Bioengineered 4:4, 236–243; July/August 2013; © 2013 Landes Bioscience

Identification of volatile compounds produced by the bacterium *Burkholderia tropica* that inhibit the growth of fungal pathogens

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Keywords: *Burkholderia tropica*, volatile compound, antagonism, phytopathogenic fungi

It has been documented that bacteria from the Burkholderia genera produce different kinds of compounds that inhibit plant pathogens, however in *Burkholderia tropica,* an endophytic diazotrophic and phosphate-solubilizing bacterium isolated from a wide diversity of plants, the capacity to produce antifungal compounds has not been evaluated. In order to expand our knowledge about *Burkholderia tropica* as a potential biological control agent, we analyzed 15 different strains of this bacterium to evaluate their capacities to inhibit the growth of four phytopathogenic fungi, *Colletotrichum gloeosporioides*, *Fusarium culmorum*, *Fusarium oxysporum* and *Sclerotium rolffsi*. Diverse analytical techniques, including plant root protection and dish plate growth assays and gas chromatography-mass spectroscopy showed that the fungal growth inhibition was intimately associated with the volatile compounds produced by *B. tropica* and, in particular, two bacterial strains (MTo293 and TTe203) exhibited the highest radial mycelial growth inhibition. Morphological changes associated with these compounds, such as disruption of fungal hyphae, were identified by using photomicrographic analysis. By using gas chromatography-mass spectroscopy technique, 18 volatile compounds involved in the growth inhibition mechanism were identified, including α-pinene and limonene. In addition, we found a high proportion of bacterial strains that produced siderophores during growth with different carbon sources, such as alanine and glutamic acid; however, their roles in the antagonism mechanism remain unclear.

Introduction

The genus *Burkholderia* comprises around 60 different species, and their taxonomic classifications have shown the existence of two large groups. In the first group, there are diverse pathogenic species, such as *B. mallei* and *B. pseudomallei* and other species belonging to the *B. cepacia* complex.[1,2] In the second group, more than 25 related environmental nonpathogenic species have been identified, most of which preferentially associate with plants. In general, diverse and interesting properties have been described for members of this genus, such as their ability to form symbiotic interactions with plants. For example, *B. phymatum* has the capacity to nodulate and fix nitrogen with the *Leguminosae Mimosa caesalpiniifolia*.[3,4] Some *Burkholderia* species show a potential to increase plant nutrient availability via nitrogen fixation and/or phosphate solubilization, e.g., *B. unamae* and *B. tropica*;[5] others, such as *B. uname*, have a catabolic potential to degrade aromatic compounds.[6] Some species exhibit the ability to colonize the rhizosphere in several plants and promote plant growth, e.g., *B. phytofirmans*.[7] Finally, there is the ability of *Burkholderia* species to inhibit the growth of multiple soil-borne pathogens on different crops by different antagonistic mechanisms, such as production of antibiotics or siderophores.[1,8,9] Nevertheless, the roles of siderophores and other antagonistic mechanisms have been poorly described for this genus, except for the clinical isolates *B. cepacia* and *B. vietnamensis*, for which ornibactin and cepabactin (siderophores of the hydroxamate class) and pyochelin and salicylic acid (catechol type) have been identified.[10,11] In this work, we analyzed the antagonistic activities of 15 different strains of *B. tropica* against plant pathogenic fungi under different conditions and, in particular, the role of bacterial volatile compounds and siderophores in fungal growth inhibition. *B. tropica* was selected because of its ability to fix nitrogen, solubilize phosphates and produce siderophores;[5] moreover, this bacterium associates with corn, teocinte and sugarcane plants from different geographical regions from Mexico.

Results

***B. tropica* is an antagonist of plant pathogenic fungi.** In order to determine the effects of the bacterial strains on fungal mycelial growth, dual-culture antagonism assays were performed on PDA plates. Fifteen *B. tropica* strains and four plant pathogenic fungi, *Colletotrichm gloesporioides*, *Fusarium culmorum*, *F. oxysporum* and

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Submitted: 01/09/13; Revised: 01/25/13; Accepted: 01/29/13
http://dx.doi.org/10.4161/bioe.23808

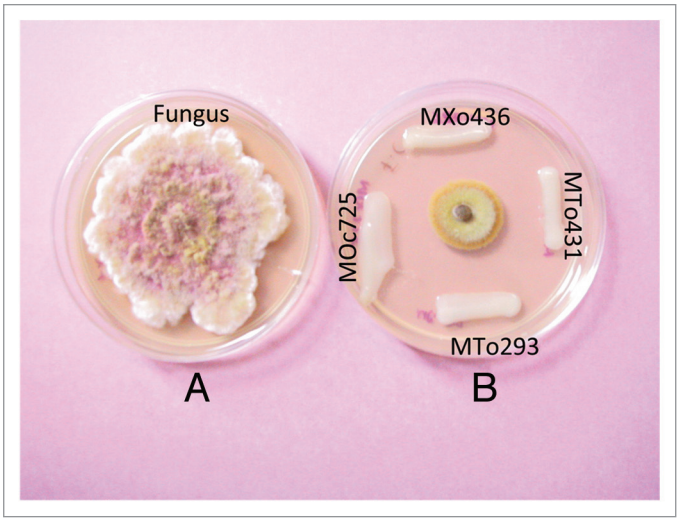

Figure 1. Antagonism assay of *B. tropica* strains against *F. culmorum* in PDA medium. The panels show the fungus without antagonist bacterial strains (**A**) or with four bacterial strains, MOc725, MXo436, MTo431 and MTo293 (**B**) (n = 3).

