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Abbreviations : ABA, abscisic acid; PGPR, plant growth-promoting rizobacteria

Declarations of interest : none

No graphic requires color reproduction

Abstract

Plant growth-promoting rhizobacteria (PGPR) naturally aid plant growth, development and tolerance to stress. Yield increase by the commercial isolate *Azospirillum lipoferum* CRT1 was recently attributed to an enhanced sprouting success. In order to provide the first biochemical and physiological analysis of sprouting enhancement by PGPR, seed germination and metabolism were followed by time-lapse photography and GC/MS-based metabolomics, respectively, after inoculating two differentially-responding maize cultivars with *A. lipoferum* CRT1. Bacterial growth on the seeds and plantlet development were also determined. Bacterial inoculation of the seeds of one cultivar led to a 6-8 h hastening of radicle emergence, increased surface bacterial counts, lower contents of energetic primary metabolites before radicle emergence and increased photosynthetic yield, and root surface area, in 3-leaf plantlets. None of these changes were observed on the other maize cultivar that rather accumulated greater levels of stress-related metabolites shortly after radicle emergence. Bacterial counts and cell division-driven central root growth increased in parallel and similarly on both cultivars. *A. lipoferum* CRT1 stimulated pre-germinating or defense events in a cultivar-dependent manner in maize after rapid (less than 24 h) recognition with initially resting seeds. This PGPR isolate therefore bears agronomic potential as a biopriming agent.

Key words: Azospirillum, biopriming, germination, maize, PGPR, seed metabolism.

1. Introduction

Over the past few decades, inoculation of seeds with plant growth-promoting rhizobacteria (PGPR) has proved to be an effective and ecologically-friendly agro-engineering practice to increase the durability of food production and limit its ecological impact (Duhamel and Vandenkoornhuyse, 2013; Gupta et al., 2015). PGPR constitute a diverse group of soil bacteria that naturally associate with plant roots for a host-specific reciprocal benefice whereby PGPR have prime access to root exudates and plant host growth and tolerance to stress are enhanced. A meta-analysis of 91 field and 175 pot studies conducted between 1981 and 2008 on the inoculation of cereal seeds with *Azospirillum* isolates has for example concluded to an average 10-15% increase in grain and forage yields respectively (Veresoglou and Menexes, 2010) making of this innocuous PGPR genus a prime target for agronomic application developments. The physiological and molecular mechanisms leading to yield increases by *Azospirillum* have been the subject of numerous studies.

Most isolates of *Azospirillum* elicit lateral root growth under field and laboratory conditions (Couillerot et al., 2012) to increase the host plant root system surface area and therefore its capacity to prospect for soil minerals and water. Auxin production by some *Azopsirillum* strains has been hypothesized to contribute to lateral root growth (Bruto et al., 2014). Mineral nutrition can be further improved by the expression of the bacterial gene *nifH* responsible for the ammonification of gaseous nitrogen (Brock et al., 1993) or by the release of bacterial siderophores (Pedraza, 2015). Probably needed to sustain root growth, photosynthetic activity is enhanced by *Azospirillum* inoculation (Rozier et al., 2016). Although it is tempting to conclude that increased mineral nutrition and photosynthesis lead to increased yield at harvest, no such direct link has been established and some studies have challenged this simplistic explanation. Nitrogen fixation has for example been shown to be too meager to justify yield increases (Orhan et al., 2006; Pérez-Montaño et al., 2014) and co-inoculating a strong nitrogen-fixing agent like *Azotobacter* did not further increase *Azospirillum*-based yield enhancement of cereals (Veresoglou and Menexes, 2010).

A recent study conducted two consecutive years and four fields with contrasted soil or farming practices suggested that improved maize sprouting by the commercial PGPR strain *A. lipoferum* CRT1 was the main cause of yield increases. Indeed, no correlation was found between yield enhancement and the ability of the bacterium to modify its host root-to-shoot biomass ratio (via enhanced root growth) or photosynthetic potential (Rozier et al., 2017). The content of primary metabolites involved in nitrogen or phosphorus metabolism were also not

modified in proportion to yield modifications. However, increased yield correlated with 6-leaf plantlets being larger in all of their proportions, a phenomenon that was already visible on plant radicle length under artificial growth conditions as early as 48 h *post*-sowing. Since increased yield was most pronounced in fields where sprouting success was below 80%, these authors hypothesized that accelerated plant development during sprouting allowed faster access to lower, and more secure, soil layers to increase seedling survival under unfavorable climate conditions and, as a consequence, mature plant density and final yield (Rozier et al., 2017). Improved sprouting by PGPR was also observed by several independent investigations (Raj et al., 2004; Cassán et al., 2009; Kaymak et al., 2009; Nezarat and Gholami, 2009; Noumavo et al., 2013; Rozier et al., 2017). A 22.44% increase of maize sprouting was, for example, observed after a co-inoculation with *Pseudomonas fluorescens* and *P. putida* and a 20.39% increase after *Azospirillum* spp. inoculation (Noumavo et al., 2013). Maize and soybean sprouting success was also improved by *Bradyrhizobium japonicum* E109 and *A. brasilense* Az39 inoculation (Cassán et al., 2009). Nevertheless, precise developmental steps affected by PGPR during sprouting have not yet been determined and a link between seed germination and the growth-promoting activity of PGPR remains to be established.

In order to provide the first detailed characterization of the impact of *Azospirillum* on plant seed germination, radicle emergence and growth were followed by time-lapse photography using the same commercial inoculant, *A. lipoferum* CRT1, as used by Rozier et al., 2017. Seed primary metabolite and starch contents were estimated throughout the germination process as well as bacterial development on the surface of the seeds. To ensure that any modification in the germination processes was the consequence of the phytostimulatory activity of the bacterial partner and not of the exogenous addition of a large number of live bacteria on a seed, all experiments were conducted on two maize cultivars, one that displayed sprouting promotion and one that displayed no phytostimulation at this stage. The impact of *A. lipoferum* CRT1 inoculation on the root length and photosynthetic yield of 3-leaf maize plantlets was also estimated on these two maize genotypes.

2. Materials and methods

2.1. Bacterial culture

Pre-culture and culture conditions from a glycerol stock of the PGPR strain *Azospirillum lipoferum* CRT1 (Fages and Mulard, 1988) were similar as in (Rozier et al., 2016). The adequacy of the bacterial suspension was evaluated by PCR with the *A. lipoferum* CRT1 specific primers F1676-Q1 (5'-ATCCCGGTGGACAAAGTGGA-3') and 1837-Q2 (5'-GGTGCTGAAGGTGGAGAACTG-3'). The proper mobility and the absence of contaminants were checked by light microscopy. The final bacterial suspension was diluted to a concentration of 1.10^7 cells/mL with sterile UP water and immediately used to inoculate maize seeds.

