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ORIGINAL ARTICLE

Bioprospecting foliar endophytic fungi of *Vitis labrusca* Linnaeus, Bordô and Concord cv.

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Abstract Endophytic fungi colonize the interior of plant tissues and organs, establishing an intimate mutualistic association with no visible symptoms. The fungi may help protect the plant against herbivores and pathogens, making them potentially useful endophytes in the biological control of diseases and agricultural pests. The biotechnological interest in these organisms has stimulated research related to the bioprospecting of endophytic fungi. Grapevine is among the oldest of plants cultivated by man, with the grape being one of the most highly consumed fruits in the world. Diseases cause significant damage to grape cultures, making their integrated control important to reduce the use of pesticides and, consequently, environmental and human contamination. The rustic species *Vitis labrusca* L. (Vitaceae), used in the preparation of juices and wines, is highly resistant to fungal diseases. We isolated leaf endophytic fungi of the Bordô and Concord cultivars (*V. labrusca* L.), which were ordered into 68 and 62 morpho-groups of the Bordô and Concord cultivars, respectively. We used scanning electron microscopy to confirm the presence of endophytes in the leaves. Endophytic diversity was analyzed based on sequencing the ITS1-5.8S-ITS2 region of rDNA, allowing the identification of fungi belonging to genera including *Cochliobolus*, *Bipolaris*, *Fusarium*, *Alternaria*, *Diaporthe*, *Phoma* and *Phomopsis*. Phylogenetic analysis confirmed the identity of the endophytes. The biotechnological potential of endophytes was tested in vitro for the control of pathogenic fungi of grapevines including *Alternaria* sp., *Sphaceloma* sp. and *Glomerella* sp. Inhibition percentages above 50 % as demonstrated by some isolates demonstrate their potential for biological control.

Keywords Endophytes · Grapevine · Biological control · Sequencing of rDNA · Phylogenetic analysis

Introduction

The grape is one of the most important fruits grown in the world, and its worldwide production surpasses 67 million tons annually (Faostat 2012). The grapevine belongs to the Vitaceae family, consisting of approximately 14 genera and 900 species (Soejima and Wen 2006). The *Vitis* genus is the most important economically because its species are used most commonly in agriculture. The Bordô and Concord cultivars of the species *Vitis labrusca* L. are hardy grapes that are resistant to fungal diseases. These two cultivars are used for in natura consumption and primarily for the production of juices and wines (Sousa 1996).

Among the most serious problem faced in vine cultivation are fungal diseases that cause losses in production and fruit quality (Fan et al. 2008). Endophytic microorganisms may be promising alternatives to the use of pesticides in controlling fungal threats. Endophytes are microorganisms that inhabit the interior of plant tissues during all or part of their life cycle, without causing any visible symptoms (Petrini 1991; Azevedo et al. 2000). However, endophytes are relatively unexplored with regards to their production of useful natural compounds for agriculture, industry and medicine (Strobel et al. 2004). The ability of endophytes to produce substances in vitro that

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inhibit the growth of other species of microorganisms has stimulated research on the bioprospecting of endophytic microorganisms and their use in biological control (Arnold 2008).

The mechanisms underlying the endophyte–host relationship are not well understood (Kogel et al. 2006). Schulz and Boyle (2005) suggest that these are not neutral interactions, with the asymptomatic colonization requiring a balance of antagonisms between the fungal endophyte and the host. According to the review of Rodriguez and Redman (2008), all plants in natural ecosystems are thought to be symbiotic with mycorrhizal and/or endophytic fungi. Collectively, fungi express several different symbiotic lifestyles that are defined by fitness benefits to plant hosts and symbionts. The range of symbiotic lifestyle expression from mutualism to parasitism has been described as the symbiotic continuum. Within each group of fungal symbionts there are isolates and/or species that span the symbiotic continuum by expressing different lifestyles. Several studies focusing on the isolation of endophytes from asymptomatic plant tissues indicate that individual species express either mutualistic, commensal, or parasitic lifestyles when re-inoculated back onto the original host species (Rodriguez and Redman 2008).

Advances in the identification of fungi followed the development of sensitive and specific techniques of molecular biology employed in the differentiation of species, such as amplification of the internal transcribed spacer (ITS) of ribosomal DNA (rDNA) using the polymerase chain reaction (PCR); rDNA-ITS sequences can be sequenced and compared for homology with sequences available in databases (Magnani et al. 2005).

Another important tool to assist our understanding of endophyte–host interactions is the use of scanning electron microscopy. This technique is advantageous for its high resolution and the possibility of in-depth analysis of various materials (Pamphile et al. 2008a).