Sclerotum rolffsi, were selected. All 15 tested strains significantly inhibited the mycelial growth, showing radial inhibition ranging from 6 to 74% in comparison with the control, when they were cocultured with fungi in dual-culture assays. In all cases, the inhibitory activity was evidenced by the limited growth or by the complete absence of fungal mycelia in the inhibition zone in front of a bacterial streak. The inhibition patterns observed for each fungal species are shown in **Figure 1** and are summarized in **Table 1**. *F. culmorum* exhibited an elevated level of antagonism by all strains assayed (59 to 74% of inhibition), and the mycelia changed from pink to yellow. Seven bacterial strains, MXo436, MXo437, MCu812, MMi786, MOc725, TTe203 and MTo293, exhibited the largest antagonistic effects over this fungus. *F. oxysporum* showed a radial inhibition of around 50%, and five bacterial strains showed a higher antagonistic effect (MXo436, MXo437, CBN724, MOc3413 and MOc235); *S. rolffsi* exhibited on average a radial inhibition of 46%, and the strains MTo293 and MTo16 showed the biggest antagonistic effects; *C. gloeosporioides* showed minor growth inhibition by all evaluated strains (between 6 and 36% inhibition), with the bacterial strains MTo293 and TTe203 showing the largest antagonistic effects over this fungus. Finally, *B. cepacia*, used as a positive control, showed a high antagonistic effect against *F. oxysporum*, *F. culmorum* and *S. rolffsi*, as we expected, whereas for *C. gloeosporioides* it showed less inhibition.

In summary, these data suggest that seven bacterial strains were able to inhibit strongly the growth of sensitive fungi, such as *F. culmorum*, whereas five strains inhibited the growth of *F. oxysporum* and two strains were mainly associated with growth inhibition of *S. rolffsi*. *C. gloeosporioides* seems to be the fungus with the lowest sensitivity to the bacterial strains tested, because all bacterial strains were less inhibitory. One strain, MTo293, was able to inhibit three out of four fungi (**Table 1**), suggesting that its antagonistic compounds could affect diverse phytopathogenic organisms. Alternatively, strains MXo436 and MXo437 were able to inhibit in large proportions only two fungi. Another interesting result was the inhibition specificity associated with strain MMi786 toward the fungus *F. culmorum*, as it was not able to inhibit the growth of additional fungi. Indeed, in two additional fungi tested, this bacterial strain showed the lowest inhibition percentages. Finally, the antagonistic property associated with a bacterial strain did not seem to reflect its association with the source of origin, as the highest inhibition was associated with strains isolated from maize, teocinte and coffee (MXo436, TTe203 and CBN724, respectively).

Production of antifungal volatile compounds by *B. tropica*. It has been demonstrated that volatile organic compounds produced by soil bacteria can influence growth of fungi.[12-15] Therefore, in this section we discuss the potential roles of volatile compounds associated to five strains, selected on the basis of their antagonistic capacities against the four fungi. From our analysis, we found that volatile compounds produced by *B. tropica* exhibited fungistatic and fungicidal activities against *F. culmorum*, *F. oxysporum*, *S. rolffsi* and *C. gloesporioides* and the growth inhibition was visible after the fourth day of incubation (**Table 2**). The strongest effect on growth inhibition (86 ± 4.5%) was observed in *S. rolffsi* with the MTo293 strain, followed by the MTo431, TTe203, MOc725 and CBN516 strains (**Fig. 2A and Table 2**). *C. gloeosporioides* was strongly inhibited (86 ± 3.2%) by strain TTe203, followed by the MOc725, MTo293 and MTo431 strains (**Fig. 2B**). *F. oxysporum* was inhibited by TTe203 (54%), followed by the MOc725 and MTo293 strains (**Table 2 and Fig. 2C**). Finally, *F. culmorum* showed the highest inhibition level, with strain TTe203 exhibiting a radial growth of only 22%, while the other four bacterial strains (MTo431, MOc725, CBN516 and MTo293) produced only around 12% inhibition of radial growth (**Table 2 and Fig. 2D**). The inhibition of *F. culmorum* was more evident when the mycelium quality was evaluated, as it changed color from pink in the absence of the bacterial strain to white in the presence of the bacterial strain and the texture changed from cotton-like without the bacterial strain to flattened and slender in the presence of the bacteria. In summary, the bacterial strains MTo293 and TTe203 exhibited the strongest inhibitory activities in almost all the fungi tested. In addition, *S. rolffsi*, a soil-borne plant pathogen, demonstrated the highest sensitivity to volatile compounds under the tested conditions.

