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Plant defense stimulation by natural isolates of *Bacillus* depends on efficient surfactin production

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Abstract

Some plant-associated *Bacilli* can induce systemic resistance (ISR) in the host, which contributes to their protective effect against phytopathogens. Little is known about the variety of elicitors responsible for ISR that are produced by *Bacillus* strains. Working with a particular strain, we have previously identified the surfactin lipopeptide as main compound stimulating plant immune-related responses. However, with the perspective of developing *Bacilli* as biocontrol agents, it is important to establish whether a central role of surfactin is generally true for isolates belonging to the *B. subtilis*/*amyloliquefaciens* complex. To that end, we set up a comparative study involving a range of natural strains. Their secretomes were first tested for triggering early defense events in cultured tobacco cells. Six isolates with contrasting activities were further evaluated for ISR in plants based both on macroscopic disease reduction and on stimulation of the oxylipin pathway as defense mechanism. A strong correlation was found between defense-inducing activity and the amount of surfactin produced by the isolates. These results support a widespread role of surfactin as a non-volatile elicitor formed by *B. subtilis/amyloliquefaciens* and screening for strong surfactin producers among strains naturally secreting multiple antibiotics, could be an efficient approach to select good candidates as biopesticides.

Introduction

Plant roots constantly release a variety of organic compounds which diffuse in the few millimeters of the surrounding soil layer thereby creating a niche favorable for the development of very diverse microbial populations. Among these communities inhabiting the so-called rhizosphere, some bacteria (termed Plant Growth Promoting Rhizobacteria, also PGPR) may provide beneficial effects to the host plant by directly promoting its growth or by protecting it against phytopathogens (Lugtenberg and Kamilova, 2009). Commercialized as biopesticide products, these bacteria constitute potent new tools for sustainable agriculture (Choudhary and Johri, 2009).

PGPR may act through several mechanisms for disease protection including competition with pathogens for ecological niche/substrate in the rhizosphere and production of antibiotics with direct inhibitory activity on infectious agents (Bhattacharyya and Jha, 2012). This direct antagonism of pathogen growth is viewed as the most powerful and best characterized mechanism. Soil *Bacilli* and *Paenibacilli* (such as *Paenibacillus polymyxa*) actually devote from 4 to 8% of their genome to antibiotic synthesis and therefore display the potential to produce a vast array of structurally diverse antimicrobial compounds (Chen *et al.*, 2009; Rückert *et al.*, 2011; Stein, 2005). Certain isolates of *Bacillus*/*Paenibacillus* are among the most efficient microbial biocontrol agents (McSpadden Gardener, 2004) and some strains have been developed to reach the market level.

A third mechanism through which rhizobacteria may inhibit phytopathogens is the strengthening of the host plant's defensive potential via stimulation of its immune machinery. Historically, this last mechanism has first been discovered in response to pathogens. Upon perception of specific compounds secreted/harbored by the attacker (Pathogen Associated

Molecular Patterns, PAMPS), plants can actually evolve an array of defense mechanisms to counteract infection. This immune response is first manifested locally in the pest-colonized organ but may also occur systemically in the non-infected tissues via molecular signaling leading to the systemic acquired resistance (SAR) phenomenon (Durrant and Dong, 2004; Luna *et al.*, 2012; Shah, 2009; van den Burg and Takken, 2009). Interestingly, certain non-pathogenic rhizobacteria can also trigger a phenotypically similar immune response called PGPR-induced systemic resistance (ISR). A variety of elicitors are emitted by PGPR and are perceived by plant cells to initiate the ISR phenomenon. The number of these molecules that are known has globally increased these last decades (De Vleesschauwer and Höfte, 2009; Jankiewicz and Kołtonowicz, 2012; Van Loon, 2007) but remains quite limited in the case of Gram positive species. For *Bacillus* strains, volatile compounds such as 2,3-butandiol and acetoin, 2-aminobenzoic acid and lipopeptides are the sole metabolites that were identified as elicitors of plant defense responses to date (Henry *et al.*, 2012; Jourdan *et al.*, 2009; Ongena and Jacques, 2008; Ryu *et al.*, 2004; Yang *et al.*, 2011).

The main lipopeptides (LPs) synthesized by *B. subtilis/amyloliquefaciens* belong to the surfactin, iturin and fengycin families. They vary in the type, number and sequence of amino acid residues as well as in the nature of the peptide cyclisation. Moreover, within each family, several homologues differing in the length and isomery of the fatty acid chain are usually co-produced by *Bacillus* strains (Ongena and Jacques, 2008). These compounds have been demonstrated to play a crucial role in the biocontrol potential of numerous isolates (reviewed in (Ongena and Jacques, 2008; Raaijmakers *et al.*, 2010). LPs may contribute to the ability of the bacterium to efficiently colonize surfaces of plant roots. They also display strong and specific antibiotic activities allowing direct antagonism against various pathogens. In addition, the potential of surfactin as plant resistance inducer has been demonstrated in our laboratory either using overproducing mutants from a specific strain or by applying the pure compound (Ongena *et al.*, 2007; Jourdan et al., 2009). Disease reduction induced by surfactin was associated with defense-related metabolic changes either occurring very early after perception such as ion fluxes across the plasma membrane and oxidative burst generating reactive oxygen species (ROS) or expressed later as defense mechanisms *sensu stricto* that limit or inhibit further pathogen penetration into the host plant tissues (Jourdan *et al.*, 2009; Ongena *et al.*, 2007; Henry et al., 2011).

The huge potential of members of the *B. amyloliquefaciens*/*subtilis* species to trigger ISR does not seem to reflect the very limited number of their metabolites identified so far as elicitors. Other molecules than those cited above could thus putatively act as stimulators of systemic resistance. Alternatively, the ISR potential of *Bacillus* strains may rely on the potential to secrete/harbor the same specific compound. In this context, the present work was initiated to evaluate the importance of surfactin for the elicitation of ISR by various *Bacillus* strains. As a basis for our bottom-up strategy, we selected a range of rhizosphere isolates in function of their low, intermediate or high production of the lipopeptide under specific *in vitro* conditions. Their corresponding supernatant extracts were tested for their capacity to stimulate the oxidative burst (considered as marker of early defense response) in cultured tobacco cells and ISR in tobacco plants. A limited number of strains were further evaluated for their potential to trigger systemic resistance in tomato and disease reduction observed macroscopically was associated with the expression of molecular defense mechanisms stimulated in the host plant. Differential disease reduction by the various strains in tomato was also interpreted in light of their rhizosphere fitness and *in situ* surfactin production.

Results

Induction of oxidative burst in tobacco cells

For this comparative study, we selected seventeen *Bacillus* strains isolated from the phytosphere and belonging to the species *Bacillus subtilis (B.s.), Bacillus amyloliquefaciens (B.a.), Bacillus pumilus (B.p.)* and *Paenibacillus polymyxa (P.p.).* These isolates were selected according to their different lipopeptide signatures which were determined in agitated cultures using a rich medium optimized for production of these compounds. Some isolates did not produce any LPs; others produced two or all three families LPs, including iturins (or their bacillomycin-type variants), fengycins and surfactins, in specific relative proportions. This is illustrated in figure 1 presenting HPLC-MS analyses of culture extract for six representative strains among those included in this work. Among strains producing all three famillies of LPs, some isolates such as *B.a.* S499 produced mainly surfactins and only low levels of other LPs while others efficiently secreted both iturins and fengycins (QST713, FZB42) or iturins (98S). For clarity purposes, the terms surfactin, iturin and fengycin will be used throughout the following text in the singular form to designate the groups of surfactin, iturin and fengycin homologues with different fatty acid chains. Some strains (such as BNO1) did not produce any lipopetides of these families and their chromatograms only show background noise. For the purpose of this study, the *Bacillus* isolates were grown under pellicle/biofilm forming conditions in order to get closer to the natural context. Indeed, rhizobacterial micro-colonies developing on plant roots are considered as biofilm-related structures and not planktonic individual cells (Bais *et al.*, 2004; Ramey *et al.*, 2004). Surfactin production under these conditions was also quantified by LC-MS analysis and results are presented in figure 2A.

In a first approach, we wanted to establish a possible correlation between surfactin concentrations in crude supernatants of the various strains with the potential of these extracts to induce some early defense responses in plant cells. To that end, equal volumes of the various supernatants were tested on cultured tobacco cells for their oxidative burst-inducing activity. This phenomenon, measured through a luminol-based chemiluminescence assay, has been previously identified as readily quantifiable marker of defense response following surfactin elicitation (Henry *et al.*, 2011; Jourdan *et al.*, 2009). As shown in figure 2B, only supernatants obtained from cultures of strains *B.a.* S499, *B.s.* 98S, *B.s.* 76 and *B.s.* 23 significantly stimulated ROS accumulation in tobacco cells compared to water control. For these samples, treatments corresponded to final surfactin concentrations in contact with the tobacco cells of 13.8 µM, 9.5 µM, 11.6 µM and 7.4 µM respectively. It has been shown previously that surfactin is effective at triggering a consistent oxidative burst response in tobacco cells in the specific range of concentrations 5-20 µM (Jourdan *et al.*, 2009). These data thus provided a first indication about the importance of the lipopeptide for triggering early defense events since supernatant samples prepared from the other strains, which did not reach the threshold in surfactin concentration, were also not active at inducing oxidative burst. Moreover, the amplitude of oxidative burst response observed for the four supernatant samples was in the same range as observed by treating tobacco cells with 10 µM pure surfactin (Jourdan et al., 2009). In support to the dose-response relationship observed, a linear correlation could be established between surfactin concentration in the sample applied to tobacco cells and the H_2O_2 production level triggered in plant cells ($R^2 = 0.9515$) (Supplementary data 1).

In a second experiment, the four active culture supernatants were diluted in order to achieve a 10 µM surfactin concentration on tobacco cells. They were further serially diluted until 0.2 µM on plant cells and tested for ROS stimulation activity in comparison with the effect of the purified lipopeptide used at the same concentrations. Statistical comparison was conducted between several surfactin concentrations for a same strain. All concentrations except 0.2 µM triggered a significant response in tobacco cells compared to water control. For all strains, the highest concentration (10 µM) significantly triggered the strongest H_2O_2 production. Concentrations of 5, 2, and 1 µM caused an intermediate response between 10 µM and water control. Such asymptotic decrease in activity with increasing dilutions corresponded well to the one observed upon treatment with pure surfactin (Fig. 3). The presence of another ROS-eliciting molecule that would have been active in a different concentration range was thus unlikely.

