Effects of bacteria-free filtrate from *Bacillus megaterium* strain L2 on the mycelium growth and spore germination of *Alternaria alternata*

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(Received 6 April 2015; accepted 29 June 2015)

Strain L2 (a *Bacillus megaterium* species that was isolated from tobacco’s rhizosphere) was applied to inhibit the pathogen *Alternaria alternata* in an attempt to reduce tobacco brown spot disease. This study focused on inhibition experiments, in which culture filtrate inhibited *A. alternata*’s mycelium growth, its spore germination rate and its spore output. The results indicated that an approximately 1.5%–20% concentration of the bacteria-free filtrate, could effectively inhibit hyphae growth. Additionally, bacterial suppression of germ tubes and mycelium growth was observed, rather than lysis. Furthermore, the filtrate caused some of the germinal tubes to undergo vacuolization and even rupturing. The inhibitory effect was associated with the change of the concentration: the greater the concentration, the greater the impact on *A. alternata*.

**Keywords:** *Bacillus megaterium*; antagonists; PGPR; *Alternaria alternata*; tobacco

**Introduction**

*Alternaria alternata* is a brown spot pathogen that was first discovered in the United States in the mid-1950s.[1] Furthermore, the severe leaf spot disease is an economically important disease that develops on mature tobacco leaves during the middle to latter part of the growing season.[2] Unfortunately, the pathogen causes dire economic consequences. For example, since *A. alternata* directly damages the tobacco yield and quality, the pathogen has caused estimated yearly losses of 3.9 to 21 million dollars to tobacco growers in North Carolina since 1959.[3]

Controlling the disease should be an apparent priority, yet this has proved to be a difficult task. Furthermore, all flue-cured tobacco cultivars are susceptible to the disease.[4] Over the past years, chemical-control methods have been the primary means of combating the brown spot.[5] However, many of the chemicals that are used to control plants’ fungal and bacterial diseases are detrimental to animal and human health alike. The truth is that the chemicals persist and eventually accumulate in the natural ecosystems. Consequently, chemical control may not be the most suitable means due to its high cost and environmental risks, associated with its application. As an alternative, several examples of controlling foliar fungal pathogens with bacteria have been reported.[6] It is therefore desirable to replace these chemical agents with biological approaches that are more environmentally friendly.[7,8]

Thus, biological control would be highly preferred for disease control in the future.[9]

One option for biological control of the disease is a subset of the total rhizosphere bacteria. It is part of healthy plants’ natural microflora and significantly assists health and general soil suppressiveness. In recognition of their beneficial nature, rhizobacteria were termed as plant growth-promoting rhizobacteria (PGPR). As their name suggests, PGPR benefit the host by stimulating plant growth; however, they can also function as a mean of biological disease control.[10] The rhizobacteria also directly reap benefits from this relationship, because plant roots secrete metabolites that the bacteria can utilize as nutrients.[11] This indirect promotion stimulates the biocontrol nature of PGPR, which can suppress deleterious effects or prevent phytopathogen damage.[7,12–19] Accordingly, efforts to select and apply PGPR for controlling specific soilborne fungal pathogens have been reviewed.[7,20]

In this study, a beneficial microbe (that we termed strain L2) was separated from the rhizosphere of tobacco and rhizosphere soil. Additionally, the mechanism of action was preliminarily investigated and the generation stability of antibacterial substances was inspected. Breed evaluation indicated that the strain L2 is *Bacillus megaterium*, which is a part of PGPR. We primarily observed and measured the inhibition effect of cell-free filtrate against *A. alternata* in the Bijie, Guizhou province, in
order to understand better how to biologically control the tobacco disease.

**Materials and methods**

**Materials**

The microorganisms used in the study were the *B. megaterium* strain L2 and *A. alternata*. The indicator fungi, *A. alternata*, was isolated and preserved by The Institute of Fungi Resource, College of Life Science, Guizhou University. The strain L2 was also isolated by The Institute of Fungi Resource, Guizhou and then preserved in the China Center for Type Culture Collection (Address: Wuhan University, WuHan, China). In order to sufficiently utilize the abundant microbe resources in the Guizhou province, we isolated the strain L2 of *B. megaterium* from 131 mature period soil samples from the eight prefectures (Qianxi, Dafang, Jinsha, Nayong, Hezhang, Zhijin, Weining counties and Bijie city), Bijie, Guizhou Province. The strain preservation number in the China Center for Type Culture Collection is CCTCCNO: M 2012381.

