The endophytic fungus *Albifimbria verrucaria* from wild grape as an antagonist of *Botrytis cinerea* and other grape pathogens

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Abstract

Gray mold, caused by *Botrytis cinerea*, is one of the most prevalent fungal diseases in table and wine grapes, affecting grape quality and yields. In this study, we isolated several endophytic fungi including *Alternaria alternata*, *Bipolaris cynodontis*, *Phoma* sp., and *Albifimbria verrucaria* from leaves of Amur grape (*Vitis amurensis* Rupr.) cultivar 'Shuangyou' and investigated their biocontrol activity against *B. cinerea*. *In vitro* dual assay showed that *A. verrucaria* isolate SYE-1 inhibited growth of *B. cinerea*. The isolate also had a wide range of biocontrol activity against *Lasiodiplodia theobromae* and *Elsinoë ampelina*. Mycelial growth and conidium germination of *B. cinerea* were significantly inhibited by metabolites of *A. verrucaria* in agar plates and culture extracts of *A. verrucaria* from liquid culture. The isolate produced a total chitinase activity of 0.4 U/ml after incubation for 10 days in Czapek's liquid medium. In addition, application of culture extracts of *A. verrucaria* prior to *B. cinerea* inoculation significantly reduced disease severity on grape leaves of the susceptible 'Red Globe' cultivar. Taken together, our results indicate that *A. verrucaria* has potential as a biocontrol agents to control grape gray mold.

INTRODUCTION

Gray mold caused by *Botrytis cinerea*, is one of the most common fungal diseases in grape (Ciliberti et al. 2015). Necrotic lesions and soft rot are the most typical symptoms on leaves and berries, followed by a rapid appearance of gray masses of conidia (Williamson et al. 2007). *B. cinerea* causes serious losses in grape quality and yields during ripening and postharvest handling of grape berries (Hong et al. 2012). Although genetic and agronomic approaches have been used to control gray mold, chemical control using fungicides, such as benzimidazoles and dicarboximides (Elad 1994), is still the main approach for disease management (Angelini et al. 2014). However, environmental risks have restricted the use of pesticides, and therefore alternative approaches for plant disease control need to be developed. Biocontrol agents could be used as an alternative to chemical pesticides in viticulture.

In grapevine, different biocontrol agents function against diverse pathogens. *B. cinerea* can be controlled by various antagonists, including filamentous fungi (Elad 1994; Buxdorf et al. 2013), bacteria (Calvo et al. 2007; Martínez-Hidalgo et al. 2015), and yeasts (Raacke et al. 2006; Santos et al. 2004). For instance, endophytic bacteria, *Pseudomonas* sp. from onion and *Bacillus* strains from tomato can reduce disease severity of *B. cinerea* (Barka et al. 2002; Kefi et al. 2015). Possibly induced resistance, antibiosis, and direct parasitism by microbial antagonists may suppress the activity of pathogens on horticultural plants (Sharma et al. 2009). Among different microbial antagonists, endophytes are well recognized to provide benefits to their hosts, which include protecting host plants from pathogen attack (Kuldau and Bacon, 2008). However, little is known about endophytic fungi that function against fungal phytopathogens in grapevine.

Endophytic fungi that colonize plant tissues are mostly non-pathogenic. They often

form mutualistic associations that benefit plants by strengthening defense against biotic stressors (Rodriguez et al. 2009). Moreover, endophyte-colonized plants activate defense responses to virulent pathogens more rapidly than do noncolonized plants (Redman et al. 2001). Recent studies have shown that some endophytic fungi-induced resistance against biotic stressors is mediated by signaling molecules, and plant hormones such as salicylic acid, jasmonic acid (Buxdorf et al. 2013), and gibberellin (Cosme et al. 2016). However, the establishment of an endophytic fungal relationship largely depends on host genotype (Horton et al. 2014). Chinese wild grape germplasm has been identified as highly resistant to *Erysiphe necator*, *Elsinoë ampelina*, and *B. cinerea* (Gao et al. 2012; Wan et al. 2015; Wang et al. 1995; Yu et al. 2013). Thus, research relating to fungal endophytes and host specificity in native wild grapes will likely provide new evidence on potential biocontrol agents.

This study aimed to identify new fungal endophytes, especially beneficial endophytes that function against fungal phytopathogens of grape. Accordingly, we isolated endophytic fungi from leaves of Chinese wild grape and examined their potential as biocontrol agents against grape gray mold.

MATERIAL AND METHODS

Fungal isolation. Grape leaves were collected from 3-year-old Amur grape *V. amurensis* cv. Shuangyou in a germplasm nursery of Northwest A&F University, Shaanxi, China. Fungal isolation was performed as described by González et al. (2011). Fifty asymptomatic leaves were collected from the second or third nodes from the current-year shoot apex, and washed three times with sterile water, immersed in

3% sodium hypochlorite for surface sterilization, then washed again three times with sterile water. Sterilized small leaf pieces (5×5 mm) were placed on potato dextrose agar (PDA) plates and incubated at 25°C under 12-h daily illumination.

Strains and growth conditions. Isolated strains were maintained in solid culture on PDA at 25°C under 12-h daily illumination and transferred to fresh medium monthly. *Botrytis cinerea*, *Lasiodiplodia theobromae*, and *Elsinoë ampelina* were isolated from infected grape (*Vitis vinifera* cv. Red Globe) and cultured on PDA plates under the same conditions. In addition, we used tomato and cucumber strains of *B. cinerea* kindly provided by Prof. Shuxia Chen (Northwest A&F University, China). Liquid cultures of *A. verrucaria* were maintained in Czapek medium for 15 days at 25°C under 12-h daily illumination on a rotary shaker at 100 min^{-1}. Colony morphology and mycelium were observed under a stereo microscope (SZX16, Olympus, Japan) and photographed with a digital camera (G10, Canon, Japan). Conidia were viewed under a compound microscope (BX53, Olympus, Japan).

Molecular identification. Fungal identification was confirmed by the nuclear rDNA internal transcribed spacer (ITS) region. Genomic DNA of the isolated endophytes was extracted using the CTAB method as previously described (Damm et al. 2009). The ITS region were amplified using the primer ITS1/ITS4 (White et al. 1990). Obtained sequences were compared by Blast against the NCBI nr database, and top matches were downloaded. Sequences were aligned using ClustalX version 1.83 (Thompson et al. 1997). A neighbor-joining tree was constructed using PAUP version 4.0b10 (Sinauer Associates, Sunderland, MA) and viewed using Treeview

(University of Glasgow, Glasgow, UK) software. Bootstrap values were evaluated using 1000 replicates to test branch strength.

Inoculation of *A. verrucaria* and morphological observation. To investigate *A. verrucaria* development on the leaf surface, conidia of the fungus were suspended in sterile water, and the concentration was adjusted to 1×10^6 conidia/ml with a hemacytometer. The second and third leaves of *V. vinifera* 'Red Globe' were selected randomly from the top of the shoot apex with similar age and size in vines. The grape leaves were surface-sterilized with 1% hypochlorite solution for 5 min, rinsed three times with sterile water, and then sprayed with 1×10^6 conidia/ml of *A. verrucaria*. Detached leaves were placed on wet Whatman filter papers in a plastic box, covered with a clear plastic film to maintain humidity, and then placed in a growth chamber with a 12-h photoperiod at 25°C.