In additional assays, we have fractioned crude cell free extracts on C18 cartridges in order to separate surfactin from less hydrophobic compounds present in the four active supernatants. Adsorbed material was step-wise eluted with increasing concentrations of methanol (0 %, 20 %, 40 %, 60 % and 100 % in water) and each fraction was tested for its ability to elicit an oxidative burst response. In all cases, only fractions eluted with pure methanol and containing surfactin triggered a response of tobacco cells (data no shown). This did not allow to rule out the possibility of another very hydrophobic molecule with similar dose/response relationship but collectively the data obtained with cultured cells supported the hypothesis that surfactin is the only elicitor contained in all the supernatants tested.

Induced systemic resistance in tobacco plants

Statistical analysis of data in figure 2A indicated a clear and statistically significant, segregation between producers and non producers of surfactin. Among producers, there is also some tendency to differentiate strains between medium and strong producers though this is not statistically significant for most strains. Nevertheless, supernatant extracts from six strains were selected on the basis of this categorization to further test their potential to induce systemic resistance in tobacco plants: *B.a.* S499 and *B.s.* 98S as strong producers of surfactin, *B.a.* FZB42 and *B.a.* QST713 as intermediate producers, and *P.p.* 56 and *B.s.* BNO1 as non-producers (Fig. 2 A).

In these assays, the extract tested for ISR-inducing activity and the pathogen were applied on different tissues to avoid direct antagonistic effects. Five-week old plants cultivated in hydroponic conditions were thus treated at the root level with equal volumes of the corresponding supernatant extracts and infected 48 h later, on detached 6th and 7th leaves (leaves were detached to achieve more reproducible infection rates in our conditions, see Methods section), with a spore suspension of *Botrytis cinerea*. Disease reduction was rated 3 days post-infection according to the percentage of spreading lesions that grew out of inoculation zones in treated plants compared to controls. Statistical analysis of disease reduction data (Fig. 4) showed that supernatant samples prepared from cultures of the strongest surfactin producer (S499) significantly triggered the most efficient protection of tobacco plants, with an average reduction of disease incidence of 41 %. By contrast, medium producers FZB42 and QST713 only triggered a weak protection not statitically different from the non producer strains. Meanwhile, 98S displayed an intermediate disease reduction between S499 and medium producers (Fig. 4). On the other hand, statistical analysis of disease incidence data showed that only S499 and 98S treatments trigger a significant protection compared to water control. A correlation could be plotted between the LP concentration of the supernatant samples and the triggered protection on leaves ($R^2 = 0.952$) (Supplementary data 2). These data showed that a dose-response relationship also occured between surfactin concentration and protection of tobacco against *B. cinerea*.

ISR triggered in tomato plants by living cells

We next wanted to test the disease control potential of living cells from the six strains against the same pathogen but using tomato as host plant. *Solanum lycopersicum* was chosen first in order to extend our observations to another species of the *Solanaceae* and second to allow further study of the defense mechanisms possibly related to ISR (see below). In these assays, bacterial cells were inoculated in the peat substrate and disease severity caused by

Botrytis cinerea was rated on the fourth leaves 5 weeks after bacterization and 3 days after infection.

Statistical analysis on *Botrytis* infection incidence indicated that all surfactin producing strains significantly protected the plants from disease, while non producers were completely unefficient. Also, disease reduction data presented in figure 5A show that both strong (S499 and 98S) and medium (QST713 and FZB42) producers were capable of inducing ISR to a significantly higher level than non producers (reduction in occurrence of spreading lesions as shown in figure 5B) but no statistically significant difference was observed between strong and medium producers of surfactin. However, considering average values for disease reduction (Fig. 5A), S499 and 98S were more efficient at triggering ISR (34 ± 5% and 31 ± 7% disease reduction) than QST713 and FZB42 (22 ± 3% and 26 ± 3%). However, the variability inherent to these ISR assays did not allow us to distinguish a statistically significant difference between the two groups. Differences in ISR potential between intermediate and very efficient surfactin producers are more contrasted by considering the percentages of strongly, moderately and slightly diseased plants in each treatment. Globally, plants treated with intermediate producers such as QST713 were more often strongly diseased and less frequently lightly diseased as compared to plants treated with strong producers like *B.a.* S499 (Fig. 5C) (Detailed data for the three other strains were not included in figure 5C for clarity purposes but are presented in supplementary data 3). Again, treatment with *P.p.* 56 (and *B.s.* BNO1) provided results similar to control plants.

Protection levels reached by S499 (34 ± 5%) in this experiment are quite similar to the ones observed after treatment of tomato plants with 10 µM purified surfactin (32 ± 13% , mean value calculated also from 3 independent ISR assays using each 10 plants per treatment and performed under hydroponic conditions).

Root colonization and surfactin production in recomposed exudates

We next wanted to establish whether this differential ability of the strains to trigger ISR may be due to differences in their ability to colonize the tomato rhizosphere and/or in their potential to produce elicitors *in planta*. However, an accurate determination of populations established on roots in these ISR assays was not possible due to the lack of suitable screening markers for most of these natural isolates. Alternatively, we used a gnotobiotic system in Petri plates filled with nutrient agar on which bacterized tomato plantlets were grown without contamination by auxiliary microflora. Microscope visualization and plate counting of biofilm-related populations formed by the various isolates after 14 d of incubation indicated that all strains efficiently colonized plant roots to a relatively similar level, execpt for BNO1 which displayed a significantly lower growth level (Fig. 6).

In addition, the studied strains were tested for their ability to use a medium simulating tomato root exudates for the production of surfactin. This experiment was based on the fact that the composition in organic substrates of exudates is very specific and the nutritional status is a key factor clearly impacting antibiotic production by root colonizing bacteria as we have previously shown for *Bacillus* lipopeptide production (Nihorimbere *et al.*, 2012; Nihorimbere *et al.*, 2009). In order to get closer to this natural nutritional context, bacteria were cultivated in a medium (referred as recomposed exudates, RE) containing, as sole carbon sources, the sugars, organic acids and amino acids typically found in tomato root exudates as determined by Kamilova and collaborators (2006). As shown in table 1, this RE medium supported growth of the six isolates to a similar level (ANOVA, $\alpha = 0.05$) which is in agreement with the similar colonization trends mentioned above. As revealed by LC-MS analysis of supernatants collected from these RE cultures, surfactin secretion was also efficient, with strains S499 and 98S producing the highest amounts of this LP and no trace of the lipopeptide was observed in extracts from *P.p.* 56 or BNO1. In this experiment, FZB42 produced a very low amount of surfactin while QST713 displayed an intermediate production level (Table 1). This means that surfactin productivity per cell unit could be enhanced in the first two isolates in accordance with their higher efficiency to trigger systemic protection.

Priming of the oxylipin pathway in resistant plants

Many works have reported the involvement of phenylpropanoid and/or oxylipin pathways as defense mechanisms stimulated in the host plant during PGPR-triggered ISR (Choudhary, 2011; De Meyer and Hofte, 1997; Mariutto *et al.*, 2011; Ongena *et al.*, 2004; Vanitha and Umesha, 2011). We therefore wanted to evaluate the possible induction of both pathways in leaves of plants treated with strains *B.a.* S499, *B.a.* QST713 and *B.s.* BNO1 as representatives of the three groups displaying differential ISR activities. However, no clear difference in the activity of phenylalanine ammonia lyase (PAL, entry enzyme of the phenylpropanoid pathway) could be observed between control and *Bacillus*-treated plants, whatever the isolate tested and the timing prior or post pathogen challenge (data not shown). By contrast, the activity of the lipoxygenase enzyme (LOX, entry enzyme of the oxylipin route) was significantly induced in *B.a.* S499-colonized plants two days after pathogen challenge but not before infection (Fig. 7A). The level of LOX activation correlated well with the one of ISR response since a slight but not significant increase in activity was observed in leaves of plants treated with the weaker resistance-inducer *B.a.* QST713 and no significant modification of LOX activity compared to control was observed for *B.s.* BNO1 (Fig. 7A). Moreover, enhanced LOX activity in S499-treated plants timely correlated with a significant accumulation of transcripts of genes corresponding to the two isoforms, *TomLOXD* and *TomLOXF* (Fig. 7B), that are associated with PGPR-induced ISR (Mariutto *et al.*, 2011). Interestingly, the enhanced LOX expressions resulted in a significant accumulation of their products 13-hydroperoxy-octadecadienoic (13-HPOD) and 13-hydroperoxy-octadecatrienoic

(13-HPOT) acids, two days after infection (Fig. 7C). In a way very similar to S499, root treatment with pure surfactin primed the plant to react more promptly to *B. cinerea* attack on leaves. As shown in figures 7A and 7B, elicitation with surfactin actually resulted in a transient but significant increase in LOX enzyme activity in infected leaves two days after pathogen challenge. Also the same hydroperoxide products, as in S499 treated plants, significantly accumulated in surfactin-elicited tomatoes upon *B. cinerea* infection (Fig. 7C). Again such stimulation was transient since those differences were no longer observed at 4 days post infection (data not shown).

Discussion

Surfactin biosynthesis is widespread among plant associated *Bacillus* isolates. This is not surprising considering the various benefits it provides for the ecological fitness of the producing bacterium. Indeed, efficient root colonization partly relies on the potential of bacterial cells to move on the root surface to reach new microniches with greater amounts of nutrients and on the ability to evolve on roots as biofilm-related structures (Bais *et al.*, 2004; Ramey *et al.*, 2004). As a matter of fact, *Bacillus* swarming (one of the two types of motility) on solid surface has been clearly linked to the secretion of surfactin acting as wetting agent (Hamze *et al.*, 2009; Kinsinger *et al.*, 2003; Raaijmakers *et al.*, 2010). It is also obvious that surfactin positively influences biofilm formation by *Bacillus* (Hofemeister *et al.*, 2004; López and Kolter, 2010). The lipopeptide likely plays a signaling role, driving cell subpopulations to produce extracellular matrix (López *et al.*, 2009)

In addition, a pivotal role of this compound in plant-*Bacillus* interactions is further illustrated by its host immunization activity resulting in enhanced resistance towards phytopathogens. Involvement of surfactin as ISR elicitor has been demonstrated in bean and tomato (Ongena *et al.*, 2007; Ongena *et al.*, 2005a; Ongena *et al.*, 2005b) but also in *Arabidopsis thaliana* (Ongena, unpublished data) and melon plants (García-Gutiérrez *et al.*, 2012). It thus appears that this molecule is active at triggering ISR in a broad range of host plants. Such a low specificity may be explained by the fact that the perception of surfactin by plant cells may rely on an uncommon mechanism based on interaction with the lipid bilayer fraction of plasma membrane rather than via recognition by some proteic receptor as established for other elicitors (Henry *et al.*, 2011).