Many studies have focused on endophytes in plants belonging to the angiosperms and conifers (Arnold 2007). The occurrence of endophytic fungi has been reported in the genus *Vitis*. However, most studies have concentrated on fungi associated with *V. vinifera* (Mostert et al. 2000; Burruano et al. 2008; Casieri et al. 2009; Gonzáles and Tello 2011; Pancher et al. 2012). Studies related to the bioprospecting of endophytic fungi in *V. labrusca* species are less common (Lima 2010; Brum et al. 2012).

Considering the potential of endophytic microorganisms as biological controllers (Azevedo et al. 2000), the aim of this study was to isolate and characterize foliar endophytic fungi of Bordô and Concord grapevine cultivars (*V. labrusca* L.). Their biotechnological potential in the in vitro control of pathogenic fungi of grapevine *Alternaria* sp., *Sphaceloma* sp. and *Glomerella* sp. was determined.

Materials and methods

Leaf sampling

Mature and healthy leaves of Bordô and Concord cultivars (*V. labrusca*) were selected randomly and used immediately after collection. Two leaves from four different plants of each cultivar were used. The material was collected at Iguatemi Experimental Farm (IEF) belonging to Universidade Estadual de Maringá (UEM), planted in 0.048 ha located in the Iguatemi District, city of Maringá, Paraná State, Brazil (23°21′22”S, 52°4′18”W). The grapevines themselves were planted in the system known as espalier in a certifiably organic area. On the day of collection (10 August 2010), the majority of berries carried by the grapevines were classified as pellet-like according to the phenological stages of the grapevine described by Eichhorn and Lorenz (1984). The temperature in the month prior to collection ranged from 12.1 °C to 37.4 °C with an average temperature of 23.5 °C and average relative humidity of 64.8 %.

Isolation of endophytic fungi of *Vitis labrusca*

Leaves were washed under running water, 0.01 % Tween 80 aqueous solution (Synth; http://www.splabor.com.br) and two rinses in sterile, distilled water to remove residues. They were then surface-disinfected to suppress epiphytic microorganisms according to Vaz et al. (2009). Washing was performed in series with 70 % ethanol for 1 min, sodium hypochlorite (2 % available Cl^-) for 3 min, 70 % ethanol for 30 s and two washes in sterile, distilled water. The effectiveness of this method was verified by spreading 100 μL of the final water used on Petri dishes containing potato dextrose agar (PDA) culture medium (Smith and Onions 1983) pH 6.6, supplemented with tetracycline (Sigma, St. Louis, MO) (50 μg mL^{-1} in 50 % ethanol), to prevent bacterial growth.

Disinfected leaves were cut into 5-mm^2 fragments and deposited (five fragments per plate, with a total of 50 plates for each plant) on plates with PDA containing tetracycline. The plates were incubated at 28 °C for 7 days. The colonization frequency (CF) was determined by Hata and Futai (1995): CF (%)=(number of fragments colonized by fungi / total number of fragments)×100.

In the purification process, the fungal isolates were transferred to PDA plates and grown for 7 days. Then, fragments (5 mm^2) were squashed in 1 mL 0.01 % Tween 80 aqueous solution, and an aliquot of 100 μL solution was then spread on plates containing PDA and incubated for 24 h. Single colonies were transferred immediately to new plates with PDA and incubated for 7 days. If necessary, the process was repeated until pure colonies were obtained.

Molecular identification of isolated endophytic fungi

Genomic DNA was extracted as described by Raeder and Broda (1985) and modified according to Pamphile and Azevedo (2002), except that endophytes were previously grown for 7 days on plates with potato dextrose broth (PDB) medium (Smith and Onions 1983) at 28 °C under stationary conditions. The concentration and purity of the genomic DNA were determined using a GENESYS 10S UV–vis spectrophotometer (OD 260/280 nm). DNA integrity was analyzed by electrophoresis in 1 % agarose gels, using the High DNA Mass Ladder (Invitrogen, Carlsbad, CA) as the molecular weight standard. The final concentration of DNA was adjusted to 10 ng mL^{-1}.

PCR amplification of the ITS1-5.8S-ITS2 of rDNA region was performed according to Magnani et al. (2005), using the primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCCCGCTTATTGATATGC-3′) (White et al. 1990) with an initial denaturation at 92 °C for 4 min, followed by 35 denaturation cycles at 92 °C for 40 s, annealing at 52 °C for 1 min and 30 s, extension at 72 °C for 2 min and a final extension at 72 °C for 5 min.