Protection of maize plants by *B. tropica*. In order to determine the role of the bacterial strains in plant protection, plants were grown in Fahraeus nutritive solution[16] in the presence or absence of the MTo431 strain (**Fig. 3**) without carbon and nitrogen source additions. The MTo431 bacterial strain was selected because it exhibits greater antagonist activity among the strains isolated. This strain has a similar growth pattern as the MTo293 and TTe203 strains, but it is easier to grow with different carbon sources; in addition, it showed a strong antagonist inhibition mediated by volatile compounds against all the fungi tested. Under these growth conditions, without carbon and nitrogen source additions, the bacterial growth reached 10^9 CFU/ml in 7 d from initial inocula of 10^4 CFU/ml. *B. tropica* strain MTo431 was coinoculated with each of the four fungal species, *F. oxysporum*, *S. rolffsi*, *C. gloesporioides* and *F. culmorum*, and

Table 1. Antagonistic effect of *B. tropica* against four fungi

Strain	Growth inhibition (%)			
	F. culmorum	***F. oxysporum***	***S. rolffsi***	***C. gloeosporioides***
MXo436	74 ± 6.0	49 ± 1.5	41 ± 3.0	32 ± 3.4
MXo437	74 ± 0.9	49 ± 4.0	41 ± 4.4	34 ± 5.0
MCu812	74 ± 4.0	30 ± 7.0	47 ± 3.6	35 ± 7.0
CBN724	67 ± 4.0	49 ± 3.0	47 ± 2.0	29 ± 4.6
CBN516	67 ± 6.0	47 ± 5.0	44 ± 3.7	34 ± 5.1
MOc3412	59 ± 5.0	35 ± 2.3	44 ± 1.0	29 ± 5.0
MOc3413	69 ± 4.0	49 ± 6.0	47 ± 1.5	32 ± 4.0
MOc235	69 ± 7.0	49 ± 4.2	44 ± 2.3	32 ± 4.0
MOc725	72 ± 2.0	28 ± 7.1	47 ± 3.0	28 ± 5.8
MTo16	62 ± 7.0	44 ± 2.0	56 ± 4.8	31 ± 5.0
MTo293	72 ± 6.0	40 ± 6.0	65 ± 4.0	36 ± 3.0
MTo431	69 ± 5.0	44 ± 4.0	41 ± 4.0	22 ± 5.0
TTe203	74 ± 4.0	41 ± 2.1	49 ± 3.7	36 ± 0.9
TTe219	64 ± 3.5	35 ± 0.9	44 ± 2.0	34 ± 1.0
MMi786	72 ± 9.0	26 ± 4.5	44 ± 8.0	6 ± 0.5
***B. cepacia* ATCC 25416**	67 ± 7.0	63 ± 2.0	56 ± 5.1	11 ± 0.8
***CCE421	49 ± 4.0	0	0	0
*Fungus with no bacteria	0	0	0	0

Values represent the percentage of growth inhibition of fungi, where zero indicates no inhibition. n = 3. ± represents standard deviations. *The negative control was each fungus in the absence of the bacterial strain. *B. cepacia* and *Burkholderia* sp CCE421 were also included as controls.

with maize plant, in a solution containing a mixture of the MTo431 strain and the fungus. Plants exclusively inoculated with the fungus showed intense fungal growth in the solution and around the maize roots, and the maize plant growth was poor. In contrast, in plants inoculated with a mixture of fungi and bacteria, the growth associated with the fungi was almost absent in the solution and in the plant roots, and the growth of the maize plant was favorable (**Fig. 3**). These results suggest that the MTo431 strain has the ability to not only antagonize *C. gloesporioides*, *F. culmorum*, *F. oxysoporum* and *S. rolffsi* in a plate assay but also in association with the plant and with root exudates as the only carbon source.

Fungi exhibit morphological changes as a consequence of the volatile compounds produced by *B. tropica*. Microscopic analysis of fungi cocultivated with *B. tropica* MTo431 showed evidence of the morphological changes resulting from the presence of bacterial volatile compounds synthesized by *B. tropica*. On one hand, *F. culmorum* in the presence of the bacteria changed its hyphal morphology, including hyphal swelling, distortion, large amounts of balloon-shaped cells and cytoplasm and protoplasm aggregation (**Fig. 4B**). On the other hand, *F. oxysporum* showed degradation of fungal cell walls, cell breakage and leakage of intracellular substances, alterations in hyphal morphology and ruptured mycelia (**Fig. 4D**). Similar results were previously reported[17,18] for *Fusarium* spp, *Colletotrichum lindemuthianum* and *Rhizoctonia solani* treated with CF66I, an antifungal compound produced by *B. cepacia* and in mycelium structures in *F. oxysporum* and *Phytium afertile*, in which lysis of fungal hyphae, vacuolization and granulation in mycelium structures have been observed.[19] In contrast, in the absence of *B. tropica* (**Fig. 4A and C**), spindly mycelia were observed for the four fungi. Therefore, these results show that volatile compounds produced by this organism possess significant fungistatic activities. Similar findings have been described for the growth and sporulation of phytopathogenic fungi, such as *Verticillium dahliae*, *Sclerotinia sclerotiorum* and *R. solani*.[13] In addition, we do not exclude the possibility that other molecules beyond volatile compounds could be also involved in the actual morphological changes. In this regard, a recent screening of antifungal active compounds from *B. cepacia* K87, identified pyrrolnitrin and two derivatives as antifungal compounds; whereas, in *B. ambifaria* a mixture of lipopeptides, where the burkholdines have been characterized, are involved in the antifungal activity.[20,21]