Small pieces (1 cm × 1 cm) were cut from the inoculated grape leaves, and then stained with trypan blue buffer (20 ml lactic acid, 20 ml glycerol, 20 ml phenol, 20 mg trypan blue, and 20 ml distilled water). The samples were boiled for 2 min in this buffer and then decolorized in 2.5 g/ml chloral hydrate overnight according to Yan et al. (2018). After rinsing three times with water, the specimens were stored in 20% (v/v) glycerol. For fluorescent observation, small pieces (1 cm × 1 cm) were cut from the grape leaves, fixed in 50% ethanol for 2 h and cleared in 20% KOH for 2 days in a 5 ml of plastic tube (Kobae et al. 2016). The specimens were soaked in phosphate buffer solution (PBS, pH 7.5) for 30 min and then stained using a PBS solution containing 20 μg/ml (g/v) fluorescein isothiocyanate-labeled wheat germ agglutinin

(WGA-FITC, Sigma–Aldrich) overnight at room temperature in the dark. Conidial development of *A. verrucaria* was observed using a light microscope from 1 to 5 days post-inoculation (dpi). Ten pieces from five inoculated grape leaves were observed each time. Each assay was performed three times.

Screening for *in vitro* activity of *A. verrucaria* against grape pathogens. Confrontation assays in vitro between *A. verrucaria* and *B. cinerea* were established as follows: Mycelial plug (5 mm diameter) from 3-day-old colonies of *A. verrucaria* were placed on PDA plates in a growth chamber with a 12-h photoperiod at 25°C. After 4 days, 5-mm agar plugs were cut from 3-day-old colonies of *B. cinerea* from grape and placed on opposite sides as previously described (Carsolio et al. 1994). Agar plugs that did not contain *A. verrucaria* served as the control. Colony diameter of *B. cinerea* was measured daily for one month. Four plates were selected for each replicate and the experiment was repeated three times on different dates.

For inhibition assays with different fungal pathogens, mycelial plugs from 3-day-old colonies of *A. verrucaria* were placed on PDA plates covered with glassine, which was removed with the fungi after incubating for 10 days. The PDA medium was turned over and the bottom sides of the medium were used to inoculate with the pathogenic fungi. Three kinds of grape pathogens (*B. cinerea*, *Lasiodiplodia theobromae*, and *Elsinoë ampelina*) and tomato and cucumber isolates of *B. cinerea* were inoculated on the bottom sides of the PDA plates with or without the *A. verrucaria*-secreted metabolites.

Conidial germination assay. For conidial germination inhibition assays, conidial

suspensions of the grape isolate of *Botrytis cinerea* were obtained using a standard protocol (Ritpitakphong et al. 2016) and also inoculated on the bottom sides of the PDA plates with or without the *A. verrucaria*-secreted metabolites. After 24 h, conidia were washed with sterile water from the PDA plates. Conidial germination percentages of 200 conidia were determined to define the number of germinated and ungerminated conidia under a microscope as previously described (Vesty et al., 2016). All plates were placed in a growth chamber with a 12-h photoperiod at 25°C. Four plates were selected for each replicate and the experiment was repeated three times on different dates.

Culture fraction assay. *A. verrucaria* isolate SYE-1 was grown on PDA medium at 25 °C for 3 days. For culture extract production, six 5-mm mycelial plugs from PDA cultures were placed in a 500 ml flask containing 200 ml of Czapek's liquid medium. The cultures were then incubated at 25 °C in a water bath shaker (100 min^{-1}) for 15 days according to Li et al. (2016). Culture extract was extracted with an equivalent volume of ethyl acetate three times from Czapek culture filtrate of *A. verrucaria*. Organic solvent fractions were collected and evaporated to dryness at 42°C (Paz et al. 2007). The dry fraction was dissolved in sterile distilled water and adjusted to a concentration of 300 μg/ml solution. Twenty-microliter solutions were used for *in vitro* assays after application to a PDA hole, which was located in the center of the PDA plates embedded with the conidia of the three *B. cinerea* isolates. Application of extracts from Czapek media without *A. verrucaria* served as the control. Four plates were selected in each replicate and the experiment was repeated

three times on different dates.

For conidial germination inhibition assays, the conidia of the three *B. cinerea* isolates were adjusted to 2×10^6 conidia/ml and then suspended in MP buffer (4% maltose and 1% peptone) as described by Zhang et al. (2015). One-third volume of the culture extract was added to 0.5 ml of conidium suspension, whereas sterile water was added to the control. All of the conidium suspensions were placed at 25°C for 24 h in a biochemical incubator. Conidial germination percentages were determined as described above. Percent germination inhibition relative to the control was analyzed as previously described (Buxdorf et al. 2013). Each assay was repeated three times.

Determination of chitinase activity. Eight 5-mm mycelial plugs from PDA cultures were placed in a 100-ml flask containing 50 ml of Czapek's liquid medium. The cultures were then incubated at 25°C in a water bath shaker (100 min^{-1}) for 10 days. The production of chitinase in the culture filtrate was measured at 2-day intervals. Chitinase activity was determinated by the colorimetric method as described by Reissig et al. (1955). The absorbance was measured at 585 nm in a UV-2450 spectrophotometer (Shimadzu, Japan). One unit (U) of enzyme activity was defined as the amount of enzyme that released 1 mg *N*-acetylglucosamine in 1 ml of culture filtrate per h.

Evaluation of *A. verrucaria* against *B. cinerea* on grape leaves. Leaves of *V. vinifera* cv. Red Globe were selected randomly from the second or third nodes from the shoot apex with similar age and size in vines. The leaves were surface sterilized with 70% ethanol for 10 s, rinsed three times with sterile water, and sprayed with the

culture extract of *A. verrucaria.* Control leaves were sprayed with sterile distilled water. Detached leaves were placed on wet Whatman filter papers in a plastic box, covered with a clear plastic film to maintain humidity, and then placed in a growth chamber with a 12-h photoperiod at 25°C. After 12 h, all detached leaves (inoculated and mock) were sprayed with 1×10^6 conidia/ml of the *B. cinerea* grape isolate (Ferrari et al. 2007) and inoculated with 5-mm mycelial plugs from 3-day-old colonies of the same isolate (Chen et al. 2004). Lesion areas were measured at 3 dpi as previously described (Adie et al. 2007). For each biological replicate, five leaves were chosen randomly from grape plants, and three biological replicates were collected on different dates.

Statistical analysis. One-way analysis of variance (ANOVA) using PROC ANOVA in SAS version 8.1 (SAS Institute, Cary, NC) was used for statistical analysis of *in vitro* inhibition assay and inoculation assay data. *P* values of 0.05 or 0.01 were used to denote statistical significance.

RESULTS

Isolate identification and in vitro assays. From the 50 surface disinfested leaves on PDA plates, 20 fungal isolates were obtained from *V. amurensis* cv. Shuangyou. The isolates were classified into four morphotypes, namely, *Alternaria alternata*, *Bipolaris cynodontis*, *Phoma* sp., and *Albifimbria verrucaria* based on ITS sequence (Fig. S1; Table S1). The results from our *in vitro* confrontation assays between four endophytes and *B. cinerea* revealed that the growth of the *B. cinerea* grape isolate was inhibited only by *A. verrucaria* (Fig. 1A, B). The colony diameter of *B. cinerea*

significantly decreased after 6 days on PDA plates in dual assay (Fig. 1C).

Characterization and development of *A. verrucaria*. *A. verrucaria* grew with compact and white mycelium and secreted flavescent metabolites when grown on PDA plates (Fig. 2A, B and C). Irregular black sporodochia were observed on the surface of colony after 15 days on PDA plates, and numerous conidia were distributed inside the sporodochia (Fig. 2D and E). The conidia were shallow green, spindle -shaped, single-celled, and contained several guttules (Fig. 2F). The conidia of *A. verrucaria* began to germinate and produce germ tubes at 1 dpi on the surface of grape leaves. Appressoria were observed from 4 dpi and penetrated the grape leaves starting from 5 dpi as observed with aniline blue staining (Fig. 3A) and WGA-FITC staining (Fig. 3B).