Some bacterial species belonging to the *Pseudomonas* and *Bacillus* genera are well known for their ability to trigger ISR but most studies reported up to now have focused on the determination of the active compound produced by one specific strain under particular conditions. In line with this, a key role of surfactin for the plant immunizing potential of the producing bacterium has been mainly evidenced in the case of strain S499 (Jourdan *et al.*, 2009). Surprisingly, no study has been done yet to generalize these observations to other strains of the same or closely related species. Results from the work described here strongly suggest that such a key role of the lipopeptide for ISR triggering can be extended to other *Bacillus* isolates. To the best of our knowledge, this is the first comparative study demonstrating the importance of a single compound for plant immunization by members of the *B. subtilis*/*B. amyloliquefaciens* group. We used various approaches to come to this conclusion.

Firstly, both early defense-inducing and ISR-triggering activities of the strain-specific extracts correlate very well with their surfactin content. Moreover, the amplitude of the plant response observed following treatment with active supernatant extracts is very similar to the one induced by pure surfactin at the same concentration. It is reported for oxidative burst stimulation in cultured cells (Fig. 3) but it also applies for ISR in tobacco plants since comparable levels of ISR induced by S499 and 98S extracts (approx. 25-30 % disease reduction, Figure 4) were previously observed by treating plants with 10 µM purified surfactin (Henry *et al.*, 2011).

Secondly, assays performed on tomato plants treated with living cells also show that disease protection correlates with the potential of the strains to produce this LP not only in optimized medium but also when submitted to a nutritional status mimicking the one imposed in the rhizosphere by exudation of plant roots (Table 1). The lower efficacy of strains FZB42 and QST713 to stimulate ISR in tomato compared to S499 or 98S is probably not due to a reduced rhizosphere-fitness since they form similar consistent biofilms on plantlets (Fig. 6). Interestingly, disease reduction of approx. 30% provided by the most efficient isolates S499 and 98S is very similar to the protection level observed after treatment with 10 µM purified surfactin in hydroponic conditions. Moreover, we have recently demonstrated that the lipopeptide signature of S499 is substantially modulated upon living on roots compared to laboratory conditions in favour of surfactin. Indeed, much higher proportions of this LP than fengycins and iturins were found in the tomato rhizosphere (Nihorimbere *et al.*, 2012). Such efficient surfactin production *in planta* is thus in support to the high systemic resistance-triggering potential of S499. Surfactin secretion by root colonizing cells has also be reported for QST713 and FZB42 strains (Fan *et al.*, 2011; Kinsella *et al.*, 2009). However, compared to S499, these two strains are less prone to produce surfactin in the presence of carbon sources typically found in tomato exudates and probably also less efficient at secreting this LP *in planta*. As the eliciting activity of the molecule is dose-dependent, it may explain why ISR activity of those strains on tomato is weaker (but still significant) than for S499.

Third, in support to the macroscopically observed ISR phenotype, we have also quantified some markers of this phenomenon in order to associate disease reduction with specific molecular events involved in the defense response of the host induced by the various strains. The activity of the phenylalanine ammonia-lyase enzyme (PAL) was not systemically stimulated in leaves upon root treatment with the tested *Bacilli*. It would be therefore unlikely that the observed resistance was associated with an increase in lignification or accumulation of antifungal phenylpropanoids (Fraser and Chapple, 2011). By contrast, our results illustrate a global stimulation of the LOX-initiated oxylipin pathway which converts unsaturated fatty acids into highly reactive hydroperoxides that can be further transformed by other enzymes into different defense metabolites (Shah, 2005). Both LOX genes transcription and enzymes activity were induced by strain S499. This resulted in accumulation of their products 13-hydroperoxy-octadecatrienoic and 13-hydroxy-octadecatrienoic acids retaining antifungal activity (Mariutto *et al.*, 2011; Prost *et al.*, 2005). This phenomenon was stimulated between 24 and 48 h after pathogen infection and is thus timely activated to counteract disease development. Indeed, even if there are no clearly visible symptoms at that time, a fast defensive response has to be mounted upon first attempts of *B. cinerea* penetration which occur 24h after infection under our experimental conditions (Mariutto *et al.*, 2011; Ongena *et al.*, 2007). The observed trends in LOX activity are thus in agreement with the disease reduction potential of strain S499. A very similar stimulation of the oxilipin pathway was observed upon treatment with pure surfactin (Fig. 7), again indicating that the global activity of S499 is mimicked by applying the lipopeptide alone. This provides additional evidence at the molecular level for its crucial role in ISR. No LOX stimulation was noted upon treatment with the less active strains but a certain level of disease resistance was provided by treating tomato plants with QST713 and FZB42 suggesting that other pathways than LOX and PAL can be activated to counteract *B. cinerea* infection. Considered globally, our data thus illustrate that a certain threshold in surfactin concentration in the rhizosphere is necessary to trigger ISR in tomato and it also correlates with the stimulation of defense pathways in that host.

Compared to S499, extracts prepared from FZB42 and QST713 cultures with high concentrations of iturins/bacillomycins and fengycins but lower contents in surfactin, were less active on both tobacco cultured cells and plantlets. Also, extracts from 98S with similar surfactin but higher iturin and fengycin content had similar effects on plantlets and cells as S499. In agreement with previous data (Henry *et al.*, 2011; Jourdan *et al.*, 2009), it shows that the two other main LP families are very poorly or not active as elicitors of early defense response and systemic protection in that plant. No iturin, fengycin or surfactin were detected in the inactive supernatant of *Paenibacillus polymyxa* strain 56 but this strain does efficiently secrete fusaricidins and their structurally-related LI-F variants as lipopeptide products with strong antifungal activity (Debois *et al.*, 2013). However, it appears from our data that these molecules do not retain ISR-triggering potential in the plants tested. It also applies for any other soluble compound with reduced hydrophobicity that could be secreted by all the *Bacillus* isolates tested such as aminobenzoic acid recently identified as potential ISR elicitor (Yang *et al.*, 2011).

In ISR assays involving living cells, plant resistance could also be due to the emission of butanediol and acetoin volatiles (Ryu *et al.*, 2004). GC-MS analyses revealed that all the tested strains except BNO1 are able to produce these volatiles to a similar extent upon growth on rich medium using the methodology described by Ryu and collaborators (2004) (Supplementary data 4). However, upon growth of strains on RE medium, volatile emission was markedly reduced and trace amounts of butanediol and acetoin could only be detected in the gas phase of S499 (Supplementary data 4) and FZB42 cultures but not for 98S and QST713 (data not shown). This suggests that tomato exudates are not conducive for an efficient release of those volatiles. So, globally, even if the involvement of volatile compounds cannot be completely ruled out, ISR potential of the strains tested here does not seem to correlate with some naturally higher productivity of acetoin and 2,3-butanediol *in vitro*.

In summary, the comparative approach used in this work strongly suggests that the key role played by the surfactin lipopeptide in plant defense elicitation not only applies to one particular strain (S499) but also to other isolates belonging to the *Bacillus subtilis*/*amyloliquefaciens* complex. Screening for isolates with huge potential for ISR stimulation is interesting for the development of new PGPR-based biopesticides given the importance of this plant immunization process in the global biocontrol activity provided by those beneficial microbes. As the ISR potential of *Bacillus* strains is tightly related to their capacity to efficiently produce surfactin, a first necessary step is to look for the presence of *srf* genes coding for the NRPS (non-ribosomal peptide synthase) biosynthesis machinery. This approach could be combined with PCR screening for other important biocontrol marker genes as reported by Joshi and collaborators (2006). Consistent expression of this genetic potential must be of course counterchecked by HPLC-ESI-MS analyses of lipopeptides in supernatant extracts after growth under optimized conditions for production. One step further in the screening strategy, assays for stimulation of early defense events in cultured cells may be used to evaluate the eliciting activity of the bacterial extracts. Indeed, our data clearly suggest a positive correlation between the potential to stimulate ROS production in cultivated cells and disease protection in whole plants even if not of the same species (tomato and tobacco) but of the same taxonomical family (*Solanaceae).* However, this is not sufficient and the ecological fitness of the producing isolates has also to be considered. Cells should efficiently colonize and adequately use root exudates for efficient production of the lipopeptide in order to reach a certain threshold amount in the rhizosphere. Our results strongly suggest that this rhizosphere-fitness may vary from one strain to another and many further efforts have to be made to provide insights into the physiological regulatory processes that could explain such differences.

Materials and methods

Bacterial strains

Bacillus strains S499 (Ongena *et al.*, 2005a; Ongena *et al.*, 2005b) and GA1 (Arguelles-Arias *et al.*, 2009) have been previously described, and strains FZB42 and QST713/QST2808 were kindly provided, respectively, by R. Borriss of Humboldt University, Berlin, and by J. Margolis of the Agraquest society. The BNO1 and ATCC strains 6633 and 21332 were obtained from the laboratory collection. *Bacillus* strains 98S, 23, 67, 164, 98r, 104, III and *Paenibacillus* strain 56 were provided by Prof. B. McSpadden Gardener from Ohio State University.

Agitated cultures

Bacteria were streaked on Luria medium (per liter : peptone, 8 g ; yeast extract, 4 g ; NaCl, 4 g ; glucose, 0.8 g ; agar, 12.5 g) and incubated overnight at 30 °C. These colonies were used to inoculate erlenmeyer flasks (250 mL) containing 50 mL of a medium optimized for lipopeptide production, Opt medium (Jacques *et al.*, 1999). These flasks were shaken during 3 days at 30 °C.

Biofilm cultures

Static cultures were conducted in 96 well plates (conical sterile non-cell-culture-treated plates, Sterilin). Each well contained 150 µL of Opt medium (Jacques *et al.*, 1999). For inoculum preparation, strains were streaked on Luria medium and incubated overnight at 30

°C. These colonies were used to inoculate agitated overnight precultures which were conducted in Opt medium at 30 °C. These precultures were stopped when their OD_{600nm} was between 0.5 and 1. The obtained active bacterial populations were centrifuged and resuspended in saline solution (9 g of NaCl per liter) to an OD_{600nm} of 0.1. A volume of 5 µL of this inoculum was applied to each well and inoculated plates were incubated statically for 19 hours at 30 °C.

LC-MS quantification of lipopeptides

Samples were analyzed by reverse phase HPLC–MS (HPLC Waters Alliance 2695/diode array detector) coupled with a single quadrupole mass spectrometer (Waters SQD mass analyser) on an X-terra MS (Waters) 150 X 2.1 mm, 3.5 µm column. We used a method, based on acetonitrile gradients, which allowed the simultaneous detection of all three lipopeptide families. Elution was started at 45 % acetonitrile (flow rate of 0.65 mL/min). After 10 minutes, the percentage of acetonitrile was brought up to 95 % and held for 9 minutes. Then, the column was stabilized at an acetonitrile percentage of 45 % for 5 minutes. Compounds were identified on the basis of their retention times compared with authentic standards and the masses detected in the SQD. Ionization and source conditions were set as follows: source temperature, 130 °C; desolvation temperature, 280 °C; nitrogen flow, 600 $L.h^{-1}$; cone voltage, 100 V.