2.2. Seed inoculation

Seeds of the maize cultivars FriedriXX (RAGT) and FuturiXX (RAGT) were calibrated for weight and shape to homogenize their germination responses. The experimental design involved four treatment conditions : cultivars FriedriXX or FuturiXX that were either inoculated with *A. lipoferum* CRT1 or mock-inoculated with sterile UP water. Mock treatments with water acted as controls to ensure that *A. lipoferum* and not another microorganism originally present on the seeds was the initiator of the described effects. Each treatment was carried in sterile 12x12 cm square Petri dishes containing 20 seeds each. Two layers of Whatman paper (Chromatography paper, 3 mm Chr, Whatmann international Ltd Maidstone, England) were pre-wetted with sterile UP water and placed at the bottom of the Petri dishes. The seeds were laid (sown) on the wet papers. All seeds were similarly inoculated with either *A. lipoferum* CRT1 or sterile UP water by three successive additions of 50 µL deposited alternatively on the different sides of the seed at 1 h intervals. This procedure allowed a complete covering of the seed with the treatment solution. It also prevented a temporary drying of the surface of the seeds (and the freshly deposited bacteria) by the fast imbibition of the seeds that was complete 3 h after the start of water contact. Petri dishes were kept closed during the inoculation process.

Each assay was replicated successively three times with three independently-prepared bacterial cultures (three biological replicates).

The seeds were not surface-sterilized before the experiment because the objective of this study was to provide a rational explanation for field observations (Rozier et al., 2017) were non-sterile seeds were used. Surface-sterilized seeds also generate plantlets that are prone to wilting under artificial growth conditions, a phenomenon that is rare with non-sterilized seeds. The absence of *A. lipoferum* CRT1 in the original batches of seeds used in

this study was confirmed by PCR with specific primers (see above section on bacterial culture).

2.3. Follow-up of the germination process

For the four treatment conditions, two closed Petri dishes (two technical replicates) containing 20 inoculated seeds each (total of 2x20=40 seeds per condition) were kept closed in a water-saturated atmosphere at 23°C with dim natural light (5 555 Lux - 99 $\mu mol.m^{-2}.s^{-1}$ Photosynthetic Photon Flux Density at mid-day) during the entire germination process. Every 2 h, a camera (Canon 550D) was activated by a remote controller coupled to an intervalometer. A graduated ruler was laid next to the seeds to measure the length of the radicles on the pictures.

2.4. Bacterial density measurement on the seeds

For the four treatment conditions, two closed Petri dishes (two technical replicates) containing 20 inoculated seeds each (total of 2x20=40 seeds per condition) were maintained in a water-saturated atmosphere at 23°C with dim natural light (5 555 Lux - 99 $\mu mol.m^{-2}.s^{-1}$ Photosynthetic Photon Flux Density at mid-day) during the entire germination process. At 0 h, 24 h, 44 h and 72 h *post*-sowing, five seeds of each Petri dish were sampled and placed in 3 mL of a tryptone-NaCl solution (1 and 8.5 g/l respectively) in a 15 mL Falcon tube. Tubes were shaken by a FastPrep System (FastPrep 24™ 5G, MP Biomedicals, Santa Ana, USA) for 40 s at 6.0 m/s. Serial 10-fold dilutions of the supernatants of the bacteria-inoculated seeds were performed and 100 µL of the 10^{-2}-10^{-4} dilutions were spread in duplicate on Red Congo agar medium. For the mock-inoculated conditions, the undiluted supernatant was spread. The CFU (colony-forming units) were counted, averaged among technical replicates (seeds originating from the two Petri dishes having received the same treatment) and the results expressed as CFU per seed. PCR were performed on random colonies using the same set of primers as described above for bacterial culture characterization to ensure that the enumerated colonies corresponded to *A. lipoferum* CRT1.

2.5. Starch and primary metabolite content measurements

For the four treatment conditions, two closed Petri dishes containing 20 inoculated seeds each (total of 2x20=40 seeds per condition) were maintained in a water-saturated atmosphere at 23°C with dim natural light (5 555 Lux - 99 µmol.m^{-2}.s^{-1} Photosynthetic Photon Flux Density at mid-day) during the entire germination process.

The dry weight (DW) of the seeds of each treatment condition was determined at the inoculation step, and 44 h and 72 h after the start of the inoculation (*post*-sowing) by weighting ten seeds after being milled and stored 24 h at 110°C.

Five maize seeds (five independent seeds treated with the same bacterial culture) of each treatment condition were then harvested at sowing and 44 h, and 72 h, *post*-sowing. Their radicles were removed if visible and the rest frozen in liquid nitrogen in order to halt metabolism. Individual seeds were crushed in liquid nitrogen with a mortar and a pestle. From the powder of each seed, 100 mg was used for the analysis of starch content and 80 mg for primary metabolite analysis (list of annotated metabolites in Table S1).

Starch content was estimated with an enzyme assay kit for total starch measurement (AA/AMG, Libios, Pontcharra sur Turdine, France) according to the manufacturer instructions. Results were expressed as percent (w/w) starch per unit of seed dry weight (% DW). The entire assay was replicated a second time (biological replicate) with an independent bacterial cultures.

All primary metabolites listed in Table S1 were subjected to the same extraction and derivatization steps and were analyzed simultaneously on each extract. Extraction, derivatization and analyses were adapted from previously published protocols (Rozier et al. 2016; 2017). In short, eighty milligrams of each milled seed (n=5, 4 conditions) were transferred into a 2 mL Eppendorf tube set on ice and mixed with 300 µL of cold methanol (-20°C) and 7 µL of ribitol (1 mg/mL - Sigma-Aldrich, St Louis, USA) as internal standard. The samples were then heated at 70°C during 15 min under stirring and cooled at room temperature. Two hundred µL of $CHCl_3$ was added to each tube and the tubes vortexed and incubated 5 min at 37°C under stirring before adding 400 µL of UP water. The samples were vortexed and centrifuged 5 min at 14 000 rpm. Four hundred µL of the supernatants were introduced in a new 1.5 mL Eppendorf tube and centrifuged again 5 min at 14 000 rpm. The supernatants were dried 4 h in a Speed Vacuum (Centrivap Concentrator, Labconco, Kansas city, USA) and stored in a desiccator until the derivatization step. A control sample that lacked seed powder was constructed in parallel.

Just before derivatization, the samples were dried during 30 min in a Speed Vacuum. They were then solubilized in 40 µL of methoxylaminehydrochloride (20 mg/mL in pyridine -

Macherey-Nagel, Düren, Germany), vortexed and incubated 1h30 at 30°C under stirring before introducing 70 µL of MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide - Macherey-Nagel, Düren, Germany). The samples were vortexed, incubated 30 min at 37°C, transferred into vials and immediately analyzed by GC-MS.

GC/MS analyses (Agilent 7890A and 7000A, Santa Clara, USA) were carried out as described (Rozier et al., 2016). Metabolite annotations were performed with TagFinder (Luedemann et al., 2008) and baseline correction of the chromatogram with MetAlign (Lommen and Kools, 2012). Peak areas of each chromatogram were estimated with the MassHunter software (Agilent, Santa Clara, USA). For every annotated compound, peak areas were normalized with those of the internal standard and expressed as percent of the maximum value among all conditions.

