. The dominant compound was DIMBOA, with concentrations up to 31 µg g⁻¹ fresh root weight (FW) in exudates from 7 days old roots (Figure 1). Levels of DIMBOA exudation showed a statistically significant linear decline in aging plants (Figure 1: linear regression, Line A, F₁₁₅ = 17.74; p = 0.001. Line B, F₁₁₅ = 7.387; p = 0.018). The other plant-derived BXs in root exudates from BX1 expressing plants were the BX glucosides DIMBOA-glc and HDMBOA-glc. Concentrations of these compounds did not exceed 3 µg g⁻¹ FW and remained constant over time.

P. putida is tolerant to DIMBOA

To examine the effect of DIMBOA on plant-beneficial rhizobacterial growth, we assessed in vitro growth of P. putida KT2440 bacteria in the presence of increasing concentrations of DIMBOA. P. putida KT2440 displayed similar growth rates up to 0.5 mM DIMBOA. By contrast, DIMBOA strongly affected growth rates of the ubiquitous soil bacterium Agrobacterium tumefaciens [36], effects were already apparent at 0.01 mM and became proportionally more pronounced at 0.1 and 0.5 mM DIMBOA (Figure 2A). Hence, P. putida KT2440 appears relatively tolerant to DIMBOA in comparison to other soil bacteria. For all subsequent experiments, DIMBOA was employed at concentrations of 3 µg mL⁻¹ (0.023 mM). This relatively low concentration has no detrimental effect on P. putida growth (Figure 2A) and is quantitatively consistent with our root exudation experiments (Figure 1).

P. putida accelerates DIMBOA breakdown

To study whether the observed tolerance of P. putida KT2440 to DIMBOA is based on BX catabolism, we studied the effect of P. putida KT2440 on stability of DIMBOA and its direct break-down product, 6-methoxy-benzoazolin-2-one (MBOA). In two independent experiments, DIMBOA concentrations were consistently reduced at a significantly greater rate, whereas MBOA accumulation was significantly reduced in the presence of P. putida bacteria (Figure 2B). These results demonstrate that P. putida KT2440 accelerates breakdown of DIMBOA. The reduced accumulation of MBOA in the presence of P. putida could be explained by a more rapid metabolic break-down of this compound, but could also suggest DIMBOA degradation via products other than MBOA. Although it is not possible to distinguish which of these processes is responsible for the observed compound dynamics in the presence of P. putida, our results clearly show that P. putida has the metabolic capacity to degrade DIMBOA and reduce overall BXs quantities in its environment.
Impact of DIMBOA on the P. putida transcriptome

The above in vitro analyses suggest that DIMBOA is metabolised by P. putida. To assess the global impact of DIMBOA on P. putida KT2440, whole-genome gene expression patterns were profiled at 1 hour of exposure to 5 μg mL−1 DIMBOA in M9 growth medium, deposited in NCBI’s Gene Expression Omnibus (GSE36489). Using KT2440-specific cDNA microarrays and a false discovery rate of 0.85% (Δ = 1.2) [35], we identified 55 genes showing consistently increased levels of transcription in response to DIMBOA treatment across three independent experiments. No genes were identified as significantly repressed by DIMBOA. A total of 36 genes could be ascribed predicted functions, whereas 19 genes encoded hypothetical proteins of unknown function (Figure 3). The 36 DIMBOA-inducible genes with identifiable function are further detailed in Table S1. Two groups of genes were of particular interest with respect to P. putida behaviour in the rhizosphere. One group of genes are typical of those associated with degradation of N-heteroaromatic compounds (Table S1), and are consistent with the accelerated breakdown of DIMBOA by P. putida KT2440 (Figure 2B). A second group of genes are indicative of bacterial motility (Figure 3; Table S1), thereby suggesting a chemotactic response of P. putida KT2440 to DIMBOA.

DIMBOA induces positive chemotaxis by P. putida

Based on the outcome of the transcriptome analysis, we examined the possibility that DIMBOA acts as a chemo-attractant for P. putida KT2440. A capillary-based assay was used to assess chemotactic behaviour to DIMBOA [11]. Significantly more cells (p = 0.022; t-test) were attracted into capillaries containing 5 μg mL−1 DIMBOA compared to tubes with motility buffer alone (Figure 4). The average number of DIMBOA-attracted cells was statistically similar to the average number of cells that were attracted to the positive control tubes, containing 0.1% w/v casamino acids (Figure 4). Hence, P. putida KT2440 is attracted to DIMBOA in vitro.