***In vitro* inhibition of *B. cinerea* by *A. verrucaria*.** *In vitro* confrontation assays between *A. verrucaria* isolates and the grape isolate of *B. cinerea* revealed that the growth of *B. cinerea* was inhibited by *A. verrucaria* (Fig. 4A). The colony diameter of *B. cinerea* significantly decreased 3-fold on 4-day-old PDA plates when compared with the control in dual assay (Fig. 4B). An antifungal circle was observed on 30-day -old PDA plates during the confrontation assays. In addition, the mycelial growth of the two other grape pathogens, *Lasiodiplodia theobromae* (*Lt*) and *Elsinoë ampelina* (*Ea*) was inhibited completely on the bottom sides of the PDA plates with *A. verrucaria*-secreted metabolites, and no such inhibition was observed on the control plates (Fig. 4C). Similarly, the other two *B. cinerea* isolates, from tomato (*BcT*) and cucumber (*BcC*) were also inhibited (Fig. S2). No germinated conidia of the grape

isolate of *B. cinerea* were observed on these plates (Fig. 4D).

In addition, culture extract of *A. verrucaria* could completely inhibit conidium germination of *Bc*G (Fig. 5A and B) and *Bc*T, whereas germination inhibition for *Bc*C also reached 92.3% (Fig. 5C). The time course of chitinase production by *A. verrucaria* SYE-1 is shown in Fig. 5D and reached 0.4 U/ml after incubation for 10 days. We obtained inhibition halos of 0.9, 1.1, and 1.2 cm, respectively, for *Bc*C, *Bc*T, and *Bc*G by measuring the diameter of growth inhibition around PDA holes, containing culture extract of *A. verrucaria*, and no inhibition with extracts from Czapek medium as a control (Fig. 6).

Effects of *A. verrucaria* extracts against *B. cinerea* on grape leaves. Based on the lines of evidence from the above assays, it is reasonable to conclude that *A. verrucaria* inhibits *B. cinerea* as a biocontrol agent *in vitro*. To investigate its antifungal ability *in vivo*, we examined the inhibition effect of *A. verrucaria* culture extracts against *B. cinerea* on leaves of grape. As shown in Fig. 7, lesion area as observed at 3 dpi was significantly reduced when leaves were sprayed with culture extract of *A. verrucaria* 3 days prior to inoculation with *B. cinerea* (conidium suspension and mycelial plug) as compared with the leaves sprayed with sterile water (control).

DISCUSSION

The fungal isolates obtained in this study are previously known grapevine endophytes, including *A. alternata* with high frequency (Kecskeméti et al. 2016; Pancher et al. 2012), as well as *Phoma* sp. and *B. cynodontis* with relative low frequency (González

and Tello 2011; Mostert et al. 2000). To our knowledge, this is the first report of *A. verrucaria* isolated as an endophyte from grapevine. Among these, *A. alternata* has been reported as a potential biocontrol agent for grape downy mildew (*Plasmopara viticola*) (Musetti et al. 2006).

The isolate *A. verrucaria* SYE-1 obtained from Chinese wild grape (Amur grape) may be considered as a potential candidate biocontrol agents. *A. verrucaria* is a synonym of *Myrothecium verrucaria*; several species of the genus *Myrothecium* were identified as endophytes in herbs, such as *M. inundatum* and *M. roridum* (Banerjee et al. 2010; Lin et al. 2014), whereas we isolated *A. verrucaria* from the woody host in the current study. Early studies showed that *A. verrucaria* functions as a bioherbicide against weeds (Walker and Tilley 1997), an antibiotic against bacteria (Zou et al. 2011) and a nematicide against root-knot nematode (Fernández et al. 2001), but rarely shows an antifungal effect (Härri et al. 1962). Here, we provided new evidence that *A. verrucaria* could act as an antifungal agent against *B. cinerea* and thus is capable of controlling grape gray mold. On the one hand, *in vitro* assays showed that *A. verrucaria* could inhibit mycelial growth and conidium germination of grape pathogens and of isolates of *B. cinerea* from other hosts. Additionally, application of *A. verrucaria* before pathogen inoculation significantly reduced the development of gray mold on leaves of susceptible grape. The antifungal ability may be attributable to the activity of secreted metabolites (Brakhage 2013).

A variety of bioactive metabolites of *A. verrucaria* have been identified as antimicrobial natural products, such as antibacterial verrucamides A-D (Zou et al.

2011), antitumor antibiotic myrocin C (Nakagawa et al. 1989), and antimicrobial diterpenoids (Hsu et al. 1988). It is worth noting that perhaps activation of unknown metabolites of *A. verrucaria* is more effective than the metabolites extracted in the current study. Therefore, an enhanced suppression of *B. cinerea* could result from the secreted antifungal metabolites that might prevent conidium germination and mycelium growth as indicated by our *in vitro* experiments. Another possible explanation is that chitinase produced by *A. verrucaria* might function to degrade the conidia and mycelia of *B. cinerea*. Our results are consistent with previous findings, showing that chitinase of *A. verrucaria* results in degradation of fungal mycelia (Vyas and Deshpande 1989). Nevertheless, the role of *A. verrucaria* as a biocontrol agent against grape disease is only a subset of its capability. *A. verrucaria* also has the ability to catalyze cyanamide to urea, which serves as a nitrogen source (Maier-Greiner et al. 1991), indicating that it not only mitigates the negative effects of pathogens, but also may alleviate nutrient stress.

Although many biocontrol agents have been found effective against *B. cinerea* in grape (Elmer et al. 2006), only few of these microbial antagonists have been obtained from grape. In the natural ecosystems, grape plants form symbiotic association with *A. verrucaria*, which may result in a greater positive effect on its host than would be observed with isolates from other plants. This could be explained by the fact that symbioses require well-matched architectural, physiological, morphological, and life history characteristics of both fungus and its host (Saikkonen et al. 2004). Several reports have shown the potential of *A. verrucaria* in biological control of weeds

(Sutton and Peng 1993; Zhang et al. 1994). In fact, a caution against the use of *A. verrucaria* has been issued because certain metabolites of this fungus are known to be toxic to mammalian cells (Anderson and Hallett 2004). Nevertheless, such toxic metabolites were not found in kudzu plants (*Pueraria montana*) after treatment with *A. verrucaria* spores (Abbas et al. 2001). Further research is needed to clarify and minimize environmental risks associated with the use of *A. verrucaria* or its metabolites.

In conclusion, we demonstrate that the endophyte *A. verrucaria* from Amur grape possesses a marked ability to suppress grape gray mold and such antifungal ability may also be effective in controlling *B. cinerea* from other hosts. Further investigations are in progress to explore its mode of action and clarify the mechanism by which *A. verrucaria* and associated molecules affect *B. cinerea* on grapevine plants.

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LITERATURE CITED

Abbas, H. K., Tak, H., Boyette, C. D., Shier, W. T., and Jarvis, B. B. 2001. Macrocyclic trichothecenes are undetectable in kudzu (*Pueraria montana*) plants treated with a high-producing isolate of *Myrothecium verrucaria*. Phytochemistry 58:269-276.

Adie, B. A., Pérez-Pérez, J., Pérez-Pérez, M. M., Godoy, M., Sánchez-Serrano, J. J.,

Schmelz, E.A., and Solano, R. 2007. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. Plant Cell 19:1665-1681.

Anderson, K. I., and Hallett, S. G. 2004. Herbicidal spectrum and activity of *Myrothecium verrucaria*. Weed Sci. 52:623-627.