A total of 126 metabolites was annotated in all samples that encompassed the two cultivars, three time-points and five repeats (list of annotated metabolites in Table S1). Based on the large time distribution window of radicle emergence of the batches of seeds, three time points were chosen to represent the metabolomes of resting seeds (at sowing - t=0 h) and seeds at late pre-germination (first seeds showing radicle emergence - t=44 h) and early *post*-germination (the radicle has emerged from all seeds - t=72 h) stages.

2.6. Physiological measurements on 3-leaf plantlets

Seeds of both cultivars that were either inoculated with *A. lipoferum* CRT1 or with UP water (8 seeds for each of the four treatments conditions) as described above under 'Seed inoculation' were then sown in pots (5 cm diameter and 18 cm in height) filled with a natural soil from a commercial maize field (luvisoil with a clay-loam texture devoid of limestone and consisting of 26.9% sand, 38.3% loam and 34.7% clay with 324.9 g of water per kg of soil at field water holding capacity) and grown indoor as described (Rozier et al., 2016). Eleven days later, the plantlets had reached the 3-leaf stage. The photosynthetic potential of the second leaf from the base was measured with a portable photosynthesis yield analyzer (Mini-PAMII, Walz, Germany) equipped with the clip holder 2035-B as described (Rozier et al., 2016). The plants were then uprooted and their root system imaged with a scanner (Expression 1680, Epson, Suwa, Japan). The cumulative root length was estimated with the WinRhizo software (Regent Instruments Inc., Quebec, Canada) as described (Rozier et al., 2016).

2.7. Statistical analyses

All statistical analyses were carried out with the open source software R using the RVAidememoire, mixOmics and ade4 packages (downloaded on March 2016 - R Development Core Team 2008). Non-parametric two-by-two Wilcoxon mean comparison tests were conducted to assess significance levels of differences in metabolite content means between inoculated and mock-inoculated conditions for each cultivar. Significance levels were recorded at $p<0.1$ (‘), $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***).

3. Results

3.1. Impact of *A. lipoferum* CRT1 on seed germination

The first seeds of the maize cultivars FriedriXX and FuturiXX that started to germinate (marked by radicle emergence) did so 42 h and 46 h *post*-sowing respectively and all seeds of these lots of seeds had germinated 62 h and 78 h *post*-sowing, respectively (data not shown). Imbibition time was similar for both cultivars and was roughly 3 h, most water uptake taking place within the first hour (data not shown).

The presence of *A. lipoferum* CRT1 on the seeds of FriedriXX hastened radicle emergence by 6-8 h, reducing by as much the times when the first and last seeds germinated. This is evidenced by a longer mean radicle length at times when no, or few, of the non-inoculated seeds had germinated (Fig. 1). This increase in radicle length was nevertheless short-lived and was only significant from 40 h to 52 h (less than 50% germination of non-inoculated seeds) *post*-sowing. It was maximum 44 h *post*-sowing (23-fold increase - $p<0.002$, Wilcoxon non-parametric test). As the percent of non-inoculated seeds that had germinated neared 100% (62 h *post*-sowing) the mean radicle length of the two lots of seeds were similar.

To the opposite, bacterial inoculation had no effect on the timing of the emergence of the first radicle and on the mean radicle length of the maize cultivar FuturiXX (Fig. 1).

All of these results suggest that *A. lipoferum* CRT1 inoculation leads to an acceleration of radicle emergence in a cultivar-specific manner, and that it has no impact on radicle growth after its initial, rapid, protrusion from the seed.

3.2. Impact of *A. lipoferum* CRT1 on seed starch content

Because starch degradation is a common *post*-germination marker, starch content was measured during the first 72 h that followed sowing. No difference was seen between the non-inoculated seeds of both cultivars at sowing (69.97 ± 5.37% dry weight (DW) and 67.68 ± 4.77% DW, respectively) and contents remained constant up to 3 days *post*-sowing (Fig. 2). Inoculation with *A. lipoferum* CRT1 had no significant impact on seed starch content ($p>0.05$ – Wilcoxon test) of both cultivars.

3.3. Impact of *A. lipoferum* CRT1 on seed small metabolites

Unlike starch, many small metabolite contents are modified at each step of the *pre*- and *post*-germination processes. A GC/MS-based metabolomic analysis of the seeds was therefore performed in order to unveil the specific effects of *A. lipoferum* CRT1 on the seed germination metabolism (see Table S1 for a list of annotated substances). A representation on its first two axes of a principal component analysis (PCA) of the soluble metabolite contents data matrix revealed that the metabolomes of the resting seeds (at sowing - t=0 h) of the two maize cultivars were different (Fig. 3). Both were modified similarly by the germination process 44 h and 72 h *post*-sowing since distribution ellipses (95% distribution estimated from the variability among the five repeats) roughly moved in a similar direction on the PCA plot to only gain partial overlap at 72 h. In agreement with a more precocious germination, the metabolome of FriedriXX seeds was subject to more changes to the 44 h *post*-sowing.

Unsurprisingly, *A. lipoferum* CRT1 had no impact on the seed metabolomes at the time of its deposition on the seeds (Fig. 3). Nevertheless, it impacted the metabolomes of the seeds of both cultivars 44 h and 72 h *post*-inoculation. Ellipse separation (and hence metabolome change) was the greatest for inoculated FriedriXX seeds between 0 h and 44 h and was so weak for this inoculated cultivar between 44 h and 72 h that the metabolomes of bacteria-inoculated seeds at 44 h, 72 h and of mock-inoculated seeds at 72 h were nearly indistinguishable. Inoculation with *A. lipoferum* CRT1 therefore appeared to have hastened germination-induced metabolic changes in FriedriXX. It, however, elicited changes that were different from those induced by germination in the other cultivar, FuturiXX at 44 h and 72 h. Ellipses of bacteria-inoculated seed metabolomes of this cultivar indeed did not follow the same pattern of displacement by inoculation on the PCA plot as for FriedriXX.

In total, the contents of 67 substances were found to be significantly affected by bacterial inoculation ($p<0.1$ – Wilcoxon mean comparison test) (Table 1). In FriedriXX, energy-related metabolites such as sugars and TCA cycle components were mostly affected 44 h after bacterial inoculation while mostly amino acid metabolism-related metabolites were affected 72 h *post*-inoculation. The concentration of all of these metabolites was decreased by bacterial inoculation. FuturiXX was subjected to fewer metabolic changes that were not focused on any specific metabolic pathways. Most of them displayed concentration increases. Noteworthy, three metabolites which contents were increased 72 h *post*-sowing/inoculation were related to plant stress. These were the phytohormone abscisic acid (ABA), the key precursor of phenolic phytoalexins, 4-hydroxycinnamic acid (*p*-coumaric acid), and the microbial, non-plant, element N-acetylglucosamine.