Chemical analysis of volatile compounds. In order to identify the chemical nature of the volatile compounds previously described, we used GC coupled to MS for *B. tropica* strain MTo431. The volatile profile of this strain was compared against noninoculated medium control profiles. Based on a double dish system, 18 different compounds were identified, including sulfur metabolites, dimethyldisulfide (DMDS), toluene and terpenoid compounds, such as α-pinene, limonene and ocimene (**Table 3**). Several of these volatile compounds, such as DMDS, methyl ketone and toluene, have been previously detected in the headspace of cultures of many strains of *Pseudomonas* spp and from *B. cepacia* cultured in trypticase soy.[22] Although diverse volatile compounds, for example, DMDS, are able to inhibit spore germination in other fungi, such as *Paecilomyces lilacinus*,

Table 2. Effects of volatile compounds on fungal radial growth

	Growth inhibition (%)			
Strain	***F. culmorum***	***F. oxysporum***	***S. rolffsi***	***C. gloeosporioides***
MTo293	11 ± 1.5	32 ± 3.7	86 ± 4.5	71 ± 3.9
MTo431	14 ± 1.5	19 ± 2.1	83 ± 1.3	63 ± 4.6
MOc725	14 ± 1.1	41 ± 3.4	59 ± 2.3	74 ± 2.0
CBN516	11 ± 0.5	38 ± 1.7	57 ± 0.9	28 ± 1.7
TTe203	22 ± 3.7	54 ± 3.4	66 ± 2.0	86 ± 3.2
**A. brasilense* sp7	2 ± 0.1	5 ± 0.6	10 ± 0.7	9 ± 0.8
***B. cepacia* ATCC 25416	16 ± 1.7	45 ± 0.5	42 ± 1.0	30 ± 1.5
Fungus with not bacteria*	0	0	0	0

*Negative controls, **positive control.

Pochania chlamydospora and *Chlonostachys rosea*,[23] in this work we found novel compounds, including α-pinene, ocimene, limonene and fencona, which are typically associated with plants and fruits with antibacterial properties,[24] suggesting that they play an important role in the antagonistic antifungal mechanism associated with this bacterium.

***B. tropica* has the capacity for siderophore production and is influenced by carbon sources.** It has been reported that siderophores, chitinase, proteases and cyanhydric acid (HCN) are used by bacteria to inhibit fungal growth;[25] however, none of the 15 bacterial strains produced either chitinase, HCN or proteases under our culture conditions (see **Supplemental Materials**). In order to determine if siderophores play an antagonistic role, their production was evaluated in all 15 *B. tropica* strains by the universal assay on CAS agar plates, using diverse carbon sources (see Materials and Methods). The halo diameter produced by iron chelation, as a product of 72 h of incubation, was considered a direct measure of siderophore production. Based on this approach, we found that all analyzed strains formed an orange halo on CAS plates (94 to 100% of strains), suggesting that siderophore production is a common characteristic of *B. tropica* (see **Fig. S1**) that is associated with the carbon source. In this regard, three main groups of carbon sources influencing siderophore production were identified. The first group corresponds to the carbon sources glutamic acid, succinic acid, ornithine, alanine, aspartic acid, mannitol and lysine, in which bacteria produce a halo diameter of between 12 and 28 mm. Indeed, these compounds resulted in the biggest halos under all growth conditions and were the best carbon sources for producing siderophores. The second group includes glucose, fructose and glycerol as carbon sources and bacterial diameters oscillated between 2 and 12 mm, corresponding to medium-sized halos (see **Figs. S1 and S2**). Of these carbon sources, glucose produced the smallest halo, and with fructose and glycerol as carbon sources only one strain (TTe203) produced a halo (between 11 and 16 mm). Finally, in the third group, which included malic acid, only three strains were able to produce siderophores, with bacterial halo diameters of 3, 4 and 26 mm (see **Figs. S1 and S2**). In addition, the high production of siderophores when using glutamic acid or succinic acid as carbon source correlated with previous results where siderophore production was increased by succinic acid in cultures of *Pseudomonas* sp strains GRP3A and PRS9 min, *Pseudomonas chlororaphis* ATCC 9446 and *P. aeuruginosa*.[26-28] These data suggest that the production of siderophores is dependent on the carbon source, with succinic and glutamic acids inducing the highest siderophore production of the carbon sources evaluated.