**Media**

The potato dextrose agar (PDA) medium consisted of the following: 200 mL potatoes extract (200 g potatoes were extracted in 1 L boiled water for 30 min and then were filtered through cotton gauze; after the extraction, the required distilled water was added to offset the evaporated water), 20 g glucose and 20 g agar, pH 7.0.

The nutrient agar (NA) medium consisted of the following: 10 g beef extract, 10 g peptone, 10 g NaCl, 20 g glucose, 17 g agar and 1000 mL distilled water, pH 7.0/C0 7.2.

The nutrient broth (NB) medium consisted of the following: 10 g beef extract, 10 g peptone, 10 g NaCl, 20 g glucose and 1000 mL distilled water, pH 7.0/C0 7.2.

The oat media plates consisted of the following: 20 g oat meal, 1 mL trace element solution, 18 g agar and 1000 mL distilled water, pH 7.0–7.2.

The trace element solution consisted of the following: 0.1 g FeSO₄·7H₂O, 0.1 g MnCl₂, 0.1 g ZnSO₄ and 100 mL distilled water.

**Methods**

**Bacteria-free filtrate preparation**

The strain L2 (stored at 4 °C) was inoculated onto plates with NA medium and then cultivated at 30 °C for 48 h. Next, the resulting mixture was inoculated into an NA culture solution and cultivated on a rotary shaker at 30 °C for 3 d (130 r/min). The medium filtrate solution was centrifuged at 5500 r/min for 25 min. The supernatant was collected and filtrated via a 0.22 μm bacterial filter to remove any bacteria. The bacteria-free medium filtrate was prepared and stored until later use.

**Experiments exhibiting the inhibitory effects of strain L2 against Alternaria alternata**

**Alternaria alternata mycelial growth inhibition**

After 3 d of cultivation in NA medium, the strain L2 was prepared via a colony diameter method onto culture plates (the final concentrations (V:V) in PDA were 1.5%, 3%, 6%, 10%, 15% and 20% bacteria-free filtrate) with PDA medium. An *A. alternata* colony (3 mm in diameter) was selected and inoculated onto the centre of the PDA plates; it was then cultivated in a thermostatic incubator at 28 °C. Then, *A. alternata*’s growing conditions at 3, 5 and 7 d were observed in order to measure and record the colony diameters (mm). For a control, an *A. alternata* colony with the same diameter was inoculated onto a PDA plate, and no L2 medium filtrate solution was added. Each experiment was repeated three times at 28 °C. The inhibited *A. alternata* colonies were selected for a microscopic (CX31 Olympus) observation of the mycelial growth. The inhibition rate of the mycelial growth was also observed,

\[
\text{Inhibition rate \%} = \frac{\text{Control colony diameter (mm)} - \text{Treated colony diameter (mm)}}{\text{Control colony diameter (mm)}} \times 100%.
\]

The nutrient agar (NA) medium consisted of the following: 10 g beef extract, 10 g peptone, 10 g NaCl, 20 g glucose, 17 g agar and 1000 mL distilled water, pH 7.0–7.2.

The nutrient broth (NB) medium consisted of the following: 10 g beef extract, 10 g peptone, 10 g NaCl, 20 g glucose and 1000 mL distilled water, pH 7.0–7.2.

The oat media plates consisted of the following: 20 g oat meal, 1 mL trace element solution, 18 g agar and 1000 mL distilled water, pH 7.0–7.2.

The trace element solution consisted of the following: 0.1 g FeSO₄·7H₂O, 0.1 g MnCl₂, 0.1 g ZnSO₄ and 100 mL distilled water.

**Spore germination inhibition rate**

The bacteria-free filtrates (100%, 60% and 20% (V:V), diluted with sterile water) were mixed with *A. alternata* spore suspensions at a ratio of 1:1 (V:V) (final contents of the bacteria-free filtrates in PDA were 50%, 30% and 10%). Moreover, to serve as control, an NA culture solution that did not have bacteria added was mixed with the *A. alternata* spore suspension at the same proportions. The mixtures were cultured at 26 °C on concave slides. The spore germination was microscopically (CX31 Olympus) observed after 6, 12 and 24 h. The germination was determined when the length of a germ tube exceeded half of the small-end diameter of the conidia. Each observation was repeated three times for computing the spore germination inhibition rate. At least 150–200 spores were examined in each visual field.