Tobacco cells cultures

The suspended tobacco cells, *Nicotiana tabacum* strain BY–2, were cultured in 250 mL flasks containing 50 mL of plant cell medium (Jourdan *et al.*, 2009). The flasks were incubated in the dark under agitation (90 rpm) at 25 °C. The tobacco cells were subcultured every 7 days; 1.6 mL of a mother culture were transferred to a flask containing fresh medium.

Oxidative burst detection on tobacco cells

We quantified H_2O_2 production, in five day old tobacco cells suspensions after exposition to elicitors, through a chemiluminescence assay. Cells were filtered, rinsed and resuspended in Hepes medium to a cell density of 60 to 90 mg/mL (wet weight of the suspension recovered by filtration) (Jourdan *et al.*, 2009). Then, they were incubated for one hour to reduce stress.

First, the base level of H_2O_2 production of this specific plant cell population was measured. The assay was conducted according to Jourdan and colleagues (2009) except 50 µL of cell suspension were used instead of 100 µL and the luminescence signal was integrated, at one measurement per second, over the first 10 s after the beginning of the reaction. After taking this measurement, the elicitor or sample was added to the suspension and H_2O_2 production was measured every 2 minutes starting at the third minute after adding the elicitor. The accumulation of H_2O_2 caused by each sample was calculated as being the ratio of the highest observed concentration of hydrogen peroxide on the base level concentration (H_2O_2 level before addition of the elicitor).

Sample preparation for oxidative burst

In the comparative assay including 17 strains, crude supernatants were all diluted 6.67 times with distilled water before application to tobacco cell to bring them into an adequate concentration range (0 to 20 µM of surfactin on the tobacco cells). For each strain, 75 µL of this diluted supernatant were added to 500 µL of tobacco cell suspension.

For the oxidative burst on diluted samples, supernatants or purified surfactin were mixed with distilled water to obtain the range of tested concentrations. For each dilution, 90 µL were applied to 600 µL of tobacco cells (same volume ratio as above).

For the tests on fractioned samples, supernatants were treated as follows. Each sample was loaded onto a reverse phase (C18) cartridge (Grace, Maxi-Clean^{TM} SPE 300mg) which was then flushed with air (sample WATER 1), rinsed with water and flushed again (sample WATER 2). The cartridge was then successively eluted with 20, 40, 60 and 100% methanol in water and flushed with air between each solvent. The volumes of loaded sample and of each solvent were identical. Fractions were dried using a speed-vac apparatus and resuspended in their original methanolic solvent obtaining a concentration factor of 7.1. For each treatment, 14.50 µL of this concentrated sample were applied on 400 µL of tobacco cell suspension, except for the concentrated 100% methanol fraction for which 11.52 µL were applied. Thus, the concentrations of metabolites applied on the tobacco cells were about 4 and 5 times lower as those found in the crude supernatant.

Cultivation and preparation of *Botrytis cinerea*

The pathogen used in this work was *Botrytis cinerea* strain R16. This fungus was maintained by subculturing it every two weeks on PDA medium (Potato dextrose agar). Fifteen days old cultures were scrapped using salt peptone water (per liter: NaCl, 5 g ; peptone, 1 g ; Tween 80, 2 mL) which was then filtered to eliminate mycelium debris. The obtained spore suspension was centrifuged and spores were resuspended in preserving liquid (8.5% defatted milk and 10% glycerol in water). This new sample was stored at - 80 °C. When needed, this sterile suspension was thawed out in a refrigerator and centrifuged. Spores were then washed with deionised water (to eliminate storage liquid) and resuspended in infection solution (see below).

ISR on cut tobacco leaves

Bacterial supernatants were prepared by cultivating the strains under biofilm conditions, for 3 days at 30 °C, in static flasks containing Opt medium (20 mL medium in a 100 mL flask). Active cell populations used to inoculate these cultures were obtained by streaking strains on Luria medium and incubating overnight at 30 °C.

The biofilm cultures were centrifuged and processed to eliminate residual sugars. This step was necessary as pure supernatants caused important contaminant growth around the tobacco roots. Sample clean-up was accomplished by following the same procedure as for the samples in the oxidative burst assay exempt the only solvents used here were water and pure methanol. After this procedure, cleaned-up samples were concentrated using a speed-vac apparatus at 25 °C and 30 mbars for 24 hours. The obtained centrifugation sediments were resuspendre in methanol.

Tobacco plants were cultivated in rock wool in commercial hydroponic systems (Araponics©). Hoagland solution was used as a nutrient base (mg per liter : $Ca(NO_3)_2.4H_2O$, 825 ; KNO_3, 316 ; KH_2PO_4, 170 ; $MgSO_4.7H_2O$, 513 ; H_3BO_3, 1.40 ; $MnCl_2.4H_2O$, 0.90 ; $ZnSO_4.7H_2O$, 0.10 ; $CuSO_4.5H_2O$, 0.05 ; $NaMoO_4.2H_2O$, 0.02 ; EDTA.2Na, 5.20 ; $FeSO_4.7H_2O$, 3.90). Two seeds were sown per seed-holder and double germinations were separated 2 weeks after seeding. In total, plants were incubated 5 weeks at 30 °C with a circadian cycle of 16 hours of light - 8 hours of darkness.

After this growth period, plants were transferred in 5 mL polystyrene tubes which had been darkened using aluminum foil and contained the treatments. These consisted of the cleaned-up concentrated samples diluted up to their initials concentrations using Hoagland solution. The tubes containing the tobacco plants were placed in Araponics systems which contained some water to promote ambient humidity. This system was incubated for 24 hours at 22 °C, under a circadian cycle comprising 10 h of light and with a humidity regulated to approximately 60 %. After this period, the sixth and seventh leaf of each plant were cut off and placed in 12 wells plates, their petioles immersed in 100 times diluted Hoagland solution. The plates were placed in small boxes containing tap water for humidity and were incubated at room temperature. After 24 hours, each leaf was infected with four drops of *Botrytis cinerea* suspension, prepared as described here above, containing 5.10^5 spores per mL of tobacco infection solution (50 mM glucose and 34 mM KH_2PO_4). Infected leaves were incubated in the same system for 3 days. We used this detached-leave system because it provided much more consistent infection rates (and therefore ISR results) throughout independent assays than working with whole tobacco plants.

Lesions were scored as infected when the fungus had caused damage outside the initial inoculation spot. Protections percentages were calculated by the formula (%) = $100(1- x/y)$ in which x and y represent the total number of infected lesions on the leaves of treated and untreated plants, respectively.

ISR induction in tomato plants

Bacterial strains were streaked on solid Luria medium and incubated overnight at 30 °C. These colonies were used to inoculate 100 mL flasks containing 20 mL of optimized medium which were then agitated (150 rpm) for 24 hours at 30 °C. Next, 150 µL of the obtained culture were spread out on solid Luria medium. These Petri dishes were then again incubated overnight at 30 °C. From this point on, manipulations were conducted under clean but non sterile conditions.

The obtained active bacterial populations were scraped of the plates using a sterile solution of $MgSO_4.7H_2O$ (10 mM). The optical density of the suspension was determined and it was diluted using Hoagland solution to a cellular density of 1.10^7or 5.10^8 CFU/mL for soil and seed inoculation respectively. Tomato plants (cvMerveille des Marchés) were inoculated, cultivated and infected with *B. cinerea* R16 as described in Mariutto and colleagues (2011).

For the induction of ISR with surfactin, tomato seeds were sown in rockwoll and, after germination, were transferred in plastic boxes containing Hoagland hydroponic solution.

Three weeks after sowing, plants were treated with 10 µM surfactin by adding the elicitor in the hydroponic solution. Three days after induction of resistance, plants were transferred to saturated humidity conditions and infected as described in Mariutto and collaborators (2011). Infection rates were scored as for the cut tobacco leaves (see above).

Colonization on tomato plantlets

Sterilized tomato seeds were pregerminated for 4 days on solid Hoagland medium (26 g of agar per liter, pH adjusted to 7) at 28 °C and 60 % humidity under a 16 hours of light – 8 hours of darkness circadian cycle. Each seedling was then transferred to an individual Petri dish containing a microscope slide covered with Hoagland medium and incubated under the same conditions. Seven-day-old seedlings were inoculated with 10 µL of bacterial suspension. For each strain, this suspension was obtained by centrifuging an overnight culture (Luria medium, 30 °C), washing the cells with $MgSO_4$ 10 mM and resuspending them to a density of 1.10^7 CFU/mL. After inoculation, plantlets were then incubated for 2 weeks after which colonization was evaluated through plate counting and microscopic observation of the biofilm-like structures. These last observations were conducted, the day before plate counting, using a light microscope equipped with a 10X objective. Pictures were taken in black and white mode. For plate counting, results were expressed as CFU/g rhizosphere. Plated samples were obtained from total rhizosphere including the plantlet roots, the 2 mm of agar surrounding them and all the agar present in between a dense root network.

Assay of LOX activity

LOX activity was assayed spectrophotometrically as described in Mariutto and collaborators (2011). Frozen leaf tissue powder was extracted in 100 mM sodium phosphate buffer containing 0.4 g/L $Na_2S_2O_5$ and 2.5 g/L Tween 20. After centrifugation (20,000 x g at

4°C for 10 min), the supernatant was added to oxygenated 100 mM sodium phosphate buffer pH 7.0 and linolenic acid. Enzymatic activity was determined by monitoring the appearance of hydroperoxides at 234 nm with a UVIKON XS spectrophotometer. LOX activity was calculated using an ε of 25,000 M^{-1} cm^{-1} and was expressed in micro-enzymatic units per gram fresh weight (mU/g FW).

***TomLoxD* and *TomLoxF* genes expression**

Total mRNA was extracted from frozen leaf tissue powder by Trizol method according to the protocol recommended by the manufacturer (Gibco BRL, Grand Island, NY, USA). Analysis of gene expression was done by semi quantitative RT-PCR (semi-Q PCR). First-strand cDNA was synthesized (Smart PCR cDNA Synthesis Kit, Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's instructions. PCR amplifications were performed with the Advantage 2 PCR kit (Clontech). Primers EF1-alphaf (CTCAAGGATGGACAGACCCG) and EF1-alphaR (AAGGGGATCTTGTCAGGGTTG) were used to amplify the cDNA of the housekeeping gene Elongation factor 1-alpha (*EF1-α*), LoxDF (GCACCAGCAGGAGTTCTTTC) and LoxDR (GCTTCTCCACACGACTCTCC) were used to amplify *TomLoxD* cDNA, and LoxFF (AAGGCATAACTCAGCTCCA) and LoxFR (TTGGGTAACTTCTGGCCATC) were used to amplify *TomLoxF* cDNA. PCR reactions contained 1 µL first-strand cDNA and each primer at 0.5 µM. Samples were amplified in a GeneAmp 9700 PCR System (Perkin-Elmer) as follows: 3 min at 94°C; 23 (for *EF1- α*) 28 (for *TomLoxF*) and 29 (for*TomLoxD*) cycles of 1 min at 94°C, 1 min at 58°C (for *EF1- α*) or 60°C (for *TomLoxD* and *TomLoxF*), and 1 min at 72°C; 7 min at 72 °C. PCR products were deposited on gel and after electrophoresis, semi quantitative analysis was conducted by measuring the integrated density values (IDV) of PCR products in electrophoresis gel picture with the program AlphaEaseFC image software (AlphaInnotech, CA, USA). The band intensities of the genes of interest were normalized with the band intensities of *EF1- α*. Expression level of *TomLoxD* and *TomLoxF* in uninfected control plants was arbitrary considered as 1. Relative transcript accumulation was determined by calculating the percentage of difference in normalized band intensities between uninfected controls and each sample. Samples were obtained as for LOX enzyme expression. Data were analyzed using Student's t-test (α=0.05).