Angelini, R. M. D. M., Rotolo, C., Masiello, M., Gerin, D., Pollastro, S., and Faretra, F. 2014. Occurrence of fungicide resistance in populations of *Botryotinia fuckeliana* (*Botrytis cinerea*) on table grape and strawberry in southern Italy. Pest Manag. Sci. 70:1785-1796.

Banerjee, D., Strobel, G. A., Booth, B., Sears, J., Spakowicz, D., and Busse, S. 2010. An endophytic *Myrothecium inundatum* producing volatile organic compounds. Mycosphere 1:241-247.

Barka, E. A., Gognies, S., Nowak, J., Audran, J. C., and Belarbi, A. 2002. Inhibitory effect of endophyte bacteria on *Botrytis cinerea* and its influence to promote the grapevine growth. Biol. Control 24:135-142.

Brakhage, A. A. 2013. Regulation of fungal secondary metabolism. Nat. Rev. Microbiol. 11:21-32.

Buxdorf, K., Rahat, I., Gafni, A., and Levy, M. 2013. The epiphytic fungus *Pseudozyma aphidis* induces jasmonic acid-and salicylic acid/nonexpressor of PR1-independent local and systemic resistance. Plant Physiol. 161:2014-2022.

Calvo, J., Calvente, V., de Orellano, M. E., Benuzzi, D., and de Tosetti, M. I. S. 2007. Biological control of postharvest spoilage caused by *Penicillium expansum* and *Botrytis cinerea* in apple by using the bacterium *Rahnella aquatilis*. Int. J. Food

Microbiol. 113:251-257.

Carsolio, C., Gutiérrez, A., Jiménez, B., Van Montagu, M., and Herrera-Estrella, A. 1994. Characterization of ech-42, a *Trichoderma harzianum* endochitinase gene expressed during mycoparasitism. Proc. Natl. Acad. Sci. USA 91:10903-10907.

Chen, S., Vaghchhipawala, Z., Li, W., Asard, H., and Dickman, M. B. 2004. Tomato phospholipid hydroperoxide glutathione peroxidase inhibits cell death induced by Bax and oxidative stresses in yeast and plants. Plant Physiol. 135:1630-1641.

Ciliberti, N., Fermaud, M., Languasco, L., and Rossi, V. 2015. Influence of fungal strain, temperature, and wetness duration on infection of grapevine inflorescences and young berry clusters by *Botrytis cinerea*. Phytopathology 105:325-333.

Cosme, M., Lu, J., Erb, M., Stout, M.J., Franken, P., Wurst, S. 2016. A fungal endophyte helps plants to tolerate root herbivory through changes in gibberellin and jasmonate signaling. New Phytol. 211:1065-1076.

Damm, U., Woudenberg, J. H. C., Cannon, P. F., and Crous, P. W. 2009. *Colletotrichum* species with curved conidia from herbaceous hosts. Fungal Divers. 39:45-87.

Elad, Y. 1994. Biological control of grape grey mould by *Trichoderma harzianum*. Crop. Prot. 13:35-38.

Elmer, P. A. G., and Reglinski, T. 2006. Biosuppression of *Botrytis cinerea* in grapes. Plant Pathol. 55:155-177.

Fernández, C., Rodríguez-Kábana, R., Warrior, P., and Kloepper, J. W. 2001. Induced soil suppressiveness to a root-knot nematode species by a nematicide. Biol. Control.

22:103-114.

Ferrari, S., Galletti, R., Denoux, C., De Lorenzo, G., Ausubel, F. M., and Dewdney, J. 2007. Resistance to *Botrytis cinerea* induced in *Arabidopsis* by elicitors is independent of salicylic acid, ethylene, or jasmonate signaling but requires PHYTOALEXIN DEFICIENT3. Plant Physiol. 144:367-379.

Gao, M., Wang, Q., Wan, R., Fei, Z., and Wang, X. 2012. Identification of genes differentially expressed in grapevine associated with resistance to *Elsinoë ampelina* through suppressive subtraction hybridization. Plant Physiol. Bioch. 58:253-268.

González, V., and Tello, M. L. 2011. The endophytic mycota associated with *Vitis vinifera* in central Spain. Fungal Divers. 47:29-42.

Härri, E., Loeffler, W., Sigg, H. P., Stähelin, H., Stoll, Ch., Tamm, Ch., and Wiesinger, D. 1962. Über die Verrucarine und Roridine, eine Gruppe von cytostatisch hochwirksamen Antibiotica aus *Myrothecium*-Arten. Helv. Chim. Acta. 45:839.

Hong, Y. S., Martinez, A., Liger-Belair, G., Jeandet, P., Nuzillard, J. M., and Cilindre, C. 2012. Metabolomics reveals simultaneous influences of plant defence system and fungal growth in *Botrytis cinerea*-infected *Vitis vinifera* cv. Chardonnay berries. J. Exp. Bot. 63:5773-5785.

Horton, M. W., Bodenhausen, N., Beilsmith, K., Meng, D., Muegge, B. D., Subramanian, S., Vetter, M. M., Vilhjálmsson, B. J., Nordborg, M., Gordon, J. I., and Bergelson, J. 2014. Genome-wide association study of *Arabidopsis thaliana*'s leaf microbial community. Nat. Commun. 5:5320.

Hsu, Y. H., Nakagawa, M., Hirota, A., Shima, S., and Nakayama, M. 1988. Structure

of myrocin B, a new diterpene antibiotic produced by *Myrothecium verrucaria*. Agr. Biol. Chem. 52:1305-1307.

Kecskeméti, E., Berkelmann-Löhnertz, B., and Reineke, A. 2016. Are epiphytic microbial communities in the carposphere of ripening grape clusters (*Vitis vinifera* L.) different between conventional, organic, and biodynamic grapes?. PloS One 11:e0160852.

Kefi, A., Slimene, I. B., Karkouch, I., Rihouey, C., Azaeiz, S., Bejaoui, M., Belaid, R., Cosette, P., and Limam, F. 2015. Characterization of endophytic *Bacillus* strains from tomato plants (*Lycopersicon esculentum*) displaying antifungal activity against *Botrytis cinerea* Pers. World J. Microb. Biot. 31:1967-1976.

Kobae, Y., Ohmori, Y., Saito, C., Yano, K., Ohtomo, R., and Fujiwara, T. 2016. Phosphate treatment strongly inhibits new arbuscule development but not the maintenance of arbuscule in mycorrhizal rice roots. Plant Physiol. 171:566-579.

Kuldau, G., Bacon, C.W. 2008. Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. Biol. Control. 46:57-71.

Li, Z., Fan, Y. C., Gao, L., Cao, X., Ye, J. L., and Li, G. 2016. The dual roles of zinc sulfate in mitigating peach gummosis. Plant Dis. 100:345-351.

Lin, T., Wang, G., Zhou, Y., Zeng, D., Liu, X., Ding, R., Jiang, X., Zhu, D., Shan, W., and Chen, H. 2014. Structure elucidation and biological activity of two new trichothecenes from an endophyte, *Myrothecium roridum*. J. Agr. Food Chem. 62:5993-6000.

Lurie, S., Pesis, E., Gadiyeva, O., Feygenberg, O., Ben-Arie, R., Kaplunov, T., Zutahy, Y., and Lichter, A. 2006. Modified ethanol atmosphere to control decay of

table grapes during storage. Postharvest Biol. Tec. 42:222-227.

Maier-Greiner, U. H., Obermaier-Skrobranek, B. M., Estermaier, L. M., Kammerloher, W., Freund, C., Wülfing C., Burkert, U., Matern, D. H., Breuer, M., and Eulitz, M. 1991. Isolation and properties of a nitrile hydratase from the soil fungus *Myrothecium verrucaria* that is highly specific for the fertilizer cyanamide and cloning of its gene. Proc. Natl. Acad. Sci. USA 88:4260-4264.