3.4. Development of *A. lipoferum* CRT1 on maize seeds

In order to better understand the interaction between *A. lipoferum* CRT1 and its host seed, its growth was estimated during the germination process of inoculated seeds of both cultivars. The bacteria multiplied significantly on the surface of the seeds of both cultivars during the 72 h that followed their inoculation on the seeds (Fig. 4). However, the timing of the start of rapid bacterial division was different on both cultivars. On FuturiXX, it occurred in parallel to radicle elongation. On FriedriXX, rapid bacterial development occurred a day before radicle emerged of the first seeds and then increased more slowly to follow radicle growth. As a consequence, CFU were greater on FriedriXX before radicle emergence (t=24 h) and during early radicle emergence (t=44 h) than on FuturiXX. Bacterial density on the two cultivars was no longer significantly different 72 h *post*-inoculation when all seeds had germinated (Fig. 4).

3.5. Impact of A. lipoferum CRT1 on 3-leaf plantlets

To relate the observed germination effects to classically-measured effects of PGPR on plants, modifications of the root system architecture and photosynthetic potential were measured on 3-leaf plantlets of both cultivars. As shown on Fig. 5, *A. lipoferum* CRT1 inoculation of the seeds of FriedriXX led to a higher cumulative root length. It was not due to longer central, or adventitious seminal roots but to increased lateral root number and growth as previously described by numerous studies (Couillerot et al., 2012; Rozier et al., 2016). The

photosynthetic potential (Fv/Fm) was also increased in this cultivar. These two parameters were unaffected in FuturiXX. Cumulative leaf area was not affected at this stage in both cultivars (data not shown).

4. Discussion

It is well established that PGPR aid plants in various aspects of their development and functioning (Vacheron et al., 2013) such as the growth of their supporting root system (Pérez-Montaño et al., 2014), nitrogen, phosphorus and iron nutrition (Richardson et al., 2009), photosystem functioning (Samaniego-Gámez et al., 2016) and protection to several stresses such as drought (Vurukonda et al., 2016), cold (Kakar et al., 2016) and pathogen attack (Beneduzi et al., 2012). A few reports have also shown that sprouting is positively affected by several genera of PGPR such as *Pseudomonas, Azospirillum* and *Bradyrhizobium* (Raj et al., 2004; Cassán et al., 2009; Kaymak et al., 2009; Nezarat and Gholami, 2009; Noumavo et al., 2013; Rozier et al., 2017). Despite the potential impact of a higher germination rate on final crop yield (Rozier et al., 2017), the present study is the first one to focus on the biochemical and physiological mechanisms affected by PGPR during the first steps of the life of a plant.

Seed germination is a complex process that is terminated by the emergence of the embryo radicle through the seed coat (Bewley, 1997). It is started by water imbibition that increases seed weight in one quick step. This rise in internal water content will restore a functional hydrophobic force to help cell membranes and proteins to reconfigure properly. Mitochondria, and DNA, will be repaired to allow protein synthesis from stored and newly-made RNAs. Germination inhibitors will be suppressed and small energetic substances will diffuse throughout the seed to reach their usage targets. The radicle will then emerge thanks to rapid cell elongation followed by a slower cell division-driven growth. Once the radicle grows, seed weight increase resumes *via* active water intake. Internal polymeric energy stores such as starch, proteins and lipids will only be consumed after germination (*i.e.* after radicle emergence) to fuel seedling development (Bewley, 1997).

The time-lapse measurements of radicle growth and the metabolomic analyses of this study both agree to indicate that the commercial PGPR strain, *A. lipoferum* CRT1, speeds pre-germination events culminating in radicle elongation in a cultivar-specific manner and that it does not promote radicle growth *post*-germination *via* increased cell division. For one of the two cultivars, radicle emergence could indeed be hastened by 6-8 h in parallel to an accelerated consumption of small energetic substance. Effects of the bacteria on the seed

metabolism were then meager once radicle had emerged, a time when root growth was similar between PGPR-inoculated and non-inoculated seedlings and when starch degradation had not yet started. As such, the PGPR strain acted as a bio-priming agent. If germination success was close to 100% under the artificial conditions used in this study, it can drop significantly under field conditions due to the unpredictable nature of weather conditions. Under such circumstances, cereal seed priming is routinely conducted through a short storage at high temperature and humidity or micronutrient application to hasten the germination of all seeds and secure sprouting (Perera and Cantliffe, 1994; Mirshekari, 2012). The results of this study confirm earlier suggestions (Kaymak, 2010; Reddy, 2012) that PGPR are a valuable alternative to the canonical energy-demanding physical treatment of seeds.

Narrow, cultivar level, host-specificity is a common characteristic of the expression of the phytostimulatory action of PGPR on their hosts (Walker et al., 2011; Chamam et al., 2013; Drogue et al., 2014). This study reveals that hastened germination, increased production, and growth, of lateral roots and improved functioning of the photosynthetic apparatus followed the same pattern of cultivar specificity. Although a correlation between these apparently unrelated physiological effects cannot be established without the testing of a large cohort of maize genotypes, two similarities can be noticed between the growth responses of maize seeds and 3-leaf plantlets to *A. lipoferum* CRT1. In both cases, cell division-mediated central root growth was unaffected by bacterial inoculation so that increased root surface resulted from either root cell elongation at germination or lateral root growth in plantlets. The metabolomes of bacterially-inoculated seeds (this study) and plantlet saps (Rozier et al., 2016) both revealed a decrease in glucose content in parallel to growth stimulation. If glucose was the only common hexose to have a decreased concentration in 3-leaf plantlet sap (Rozier et al., 2016), it was not the case in pre-germinating seeds where simple sugar content lowering was less specific. In the later case, energetic metabolite content lowering may be the consequence of an accelerated germination process. But it may also be its cause. It may indeed also result from the observed more abundant bacterial growth on the surface of growth-stimulated seeds a day prior to germination. It is also increasingly recognized that common hexoses, and especially glucose, do not only serve as a reservoir of energy in plants and that they initiate a cell signaling cascade that modulates the functioning of several plant growth hormones including auxins (Gibson, 2004). Glucose, for example, impacts auxin-regulated gene transcription to modulate root growth and development in *Arabidopsis* (Mishra et al., 2009). Simple hexoses also act as germination inhibitors in maize (Dekkers and Smeekens, 2018) by repressing the biosynthesis of auxins (LeClere et al., 2010). As glucose

exerts a product feedback inhibitory action on photosystem functioning, it is possible that glucose may be a central regulator, and coordinator, of *A. lipoferum* CRT1 action in plants from the lifting of dormancy to vegetative development. It may also explain why this isolate generates auxin-type phenotypes in its hosts despite not having *ipdC* (C. Prigent-Combaret pers. comm.), a key gene for auxin biosynthesis in other *Azospirillum* (Bruto et al., 2014).