DIMBOA attracts P. putida to the rhizosphere

Having established that DIMBOA induces chemotaxis-associ- ated genes in P. putida KT2440, and that P. putida KT2440 is attracted to DIMBOA in vitro, we investigated whether these responses are biologically relevant in the maize rhizosphere. To this end, GFP-expressing cells of P. putida FBC004 were mixed into the soil prior to planting seeds of either DIMBOA-producing wild-type plants (BX1), or BX-deficient bx1 mutant plants. After 7, 14 and 21 days of growth, roots of BX1 and bx1 plants from two independent genetic lines were collected and analysed for colonisation by P. putida FBC004 and other (non-GFP expressing) culturable rhizobacteria.

The first experiment was performed with soil that had been autoclaved once before the start of the experiment (Figure 5A), presenting a relatively low competition environment for introduced P. putida. Two-factor ANOVA of rhizosphere colonisation of plants from Line A revealed a statistically significant interaction between plant genotype (BX1 versus bx1) and bacterial cell type (P. putida versus others) at all three time-points (7 days: F1,14 = 9.151, p = 0.009; 14 days: F1,14 = 43.432, p<0.001; 21 days: F1,14 = 7.977; p = 0.014), even though a statistically significant main effect of BX genotype could not be detected. Inspection of the data revealed that more P. putida cells were recovered from roots of DIMBOA-producing BX1 plants than from roots of DIMBOA-deficient bx1 plants; this was not the case for numbers of other culturable rhizobacteria. Holm-Šidák comparisons confirmed significantly higher P. putida cell numbers in rhizosphere washes from BX1 roots compared to that from bx1 roots (statistical probabilities are presented in Figure 5A). For line B, a significant main effect of BX genotype was evident at day 7 (F1,16 = 18.163; p<0.001) and day 14 (F1,16 = 19.776; p<0.001), but not at day 21 (F1,16 = 3.775; p = 0.070). However, a statistically significant interaction between
Figure 2. Tolerance of *Pseudomonas putida* KT2440 to DIMBOA. A. *In vitro* growth of *P. putida* KT2440 is not affected by up to 0.5 mM DIMBOA, whereas the ubiquitous soil bacterium *Agrobacterium tumefaciens* is increasingly affected at concentrations of 0.01 mM DIMBOA and greater. Growth was quantified by determining average OD$_{600}$ values ($n=5$). B. In the presence of *P. putida* KT2440, DIMBOA degradation is significantly accelerated, but accumulation of MBOA is significantly reduced. Shown are best fitting polynomial regressions ±99% confidence intervals. DIMBOA and MBOA quantities are expressed as relative peak areas (HPLC-DAD), normalised to DIMBOA peak areas at the start of each experiment.

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BX genotype and rhizobacterial cell type was apparent at all three time-points, including day 21 ($F_{1,16} = 6.122; p = 0.025$). Again, Holm-Sidak comparisons indicated significantly greater numbers of *P. putida* cells in the rhizosphere of *BX1* plants compared to *bx1* plants at all three time-points (Figure 5A). Hence, *BX1*-dependent exudation of DIMBOA stimulates rhizosphere colonisation by *P. putida* bacteria.

To investigate whether BX-dependent attraction of *P. putida* is also apparent in a more competitive soil environment, we repeated the experiment in non-autoclaved soil (Figure 5B). A significant main effect of BX genotype was observed at day 7 in seedlings from line A ($F_{1,10} = 6.725; p = 0.027$), but not at any later growth stage. Holm-Sidak comparisons confirmed significantly higher numbers of *P. putida* cells in the rhizosphere of 7-day-old *BX1* seedlings, but no statistically significant difference in the number of other culturable rhizobacteria (statistical probabilities are presented in Figure 5B). For line B, a significant main effect of BX genotype was observed at 7 days ($F_{1,11} = 4.904; p = 0.049$). Although no significant main effect of BX genotype was observed at 14 days ($F_{1,12} = 4.547; p = 0.054$), there was a significant interaction between BX genotype and rhizobacterial cell type at this time-point ($F_{1,12} = 6.425; p = 0.026$). Subsequent Holm-Sidak comparisons confirmed significantly increased numbers of *P. putida* in the rhizosphere of *BX1* plants at both 7 and 14 days (Figure 5B). At 21 days there was no longer a statistically significant main effect of plant genotype, nor was there a statistically significant interaction between plant genotype and rhizobacterial cell type (Figure 5B). Together, these data indicate that BX exudation in non-autoclaved soil stimulates rhizosphere colonisation by *P. putida* of relatively young seedlings. This BX effect becomes variable by 14 days and is absent in 21 day-old plants.