After the L2 bacteria-free filtrates were diluted with sterile water, we determined five concentrations: medium
filtrate stock solution, 1:2, 1:4, 1:8 and 1:16 (V_bacteria-free filtrates:V_sterile water). Moreover, an *A. alternata* conidia suspension was prepared (*A. alternata* colony after 7 d was scraped into the sterile water and then the hypha was filtered by filtration), with each microliter containing 400 spores. The mixtures were cultured on concave slides at 26°C. After 8–10 h, the conidia’s germination was observed under a microscope (CX31 Olympus). The conidia suspension that had medium filtrate broth added to it was used as a control.

Germination rate: $\text{Total germination rate} \% = \frac{\text{Germinated spores (spores/mL)}}{\text{Total spores (spores/mL)}} \times 100\%$

Germination inhibition rate: $\text{Germination inhibition rate} \% = \frac{\text{Germinated control spores (spores/mL)} - \text{Germinated treated spores (spores/mL)}}{\text{Germinated control spores (spores/mL)}} \times 100\%$

Sporulation: $\text{Control spores quantity (spores/mL)} - \text{Treated spores quantity (spores/mL)} \times 100\%$

**Alternaria alternata** spore number reduction and sporulation inhibition rate

The inhibitory effects of the bacteria-free filtrates on *A. alternata* spore production were measured via the mycelial soaking method [21] at different dilution factors: $\times 1, \times 10, \times 20, \times 40$ and $\times 80$. The *A. alternata* spores were coated onto oat media plates at 26°C for 2 d. The mycelial plates were soaked in 20 mL of the bacteria-free filtrates for 1 h. After the redundant liquids had been poured out, the plates were blow-dried aseptically and then cultured in the incubator at 26°C. The control was soaked in the NB culture solution. Each process was repeated three times. At 7 and 14 d, the plates were washed with a 2% sterile glucose liquid and then water was added to 20 mL. The spores were counted, and the inhibition rate was calculated. The conidiophores and conidia chains were observed under a microscope (CX31 Olympus).

**Results and discussion**

**The effect of bacteria-free filtrate on Alternaria alternata mycelial growth and morphology**

The L2 bacteria-free filtrates in concentrations of 1.5%, 3%, 6%, 10%, 15% and 20% (V:V) severely inhibited the growth of *A. alternata*’s mycelia (Figure 1). In the presence of the filtrates, they grew slowly. However, as the bacteria-free filtrates content was increased, the fungal colonies grew in an inversely proportional manner (Table 1). After 7 d, the inhibition rate reached 76%, which expressed the good effect of *A. alternata* on the mycelial growth inhibition. Under a microscopic observation, the *A. alternata* mycelial growth in the medium containing the bacteria-free filtrate, was obviously affected (Figure 2(A)). The mycelia were swollen (Figure 2(B)), deformed to tubercles (Figure 2(C)) and had obviously gathered bioplasm (Figure 2(D)). Then, the mycelia became swollen into bead-like shapes, the cells ruptured and the cytoplasm oozed out. Moreover, even at low concentrations (1.5%), the bacteria-free filtrate led to the aforementioned changes in the young mycelia (Figure 2(E)). In contrast, the mycelia in the control group were uniformly thin and long with smooth tops (Figure 2(F)). This result was the same as the one in the study of Lin et al. [22] and
had a significantly better inhibition effect when compared to the *Solanum nigrum* extract component.

**Inhibition of Alternaria alternata spore germination by bacteria-free filtrate**

Figure 3 illustrates how the L2 bacteria-free filtrates inhibited the germination of the *A. alternata* spores. During the germination, the spores first expand upon imbibing water and then grow germ tubes. The standard germination is when the germ tubes’ length exceeds half of the spore’s diameter. Clearly, after being treated with the stock solution, the majority of the spores was vacuolated and ruptured with cytoplasm exosmosis and thus could not develop into normal mycelia (Figure 3(A)). After treatment with 1:4 bacteria-free filtrates, the spores germinated abnormally and the germinating sites expanded and developed into round bubbles (Figure 3(B)). Regarding the germination time, the spores that were treated with the 1:4 bacteria-free filtrate germinated much more slowly than the control group, but if the abnormal germ tubes were subsequently treated with the stock solution, the previously-inhibited germ tubes were also vacuolated and ruptured along with the increased concentration of bacteria-free filtrates (Figure 3(C)). In contrast, the mycelia in the control group were uniformly thin and long with smooth tops (Figure 3(D)). Therefore, treating *A. alternata* spores with the L2 bacteria-free filtrates ultimately led to two consequences: (1) the spores vacuolated and ruptured, (2) the germ tubes developed abnormally and could continue to grow, but the germination time was significantly longer than a normal germination time. In conclusion, the bacteria-free L2 filtrates could obviously restrain the spore germination.