Quantification of fatty acid hydroperoxides

Fatty acid hydroperoxides were extracted from plant tissues, using a method described in Fauconnier and collaborators (2008) with some modifications, and quantified by UPLC-DAD (Ultrahigh Pressure Liquid Chromatography- Diode Array Detector). Powdered frozen leaf tissue (0.5 g) was extracted in 10 mL of extraction medium (isohexane/2-propanol, 3/2 (v/v); 0.0025% (w/v) butylatedhydroxytoluene). Ricinoleic acid was used as internal standard. After homogenization, the extract was centrifuged at 1,300 g at 4 °C for 10 min. The upper phase was collected and added to a 6.7 % (w/v) solution of potassium sulfate to reach a volume of 20 mL. After 10 min of shaking at 4 °C, the extract was centrifuged at 1,300 g at 4 °C for 10 min. The upper phase was collected, dried under nitrogen stream, and dissolved in hexane. UPLC analysis was carried out on a reverse phase column (Acquity UPLC BEH 1.7 µm, C18, 2.1 x 100mm, Waters) using water, acetonitrile and phosphate buffer (pH 2) as solvents in the following gradients: 70:20:10 at the beginning; then 45:45:10 until 6 min and stabilized until 12 min; 37:53:10 until 20 min and stabilized until 25 min; 5:95:0 until 28 min and maintained until 29 min; 0:100:0 until 32 min and stabilized during 5 min; finally the gradient was returned to its initial value and maintained during 3 min. The flow rate was 0.35 mL/min and compounds were detected at 234 nm. The retention time of the observed fatty acid hydroperoxides was compared with those of commercial 13- and 9- HPOD/HPOT (Larodan). Samples collection and data analysis were conducted as for LOX gene expression.

Analysis of volatiles production

Strains were cultivated on solid medium in glass vials. In a first experiment, isolates were grown for 24 hours on a volatile production medium at 36 °C to place strains in optimized production conditions (Ryu *et al.*, 2004). In a second experiment, strains were cultivated on recomposed exudates medium at 25 °C for 48 hours to simulate rhizosphere conditions. For inocula preparation, strains were streaked on Luria medium and incubated overnight at 30 °C. The obtained colonies were scraped of the plates with saline solution (9 g/L), centrifuged and resuspended to an OD_{600nm}= 0.5. A volume of 100 µL of inoculum was applied to each vial. After incubation, the headspaces of all cultures were sampled with SPME fibers (with divinylbenzene, carboxen and polydimethylsiloxane coating of 50/30 µm X 1 cm) at 35 °C for 1 hour. Loaded fibers were analyzed with a gas chromatograph (THERMO) coupled to a mass spectrometer (Finnigan Trace MS). Desorption was conducted at 225 °C and injections were conducted in splitless mode. A Optima 5 Accent (Macherey-Nagel), 30 m x 0.25 mm ID 0.25 µm column was used. Carrier flow was set to 1 mL/min. Oven temperature program was as follows: 40 °C during 2 min, then a ramp of 5 °C/min up to 60 °C, followed by a second ramp of 120 °C/min until 280 °C. This temperature is maintained for 6 minutes. Scanning range of the MS was set to 35-350. Identification of the acetoin and 2,3-butanediol peaks was based on retention time compared with authentic standards (Sigma-Aldrich) and on MS spectra.

Statistical analysis

Data were analyzed by ANOVA ($P < 0.05$, Minitab software). Means from the different treatments were compared using Newman and Keuls' test (least significant difference at $\alpha = 0.05$).

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Author Contributions

HC and MM carried out the experimentations and the analysis and interpretation of data. HC, MM and MO wrote the manuscript. GH participated in early events experiments. CF carried out LC/GC-MS analyses. NV assisted with ISR experiments on tomato (molecular and macroscopic approaches). PT, JD and MO conceived the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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Table 1. Bacterial population and surfactin concentration in the culture broth of the six selected *Bacillus* strains upon culture in recomposed exudates medium. Means and standard deviations were calculated from two independent cultures. Statistical analysis on the two repeats showed aggregation of means in several significantly different groups represented by different letters.

	Bacterial population (10^8 CFU/mL)	**Surfactin production (µg/mL)**
***B.a.* S499**	5.4 ± 2.4 a	149 ± 22 a
***B.s.* 98S**	3.8 ± 0.5 a	145 ± 67 a
***B.a.* FZB42**	5.6 ± 3.0 a	18 ± 2 b
***B.a.* QST713**	3.9 ± 0.8 a	82 ± 10 ab
***P.p.* 56**	1.3 ± 0.4 a	0 ± 0 b
***B.s.* BNO1**	1.5 ± 0.7 a	0 ± 0 b

Figure captions

Figure 1. LC-ESI-MS profiling of lipopeptides secreted by some of the isolates used in this work after cultivation under optimized conditions (see Methods section). For each lipopeptide family, several peaks are detected which correspond to the various co-produced homologues differing in the length/isomery of the fatty acid tail. Labelled ions mostly correspond to the $[M+Na]^+$ form of the compounds. For each strain, Y axes in the mass spectra for surfactins, iturins and fengycins represent total ion current values (relative abundance) and were linked at the same scale to allow comparison of the relative intensities of the ions correponding to the three LP families based on peak area/height. Data were obtained from analysis of extracts prepared from one culture but similar MS spectra and LP profiles were obtained in two repeats.

Figure 2. Relationship between surfactin content and early event triggering for *Bacillus* supernatants. **A.** Surfactin concentrations added to tobacco cells when applying supernatants of the studied *Bacillus* strains. Bacteria were cultivated as biofilms in optimized medium in 96 well-plates. Equal volumes of the various supernatants were tested on cultured tobacco cells. Means and standard deviations were calculated from the results of three independent cultures. **B.** Quantification of the oxidative burst (accumulation of H_2O_2 in the medium by the tobacco cells) caused by each *Bacillus* supernatant. Accumulation of H_2O_2 is a ratio calculated as explained in M&M. Means and standard deviations were obtained from three repeats of the oxidative burst experiment. In both panels, statistical analysis conducted on the three repeats showed aggregation of means in several siginificantly different groups represented by different letters on the mean bars.

Figure 3. Early event triggering potential of serial dilutions of active *Bacillus* supernatants. Measurement of the oxidative burst response of tobacco cells (accumulation of H_2O_2 in the medium) triggered by diluted supernatants of the four active *Bacillus* strains and several dilutions of pure surfactin. Accumulation of H_2O_2 is a ratio calculated as explained in M&M. Means and standard deviations were calculated from the results of three independent oxydative burst experiments. Statistical analysis on the three repeats, conducted between several surfactin concentrations for the same strain, showed aggregation of means in several significantly different groups represented by different letters on the mean bars.

Figure 4. A. Differential protection of tobacco leaves by six *Bacillus* supernatants against *B. cinerea* infection. Plants were treated at the root level with extracts of biofilm cultures (supernatants depleted of sugars by C18 fractioning), after incubation leaves were detached and infected with the pathogen. Disease reduction is calculated compared to water treated control plants. Protections percentages were calculated by the formula (%) = 100(1− x/y) in which x and y represent the total number of infected lesions on the leaves of treated and untreated plants, respectively. Each trial included five plants per treatement, two leaves were inoculated for each of these plantlets (6th and 7th leaf). The experiment was conducted twice. As data for the five plants per treatement were pooled in each expeirment, means and standard deviations were calculated on four data points each (two types of leaves and two independent experiments). Statistical analysis on the four repeats showed aggregation of means in several significantly different groups represented by different letters on the mean bars. **B.** Illustration of the symptoms caused by *Botrytis cinerea* on water control and *B.a.* S499 supernatant-treated tobacco plants.

Figure 5. Differences in triggered protection in tomato plants for six *Bacillus* strains against *B. cinerea* infection. **A.** Disease reduction on tomato leaves inoculated with *Botrytis cinerea* after root inoculation with live cell of the six selected *Bacillus* strains compared to uninoculated control plants. Protections percentages were calculated by the formula (%) = 100(1− x/y) in which x and y represent the total number of infected lesions on the leaves of treated and untreated plants, respectively. Means and standard deviations were calculated on the basis of three independent experiments, containing each 7 plants per treatment. Statistical analysis on the three repeats showed aggregation of means in several significantly different groups represented by different letters on the mean bars. **B.** Reduction in occurrence of typical spreading lesions as compared between diseased control and plants inoculated with S499 before infection. **C.** For three selected strains of *Bacillus*, percentage of plants, in the experiment described in A, presenting 0 to 3 lesions (low disease severity, white bars), 4 to 6 lesions (intermediate disease severity, grey bars) or 7 to 10 lesions (high disease severity, black bars). Presented data are means of three independent experiments.

Figure 6. A. Comparaison of biofilm formation on tomato roots by six *Bacillus* strains. Illustration of the observed biofilm (circled in black) for the six selected *Bacillus* strains, 2 weeks after inoculation on tomato plantlets grown *in vitro* on Hoagland agar. Pictures were taken using a light microscope equipped with a 10X objective. Presented photographs are representative of observations conducted on three plantlets per treatment. **B.** Colonization levels of the studied strains on the *in vitro* plantlets. Presented data are means and standard deviations calculated from plate counts on 3 plantlets (one plate count per plantlet). Statistical analysis on the three repeats showed aggregation of means in several significantly different groups represented by different letters on the mean bars.