PCR products were purified with the GFX PCR DNA kit and Gel Band Purification (Amersham Biosciences, Piscataway, NJ), according to the manufacturer's instructions, prepared for second sequencing reactions according to Magnani et al. (2005) using the primer ITS4. PCR reactions were carried out in a thermocycler programmed to perform an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 60 °C for 1 min and a final extension at 60 °C for 5 min. Sequencing was performed in a MegaBACE TM 1000 sequencer (Amersham Biosciences), with injection and electrophoresis conditions of 1 kV/90 s and 7 kV/240 min, respectively.

The nucleotide sequences were analyzed and edited, and compared to those deposited in GenBank (http://www.ncbi.nlm.nih.gov). For research into genera or species, the BLASTN program was used. Determination was based on the best result obtained for identity.

For the phylogenetic analyses, a dendrogram was constructed with the sequences obtained along with those available in GenBank. Sequences were aligned using Clustal X (Thompson et al. 1997), and the dendrogram was constructed using MEGA program version 5 (Tamura et al. 2011) with grouping by the neighbor-joining (NJ) method (Saitou and Nei 1987), using p-distance for nucleotides with the option of pairwise gap deletion and a bootstrap with 10,000 repetitions.

In vitro antagonistic activity of endophytic isolates against pathogenic fungi

Tests were performed with pathogenic fungi of grapevine provided by EMBRAPA Grape and Vine of Bento Gonçalves – RS. Fungi were: *Alternaria* sp. (CNPUV 674), responsible for leaf blight; *Glomerella* sp. (CNPUV 378), responsible for ripe rot of grapes; and *Sphaceloma* sp. (CNPUV 102), which causes anthracnose.

A paired culture technique described by Campanile et al. (2007) was used. Endophytes and phytopathogenic fungal mycelial-disks (5 mm) were inoculated at a distance of 2 cm on opposite sides of Petri dishes (9 cm) containing PDA. Negative control plates had each pathogen disk inoculated in dual culture with a disk of PDA medium. The tests were performed in triplicate and all plates were incubated at 28 °C for 7 days. The inhibition percentages were calculated according to Reyes Chilpa et al. (1997) as cited by Quiroga et al. (2001): IP (%)=(average diameter of the pathogen colony in control − average diameter of the pathogen colony in the treatment / average diameter of the pathogen colony in control)×100.

The competitive interactions between endophytic and pathogenic fungi were characterized based on the scale of Badalyan et al. (2002) where three types (A, B and C) and four subtypes (CA1, CA2, CB1 and CB2) of interaction are possible: A=deadlock with mycelial contact; B=deadlock at a distance; C=replacement, overgrowth without initial deadlock; CA1 and CA2=partial and complete replacement after initial deadlock with mycelial contact; and CB1 and CB2=partial and complete replacement after initial deadlock at a distance.

Scanning electron microscopy

During the process of isolation of endophytic fungi, each foliar sample of *V. labrusca* was cut into two portions: one for isolation test and the other for microscopic observation. The latter portions were incubated for 3 days and ruptured by the freeze-fracture process then subjected to scanning electron microscopy (SEM). Sample preparation was modified from that described by Pamphile et al. (2008b). A gradient of 30 %, 50 %, 70 %, 90 % and 100 % alcohol was used for the dehydration of vegetable material, and the resultant fragments were dried to critical point in a Bal-tec-CPD030 (Baltec, Los Angeles, CA), for seven cycles. Samples were mounted on stubs with the vertical position of the fragments in a conductive tape for SEM. The fragments received a thin layer of gold on Shimadzu-unit IC 50 (260 s, 50 mA, at 27 °C) for three cycles. The fragment covered with metal was observed in a field emission scanning electron microscope (SHIMADZU-SS550; Shimadzu, Tokyo, Japan) at 15 kV and 7 mm away.

Statistical analysis

Experiments were conducted in a completely randomized design, and evaluated statistically by analysis of variance

(ANOVA) and means compared by Scott-Knott test ($P<0.05$) using the statistical program SISVAR 5.3 (Ferreira 2008).

Results

Isolation and molecular identification of endophytic fungi of *V. labrusca*

The frequency of colonization of the 250 leaf fragments sampled from the Bordô cultivar was 74 %. A sample of 140 fungi was isolated and grouped randomly into 68 morpho-groups based on macroscopic characteristics including morphology and characteristics when grown on the culture media PDA: sporulation, mycelium aspect, mycelium coloration, coloration of the reverse of the Petri dish, pigmentation of the culture medium and average diameter of colony growth. Of the 250 leaf fragments sampled from the Concord cultivar, we obtained a colonization frequency of 64 % and 145 fungi were isolated and divided randomly into 62 morpho-groups. A fungus from each morpho-group was chosen randomly and purified for other experiments.