| Concentration | 3 d post-treatment (b1) | 5 d post-treatment (b2) | 7 d post-treatment (b3) |
|---------------|-------------------------|-------------------------|-------------------------|
|               | Colony diameter (mm)    | Inhibition rate (%)     | Colony diameter (mm)    | Inhibition rate (%)     | Colony diameter (mm)    | Inhibition rate (%)     |
| Control (a1)  | 23.4 ± 0.2              | –                       | 49.5 ± 0.3              | –                       | 72.5 ± 0.5              | –                       |
| 1.5% (a2)     | 16.4 ± 0.4              | 30.2                    | 36.3 ± 0.4              | 26.7                    | 47.5 ± 0.2              | 23.6                    |
| 3.0% (a3)     | 15.6 ± 0.2              | 33.6                    | 35.6 ± 0.1              | 28.1                    | 43.6 ± 0.5              | 39.9                    |
| 6.0% (a4)     | 13.7 ± 0.7              | 41.7                    | 32.2 ± 0.2              | 34.9                    | 35.8 ± 0.2              | 50.6                    |
| 10.0% (a5)    | 8.7 ± 0.4               | 63.0                    | 26.4 ± 0.4              | 46.7                    | 31.4 ± 0.1              | 56.7                    |
| 15.0% (a6)    | 7.4 ± 0.2               | 68.5                    | 21.5 ± 0.4              | 56.6                    | 17.4 ± 0.6              | 76.0                    |
| 20.0% (a7)    | 5.3 ± 0.2               | 77.4                    | 14.5 ± 0.3              | 70.7                    | –                       | –                       |

Note: The reported values are the average of three replications. 
\( R^2 = 0.934 > 0.7 \). It indicates that the model variable 1 and variable 2 fit well. 
\( P = 0.0002 \) for concentration (a); \( P < 0.001 \) for days (b); the data showed to be statistically significant through comparing the two groups as calculated by the method of significant difference.

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**Table 1. The inhibitory effects of bacteria-free filtrate on *A. alternata* mycelial growth.**

![Figure 2. Morphology of L2-inhibited *A. alternata* mycelium under a microscope. Microscopic magnification (10×) of (A) the inhibited pathogenic mycelium; (B) expansion of the pathogenic mycelium; (C) abnormal growth of the pathogenic mycelium; (D) protoplast aggregation; (E) influence of bacteria-free filtrate (1.5%) on young mycelial morphology; and (F) control.](image1)

![Figure 3. The effect of bacteria-free filtrate on *A. alternata* spore germination (640×). (A) Germ tube vacuolization; (B) abnormal germination of germ tubes; (C) germ tube vacuolization with high-concentration treatment; (D) control.](image2)
Table 2. The effect of culture filtrate on *A. alternata* spore germination.

| Conc. of bacteria-free filtrate (% V/V) | Germination rate (%) | Inhibition rate (%) | Germination rate (%) | Inhibition rate (%) | Germination rate (%) | Inhibition rate (%) |
|----------------------------------------|----------------------|---------------------|----------------------|---------------------|----------------------|---------------------|
| Control                                | 43.2                 | –                   | 72.3                 | –                   | 91.7                 | –                   |
| 10                                     | 32.8                 | 18.5                | 69.8                 | 8.7                 | 87.6                 | 11.5                |
| 30                                     | 9.8                  | 77.6                | 14.9                 | 83.6                | 31.4                 | 65.6                |
| 50                                     | 4.9                  | 91.1                | 7.5                  | 90.8                | 9.3                  | 89.9                |

Note: 150–200 conidia checked for each treatment.

Table 3. *A. alternata* spore number reduction and sporulation inhibition rate.

| Dilution factor of the bacteria-free filtrates | Spore number ($10^4$/mL) | Inhibition rate (%) | Spore number ($10^5$/mL) | Inhibition rate (%) |
|-----------------------------------------------|---------------------------|---------------------|---------------------------|---------------------|
| Control (a1)                                  | 1.21 ± 0.01               | –                   | 1.46 ± 0.01               | –                   |
| 80 (a2)                                       | 1.01 ± 0.03               | 16.5                | 1.22 ± 0.04               | 16.4                |
| 40 (a3)                                       | 0.82 ± 0.03               | 32.2                | 0.75 ± 0.02               | 48.6                |
| 20 (a4)                                       | 0.42 ± 0.02               | 65.3                | 0.44 ± 0.04               | 68.9                |
| 10 (a5)                                       | 0.35 ± 0.04               | 71.1                | 0.39 ± 0.04               | 73.3                |
| 1 (a6)                                        | 0.23 ± 0.03               | 81.0                | 0.3 ± 0.02                | 79.5                |

Note: The reported values are the average of three replications. 