Martínez-Hidalgo, P., García, J. M., and Pozo, M. J. 2015. Induced systemic resistance against *Botrytis cinerea* by *Micromonospora* strains isolated from root nodules. Front. Microbiol. 6:922.

Mostert, L., Crous, P. W., and Petrini, O. 2000. Endophytic fungi associated with shoots and leaves of *Vitis vinifera*, with specific reference to the *Phomopsis viticola* complex. Sydowia 52:46-58.

Musetti, R., Vecchione, A., Stringher, L., Borselli, S., Zulini, L., Marzani, C., D'Ambrosio, M., di Toppi, L. S., and Pertot, I. 2006. Inhibition of sporulation and ultrastructural alterations of grapevine downy mildew by the endophytic fungus *Alternaria alternata*. Phytopathology 96:689-698.

Nakagawa, M., Hsu, Y. H., Hirota, A., Shima, S., and Nakayama, M. 1989. Myrocin C, a new diterpene antitumor antibiotic from *Myrothecium verrucaria*. I. Taxonomy of the producing strain, fermentation, isolation and biological properties. J. Antibiot. 42:218-222.

Pancher, M., Ceol, M., Corneo, P. E., Longa, C. M. O., Yousaf, S., Pertot, I., and Campisano, A. 2012. Fungal endophytic communities in grapevines (*Vitis vinifera*

L.) respond to crop management. Appl. Environ. Microbiol. 78:4308-4317.

Paz, Z., Burdman, S., Gerson, U., and Sztejnberg, A. 2007. Antagonistic effects of the endophytic fungus *Meira geulakonigii* on the citrus rust mite *Phyllocoptruta oleivora*. J. Appl. Microbiol. 103:2570-2579.

Raacke, I. C., von Rad, U., Mueller, M. J., and Berger, S. 2006. Yeast increases resistance in Arabidopsis against *Pseudomonas syringae* and *Botrytis cinerea* by salicylic acid-dependent as well as -independent mechanisms. Mol. Plant Microbe. Interact. 19:1138-1146.

Redman, R. S., Dunigan, D. D., and Rodriguez, R. J. 2001. Fungal symbiosis: from mutualism to parasitism, who controls the outcome, host or invader? New Phytol. 151:705-716.

Reissig, J. L., Strominger, J. L., and Leoloir, I. F. 1955. A modified colorimetric method for the estimation of *N*- acetylamino sugars. J. Biol. Chem. 217:959-966.

Ritpitakphong, U., Falquet, L., Vimoltust, A., Berger, A., Métraux, J. P., and L'Haridon, F. 2016. The microbiome of the leaf surface of *Arabidopsis* protects against a fungal pathogen. New Phytol. 210:1033-1043.

Rodriguez, R. J., White Jr, J. F., Arnold, A. E., and Redman, R. S. 2009. Fungal endophytes: diversity and functional roles. New Phytol. 182:314-330.

Saikkonen, K., Wäli, P., Helander, M., and Faeth, S. H. 2004. Evolution of endophyte–plant symbioses. Trends Plant Sci. 9:275-280.

Santos, A., Sanchez, A., and Marquina, D. 2004. Yeasts as biological agents to control *Botrytis cinerea*. Microbiol. Res. 159:331-338.

Sutton, J.C., and Peng, G. 1993. Biocontrol of *Botrytis cinerea* in strawberry leaves. Phytopathology 83:615-621.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, and F., Higgins, D. G. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25:4876-4882.

Vesty, E. F., Saidi, Y., Moody, L. A., Holloway, D., Whitbread, A., Needs, S., Choudhary, A., Burns, B., McLeod, D., Bradshaw, S. J., Bae, H., King, B. C., Bassel, G. W., Simonsen, H. T., and Coates, J. C. 2016. The decision to germinate is regulated by divergent molecular networks in spores and seeds. New Phytol. 211:952-966.

Vyas, P., and Deshpande, M. V. 1989. Chitinase production by *Myrothecium verrucaria* and its significance for fungal mycelia degradation. J. Gen. Appl. Microbiol. 35:343-350.

Walker, H. L., and Tilley, A. M. 1997. Evaluation of an isolate of *Myrothecium verrucaria* from sicklepod (*Senna obtusifolia*) as a potential mycoherbicide agent. Biol. Control 10:104-112.

Wan, R., Hou, X., Wang, X., Qu, J., Singer, S. D., Wang, Y., and Wang, X. 2015. Resistance evaluation of Chinese wild *Vitis* genotypes against *Botrytis cinerea* and different responses of resistant and susceptible hosts to the infection. Front. Plant Sci. 6:854.

Wang, Y., Liu, Y., He, P., Chen, J., Lamikanra, O., and Lu, J. 1995. Evaluation of foliar resistance to *Uncinula necator* in Chinese wild *Vitis* species. Vitis

34:159-164.

White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR protocols. A guide to methods and applications. Innis, M. A., Gelfland, D. H., Sninsky, J. J., and White, T. J. eds. Academic Press, San Diego.

Williamson, B., Tudzynski, B., Tudzynski, P., and van Kan, J. A. 2007. *Botrytis cinerea*: the cause of grey mould disease. Mol. Plant Pathol. 8:561-580.

Yan, H. Q., Zhang, T. T., Lan, S. C., and Jiang, S. 2018. Ultrastructural study on the interaction between *Physcomitrella patens* and *Botrytis cinerea*. Plant Pathol. 67:42-50.

Yu, Y., Xu, W., Wang, J., Wang, L., Yao, W., Yang, Y., Ma, F., Du, Y., and Wang, Y. 2013. The Chinese wild grapevine (*Vitis pseudoreticulata*) E3 ubiquitin ligase *Erysiphe necator*-induced RING finger protein 1 (EIRP1) activates plant defense responses by inducing proteolysis of the VpWRKY11 transcription factor. New Phytol. 200:834-846.

Zhang, P. G., Sutton, J. C., and Hopkin, A. A. 1994. Evaluation of microorganisms for biocontrol of *Botrytis cinerea* in container-grown black spruce seedlings. Can. J. Forest Res. 24:1312-1316.

Zhang, Y., Jin, X., Ouyang, Z., Li, X., Liu, B., Huang, L., Hong, Y., Zhang, H., Song, F., and Li, D. 2015. Vitamin B6 contributes to disease resistance against *Pseudomonas syringae* pv. *tomato* DC3000 and *Botrytis cinerea* in *Arabidopsis thaliana*. J. Plant Physiol. 175:21-25.

Zou, X., Niu, S., Ren, J., Li, E., Liu, X., and Che, Y. 2011. Verrucamides A–D, antibacterial cyclopeptides from *Myrothecium verrucaria*. J. Nat. Prod. 74:1111-1116.

Supporting information

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Neighbor-joining tree of four endophytic isolates from Amur grape (*Vitis amurensis*) with bootstrap values obtained from the internal transcribed spacer. *Myriangium hispanicum* used as outgroup. Isolated endophytes are indicated with a black dot.

Fig. S2. *In vitro* inhibition of *Botrytis cinerea* by *Albifimbria verrucaria* metabolites on potato dextrose agar (PDA). Morphology of *B. cinerea* from tomato (**A**) and cucumber (**B**) after 4 days on PDA plate. **C**, Growth inhibition of *B. cinerea* from tomato (*Bc*T) and cucumber (*Bc*C) on bottom side of PDA colonized by *A. verrucaria* on the upper side of the medium.