Figure 7. Stimulation of LOX pathway in leaves of tomato plants inoculated with strong, medium and non producer strains, *B.a.* S499, *B.a.* QST713 and *B.s.* BNO1 respectively. **A.** Time course evolution of LOX activity in plants bacterized with *B.a.* S499, *B.a.* QST713 and *B.s.* BNO1. Samples were collected before infection by *B. cinerea* and one, two and four days thereafter. Data are means and standard deviations calculated from three measurements on two independently prepared extracts. In each experiment, statistical analysis on the six repeats (6 samples/ treatment) showed aggregation of means in several significantly different groups represented by different letters on the mean bars. Statistical comparaison was conducted between treatements for a same sampling time. **B.** Relative expression of *TomLoxD* and *TomLoxF* and **C.** fatty acid hydroperoxide (HPO) accumulation in *B.a.* S499 as compared to control and surfactin treated plants two days upon pathogen inoculation were respectively analyzed by semi-Q PCR (semi quantitative RT-PCR) and UPLC-DAD (Ultrahigh Pressure Liquid Chromatography- Diode Array Detector). Data are means and standard deviations calculated from three measurements on two independently prepared extracts. In each experiment, the different treatments (6 data points/ treatment) were compared by Student's t-test ($\alpha = 0.05$). The three panels of the figure show data from a representative experiment that was repeated on two independent biological replicates.

Supplementary data 1. Correlation between intensity of oxydative burst (accumulation of H_2O_2) and surfactin concentration applied to the tobacco cells based on data presented in figure 2. Samples causing a surfactin concentration beneath 4 µM on tobacco cells were not included in regression because the H_2O_2 acccumulation they induced was not significantly different from water control.

Supplementary data 2. Correlation between disease reduction, against symptoms caused by *Botrytis cinerea*, and surfactin concentration applied to the tobacco roots as described in figure 4. Only S499 and 98S triggered a siginificant protection compared to water control.

Supplementary data 3. Differences in triggered protection in tomato plants for six *Bacillus* strains against *B. cinerea* infection**.** Disease reduction on tomato leaves inoculated with *Botrytis cinerea* after root inoculation with live cell of the six selected *Bacillus* strains compared to uninoculated control plants. For each strain of *Bacillus*, percentage of plants presenting 0 to 3 lesions (low disease severity, white bars), 4 to 6 lesions (intermediate disease severity, grey bars) or 7 to 10 lesions (high disease severity, black bars). Presented data are means of three independent experiments.

Supplementary data 4. Volatiles production by the six selected *Bacillus* strains. Production of butanediol (area of the corresponding peaks detected in GC-MS) by the six *Bacillus* isolats on a specific medium as defined by Ruy and collaborators (Ryu *et al.*, 2004) and, for S499, upon growth in recomposed exsudats (RE). Volatile production on RE medium was also determined for the other strains but only trace amounts were detected and are not represented here for clarity purposes. These data are from one representative experiment.

Figure 1. Cawoy et al.

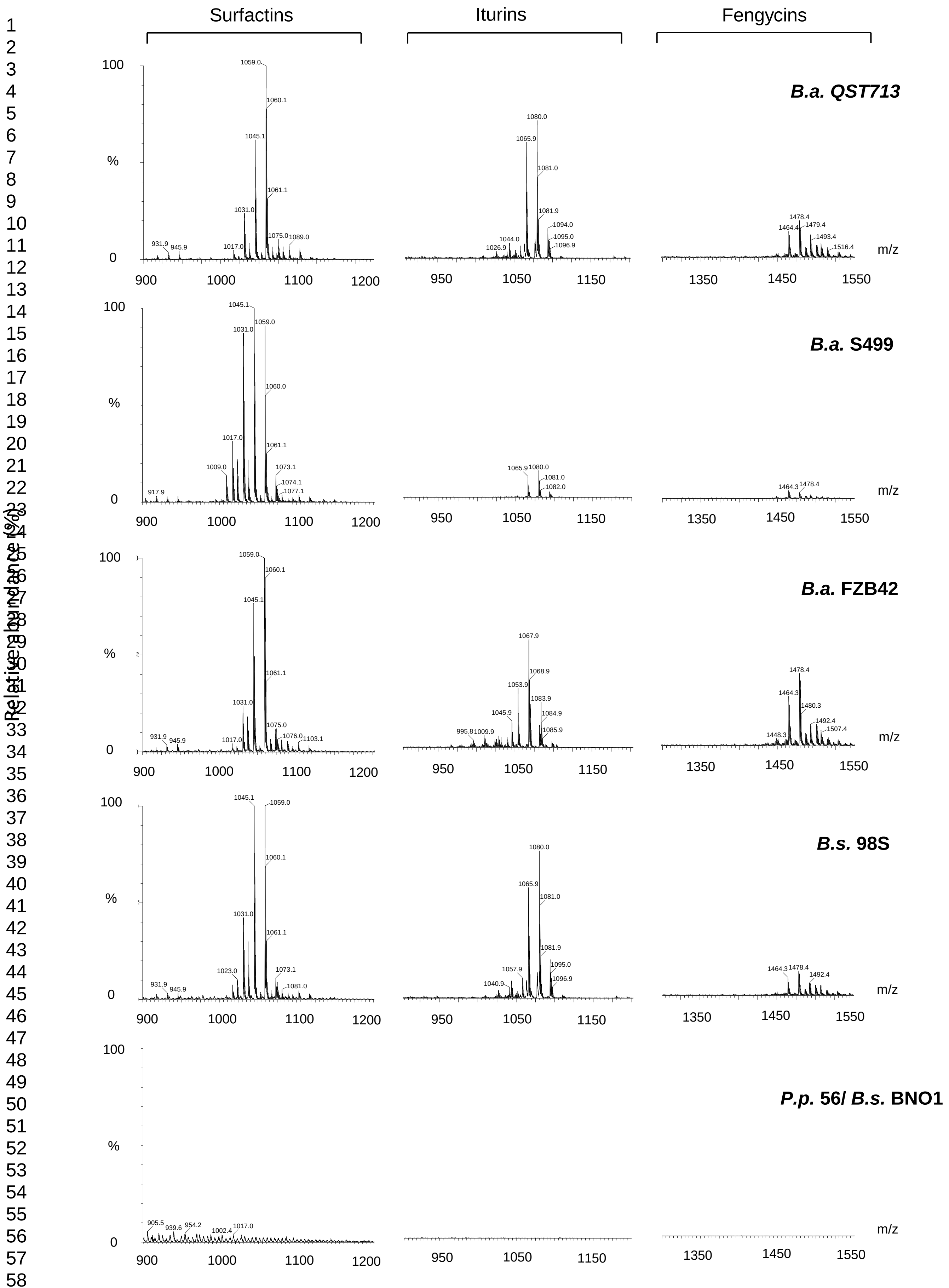
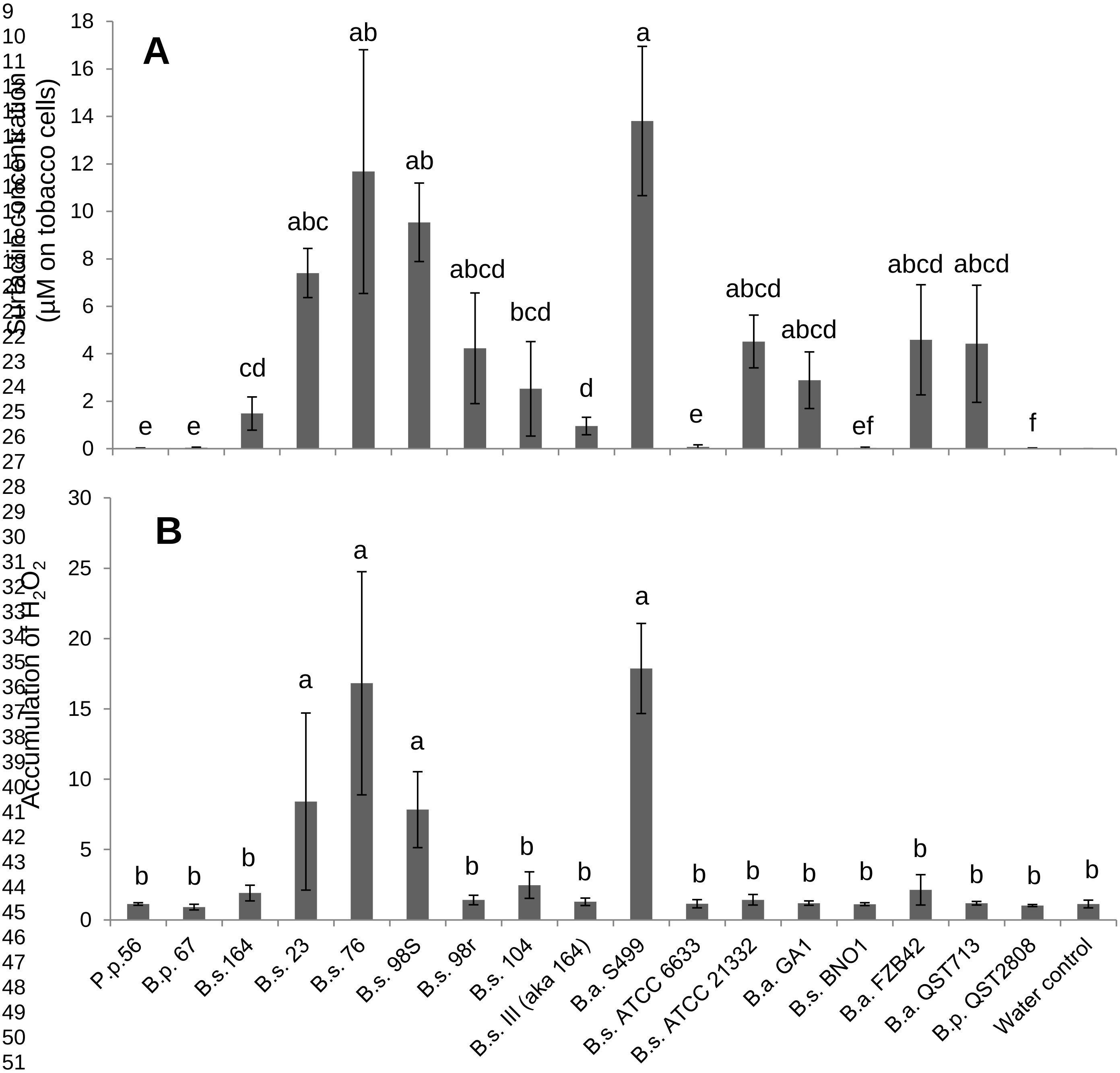


Figure 2. Cawoy et al.