Characterization of the chemical nature of *B. tropica* siderophores. In order to identify the chemical structures of the detected siderophores, the methods described by Csàcky[29] and Arnow[30] were used to measure the concentrations of hydroxamate siderophores and catechol siderophores, respectively. Glutamic and succinic acids were used as carbon sources for these experiments because they were identified as the best carbon sources for siderophore production. Based on this analysis, we detected higher production of a hydroxamate-type siderophore when glutamic acid was used as a carbon source (3 to 12 μM) compared with succinic acid (0.4 to 5 μM). Indeed, 87% of the analyzed strains secreted hydroxamate-type siderophores in succinic and glutamic acids (see **Fig. S3**). The high siderophore production levels of *B. tropica* in glutamic acid and succinic acid suggest that these organic acids may participate in the siderophore biosynthetic pathway. This idea is based on the structure of pyoverdine, in which the 3-amino-acid moiety of the chromophore is replaced with various acyl groups derived from succinate, malate or α-ketoglutarate.[26,31] Furthermore, it was described previously that glutamic acid is transformed into ornithine, which is a frequent amino acid associated with siderophore structures.[32] In contrast, with glucose or fructose as the only carbon source, we observed small halos in comparison to other carbon sources, suggesting that these two carbon sources may induce catabolic repression in the genes associated with siderophore production, as has been reported for *Escherichia coli* Nissle strain 1917.[33] Alternatively, in order to evaluate the bacterial response for siderophore production under physiological conditions, the bacterial strain MTo431 of *B. tropica* was cocultivated together with a maize plant in Fahraeus nutritive solution. This strain produces siderophores with root exudates as the carbon source (see **Fig. S4**). The siderophore production in the cocultivation assay, using maize root exudates, reached the maximal level (2.5 μM) on the second day, and then the siderophore concentration decreased. Therefore, the siderophore production correlates with the time of susceptibility of the plant to infection by a phytopathogenic organism. These results suggest that

