

Antifungal Activity of *Bacillus amyloliquefaciens* NJN-6 Volatile Compounds against *Fusarium oxysporum* f. sp. *cubense*

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***Bacillus amyloliquefaciens* NJN-6 produces volatile compounds (VOCs) that inhibit the growth and spore germination of *Fusarium oxysporum* f. sp. *cubense*. Among the total of 36 volatile compounds detected, 11 compounds completely inhibited fungal growth. The antifungal activity of these compounds suggested that VOCs can play important roles over short and long distances in the suppression of *Fusarium oxysporum*.**

Microbial antagonist strains capable of producing both non-volatile compounds and volatile compounds (VOCs), which exhibit strong inhibitory activity against plant pathogens, have received much attention (4, 14). These antagonists include bacteria, such as *Pseudomonas* spp. (5), and nonpathogenic fungi like *Trichoderma* spp. (11). The release of VOCs by soil microbes has been reported to promote plant growth (13), display nematocidal activity (7), and induce systemic resistance in crops (3). Previous researchers also found that VOCs produced by bacteria could inhibit the growth (6) and the spore germination of pathogenic fungi (10), suggesting that VOCs produced by bacteria could be a mechanism of biocontrol against some soilborne fungal diseases.

Fusarium oxysporum is a well-known soilborne fungus, and some strains of *F. oxysporum* are pathogenic to plants and are difficult to control; however, biological methods may be a reliable alternative to chemical methods for controlling soilborne fungal growth. For applications in agriculture, the *Bacillus* species are considered important biological control agents. *Bacillus amyloliquefaciens* (NJNI-6), isolated from the rhizosphere soil of healthy banana plants, acts as an efficient antagonist against *F. oxysporum* f. sp. *cubense* by producing several antibiotics (15, 16). In this study, we characterized the volatile organic compounds produced by strain NJN-6. We used solid-phase microextraction (SPME) combined with gas chromatography-mass spectrometry (GC-MS) to extract and identify the VOCs. Finally, we identified antagonistic VOCs as those that reduced the growth and inhibited the spore germination of *F. oxysporum*.

Microorganisms and culture conditions. The antagonistic strain NJN-6 was identified as *B. amyloliquefaciens* (CGMCC [China General Microbiology Culture Collection Center] accession no. 3183) by 16S rRNA sequencing (15). The fungal strain *F. oxysporum* f. sp. *cubense*, which exhibited high virulence in banana plants, was used as the target fungus.

Antagonistic assay of VOCs against fungi. One compartment of the divided plates containing modified MS medium (with 1.5% [wt/vol] agar, 1.5% [wt/vol] sucrose, and 0.4% [wt/vol] TSA [3]) was inoculated with NJN-6, except for control plates. Another compartment containing PDA medium was used for *F. oxysporum* to test growth inhibition, or 100 μ l of spore solution (10^8 CFU/ml) was spread evenly to test the ability of the VOCs to inhibit the spore germination of fungi, or 10 g of diseased banana field soil from Ledong, Hainan Province, was added to one compartment. The plates were incubated at 28°C for 3 days, and then the diam-

eters of the fungal growth in different plates were measured. Inhibition of fungal spores from soils by VOCs was determined using Komada's salts-carbohydrate (SC) medium (9) after 45 days of incubation.

Collection of VOCs with SPME and analysis by GC-MS. Three SPME fibers from Supelco (Bellefonte, PA), polydimethylsiloxane (PDMS, 7 μ m), stable flex divinylbenzene-carboxene-PDMS (DCP, 50/30 μ m), and polydimethylsiloxane-divinylbenzene (PDMS-DVB, 65 μ m), were used to collect VOCs. Strain NJN-6 was inoculated into 40 ml of MS medium in a 100-ml vial with the SPME fiber inserted into the headspace. The vial was incubated at 28°C for 3 days and then placed in hot water at 50°C for 30 min. The results indicated that the DCP 50/30 μ m SPME fiber was better than the PDMS and PDMS-DVB fibers. GC-MS analysis was performed using Trace DSQ (Finnigan). The SPME fibers were desorbed at 220°C for 1 min with an RTX-5MS column (30 m, 0.25-mm inside diameter, 0.25 μ m). Each run was performed for 24 min. The initial oven temperature of 33°C was held for 3 min, ramped up at a rate of 10°C/min to 180°C, further ramped up at a rate of 40°C/min to 240°C, and held for 4 min. The mass spectrometer was operated in the electron ionization mode at 70 eV with a source temperature of 220°C, and a continuous scan from 50 m/z to 500 m/z was used. The mass spectra of VOCs were compared with those in the NIST/EPA/NIH Mass Spectrometry Library with respect to the spectra in the Mainlib and/or Replib databases.