$R^2 = 0.941 > 0.7$. It indicates that the model variable 1 and variable 2 fit well.

$P = 0.0047 < 0.005$ for concentration (a), the data showed to be statistically significant and indicated as calculated by the method of significant difference.

Table 2 shows that the L2 bacteria-free filtrates could all inhibit the spore germination at concentrations of 50%, 30% and 10%, but at 10%, the majority of spores developed germ tubes with only a small segmentation of the mycelia. Furthermore, the germ tubes were thick-short, slightly expanded and could not develop into mycelia. Obviously, the 10% concentration still inhibited the germination of *A. alternata* spores.

**Inhibition of Alternaria alternata sporulation by bacteria-free filtrate**

According to Table 3, the bacteria-free filtrate’s inhibition of *A. alternata* spore germination declined when the dilutions increased. Different final concentrations of the bacteria-free filtrates had different inhibition effect against *A. alternata* spore germination. At greater concentrations, the inhibition was more obvious. Besides, according to the data, the inhibition effect was not observed after the bacteria-free filtrates were diluted 80 times. Furthermore, the inhibition after 14 d still had a great effect, compared with the inhibition after 7 d. Through the examination under a microscope, we found that the conidiophores and conidia chains, which were disposed to the coarse metabolites, were sparse and short. The inhibited conidiophores were teratogenic (**Figure 4(A)**). Meanwhile, the quantity of the spore germinates clearly declined and the inhibited spore chains were been teratogenic (**Figure 4(B)**).
**Final remarks**

During biological prevention, the key to bio-controlling antagonistic bacteria is to control the diseases at an early stage. The most fundamental work concerning bio-controlling methods incorporates the screening of antagonistic bacteria (the best bio-control factor).[23] Furthermore, *Bacillus* has a set of distinctive advantages that makes it suitable for a bio-control material; this claim is demonstrated in the following explanations. *Bacillus* is easily screened because it is alogous, heat-resisting, generates spores, rhabitiform and it is Gram (+). Those and other factors, may make *Bacillus* a great bio-control material in the future. On the other hand, most of *Bacillus* excellent bio-control properties are closely associated with the strong antagonistic substance that is generated by the bacterial strains. According to Elliott et al.,[24] field applied chemical pesticides did not produce any negative effects for *Bacillus* strains. This indicates that the integrated use of biological and chemical-control measures were steady. Using bio-control agents to effectively suppress plant diseases is a key to control the early development of a disease.[25]

Conduction of research works into a larger field is encouraged in order to successfully biocontrol a regulated environment. Practical field control will depend on having an adequate introduction of the control agent at the appropriate times.[7]

**Conclusions**

In this study, the bacterial suppression of germ tube and mycelium growth of *A. alternata* was observed, rather than lysis. Our results indicated that the antagonistic substances produced by L2 were effective on the young mycelia even at very low concentrations. They also obviously inhibited the germination of *A. alternata* spores so that the spores were vacuolated, ruptured, or abnormally developed, thus slowing the germination. L2 most likely produced an antibacterial substance that inhibited the early germination, the *A. alternata* spores forming and the extension of germ tubes. Therefore, the antimicrobial substances were very stable and effective. This is the first time to illustrate the inhibition of *A. alternata* spore germination by antagonistic bacterium metabolites. It clearly showed the effect of antagonistic bacterium metabolites against pathogenic fungus growth. Currently, we have applied strain L2 medium filtrate to a Guizhou Province field and have verified the effect stepwise. Further work is necessary to determine the effective substances of this bacterium for pathogen inhibition and for growth-promoting effects on plants.

**Acknowledgements**

Zhu Li and Bokai Guo contributed equally to this article and should be considered co-first authors.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

The Institute of Fungi Resource’s work that is referred here was supported by grants from The National Natural Science Foundation of China [grant number 31460486]; The Guizhou Province Science and Technology Project [grant number NY (2014)3033]; The Guizhou Province Science and Technology Project [grant number SY (2014)3053]; The Guizhou University SRT Project [grant number SRT (2013)079]; The Bijie Tobacco Company Science and Technology Project [grant number BT(2011)06].

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