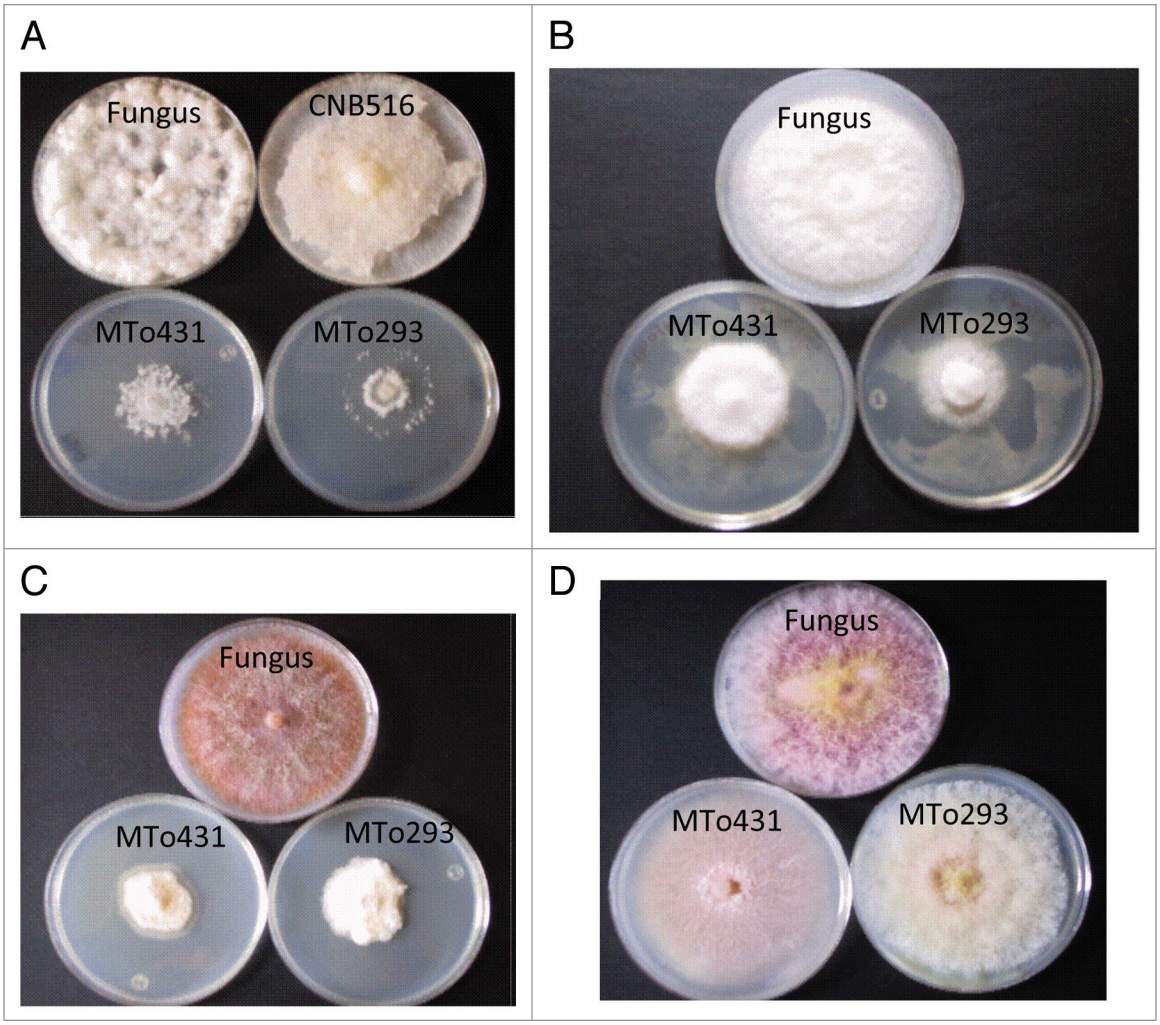

Figure 2. Growth inhibition of (**A**) *S. rolffsi*, (**B**) *C. gloesporioides*, (**C**) *F. oxysporum* and (**D**) *F. culmorum* by volatile compounds.

siderophore production also contributes to plant protection against pathogenic organisms. However, when the role of siderophores for the antagonistic effect was evaluated in dual cultures in PDA plates in the presence and absence of iron ($FeCl_3$), no significant differences were observed, suggesting that siderophores belonging to the hydroxamate and catechol classes are not directly involved in the antagonistic mechanism of *B. tropica* against fungi (see **Fig. S5**) and also suggesting that alternative classes of undefined siderophores could participate in this mechanism.

Discussion and Conclusions

To expand our knowledge about *B. tropica* as a potential biological control agent, in this work we studied its capacity to inhibit the growth of diverse phytopathogenic fungi and their antagonistic mechanism. Our results showed that *B. tropica* has the capacity to inhibit growth of *C. gloesporioides*, *S. rolffsi*, *F. culmorum* and *F. oxysporum* fungi, with *F. culmorum* the most sensitive to the antagonistic activity and *C. gloeosporioides* the least susceptible. In in vivo experiments, we showed that *B. tropica* has the ability to protect maize plants against fungal attack. *B. tropica* MTo431

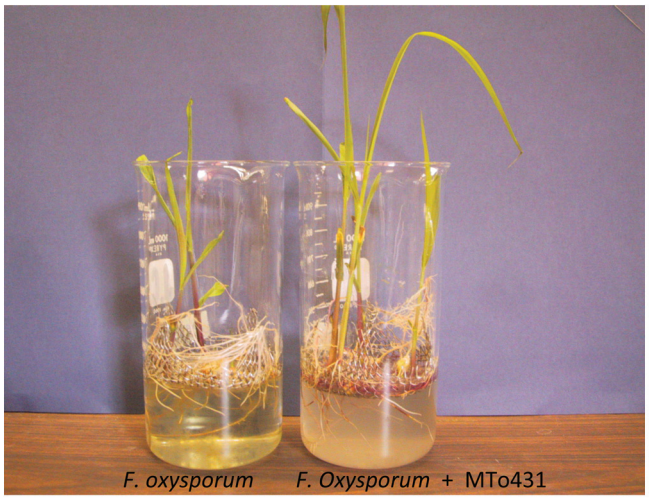

Figure 3. Antagonistic effect of strain MTo431 over the fungus *F. oxyxporum* when cocultivated with maize plant in Fahraeus nutritive solution. Bacterial strains inhibited the fungus growth and resulted in maize plant protection. Control experiments were performed by inoculating plants with only the fungus without bacteria.

Table 3. Volatile compounds detected in *B. tropica* MTo431 strain

Number	Compound	Number	Compound
1	Acetic acid	10	Methylcyclohexane
2	Methyl hexadecanoate	11	Nonane
3	Dimethyl disulfide	12	Ethylbenzene
4	Isobutylether	13	p-Xylene
5	Toluene	14	Ethyl valerate
6	Tetrachloroethyene	15	Ocimene
7	5-Cyano-1,2,3-thiadiazole	16	α-Pinene
8	Tricosene	17	D-Limonene
9	3-Methoxybutyl-1-eno	18	L-Fenchona

n = 3.

significantly reduced the growth of the four fungi tested after seven days, compared with control plants for which the bacteria were absent, and this bacterial strain had the capacity to colonize the Fahraeus solution after 11 d. These results led us to conclude that *B. tropica* can grow with root exudates as the only carbon source and is able to fully protect plants against phytopathogenic fungi. Our findings indicate that *B. tropica* is a proficient antagonist of phytopathogenic fungi under the conditions evaluated here, for which the activities of the volatile compounds seem to play an important role in fungal growth inhibition.

Materials and Methods

Bacterial strains and growth conditions. We analyzed a collection of 15 strains of *B. tropica*, including natural isolates from coffee (3 isolates), corn (10 isolates) and teocinte (2 isolates) plants from different regions of Mexico; these isolates have been previously described.[34] Four pathogenic fungi, *Colletotrichum gloeosporioides*, *Fusarium culmorum*, *F. oxysporum* and *Sclerotium rolffsi*, were provided by Dr. Oscar Mascorro-Gallardo (Universidad Autónoma de Chapingo, Mexico) and correspond to isolates from mango, ornate plants and onion from Mexico.

