The indole-alkaloid gramine shapes the bacterial communities thriving at the barley root-soil interface

Running title: Gramine as a determinant of the barley rhizosphere bacterial microbiota

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Abstract

The biosynthesis of plant allelochemicals underpinning inter-organismal relationships has been moulded by domestication and breeding selection. The indole-alkaloid gramine, whose occurrence in barley (*Hordeum vulgare* L.) is widespread among wild genotypes but virtually absent from modern varieties, is a paradigmatic example of this phenomenon. This prompted us to investigate how the exogenous application of gramine impacted on the rhizosphere bacterial microbiota of two, gramine-free, elite barley varieties grown in a reference agricultural soil. Our investigation revealed that the application of the indole-alkaloid gramine modulates the proliferation of a subset of soil bacteria with a relatively broad phylogenetic assignment. This effect is two-pronged: a limited, but significant, component of the barley microbiota responds to gramine application in a genotype- and dosage-independent manner while at the highest dosage this secondary metabolite attenuates the host recruitment cues of the barley microbiota. Interestingly, this latter effect displayed a bias for members of the phyla Proteobacteria. These initial observations indicate that gramine can act as a determinant of the bacterial communities inhabiting the root-soil interface.
Main text

Plants have evolved the capacity to shape soil microorganisms to establish symbiotic interactions with the microbiota proliferating at the root-soil interface [1]. Chief towards the recruitment and maintenance of a distinct plant microbiota is the release from roots of metabolites capable of modulating the interactions among plants, microbes and the surrounding environment [2]. Consistently, an increasing number of plant secondary metabolites have recently been implicated in the definition of the plant microbiota [3].

Interestingly, the processes of crop domestication and breeding selection, which gave rise to modern cultivated varieties, impacted on plant’s capacity to assemble a rhizosphere microbiota [4]. As wild ancestors of modern cultivated varieties may hold the capacity to adapt to marginal soil conditions, there is a growing interest in discerning the molecular mechanisms underpinning the recruitment of the microbiota in crop wild relatives and its contribution to plant’s adaptation to the environment [5]. This is particularly attractive for crops like barley (*Hordeum vulgare*) the fourth most cultivated cereal worldwide, for which modern and wild genotypes are readily available for experimentation [6, 7].

The genus *Hordeum* has evolved two main indole alkaloids with allelopathic and defensive functions, the benzoxazinoid DIBOA and gramine, whose biosynthesis is mutually exclusive within barley lineages [8]. In particular, gramine is the main allelochemical of the species *H. vulgare* which has historically been implicated in defensive responses against aphids [9] as well as foliar pathogens [10, 11]. Intriguingly, crop selection left a footprint on the biosynthesis of this secondary metabolite: modern varieties (*H. vulgare* subp. *vulgare*) fail to accumulate gramine to levels identified in their wild relatives (*H. vulgare* subp. *spontaneum*) [11, 12]. Of note, this apparent counter-selection for gramine within the domesticated material has been exerted on at least two distinct barley genes [13].
As benzoxazinoids recently gained centre-stage as main determinants of the microbiota in maize [14, 15] we hypothesized that in barley this regulatory role is played, at least in part, by gramine. To test this hypothesis, we exposed two ‘Elite’, gramine-free, barley genotypes, the cultivars Morex and Barke [13], to exogenous applications of gramine and we assessed the impact of these treatments on the taxonomic composition of the bacteria thriving at the root-soil interface using a cultivation independent approach.

To evaluate the effects on the composition of the barley rhizosphere microbe associated communities due to the external addition of gramine, we sequenced the hypervariable V4 region of the 16S rRNA gene of 45 samples, extracted from rhizosphere and bulk soils. Pre-germinated seeds of the two-row malting Barke and the six-row malting Morex, selected for their demonstrated inability to accumulate gramine in their tissues were grown for 4 weeks in a previously characterised agricultural soil designated “Quarryfield” [7, 16]. Two different concentrations of a solution of gramine, 24 µM and 46 µM, representing a range of concentration of this metabolite in a panel of wild barley genotypes [12] were applied to the pots, along with a mock control (G0), 4 days after seedling transplanting. The retention capacity of our reference soil for gramine was assessed on unplanted soil specimens (Supplementary Figure 1). At early stem elongation, plants were excavated from the soil and we generated 6983 582 high-quality amplicon metagenomic 16S amplicon sequencing reads using an established Illumina MiSeq protocol (Supplementary information).