Table S1. Numbers of isolates recovered and molecular identification of endophytic fungus isolates obtained from Amur grape (*Vitis amurensis*) based on BLAST analyses of ITS1-5.8S rDNA-ITS2 region.

Figure legends

Fig. 1. *In vitro* dual assays between four endophytic fungi and a grape isolate of *Botrytis cinerea*. A, Morphology of four endophytic fungi from leaves of *Vitis amurensis* cv. Shuangyou after 15-day growth on potato dextrose agar (PDA) medium. B, Dual assays between selected endophytes (left) and *B. cinerea* (right). C, Colony

diameter of *B. cinerea* in dual assays with four fungal endophytes after 6 days on PDA. Values are means and standard deviations of three replicates. Different letters above the bars indicate statistically significant differences ($P<0.05$) according to one-way ANOVA followed by Tukey's test.

Fig. 2. Morphology of *Albifimbria verrucaria* on potato dextrose agar (PDA). **A** and **B**, Front and back of *A. verrucaria* after 15-day growth. **C**, Hyphae of *A. verrucaria*. **D**, Magnification of (A) showing black sporodochium on colony surface. **E**, Higher magnification of (D). **F**, Conidia of *A. verrucaria*.

Fig. 3. Conidial development of *Albifimbria verrucaria* on leaves of *Vitis vinifera* 'Red Globe' at various days post-inoculation (dpi). A, Conidial germination on grape leaves after trypan blue staining. B, Conidial germination on grape leaves stained with WGA-FITC from 1 to 5 dpi. ap, appresorium; c, conidium; is, infection site; gt, germ tube. Scale bar = 10 μm.

Fig. 4. *In vitro* inhibition of a grape isolate of *Botrytis cinerea* by *Albifimbria verrucaria* metabolites on potato dextrose agar (PDA). **A**, Paired culture of *A. verrucaria* and *B. cinerea*. **B**, Colony diameter of *B. cinerea* in paired culture (A) after 4-days on PDA. **C**, Growth inhibition of grape isolate of *B. cinerea* (*Bc*), *Lasiodiplodia theobromae* (*Lt*) and *Elsinoë ampelina* (*Ea*) on the bottom side of PDA (control) and PDA with *A. verrucaria* (AV) growing on upper side. **D**, Percentage conidium germination of *B. cinerea* on bottom side of AV and control. Values are means and standard deviations of three replicates. Different letters above the bars indicate statistically significant differences ($P<0.01$) according to one-way ANOVA

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Fig. 5. Inhitition of *Botrytis cinerea* by *Albifimbria verrucaria* (AV) metabolites. A, Inhibition of conidium germination of *B. cinerea* from cucumber (*Bc*C), tomato (*Bc*T), and grape (*Bc*G) by culture extracts of AV relative to the Czapek medium fraction (control). Conidium germination of *B. cinerea* from grape in MP (maltose-peptone) buffer (B) and MP buffer containing culture extracts of AV (C) after 24-h; bar= 25 μm. D, Chitinase production by *A. verrucaria* grown in Czapek's liquid medium. Values are means and standard deviations of three replicates.

Fig. 6. Inhibition halo assays. **A**, Mycelial growth-inhibition of *Botrytis cinerea* from cucumber (*Bc*C), tomato (*Bc*T), and grape (*Bc*G) by culture extracts of *Albifimbria verrucaria* (AV) relative to Czapek medium fraction (control) after 4 days on potato dextrose agar. **B**, Diameter of inhibition halo. Values are means and standard deviations of three replicates.

Fig. 7. Biocontrol ability of *Albifimbria verrucaria* against *Botrytis cinerea* on grape leaves. **A**, Photographs of detached grape leaves treated with filtrate fraction (AV_F) of *A. verrucaria* and inoculated with spores and mycelial plugs of *B. cinerea* (*Bc*). Detached grape leaves were sprayed with filtrate fraction of *A. verrucaria* at 12 h and *A. verrucaria* spores at 3 days prior to inoculation with *B. cinerea*. Photos were taken at 3 days post-inoculation (dpi). Bar = 1 cm. **B**, Lesion area of detached grape leaves of *Bc* and AV_F+ *Bc* after inoculation with spores and mycelial plugs of *B. cinerea*. Values are means and standard deviations of three replicates. Different letters above the bars indicate statistically significant differences ($P<0.01$) according to one-way

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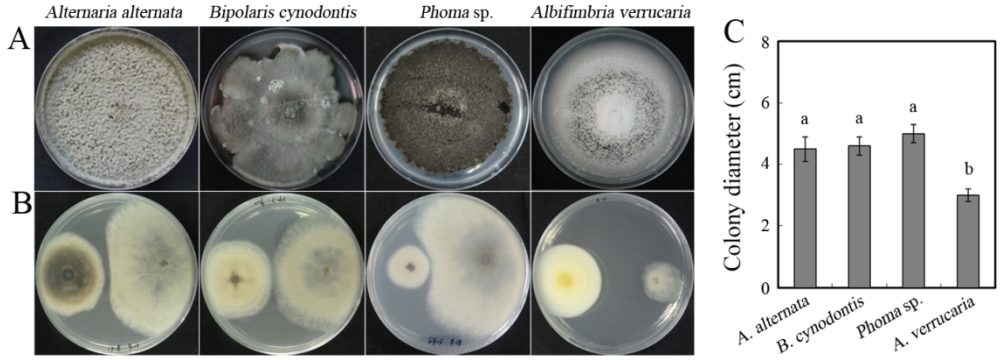

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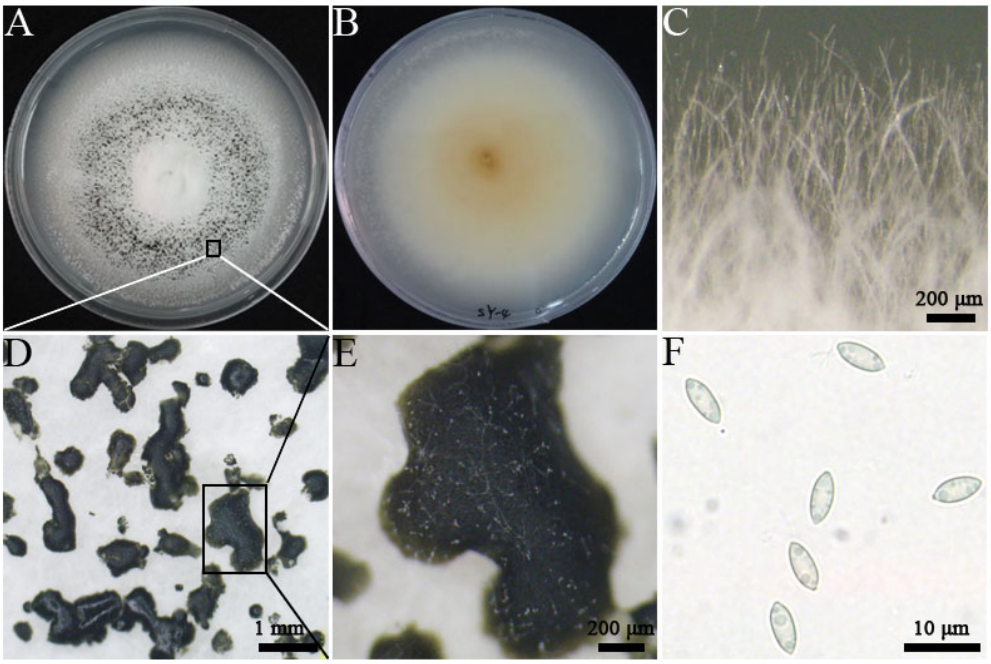

