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# *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 endophytes produce antifungal volatile organic compounds and exhibit additive plant growth-promoting effects

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ABSTRACT

Endophytic bacteria are part of the plant microbiome, which can promote the growth of plants and act as biocontrol agents against potential phytopathogens through various mechanisms, including the production of volatile compounds. In this work, we isolated and characterized two new bacterial endophytes, strains E25 and CR71, that exhibited antifungal activity and plant growth promotion. Analysis of the complete 16S ribosomal gene sequences of the strains showed high species-level identity (99%) with *Pseudomonas stutzeri* (E25) and *Stenotrophomonas maltophilia* (CR71). In in vitro assays, both strains showed excellent antagonistic action against *Botrytis cinerea* by emission of volatile organic compounds (VOCs), but not through diffusible compounds. Interestingly, the volatile cocktails emitted by E25 and CR71 were quite similar, highlighting the production of sulphur-containing compounds such as the antimicrobial volatile dimethyl disulphide (DMDS). Analysis of the pure DMDS compound showed mycelial inhibitory activity against the fungal strain. In a greenhouse experiment, inoculation of strains promoted the shoot and root length, chlorophyll content, and total fresh weight of tomato plants (*Lycopersicon esculentum* cv Saladette). Interestingly, when strains were co-inoculated, a better plant growth-promoting effect was observed. In conclusion, the co-inoculation of novel endophytic strains reported herein represents an excellent option to promote growth and achieve the biocontrol of *B. cinerea* through the production of potent volatiles such as DMDS.

## 1. Introduction

One of the main causes of losses in agricultural crops is the presence of phytopathogenic fungi. These pathogens may be crop-specific or mostly generalists such as the fungus *Botrytis cinerea*, which is responsible for grey mould disease and attacks more than 200 species of plants (Nambeesan et al., 2012; Williamson et al., 2007). In many countries, and especially in developing countries, the first option to control grey mould disease in various crops is the use of agrochemicals; however, the overuse of these compounds has been widely documented to cause various toxic effects on the environment (Adesemoye and Kloepper, 2009; Rosslenbroich and Stuebler, 2000). Therefore, it is important to search for new eco-friendly strategies to control *B. cinerea*.

The use of bioinoculants represents an excellent option to control phytopathogens while promoting plant growth and production, since, to our knowledge, negative effects on the environment, or on human or animal health have not been documented (Santoyo et al., 2012). Moreover, beneficial effects of certain bioinoculants have been reported, which have helped to diminish the employment of agrochemicals (Adesemoye and Kloepper, 2009). For example, several studies have shown the beneficial effects of engineering the plant microbiome to improve biocontrol and/or exhibit plant growth-promoting effects. As part of the beneficial plant microbiome, we here focus on the potential exploitation of bacterial endophytes for eco-friendly pest control (Sturz et al., 2000). Bacterial endophytes inhabit the interior of plant tissues without causing visible harm, and can be isolated from surface-disinfected tissues (Hardoim et al., 2008; Santoyo et al., 2016). The ability of diverse bacterial endophytes to promote plant growth occurs as a consequence of either direct or indirect mechanisms. Direct promotion of plant growth occurs when a bacterium either facilitates the acquisition of essential nutrients or modulates the level of hormones of a plant. Indirect promotion of plant growth occurs when the phytopathogenic activity is inhibited, which therefore decreases the damage to plants (Glick, 2014; Santoyo et al., 2012).

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Some reports have shown that bacteria can produce either antifungal diffusible or volatile compounds (Chaurasia et al., 2005; Hernández-León et al., 2015; Kanchiswamy et al., 2015). For example, the plant growth-promoting rhizobacteria (PGPR) *Arthrobacter agilis* UMCV2 produces volatiles with antagonistic action toward the fungal phytopathogen *B. cinerea* and the oomycete *Phytophthora cinnamomi*. Interestingly, a single compound from the volatile cocktail, namely dimethylhexadecylamine (DMHDA), was found to be responsible for inhibiting mycelial growth (Velázquez-Becerra et al., 2013). In addition, volatiles produced by *A. agilis* UMCV2 have shown the ability to promote growth in *Arabidopsis thaliana* and *Medicago truncatula* plants (Orozco-Mosqueda et al., 2013).

In another study, Huang et al. (2012) reported that dimethyl disulphide (DMDS), a volatile compound produced by the *Bacillus cereus* C1L strain, played a role as an elicitor of induced systemic resistance (ISR) in tobacco and corn plants. This compound elicited a protective response of the plant against *B. cinerea* and *Cochliobolus heterostrophus* under greenhouse conditions. However, DMDS only showed a weak effect on mycelial growth inhibition.

We previously demonstrated that *Pseudomonas fluorescens* strain UM270 is able to produce diffusible and volatile compounds against diverse fungal phytopathogens (Hernández-León et al., 2015). Interestingly, the strain UM270 produced a potent antimicrobial blend of volatiles, including DMDS and DMHDA, which have been reported as antifungals or elicitors of ISR. However, these compounds showed different levels of antagonism against the fungi: for some phytopathogens the mycelial growth inhibition reached up to 100%, whereas for others the inhibitory action was almost non-existent (Huang et al., 2012; Velázquez-Becerra et al., 2013).