The absence of a germination response of the maize cultivar FuturiXX to *A. lipoferum* CRT1 does not signify an absence of response of this cultivar to the presence of the bacteria. The rise in content of three substances in the seed metabolome 72 h *post*-inoculation indeed suggests that the germinating seeds developed a defense response towards the PGPR. One of them was abscisic acid (ABA), a substance up-regulating defense responses (Vishwakarma et al., 2017) and inhibiting germination (Dekkers and Smeekens, 2018). In agreement, 4-hydroxycinnamic acid, a central biosynthetic precursor of the phenolic defenses (Weisshaar and Jenkins, 1998) also exhibited a higher content as well as the microbial-specific component N-acetylglucosamine that is a central building block of Gram-positive bacterial and fungal cell walls (Scheffers and Pinho, 2005) and of Gram-negative bacterial membrane associated lipopolysaccharides (Gronow and Brade, 2001). The presence of this substance in the inoculated seed extracts suggests some level of microbial degradation by plant defense lytic enzymes present in cereal seeds (Roberts and Selitrennikoff, 1988; Grover, 2012). As the seeds used in this study were not sterile, it is impossible to know whether it originated from inoculated *A. lipoferum* CRT1 or previously present microorganisms. An analysis of the seed transcriptome is now required to complement the present metabolomic analysis and determine the extent of the defense response developed by the host germinating seed, a defense response that, according to the results of this study, does not prevent *A. lipoferum* CRT1 development but may be such that it impairs the induction of a growth response at all steps of the plant development.

The nature of signaling substances is, to the present day, unknown between *Azospirillum* and its hosts. The results of this study, however, suggest that they are at play as early as 24 h after the contact between *A. lipoferum* CRT1 and resting maize seeds. They also induced a unique pattern of cultivar-specific responses that either led to rapid bacterial growth and accelerated radicle emergence or to delayed bacterial development and the upgrading of seedling defenses.

Competing interests

454 The authors declare that they have no competing interests.

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Figure legends

Fig. 1. Impact of *A. lipoferum* CRT1 inoculation on maize radicle length. Two maize cultivars, FuturiXX (balls) and FriedriXX (squares) were mock-inoculated (open symbols) or inoculated (full symbols) with *A. lipoferum* CRT1. Mean radicle length +/- SD was calculated on a lot of n=20 seeds. Wilcoxon non-parametric test between inoculated and mock-inoculated seeds were conducted at each time point (* $p<0.05$; ‘ $p<0.1$). Similar results were obtained during two additional independent biological replicates conducted with independent bacterial cultures

Fig. 2. Impact of *A. lipoferum* CRT1 on maize seed starch content. Maize cultivars FuturiXX and FriedriXX were mock-inoculated (white bars) or inoculated (black bars) with *A. lipoferum* CRT1. The starch content of the seeds was estimated at 0 h, 44 h and 72 h post-sowing and was expressed as % of dry matter (DW). No significant differences were found at $p<0.05$ by using one-way ANOVA and two-by-two Wilcoxon non-parametric mean comparison tests (n=5). Similar results were obtained with a separate biological replicate generated with an independent bacterial cultures

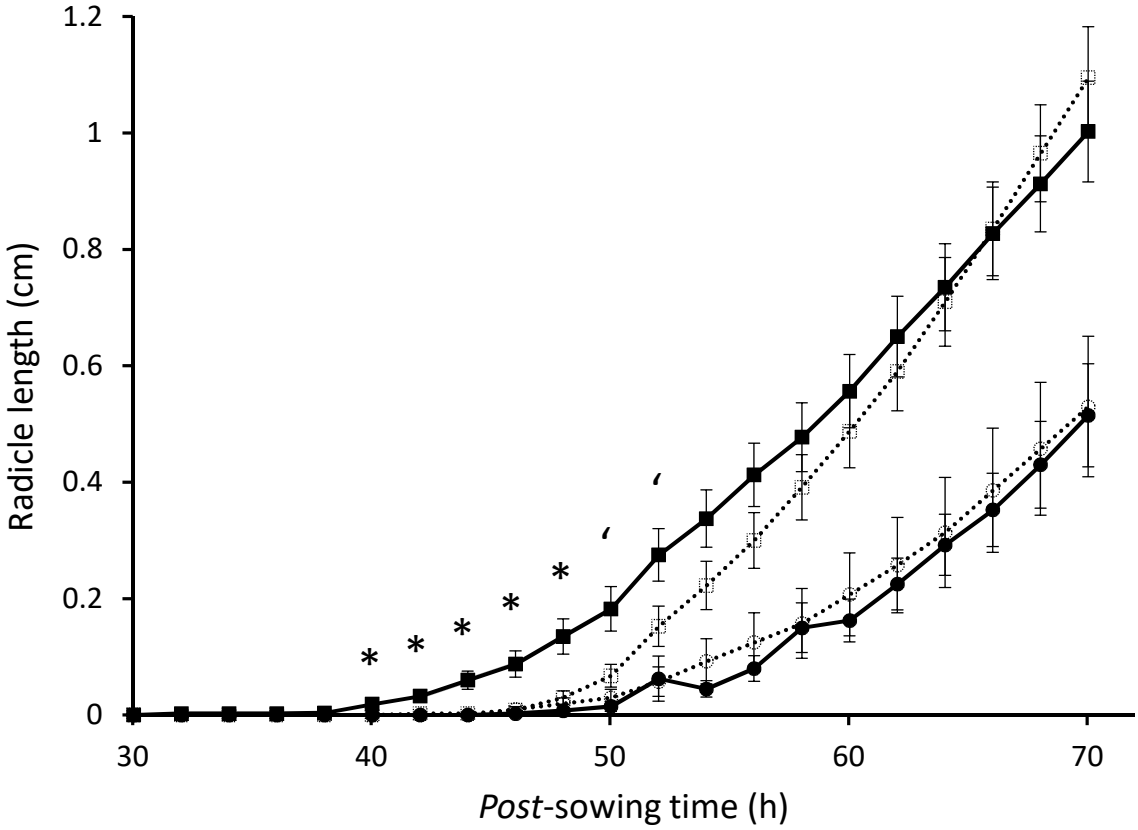
Fig. 3. Principal component analysis (PCA) plot of maize seed metabolite contents. Cultivars FuturiXX (Futu - balls) and FriedriXX (Frie - squares) were either mock-inoculated (NI - empty symbols) or inoculated (I - filled symbols) with *A. lipoferum* CRT1 and analyzed for metabolite content (n=5) at sowing (t0 - lighest grey) or at 44 h (t44 - medium grey) and 72 h (t72 darkest grey) post-sowing