**Discussion**

The rhizosphere is an energy-rich niche that is characterised by a rapid turnover of chemical compounds exuded from plant roots [37]. Before rhizobacteria can exploit these compounds in the rhizosphere, they must first locate their host and tolerate potentially toxic allelochemicals in root exudates. In this study, we provide evidence that rhizosphere-colonising *P. putida* cells are tolerant of the N-heteroaromatic allelochemical DIMBOA (Figure 2), which is exuded in relatively high quantities from roots of young maize seedlings (Figure 1). Since BXs are nitrogen-containing metabolites, it might be expected that constitutive DIMBOA exudation by seedlings must provide significant ecological benefits, outweighing the high metabolic cost. Apart from allelopathic activity by DIMBOA [13,14], our study revealed that DIMBOA also acts as a below-ground
Recruitment of Beneficial Bacteria by Maize Roots

Figure 4. Taxis of P. putida KT2440 towards DIMBOA. A capillary-based assay was used to assess chemotactic responses. Data represent average numbers of colony forming units (CFU ± SEM) from 1 µL glass capillaries containing motility buffer (control), 0.1% casamino acid (positive control), or 5 µg mL^-1 DIMBOA. Cells were extracted from capillaries after 30 minutes of incubation and enumerated on solid medium. doi:10.1371/journal.pone.0035498.g004

Our results confirm the hypothesis that DIMBOA acts as a chemo-attractant for P. putida. This hypothesis was confirmed by our subsequent chemotaxis assays, demonstrating positive taxis of P. putida KT2440 towards DIMBOA (Figure 4).
**Figure 5. Rhizosphere colonisation of DIMBOA-producing (BX1) and DIMBOA-deficient (bx1) maize lines by green fluorescent protein (GFP)-expressing P. putida and other culturable rhizobacteria in autoclaved (A) and non-autoclaved soil (B).** P. putida cells were introduced into the soil prior to planting of maize seeds. Shown are average values (CFU g\(^{-1}\) root fresh weight ± SEM; n = 6–8), corresponding to P. putida or other rhizobacteria. Cells were enumerated after 7, 14 and 21 days of plant growth by plating root surface washes onto solid agar medium. Probabilities indicate the likelihood of the differences between BX1 and bx1 plants, within one line at each time-point, occurring by chance (Holm-Šidák pair-wise multiple comparisons) when two-factor ANOVA indicated a statistically significant (α = 0.05) main effect of BX genotype and/or a statistically significant interaction between maize BX genotype and bacterial cell type.

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*P. putida* KT2440 is relatively tolerant of DIMBOA in comparison to other soil bacteria (Figure 2A). We subsequently found that *P. putida* KT2440 accelerates degradation of DIMBOA and its direct breakdown product MBOA (Figure 2B), indicating BX catabolism [20]. Such a mode of tolerance is supported by our transcriptome analysis, which revealed seven DIMBOA-inducible genes that may be associated with degradation of \(\text{N}^-\text{heteroaromatic compounds} \) (Table S1). These genes include *nuoCD* (PP4121) and *nuoG* (PP4124), which encode subunits of NADH dehydrogenase I, PP4690 encoding a Rieske 2Fe-2S family subunit of soluble dioxygenases, PP0256 encoding a molybdopterin oxidoreductase, PP4661 encoding a putative oxidoreductase, and the \(\alpha/\beta\) hydrolase-fold Superfamily genes PP4540 and PP4551, members of which catalyse degradation of the \(\text{N}^-\text{heteroaromatic compound} 1\text{H}-3\text{-hydroxy-4-oxoquinoline} \) by *P. putida* 33/1 [49]. We conclude that this mechanism of BX tolerance provides *P. putida* KT2440 with a competitive advantage over other micro-organisms in exploiting the maize rhizosphere.