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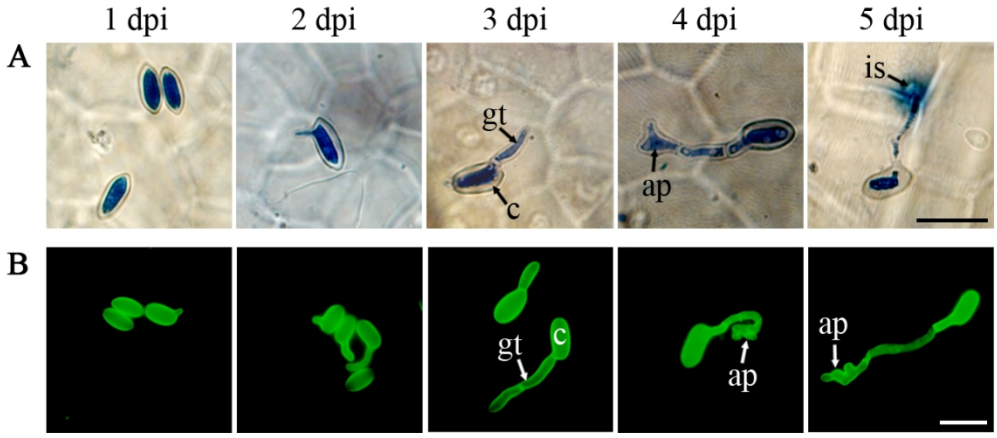

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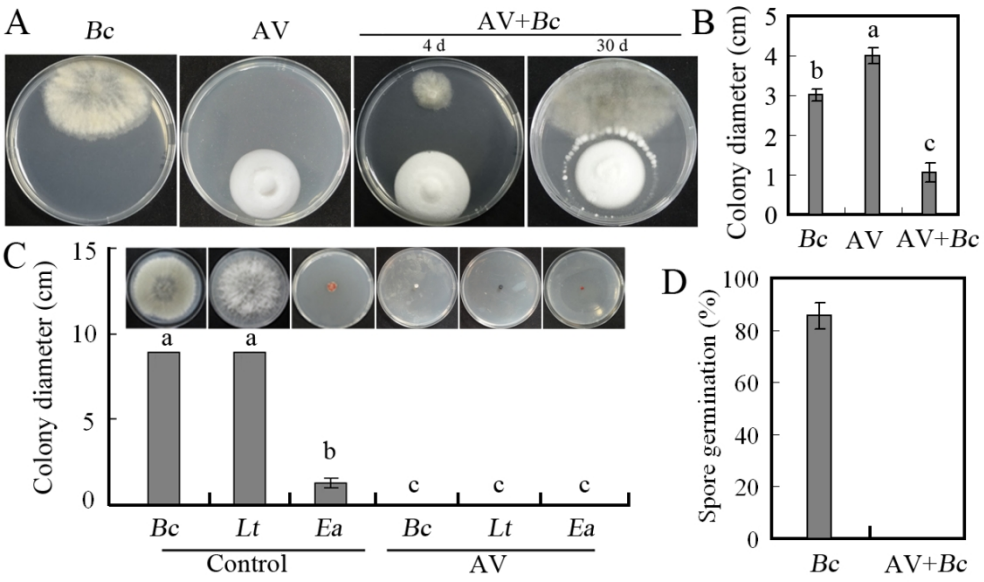

Fig. 4. In vitro inhibition of a grape isolate of Botrytis cinerea by Albifimbria verrucaria metabolites on potato dextrose agar (PDA). A, Paired culture of A. verrucaria and B. cinerea. B, Colony diameter of B. cinerea in paired culture (A) after 4-days on PDA. C, Growth inhibition of grape isolate of B. cinerea (Bc), Lasiodiplodia theobromae (Lt) and Elsinoë ampelina (Ea) on the bottom side of PDA (control) and PDA with A. verrucaria (AV) growing on upper side. D, Percentage conidium germination of B. cinerea on bottom side of AV and control. Values are means and standard deviations of three replicates. Different letters above the bars indicate statistically significant differences ($P<0.01$) according to one-way ANOVA followed by Tukey's test.

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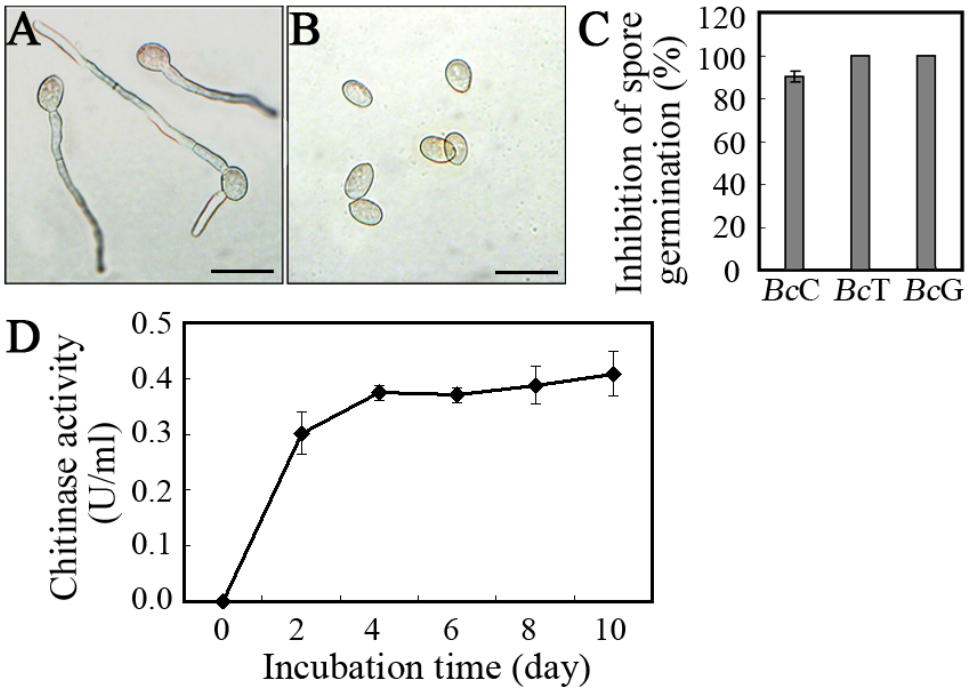