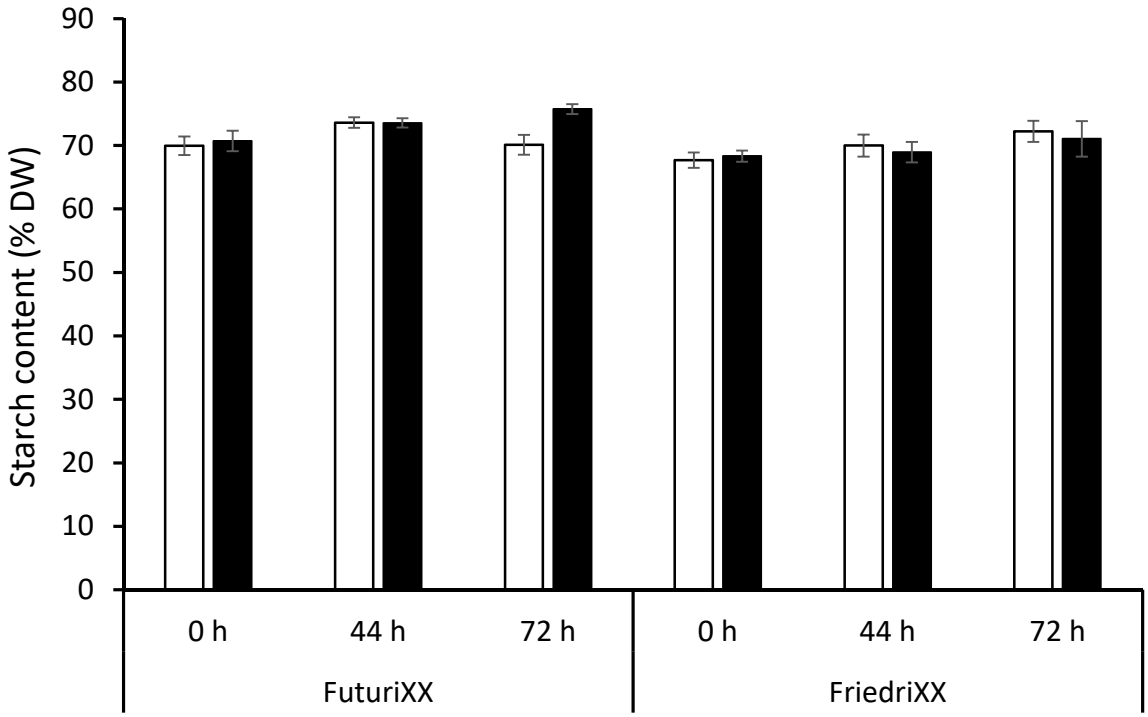
Fig. 4. Colony forming units (CFU) of *A. lipoferum* CRT1 on germinating maize seeds. Microorganisms adhering to the seeds of cultivars FriedriXX (squares) and FuturiXX (balls) were detached and the CFU of *A. lipoferum* CRT1 counted on a selective solid medium. Data represent means +/- SE (5-seed extracts and 2 counts). Significance levels of the differences between the two cultivars were assessed at each time point with the Wilcoxon mean comparison test (***, $p<0.001$; **, $p<0.01$). Similar results were obtained with two biological replicates made with independent bacterial cultures

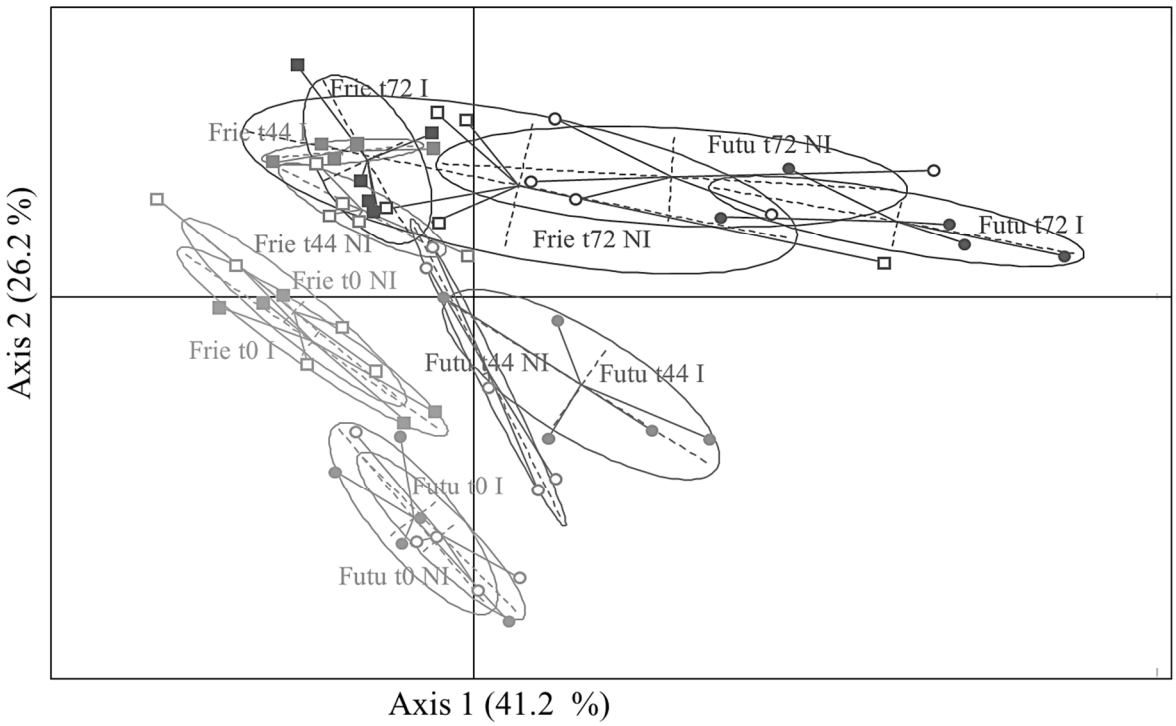
Fig. 5. Modification of cumulative root length (a) and photosynthetic potential (b) of 3-leaf maize plantlets inoculated with *A. lipoferum* CRT1. The length of the central, seminal and lateral roots was summed. The maximum quantum efficiency of photosystem II (Fv/Fm) of the second leaf from the bottom was used as an estimate of the youngest mature leaf photosynthetic efficiency. FuturiXX and FriedriXX were either mock-inoculated (white bars) or *A. lipoferum* CRT1-inoculated (black bars). Significance levels of the impact of the bacterium were assessed with the Wilcoxon mean comparison test (***, $p<0.001$; *, $p<0.05$)