Our soil-based colonisation assays revealed that *P. putida* cells colonise maize roots of DIMBOA-synthesising lines in greater numbers than roots of DIMBOA-deficient lines. Although BX-dependent rhizosphere attraction of *P. putida* occurred in both...
autoclaved and non-autoclaved soil (Figure 5), the difference in *P. putida* colonisation between *BX1* and *bx1* lines in non-autoclaved soil was only consistent between both lines during relatively young developmental stages of the plants (Figure 5B). This age-dependent decline in *P. putida* response to BXs concurs with our finding that DIMBOA root exudation declines steadily as seedling age (Figure 1). In autoclaved soil however, this age-dependence was unclear. Autoclaved soil provides a much less competitive environment for introduced *P. putida* cells than non-autoclaved soil. It is therefore possible that the lower DIMBOA exudation rates of older plants remains sufficient to attract bacteria from non-autoclaved soil to the rhizosphere. Alternatively, it is possible that HDMBOA-glc, which did not show a noticeable age-dependent decline in exudation rate (Figure 1), contributes to bacterial recruitment at later developmental stages of the host plant. In both autoclaved and non-autoclaved soil, numbers of other rhizosphere bacteria were similar between roots of *BX1* and *bx1* plants (Figure 5). The difference in response to BX-exuding roots between *P. putida* and other rhizobacteria indicates that the composition of the rhizosphere microbial community is strongly influenced by the presence of DIMBOA in root exudates of the host plant. Apart from direct anti-microbial effects, DIMBOA root exudation may have an additive effect considering that DIMBOA-exposed *P. putida* showed enhanced expression of the *phzF* gene (Table S1), which encodes an enzyme in the biosynthesis of the broad-spectrum antibiotic phenazine [50]. Other studies have revealed bacterial attraction to primary metabolites in plant roots: L-leucine and L-malate attract *P. fluorescens* to tomato roots [51], while L-malate was found to promote attraction of *Bacillus subtilis* to the rhizosphere of *Arabidopsis thaliana* [4]. To our knowledge, DIMBOA is the first allelochemical shown to act as a chemo-attractant for beneficial rhizobacteria, and may explain why *P. putida* KT2440 is such a successful coloniser of the maize rhizosphere [29]. Our discovery also strengthens the notion that certain bacteria have acquired the ability to detoxify aromatic plant compounds, allowing them to exploit the energy-rich rhizosphere of plant roots exuding allelochemical compounds. These same bacteria can be exploited for the remediation of aromatic pollutants and herbicides [10].

In summary, our study has shown that root exudation of BXs attracts plant beneficial rhizobacteria. Although BX biosynthesis is mostly developmentally regulated [12], recent evidence has revealed that BX production by maize seedlings is to a certain extent responsive to environmental stimuli [52,53]. It would therefore, be interesting to examine BX-dependent effects on rhizobacteria during adaptive interactions between above- and below-ground defences. Our study also provides important knowledge for agricultural programmes aiming at sustainable yield improvement of cereal crops. Management of soil-borne diseases has proved problematic, because plant roots are relatively inaccessible to fungicidal chemicals. Furthermore, growth promotion by excessive soil fertilisation can have detrimental environmental impacts. Selection of cereal varieties with increased capacity for BX root exudation may lead to crops with an improved ability to recruit disease-suppressive and growth-promoting rhizosphere communities, reducing the need for repeated applications of fungicides and fertilisers. However, there is evidence that the specialist herbivore Western Corn Rootworm (*Diabrotica virgifera*) uses root-exuded BXs, such as DIMBOA and MBOA, as feeding cues [40,54]. The potential for crop improvement by selection for increased BX exudation should therefore be approached with caution. On the other hand, the accelerated degradation of DIMBOA and MBOA by *P. putida* (Figure 2B) may interfere with host location by *D. virgifera*, presenting a potential opportunity for biocontrol of this pest.

**Supporting Information**

**Table S1** Functional annotation of *Pseudomonas putida* KT2440 genes with a statistically significant induction at 1 h after exposure to 5 μg mL⁻¹ DIMBOA. Presented are 36 genes with annotated functions out of a total of 55 DIMBOA-inducible genes.

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**Author Contributions**

Conceived and designed the experiments: ALN RGW JT. Performed the experiments: ALN SA JT. Analyzed the data: ALN SA JT. Contributed reagents/materials/analysis tools: ALN JT. Wrote the paper: ALN JT.

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