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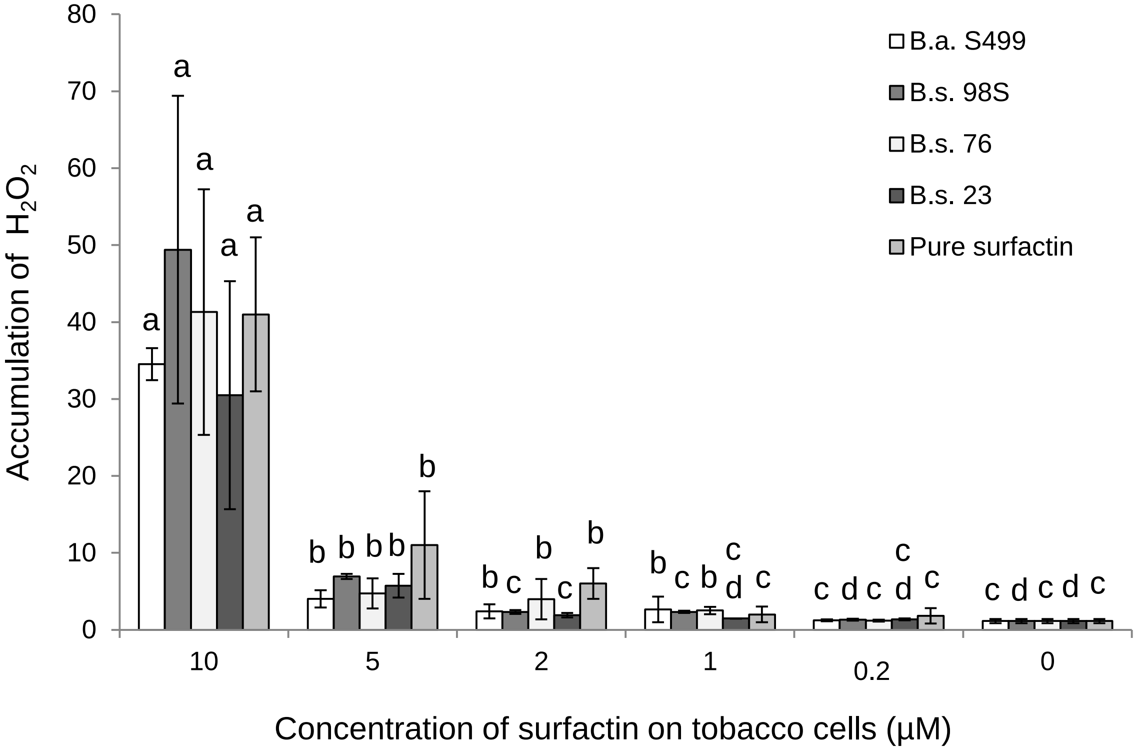
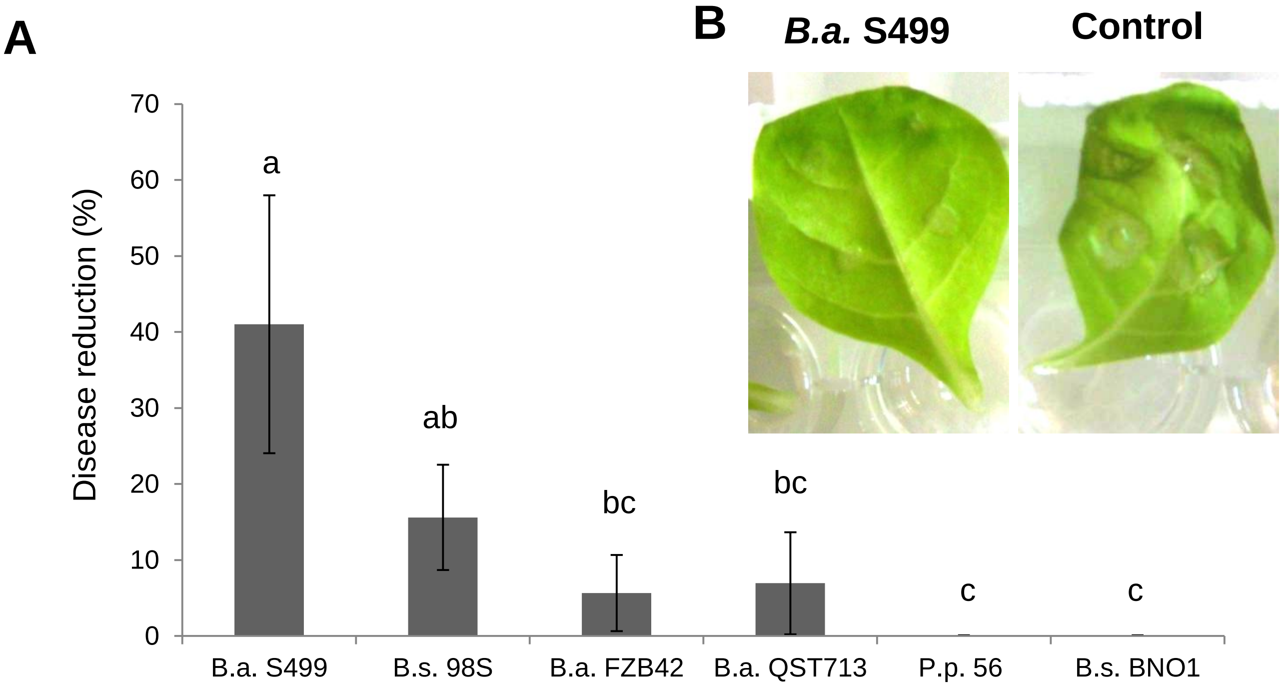


Figure 4. Cawoy et al.



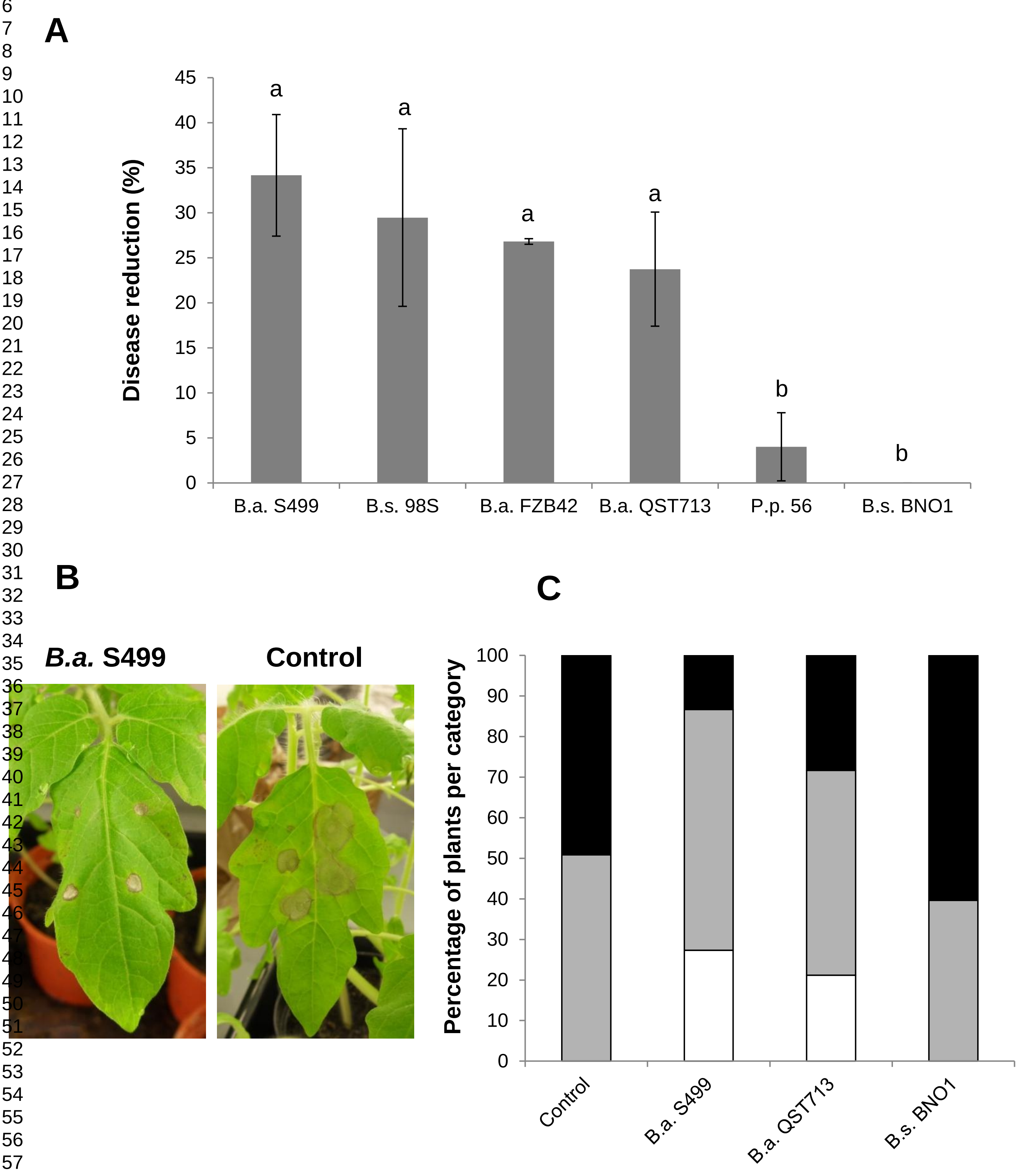


Figure 5. Cawoy et al.

Figure 6. Cawoy et al.