Antifungal activity of bacterial VOCs against *F. oxysporum*. The VOCs produced by NJN-6 reduced the mycelial growth (Fig. 1A) and the germination of spores (Fig. 1B) of *F. oxysporum* in comparison with the control plates. The VOCs decreased the length of fungal mycelia, and colonies seemed to be significantly reduced. The inhibition of *F. oxysporum* by VOCs was about 30% to 40% compared with the control after 3 days, suggesting that the bacterial VOCs were unable to kill *F. oxysporum* but had a signif-

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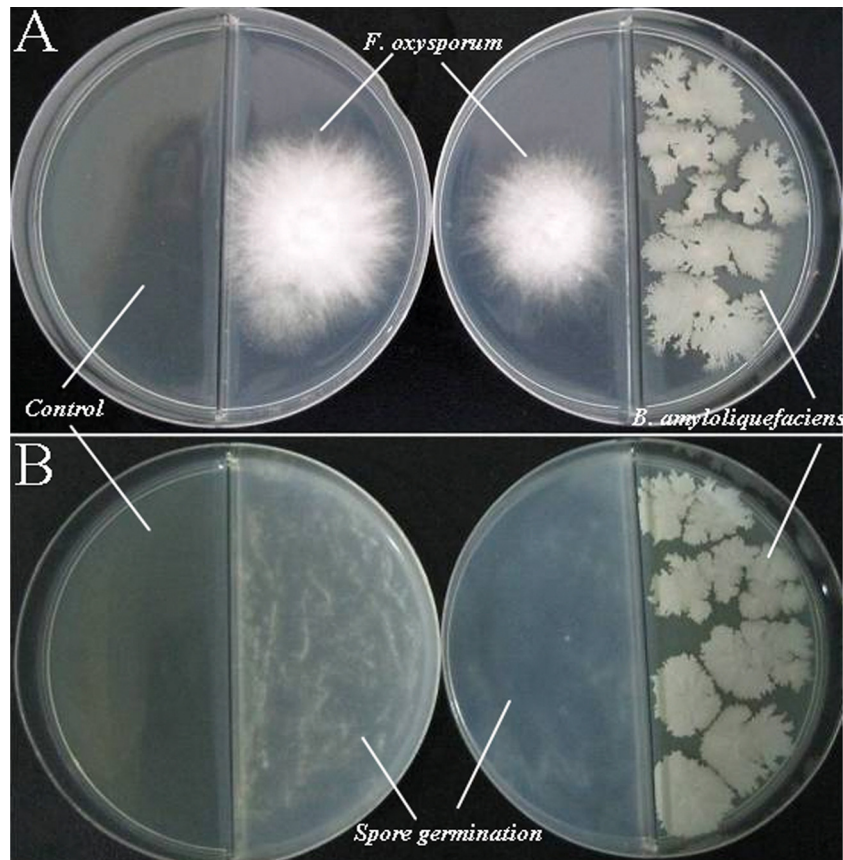


FIG 1 *B. amyloliquefaciens* NJN-6 antifungal volatile activity on divided plates. (A) Mycelial plug growth was inhibited in the presence of the bacteria streaked in the different compartments (right), compared to the control (left). (B) Volatile organic compounds produced by bacteria affected the germination of spores (right), compared to the control (left).

icantly inhibitory effect on fungal mycelia (Fig. 2). The VOCs produced by NJN-6 strongly antagonized *F. oxysporum* in soil and the quantity of *F. oxysporum* in the treated soil was 10^2 g^{-1} after 45 days while it was 10^4 g^{-1} in the control soil. Previous research has shown that abundant nutrients in the soil increased the production of bacterial VOCs, suggesting that the amount of organic