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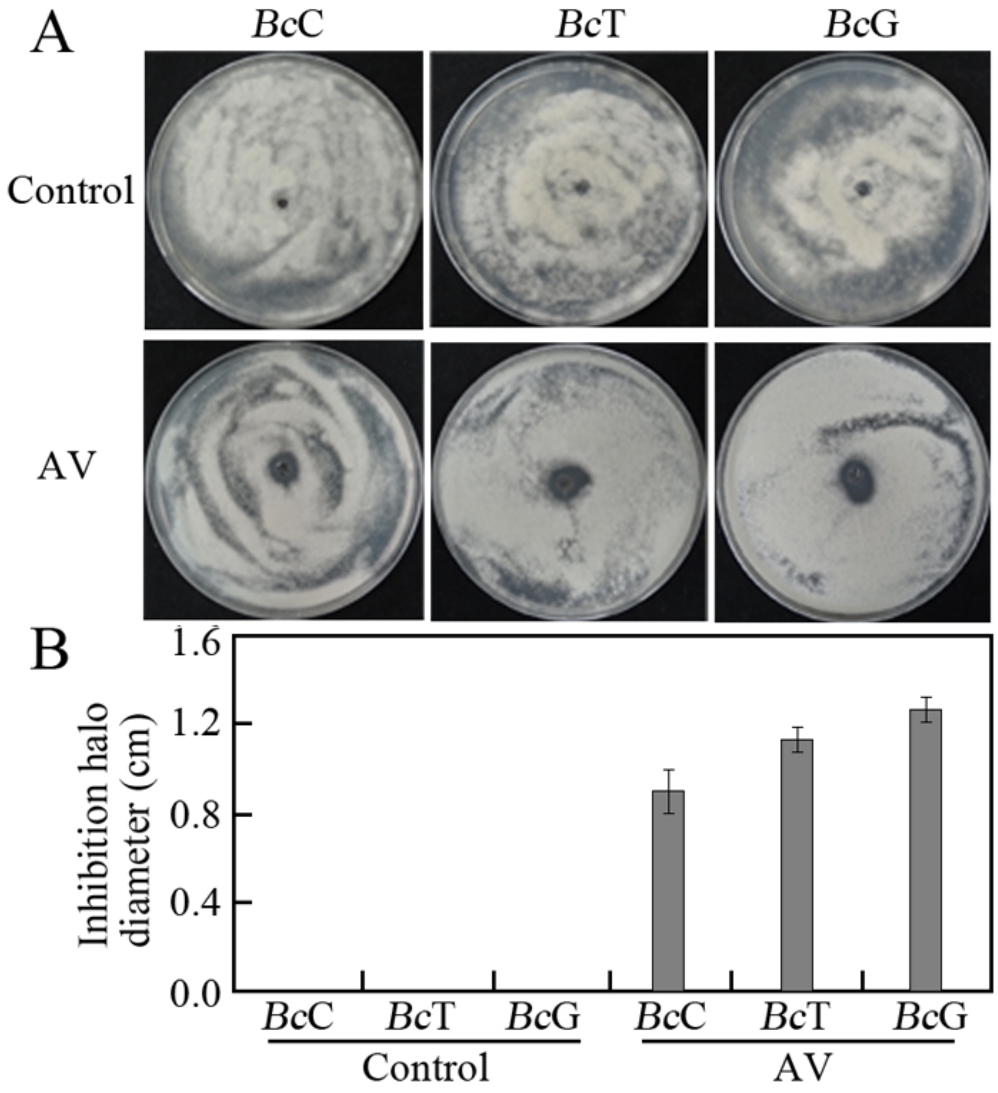

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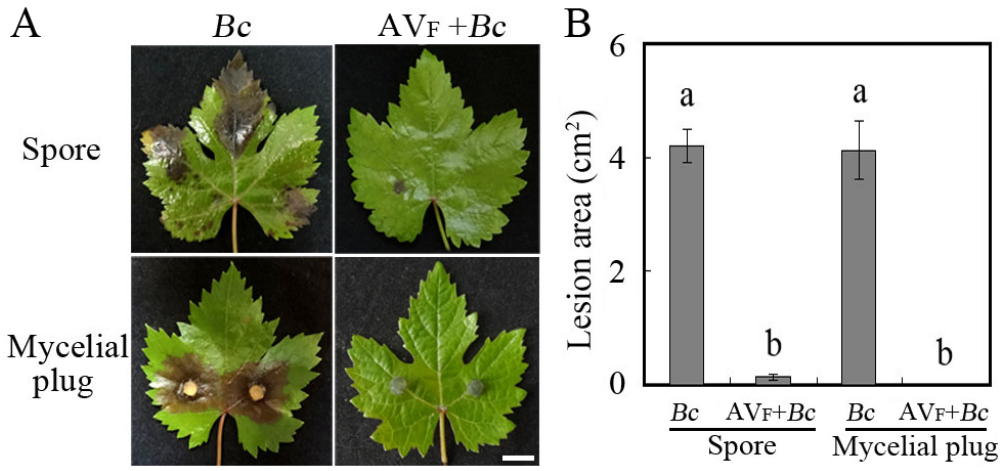

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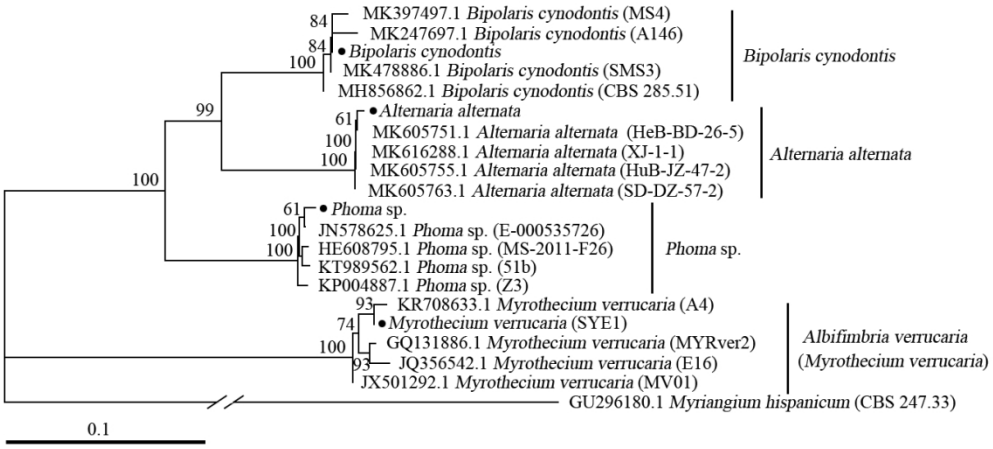

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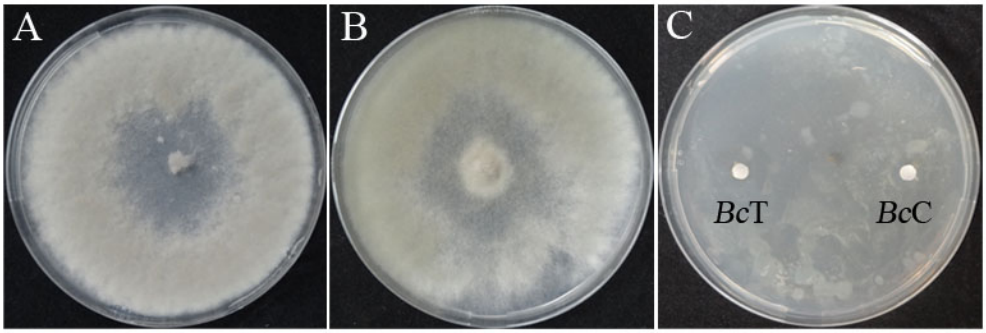

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Table S1. Numbers of isolates recovered and molecular identification of endophytic fungus isolates obtained from Amur grape (*Vitis amurensis*) based on BLAST analyses of ITS1-5.8S rDNA-ITS2 region.

Endophytes	Isolates recovered	Blast			
		Accession number	Source	Strain/isolate	Percent identity (%)
Alternaria alternata	13	MK605763	*A. alternata*	SD-DZ-57-2	100
		MK605755	*A. alternata*	HuB-JZ-47-2	100
		MK616288	*A. alternata*	XJ-1-1	100
		MK605751	*A. alternata*	HeB-BD-26-5	100
Bipolaris cynodontis	2	MK247697	*B. cynodontis*	A146	99
		MK397497	*B. cynodontis*	MS4	99
		MH856862	*B. cynodontis*	CBS 285.51	100
		MK478886	*B. cynodontis*	SMS3	96
Phoma sp.	2	JN578625	*Phoma* sp.	E-000535726	99
		HE608795	*Phoma* sp.	MS-2011-F26	98
		KP004887	*Phoma* sp.	Z3	98
		KT989562	*Phoma* sp.	51b	98
Albifimbria verrucaria	3	JX501292	*M. verrucaria*	MV01	99
(*Myrothecium verrucaria*)		GQ131886	*M. verrucaria*	MYRver2	99
		KR708633	*M. verrucaria*	A4	99
		JQ356542	*M. verrucaria*	E16	99