Upon processing of the sequencing reads in silico (Supplementary Information) and consistent with previous observations of barley plants grown in the same soil type [7, 16], we failed to identify a significant effect of either the microhabitat or treatment on the alpha-diversity
parameters of the tested communities (Supplementary Figure 2). Conversely, we observed a clear impact of the external application of gramine on the bacterial communities thriving at the root soil interface regardless of the applied concentration: both a Canonical Analysis of Principal Coordinates computed on a Bray-Curtis dissimilarity matrix (Supplementary Figure 3) and a Principal Coordinates Analysis (PCoA) built on the Weighted Unifrac distance (Supplementary Figure 4) revealed a partition of the microbiota according to the applied treatment. Regardless of the matrix used, the effect of gramine appeared more pronounced on the unplanted soil communities when compared with rhizosphere specimens. Congruently, a permutational analysis of variance computed on both matrices indicated a significant effect of a) the individual microhabitat, unplanted soil, Morex and Barke rhizosphere, respectively (R2 ~40%) b) the treatment (R2 ~5%) and c) their interaction term (R2 ~7%; Supplementary Table 1, Adonis p <0.05, 5 000 permutations) on the bacterial microbiota. Taken together, this suggests that the gramine application exerts a selective pressure on the proliferation of a defined subset of members of the microbiota, rather than on their presence/absence, and this pressure appears stronger on unplanted soil communities. It is therefore legitimate to hypothesize that, considering also the convergence of rhizosphere profiles on the computed ordination (see Supplementary Figures 3 and 4), Elite genotypes may have evolved the capacity of reverting, at least in part, the selective pressure of gramine on soil bacteria. To test this hypothesis, we implemented a set of pair-wise comparison between individual genera retrieved from unplanted soil and rhizosphere communities at the three levels of gramine tested. In agreement with our hypothesis, we discovered that the majority of genera enriched in the rhizosphere of either genotype does not respond to gramine treatment (Figures 1A and1B, Wald test, p <0.05, FDR corrected). Conversely, 15 genera whose cumulative relative abundance represented ~2.06% and 1.75% of the Morex and Barke rhizosphere communities respectively, were identified as gramine-responsive in a genotype-independent manner (Figure 1D, Wald test, p <0.05, FDR corrected). Interestingly, when we evaluated the impact of gramine on the host control of the
rhizosphere microbiota we identified an interference between the application of gramine and the endogenous host recruitment cues of the tested genotypes. For instance, plants exposed to no gramine or the lowest dosage displayed a differential enrichment between cultivars of 17 and 20 genera, respectively (Figures 2A and B, Wald test, $p < 0.05$, FDR corrected). Conversely at the highest gramine dosage the host genotype effect was limited to 5 genera differentially enriched (Figure 2C, Wald test, $p < 0.05$, FDR corrected). When we inspected the taxonomic affiliation of these differentially enriched genera, we noticed a predominance of members of the phylum Proteobacteria (Figures 2E-F).

Our results indicate that the application of the indole-alkaloid gramine is capable of modulating the proliferation of a subset of soil bacteria with relatively broad phylogenetic assignments. This effect is two-pronged: a component of the barley microbiota responds to gramine application in a genotype- and dosage-independent manner while at the highest dosage this secondary metabolite attenuates the host recruitment cues of the barley microbiota with a bias for members of the phylum Proteobacteria. It is interesting to note that gramine applications failed to trigger a sustained enrichment of members of the Actinobacteria, which can be considered as an hallmark of elite, gramine-free, barley genotypes grown in the same soil type [7] and in other modern/ancestral plant pairs [17]. As gramine biosynthesis has previously been reported as stress induced [11, 18], we anticipate that exposure to different soils, and therefore different microbiomes, is likely to amplify (or obliterate) the effect of this metabolite on edaphic microbes. We therefore propose to capitalise on these initial observations and the expanding genomic resources for barley [19, 20] to resolve the genetic basis of gramine biosynthesis and ultimately elucidate its adaptive value for plant-microbe interactions.

Data availability
The sequences generated in the 16S rRNA gene sequencing survey are deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB39836. The version of the individual packages and scripts used to analyse the data and generate the figures of this study are available at https://github.com/Stramon1um/gramine_microbiome.

**Figure legend**

**Figure 1. Gramine modulates bacterial abundances at the barley root-soil interface** A) Genera simultaneously enriched in pair-wise comparisons retrieved from unplanted soil and Morex rhizosphere, B) and from unplanted soil and Barke rhizosphere, at the three levels of gramine tested. Vertical bars denote the number of genera enriched shared or unique for each comparison, while the horizontal bars the number of genera enriched in the indicated gramine concentration. In A and B genera differentially enriched a $p < 0.05$, Wald Test, FDR corrected.

C) Heatmap of the 15 bacteria, classified either at Order or Phylum level, significantly enriched in rhizosphere samples in a genotype- and gramine dosage- (24 and 46 $\mu$M, respectively) independent manner.

**Figure 2. Gramine application attenuates the genotype effect on the rhizosphere microbiota** Ternary plots depicting bacteria distribution across the indicated microhabitats in sample exposed to A) no gramine, B) gramine 24 $\mu$M or C) gramine 46 $\mu$M. In each plot, individual dots depict individual bacteria whose size is proportional their sequencing abundances. Position of the dots within the plots reflects the contribution of each microhabitats to bacterial abundances. Coloured dots denote bacterial significantly enriched ($p < 0.05$, Wald Test, FDR corrected) either in Morex versus Barke (magenta dots) or Barke versus Morex (blue dots). Only bacteria significantly enriched in rhizosphere samples versus bulk soil included in the plots. Pie charts depicting the taxonomic assignment at class level of genera differentially enriched at D) no gramine, E) gramine 24 $\mu$M or F) gramine 46 $\mu$M. The size of the slices is proportional to the number of genera assigned to a given class in the given comparisons.
**Authors contributions**

MM, TM and DB conceived and designed the experimental approach. MM performed the experiments. JM and PH generated the 16S rRNA sequencing reads. MM and DB analysed the data. MM, TM and DB wrote the manuscript, all the authors reviewed and approved is publication.

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9. Corcuera LJ. Biochemical basis for the resistance of barley to aphids. *Phytochemistry*


Differential bacterial enrichments

- Bacteria significantly enriched in Morex
- Bacteria significantly enriched in Barke
- Bacteria not enriched

**Taxonomic affiliation**

- Actinobacteria
- Alphaproteobacteria
- Bacteroidia
- Gammaproteobacteria
- Other taxa