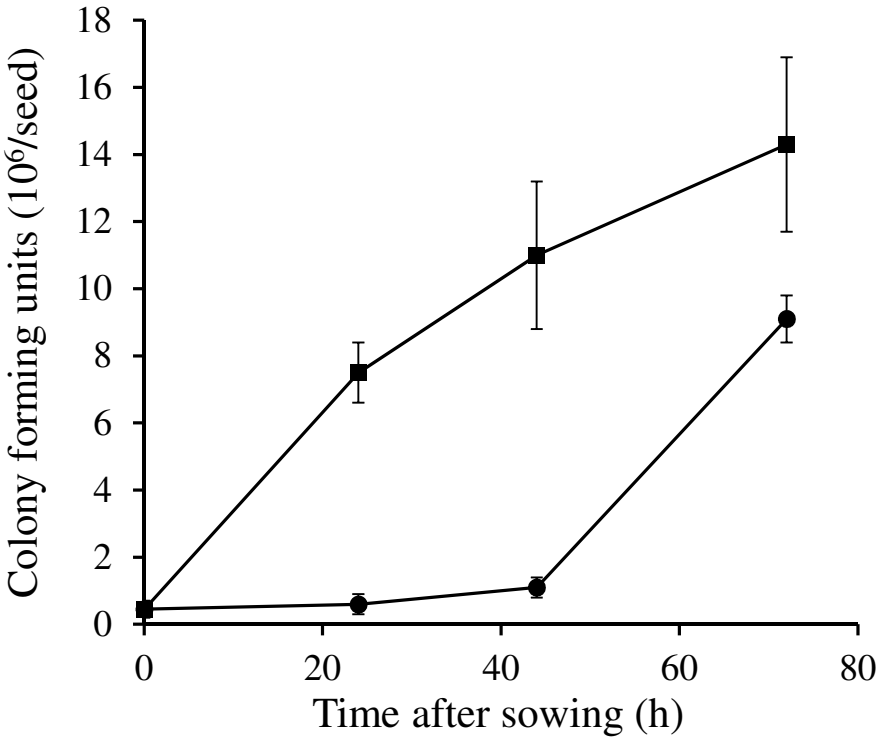
Supporting information

Table S1 List of annotated substances


1.2
1
0.8
0.6
0.4
0.2
0
Radicle length (cm)
30
40
50
60
70
Post-sowing time (h)


Starch content (% DW)
90
80
70
60
50
40
30
20
10
0
0 h
44 h
72 h
0 h
44 h
72 h
FuturiXX
FriedriXX


Frie t72 I
Frie t44 I
Futu t72 NI
Futu t72 I
Frie t72 NI
Frie t44 NI
Frie t0 NI
Frie t0 I
Futu t44 NI
Futu t44 I
Futu t0 I
Futu t0 NI
Axis 2 (26.2 %)
Axis 1 (41.2 %)


Colony forming units (10⁶/seed)
18
16
14
12
10
8
6
4
2
0
0
20
40
60
80
Time after sowing (h)

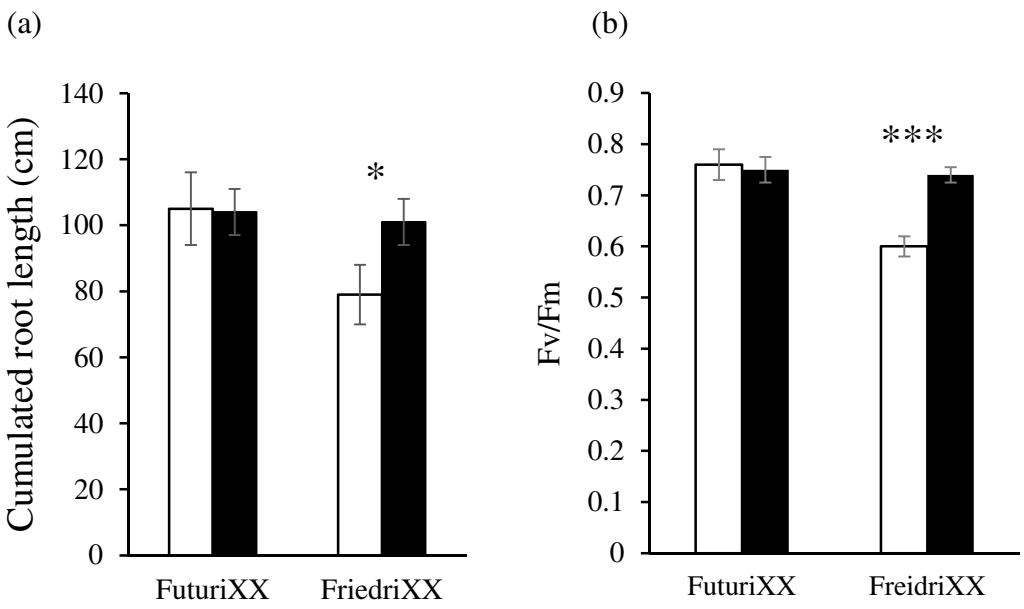

(a)
Cumulated root length (cm)
140
120
100
80
60
40
20
0
*
FuturiXX
FriedriXX
(b)
Fv/Fm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
***
FuturiXX
FreidriXX

Table 1

Metabolite content changes in maize seeds after inoculation with *A. lipoferum* CRT1.