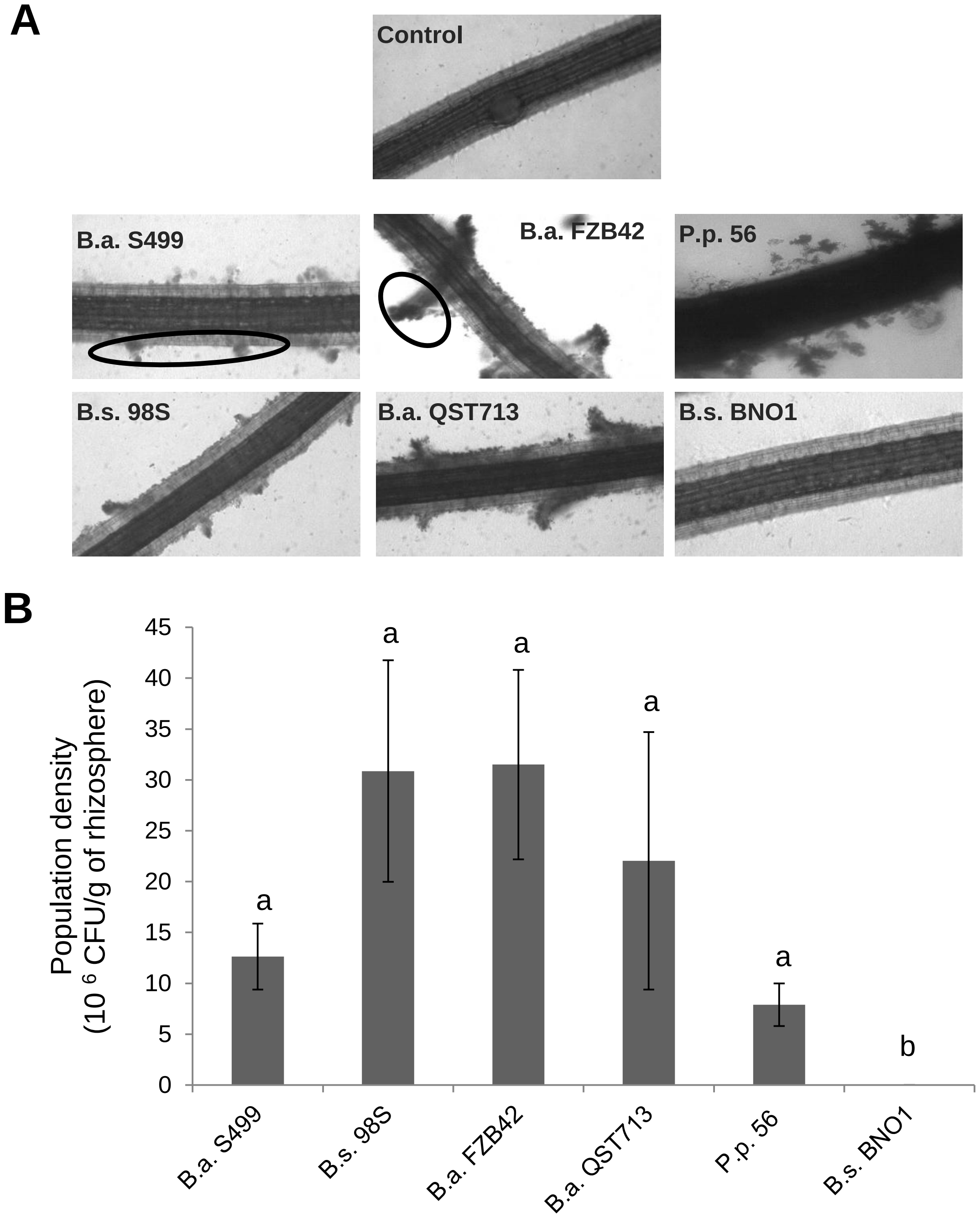
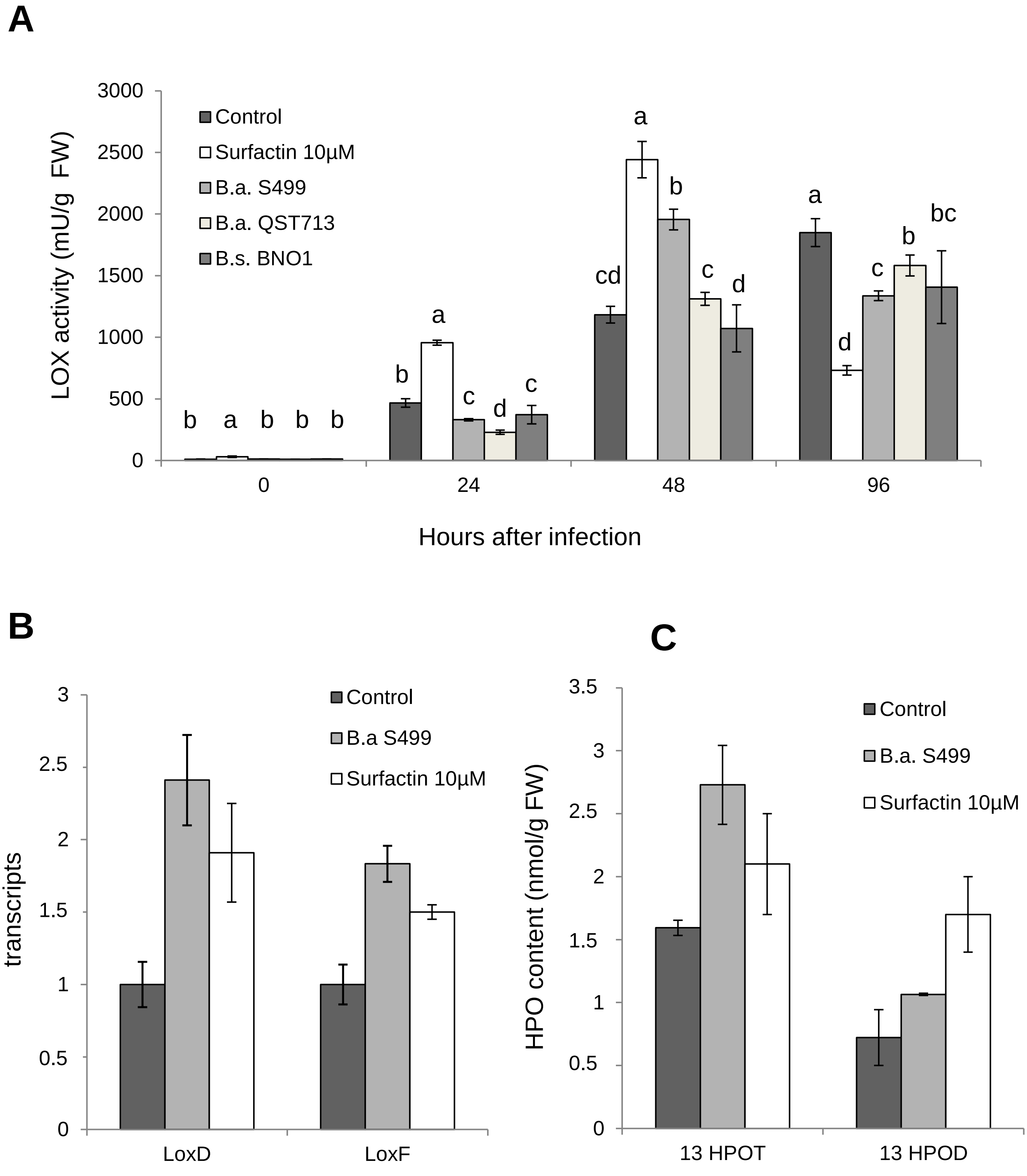
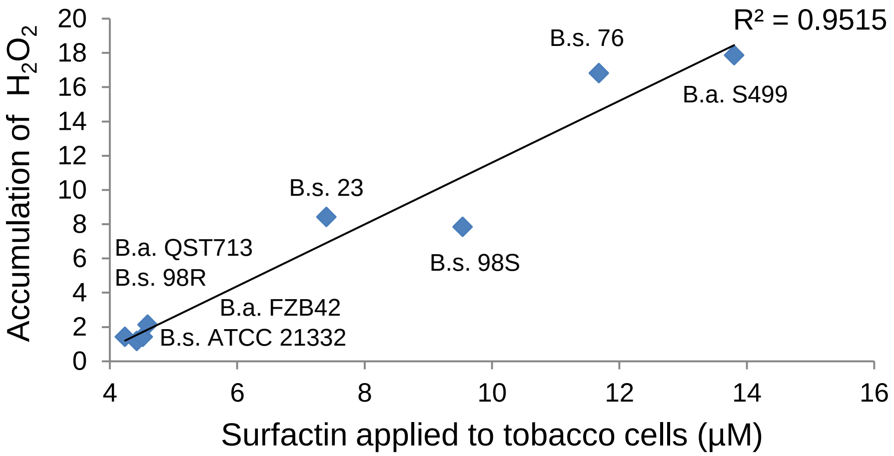


Figure 7. Cawoy et al.



Supplementary data 1. Cawoy et al.



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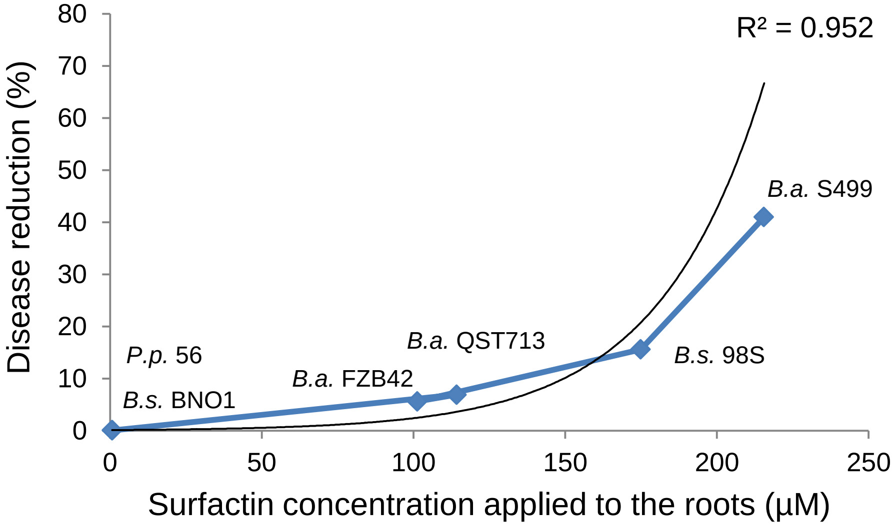
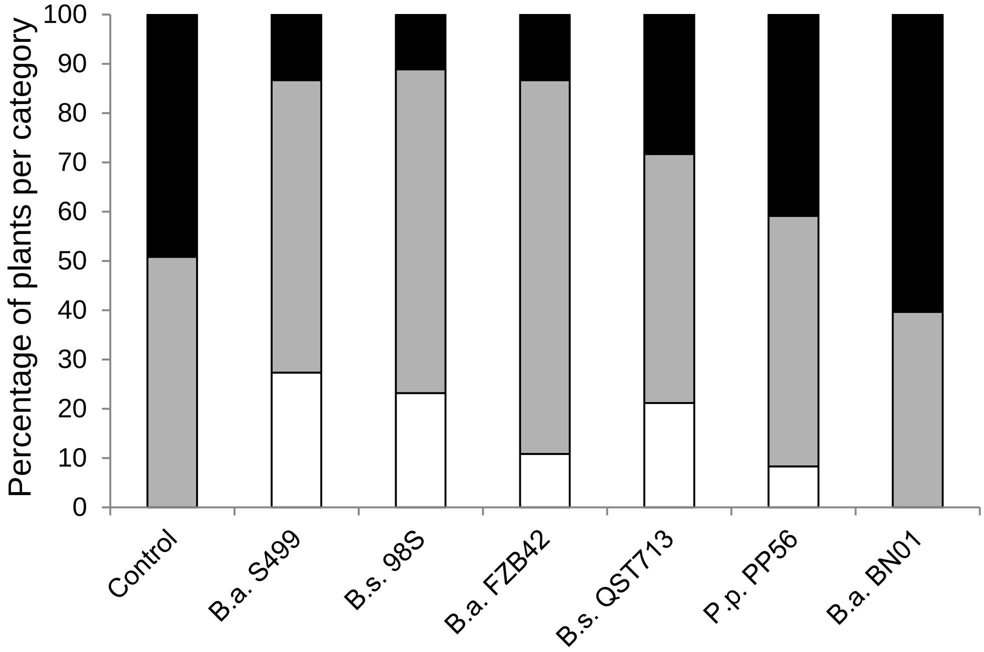


Supplementary data 3 Cawoy et al.



Supplementary data 4. Cawoy et al. MPMI