Antagonism assays with fungal pathogens. To evaluate the bacterial antagonistic effects against fungal pathogens, 15 strains of *B. tropica* were selected and streaked onto one side of a Petri dish (1 cm from the edge) containing potato dextrose agar (PDA; Difco). Six-millimeter mycelia discs from seven-day-old PDA cultures of four fungal pathogens were then placed in the middle of the Petri dishes, and the plates were incubated at 25°C for seven days. Thereafter, the zones of inhibition (in mm) were determined by measuring the distance between the edges of the fungal mycelia and the bacterial streak. All strains were evaluated in three independent replicates. The bacterial antagonistic effect against the fungi was calculated based on the radial inhibition percentages, according to the following equation:

$$\text{Radial inhibition (\%)} = \left(\frac{Rc - Ri}{Rc}\right)100$$

where *Rc* is the mean value of the fungus radius in the absence of the bacteria and *Ri* represents the fungus radius in the presence of the antagonistic bacteria.

All strains were inoculated in BSE liquid medium[34] for 16 h at 29°C with reciprocal shaking (250 rpm). The cultures were diluted with 0.1 M phosphate buffer (pH 7.5) and the optical density at 600 nm was adjusted to 0.4. Four strains were streaked per plate to evaluate the siderophore activities; this assay was performed in the presence and absence of 15 ppm $FeCl_3$.

Plant antagonism assay. Antagonism assays were performed in association with plants under conditions of sterility by using maize seeds previously germinated in a solution with mineral salts, Fahraeus nutritive solution[16] and in the absence of carbon sources. Two conditions were assayed: (1) only the fungus was inoculated and (2) the fungus was coinoculated with *B. tropica* strain MTo431.

Microscopic analysis. In order to evaluate morphological changes associated with volatile compounds, a double dish assay was performed as previously described, and fungal samples of *F. culmorum* and *F. oxysporum* were taken from areas showing high inhibition levels and stained with lactophenol-cotton blue. Samples from control plates without bacteria were also stained and examined.

Detection and extraction of volatile compounds. Volatile compounds were detected by the method reported by Jayaswal et al.[35] Bacterial strains and each fungus were cultivated in PDA medium in separate plates and then the plate with bacteria was placed over the plate with the fungus, avoiding direct contact between the two, sharing only the air. Both plates were sealed from the bottom with parafilm and plates were incubated at 29°C for 5 to 6 d. The production of volatile compounds was then determined based on inhibition of the radial growth of the fungi. In a posterior step and in order to extract the volatile compounds produced by *B. tropica*, two bacterial strains were inoculated on PDA plates and the lid was replaced with a bottom plate that contained 3 g of sterile activated charcoal (Sigma). These two plates were sealed with adhesive transparent tape and they were incubated at 30°C for 4 d for the fungi *C. gloeosporioides* and *S. rolfssi* and for 7 d for *F. oxysporum* and *F. culmorum*. All experiments were performed in triplicate and mean values are provided. After the incubation, the activated charcoal was collected and washed with 5 ml of ethyl acetate to extract all trapped volatile compounds, which were posteriorly analyzed by gas chromatography-mass spectrometry (GC-MS).

GC-MS analyses. The GC-MS analyses were performed using an Agilent GC-MS system (GC with 6890N and mass selective detector 5973N). Samples (1 μl volume) were injected in splitless mode. An Agilent Technologies HP-5MS (30 min by 0.250 mm by 0.25 min) fused silica capillary column was used. Helium (99.999%) was used as the carrier gas at an on-column flow of 1 ml/min. The temperature of the oven was programmed at 60°C for 5 min, raised at a rate of 15°C/min up to 300°C and then held for 10 min at 300°C. The mass detector conditions were the following: the transfer line from GC to MS was held at 280°C, the MS quadrupole was at 150°C and the MS source was at 230°C. The detector was operated at 70 eV. The analysis was performed in full scan mode, ranging from *m*/*z* 2 to 600.

Siderophore production assays. Siderophore production was determined by the universal chemical assay on chrome azurol S