Metabolite [a]	FuturiXX [b]		FriedriXX [b]	
	44 h	72 h	44 h	72 h
Glycolysis-TCA and related				
A105001-Lactic acid	63.7±14.9	-73.8±42.9	93.5±15.5	**-70.0±3.6 ***
A123004-Glyceraldehyde	36.5±14.7	**28.9±2.3 '**	5.5±1.4	**-31.9±2.0 '**
A176014-Aconitic acid, trans-	17.3±9.6	**36.2±2.2 '**	19.1±8.6	-58.8±14.4
A182003-Isocitric acid	17.7±4.6	**40.8±3.9 '**	16.8±6.6	-62.5±18.9
A134001-Succinic acid	0.1±2.5	25.8±10.3	**-45.2±1.6 ***	-44.5±13.1
A137001-Fumaric acid	13.9±4.3	35.9±9.1	**-69.6±2.3 ***	-24.8±8.4
Sugars and related				
A167011-Ribose	-8.5±7.1	48.5±15.2	**-23.5±0.1 ***	-0.6±0.1
A199002-Galactonic acid	6.0±3.3	17.4±13.2	**-47.6±0.3 ***	-1.0±0.2
A186001-Gulonic acid, 2-oxo-	-3.8±2.1	**10.7±0.1 ***	**-44.2±1.7 ***	-4.8±2.2
A189002-Glucose	7.8±11.0	**9.3±1.3 '**	**-40.7±1.8 ***	-4.5±6.7
A189001-Mannose	24.4±9.2	**26.5±2.1 '**	**-50.7±2.6 ***	-2.5±1.9
A233002-Glucose-6-phosphate	13.1±4.1	**44.9±2.0 ***	**-31.3±0.7 ***	-18.1±4.1
A188001-Galactose	-93.2±68.1	-45.7±15.3	**-54.5±0.6 ***	-10.4±3.7
A188004-Fructose	-95.6±48.8	-42.8±17.4	**-66.2±3.5 ***	16.8±5.2
A198006-Glucopyranose, D-	2.3±4.1	42.8±10.2	**-28.5±0.2 ***	-13.1±3.1
A267008-*beta*-D-Fructofuranosyl-(2.1)-*beta*-D-fructofuranose	-10.6±7.3	9.9±6.2	**-17.8±0.4 ***	-6.2±0.6
A211003-Ribose-5-phosphate	13.6±4.2	24.1±6.1	**-22.8±1.7 '**	40.5±7.1
A269006-Cellobiose, D-	-1.4±0.3	**9.6±1.1 '**	**-16.8±0.9 '**	-5.9±1.4
A179001-Arabinonic acid	8.8±9.5	**35.2±2.7 '**	22.4±6.5	-52.7±11.4
A231001-Mannose-6-phosphate	85.4±14.7	**58.0±2.3 '**	24.6±.1	-45.9±9.4
A193004-Glucuronic acid	NA	**38.2±1.7 '**	NA	NA
A287001-Isomaltose	32.1±9.2	**54.0±5.8 '**	NA	-47.6±9.8
A172002-Rhamnose	-39.1±0.5 *	**-26.1±0.4 ***	-5.9±1.9	-7.8±3
A204001-Galactaric acid	79.0±3.1 '	21.3±9.1	74.7±20.1	-41±10.1
Amino acids and related				
A164007-Asparagine	-97.8±16.7	42.4±16.2	**-47.3±2.0 ***	-72.7±10.0
A118002-Isobutanoic acid, 2-amino-	2.1±0.9	20.8±8.1	**-32.8±2.1 '**	-22.5±6.1
A192003-Lysine	-88.9±22.9	-14.2±6.1	**-62.7±3.2 '**	24.1±7.1
A194002-Tyrosine	-5.1±4.9	46.3±14.7	**-61.3±4.9 '**	22.1±6.9
A140001-Threonine	91.3±26.2	73.4±18.7	-21.9±6.5	**-60.6±3.5 ***
A133001-Glycine	30.0±11.4	44.8±10.0	0.8±0.2	**-46.4±0.4 ***
A153002-Pyroglutamic acid	**28.6±6.6 '**	**35.9±2.9 ***	5.7±5.7	**-34.9±2.2 ***
A129002-Leucine	9.6±3.6	**41.9±0.6 ***	33.8±8.7	**-57.4±3.8 '**
A122001-Valine	17.2±9.1	**50.4±4.2 '**	-11.4±3.1	-33.7±5.5
A142007-Methionine	-20.7±5.9	**57.6±5.1 '**	-23.8±7.8	-80.2±13.9
A145025-Glycinamide	19.6±8.3	**31.8±1.2 '**	-6.8±2.2	32.1±8.2
A164001-Phenylalanine	46.0±9.6	**37.7±2.5 '**	33.0±7.3	-41.5±9.5
Polyols				
A149002-Threitol	-5.6±12.4	42.4±13.9	**-33.7±1.2 ***	-15.6±15.7
A150002-Erythritol	-1.3±1.8	0.5±1.5	**-35.1±1.0 ***	-18.8±4.2
A194001-Galactitol	25.4±4.9	20.7±6.1	**-57.0±2.9 '**	28.9±8.3
A193002-Mannitol	12.3±3.9	-30.2±12.6	**-64.9±3.8 '**	27.5±7.5
A171001-Xylitol	-3.3±1.1	**14.6±0.4 ***	-1.5±0.9	-0.5±0.1
Nucleotides and related				
A136001-Uracil	46.8±10.5	94.5±10.6	**-39.8±2.1 '**	**-89.3±3.6 '**
A249007-Thymidine	-17.9±9.1	16.9±14.1	NA	**-28.9±1.4 '**
Hormones and polyamines				
A175002-Putrescine	83.8±10.6	18.1±4.2	11.6±6.2	**-19.4±2.1 '**

	A228007-Abscisic acid	16.6±8.2	**48.8±2.7 ***	21.9±7.2	-29.5±10.2
Phenolic compounds					
	A210001-Ferulic acid, trans-	NA	14.6±0.1	**-41.9±2.9 '**	-9.2±6.1
	A199001-Caffeic acid, cis-	35.8±10.2	44.1±0.1	**-45.6±1.1 '**	-7.3±3.1
	A180006-Cinnamic acid, 4-hydroxy-.	-34.5±9.7	**-42.5±0.2 ***	-3.9±1.5	**-65.5±2.2 '**
	A178004-Vanillic acid	-7.7±3.1	**66.9±3.2 '**	-15.7±7.3	-15.1±3.2
	A185001-Quinic acid	-94.0±13.1	8.6±10.0	**-20.9±0.8 '**	40.3±10.0
Others					
	A122003-Malonic acid	**-60.8±6.7 '**	-15.7±6.3	-27.6±7.2	37.2±7.6
	A129001-Phosphoric acid	**23.7±0.4 ***	21.4±6.7	4.5±6.7	-25.0±12.4
	A135003-Glyceric acid	**98.9±2.6 ***	17.6±9.4	-2.2±0.9	**-58.0±2.7 ***
	A106002-Glycolic acid	3.9±1.1	**72.7±2.2 '**	1.5±0.7	**-37.3±0.1 ***
	A143001-Glutaric acid	20.3±8.1	**26.3±0.1 '**	-7.4±2.1	-3.3±0.3
	A195002-Ascorbic acid	NA	**35.7±0.8 '**	NA	-30.8±6.7
	A145012-Isobutanoic acid, 3-amino-	-98.5±21.2	**30.7±1.6 ***	-27.3±7.9	16.6±5.1
	A203003-NA	NA	**11.9±2.1 '**	**-41.2±1.9 ***	6.6±3.1
	A140003-NA	14.1±2.3	31.7±1.2	**-70.3±2.3 ***	-33.1±12.9
	A155004-NA	-2.7±34.9	29.1±35.0	**-88.0±4.1 ***	-94.0±22.0
	A237001-NA	25.6±8.1	19.8±0.1	**32.4±3.7 '**	**-56.8±0.8 ***
	A230001-NA	16.4±6.1	**57.5±4.5 '**	-22.3±5.4	10.7±10.0
	A234001-NA	50.6±18.0	**87.5±3.7 '**	NA	NA
	A254003-NA	8.4±18.3	**45.7±4.2 '**	-32.5±8.4	-49.6±12.4
	A143003-NA	25.1±9.5	**34.1±2.7 '**	-11.1±7.8	-3.2±1.0
	A189019-NA	16.3±4.3	**39.5±3.9 '**	15.7±3.3	-59.0±6.8
Non-plant substances					
	A207013-Glucosamine, N-acetyl-	20.6±0.4	**20.6±0.4 ***	-12.0±0.1	-23.3±0.3

[a] Metabolites were identified by comparison to a standard. They are grouped according to their metabolite class (KEGG - https://www.genome.jp/kegg/). Compound names are preceded by their code in the Golm Metabolome Database (http://gmd.mpimpgolm.mpg.de/) and they are ordered by increasing code number within each metabolite class. Unidentified metabolites are named according to their code in the Golm Metabolome Database followed by the NA label.

[b] Ratios of mean content values of inoculated over mock-inoculated seeds of maize cultivars FuturiXX and FriedriXX at 44 h and 72 h *post*-sowing are listed and expressed as percentage values *((I - NI)/NI *100) (mean* +/− percentage relative standard error). Wilcoxon non-parametric tests were conducted for each metabolite to compare inoculated and non-inoculated plants mean values (n=5 - bold lettering - * $p < 0.05$; ' $p < 0.1$). Only substances displaying significant content changes in at least one condition are listed. NA indicates that the metabolite was detected in less than two of the five replicate samples.