Thus, in the present study, we characterized the volatile blends of two new bacterial endophytes with antifungal and plant growth-promoting activity. Both strains produced similar volatiles, including DMDS, which in a pure form was highly antagonistic against a pathogenic *B. cinerea* strain isolated from unhealthy strawberry plants. In addition, co-inoculation of both endophytic strains in tomato plants in a greenhouse experiment showed additive plant growth-promoting effects.

## 2. Materials and methods

### 2.1. Isolation of cultured endophytic bacteria

Plants of the tomatillo *Physalis ixocarpa* were collected from an agricultural field close to Morelia, Michoacán, México. The shoots and roots were washed with water to remove soil particles. Endophytic bacteria were collected from several tissues as described by Contreras et al. (2016). In brief, the roots were immersed in 70% ethanol for 30 s, washed with fresh sodium hypochlorite solution (2.5% available $Cl^-$) for 5 min, rinsed with 70% ethanol for 30 s, and finally washed five times with sterile distilled water. To further confirm the success of the sterilization process, aliquots of the sterile distilled water used in the final rinse were cultured on plates containing nutrient agar (NA) medium. The plates were examined for bacterial growth after incubation at 30 °C for 5 days. Uncontaminated roots, as detected by a culture-dependent sterility test, were used for the isolation of endophytic bacteria.

A collection of approximately 1000 strains was generated. The strains of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 were selected during preliminary analysis in the search of strains with antifungal and plant growth-promoting activities. The strains E25 and CR71 were grown at 30 °C for 24 h on NA plates and routinely maintained at 4 °C.

### 2.2. Phytopathogenic fungus

The fungus *B. cinerea* was used as the pathogenic strain, and inoculated in potato dextrose agar (PDA) at 30 °C for 3–5 days in the dark and kept at 4 °C. The same *B. cinerea* strains have been previously employed and reported in other works (Hernández-León et al., 2015; Martínez-Absalón et al., 2014).

### 2.3. Molecular characterization and phylogenetic analysis of CR71 and E25

Genomic DNA was isolated from the endophytic bacteria E25 and CR71, and the 16S ribosomal DNA subunit (rDNA) was amplified using polymerase chain reaction (PCR) with the universal bacterial primers fD1: 5´-CAGAGTTTGATCCTGGCTCAG-3´ and rD1: 5´-AAGGAGGTGATCCAGCC-3´ under previously reported PCR conditions (Hernández-León et al., 2015). PCR amplifications were performed using the TC-142 Thermocycler Techne thermal cycler (Keison Products, Chelmsford, UK) in Go Taq Master Mix tubes (Promega, Madison, WI, USA). The PCR product was further purified and the 16S rDNA regions of the bacterial isolates were sequenced. The ribosomal sequences were obtained and compared to the GenBank database using the Nucleotide Basic Local Alignment Search Tool (BLAST) program. The alignment of multiple sequences was generated with Clustal W (www.ebi.uk/Tools/clustalw2), and phylogenetic analysis of the 16S rRNA gene sequences was performed using the MEGA 4.0 program (Tamura et al., 2007). To obtain the confidence value for the set of aligned sequences, a bootstrap analysis of 1000 replicates was performed. The phylogenetic tree was constructed using the maximum parsimony algorithm. The GenBank accession numbers of the ribosomal sequences of *Pseudomonas stutzeri* E25 and *Stenotrophomonas maltophilia* CR71 are MG000977 and MF992168, respectively.

### 2.4. In vitro evaluation of the antagonistic effects of diffusible and volatile compounds

The antagonism of compounds produced by the endophytic bacteria against *B. cinerea* was evaluated in bioassays performed in Petri dishes as previously reported (Santoyo et al., 2010). In brief, the bacterial isolates were simultaneously striated with the pathogenic fungus in Petri dishes containing PDA. The bacteria were streaked in the cross-shaped dishes and a 4-mm portion of the mycelium was deposited in the centre of each of the formed quadrants on the plates. The Petri dishes were incubated in the dark at 30 °C and mycelial diameter growth was measured at day 6.

To evaluate the antifungal effect of the volatile organic compounds (VOCs) emitted by isolated bacterial endophyte strains, a bacterial inoculum of each strain ($1 \times 10^6$ CFU) was simultaneously deposited on one side of the Petri dish, and the 4-mm plug of the *B. cinerea* mycelium was inoculated in another portion of the plate. The cultures were incubated in the dark at 30 °C and the growth of the mycelial diameter was measured at day 6.

### 2.5. Preparation and antifungal bioassay with DMDS

DMDS (Sigma, St. Louis, MO, USA) was prepared as reported by Huang et al. (2012). Thus, DMDS was dissolved in ethanol to make a stock solution of 1.0 M DMDS. The DMDS stock solution was subsequently dispersed in Milli-Q water to make solutions of different concentrations, ranging from 0.1 to 1.0 µM, with an ethanol content of 10 mL/L immediately before application to Petri dishes.

The effect of DMDS on the mycelial growth of *B. cinerea* was investigated as a diffusible or volatile compound in normal or divided Petri dishes. When Petri dishes with separate compartments were used, a 4-mm-diameter portion of the mycelium was inoculated on one side of the divided dish containing PDA medium. Subsequently, the DMDS solution was added to the other side of the dish at different concentrations (0.1, 1, 10, 100, and 1000 µM). The Petri dishes were sealed with parafilm to minimize loss or volatilization of DMDS and incubated at 30 °C. After incubation for 5 days, the diameter of the fungal