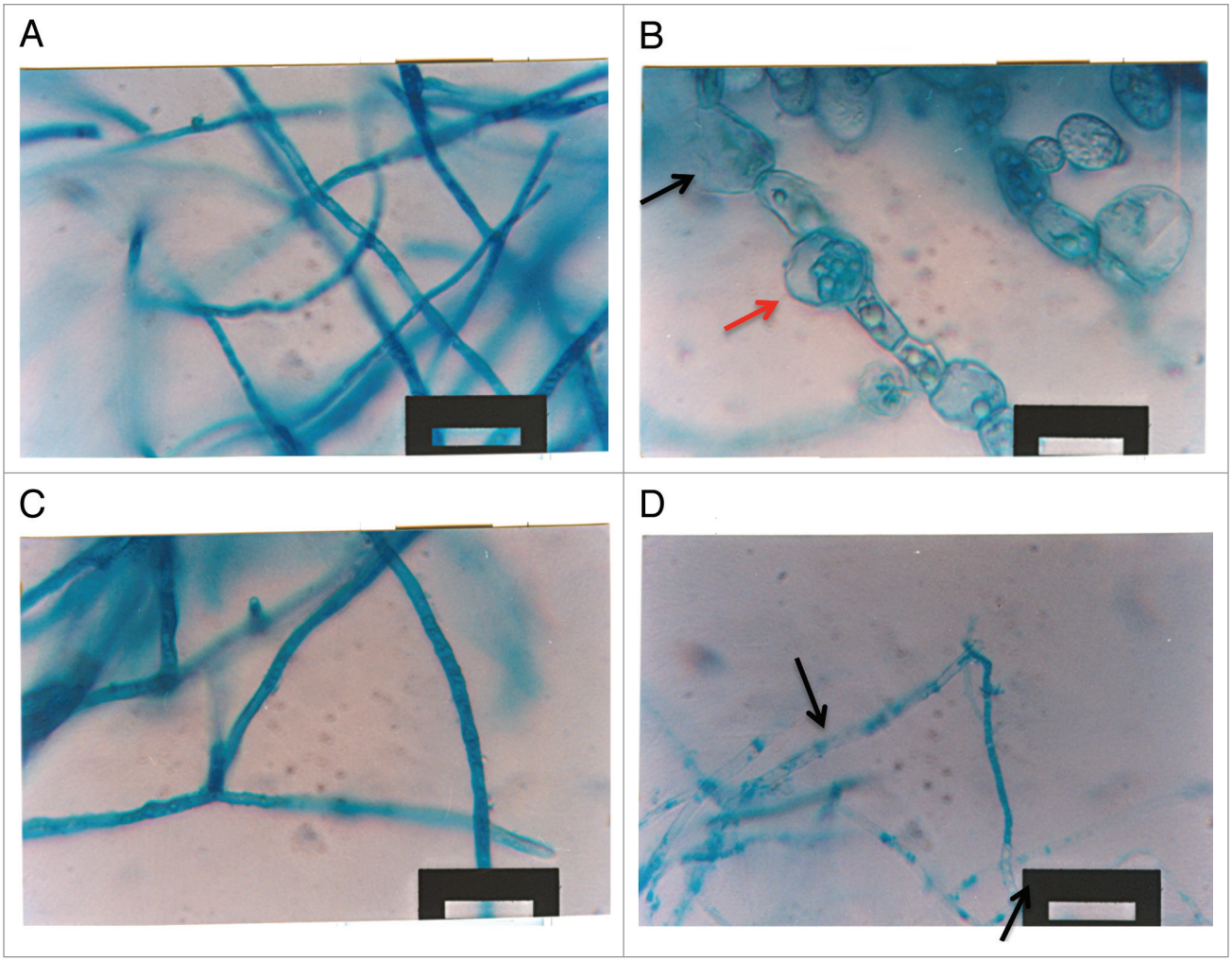

Figure 4. Morphological changes in phytopathogenic fungi by diffusible and volatile compounds produced by *B. tropica* strain MTo431. (**A**) *F. culmorum* without bacteria. (**B**) Effects of the volatile compounds on morphology of the fungus *F. culmorum*. (**C**) *F. oxysporum* without strain MTo431. (**D**) *F. oxysporum* in the presence of MTo431. The black arrows indicate hyphal swelling and red arrows denote protoplasm aggregation. The scale bar represents 1 mm.

(CAS) agar plates.[36] Two organic acids (succinc acid and malic acid), two sugars (sucrose and glucose), two polyols (glycerol and mannitol) and three amino acids (alanine, glutamic acid and ornithine) were used as carbon sources. Siderophore sequestration of iron on CAS agar changes the color of the medium from blue to orange around bacterial colonies after 72 h of incubation. Siderophore production was then measured based on the size of the orange halos formed around the colonies. In order to identify the chemical structures of the siderophores, the Csàcky method[29] was used to identify and quantify siderophores from the hydroxamate class, whereas catechol siderophores were identified and quantified by the Arnow method.[30]

Siderophore production in association with maize plants and plant protection. Maize seeds were germinated under sterility conditions in a solution with mineral salts and in the absence of carbon sources. The MTo431 strain was inoculated (10^4 CFU) in salt solution and incubated for 11 d. Siderophore production was determined by the Csàcky[29] Arnow[30] method.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We kindly thank Oscar Mascorro-Gallardo for providing fungi. We thank Juan Miranda-Rios and Tanya Romantsov for their critical reading of the manuscript. S.T-S. was supported with a doctoral scholarship (117009) by the Mexican Science and Technology Research Council (CONACYT). E.P.R. was supported by a grant (IN-209511) from DGAPA-UNAM and CONACYT grant 155116.

Supplemental Materials

Supplemental material may be found here:
www.landesbioscience.com/journals/bioengineered/articles/23808

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