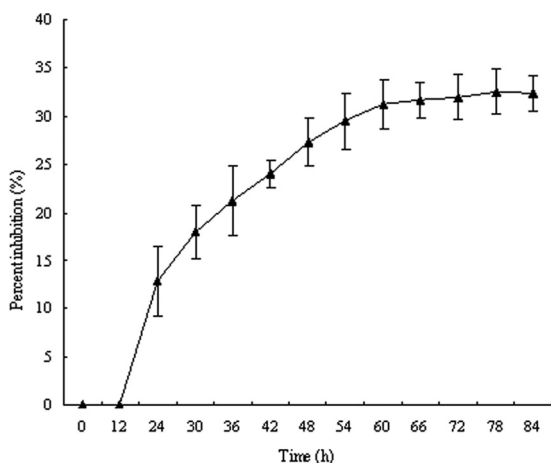


FIG 2 Inhibition of *F. oxysporum* mycelial growth by the *Bacillus amyloliquefaciens* NJN-6 antifungal volatile compounds on divided plates.

matter in the soil might affect the ability of NJN-6 to release VOCs into the soil (5, 6). Nonvolatile antibiotics, including lipopeptides, have strong antifungal activities (8, 12). However, these nonvolatile antibiotics cannot spread over long distances, and only when these antagonists directly colonize the plant roots can they prevent pathogenic fungi from infecting plants. In contrast, VOCs can spread over a long distance, and fungistatic microenvironments exist around the antagonist communities. In addition, the antifungal VOCs produced by bacteria can kill surviving spores in the soil and limit both the production and the establishment of the disease (5).

GC-MS analysis and antifungal activity of VOCs. The VOCs produced by NJN-6 strain were analyzed by GC-MS, and 36 compounds were detected, including 12 benzenes, seven alkyls, three alcohols, seven ketones, two aldehydes, three naphthyls, one ester, and one ether compound (see Table S1 in the supplemental material). The identified volatile compounds were purchased from Sigma-Aldrich and Aladdin. To check the antagonistic effect of VOCs, sterile filter paper discs containing 200 μl of each VOC were placed in one compartment while the other compartment was inoculated with *F. oxysporum* on PDA medium, and water was used as a control (5). After 3 days, the diameters of fungal colonies were measured, and percent inhibition was calculated as $(\text{control} - \text{treatment})/\text{control} \times 100$. All 36 compounds produced by NJN-6 were used for the antifungal activity assays (see Fig. S1 in

the supplemental material). The bacterial VOCs had been demonstrated to have antimicrobial activity, could promote plant growth, interacted with the host, and behaved as a signal among the bacterial communities. For example, *n*-decanal was shown to inhibit *Sclerotinia sclerotiorum* (5), allyl alcohol was shown to increase the populations of beneficial bacteria (1), and 2,3-butadienol was shown to promote plant growth (13). Phenol has well-established antiseptic uses in clinical applications because of its toxic activity for cells (2).

All 12 benzene compounds showed antifungal activity against *F. oxysporum*. The benzothiazoles phenol and 2,3,6-trimethylphenol completely inhibited the growth of *F. oxysporum* and exhibited high production levels, as indicated by the peak areas of the GCs, so it was likely that the two benzenes played an important role in the antifungal effects of the VOCs. Toluene, ethylbenzene, propylbenzene, and isopropylbenzene were less effective in inhibiting the growth of fungi. The antifungal activities of these compounds appeared to be related to their low benzene contents.

The alkyl group compounds like dodecane and tridecane did not show antifungal activities, while pentadecane and tetradecane exhibited inhibition of 22.5% and 18.4%, respectively. The alcohol, aldehyde, ester, ether, and naphthyl compounds (5 of 10 VOCs) could almost completely antagonize *F. oxysporum* at a concentration of 200 μ l *in vitro*; however, all of these compounds were produced at very low levels. Consequently, these compounds are not promising candidates as effective antifungal VOCs produced by NJN-6.

In contrast to the other VOCs, ketones were produced at high levels. The antifungal activity of ketones was negatively correlated with the number of carbon atoms in the ketones. The ketones 2-nonanone and 2-decanone exhibited 100% percent inhibition, but their contents were low in VOCs, as indicated by GC. Conversely, 2-tetradecanone and 2-pentadecanone exhibited large peaks areas on the GCs, but they did not show strong antifungal effects against *F. oxysporum*. Considering both ketone contents and antifungal effects, 2-undecanone, 2-dodecanone, and 2-tridecanone might be considered active ketone compounds.

The results of these experiments suggested a long-distance biocontrol mechanism of action of VOCs produced by *B. amyloliquefaciens* NJN-6 against *F. oxysporum*. Antagonistic activities could result from both the reduction of pathogen mycelial growth and the inhibition of spore germination. The results are useful for the better understanding of the biocontrol mechanisms by *B. amyloliquefaciens* against *F. oxysporum*.

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