Sequence analyses of ITS1-5.8S-ITS2 regions allowed identification of endophytic fungi (Table 1) belonging to seven genera: *Cochliobolus*, *Bipolaris*, *Fusarium*, *Alternaria*, *Diaporthe*, *Phoma* and *Phomopsis* (the latter two genera being the most common). The percentages of identity between the sequences obtained and those available in GenBank varied between 97 % and 100 %.

Phylogenetic analyses of the endophytic isolates of *V. labrusca* based on rDNA sequence data

Phylogenetic analysis (Fig. 1) resulted in the grouping of isolated endophytes, along with fungal sequences obtained from GenBank (NCBI), in three clades belonging to the phylum Ascomycota. Clade I belongs to the Sordariomycetes class, Diaporthales order, with representatives of the *Phomopsis* and *Diaporthe* genera. In Clade II, the Sordariomycetes class is also represented by the Hypocreales order, including the fungi *Fusarium* and *Giberella* genera. Clade III is represented by the Dothideomycetes class and Pleosporales order, including fungal *Phoma*, *Cochliobolus*, *Bipolaris* and *Alternaria* genera.

The first Clade was subdivided into two sub-clades. In the first sub-clade, isolates B17-49 [99 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], B27-116 [98 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], C53-134 [99 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], C27-07 [98 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], B63-34 [97 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], B64-46 [100 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], C40-59 [99 % identity with Sordariomycetes (JX174146.1) in BLAST analysis] and B35-107 [99 % identity with Sordariomycetes (JX174146.1) in BLAST analysis], were clustered with the *Diaporthe* sp. (anamorph=*Phomopsis*) group with 100 % bootstrapping (BP) analysis. Isolate C25-19 [99 % identity with *Diaporthe longicolla* (JQ753971.1) in BLAST analysis] was grouped with the *Diaporthe* sp. sub-clade with 100 % BP analysis. In that case, the species was identified as *Diaporthe* sp. instead of *D. longicolla*. The isolate B43-48 [99 % identity with *Phomopsis* sp. (GQ461582.1) in BLAST analysis] and the isolate B30-122 [99 % identity with *Phomopsis* sp. (GQ461582.1) in BLAST analysis] grouped with the *Diaporthe*/*Phomopsis* sub-clade with 100 % BP, showing that they are both *Diaporthe* sp. The isolates B15-03 [98 % identity with *Phomopsis* sp. (KF159989.1) in BLAST analysis], B40-119 [98 % identity with *Diaporthe* sp. (EF423554.2) in BLAST analysis], B28-47 [99 % identity with *Phomopsis* sp. (JN153054.1) in BLAST analysis] and B45-62 [99 % identity with *Phomopsis* sp. (KF153062.1) in BLAST analysis] clustered with the sub-clade of *Diaporthe*/*Phomopsis* with 100 % BP, confirming the *Diaporthe* sp. identity.

In the second sub-clade of the first clade of Sordariomycetes, isolate C22-22 [99 % identity with *Fusarium oxysporum* (FJ867931.1) in BLAST analysis] was grouped in the *Fusarium* sp./*Gibberella* sp. with 100 % BP, confirming its identity as *Fusarium* sp. Isolate C03-45 [99 % identity with *Fusarium culmorum* (KC311482.1) in BLAST analysis] was grouped with *Fusarium culmorum* with 8 % BP, indicating its identitiy as *Fusarium* sp.

In the second clade, the isolates B31-38 [99 % identity with *Phoma herbarum* (KJ767079.1) in BLAST analysis], B61-72 [99 % identity with *Phoma herbarum* (JX867222.1) in BLAST analysis], B46-95 [100 % identity with *Phoma* sp. (KJ572232.1) in BLAST analysis] and C18-40 [99 % identity with *Phoma herbarum* (KJ767079.1) in BLAST analysis] were grouped together in the *Phoma* sp. clade with 100 % BP. The isolate C07-137 [99 % identity with *Phoma exigua* (AY531684.1) in BLAST analysis] was grouped with *Phoma exigua* with 70 % BP, indicating that C07-137 belongs to *Phoma exigua* species.

The third clade of the Pleosporales group was divided into three sub-clades. The two initial sub-clades clustered with the *Cochliobolus* (anamorph=*Bipolaris*) species. Isolates B33-61 [98 % identity with *Bipolaris* sp. (GU017499.1) in BLAST analysis] and B60-132 [99 % identity with *Bipolaris* sp. (GU017499.1) in BLAST analysis] were grouped in the first sub-clade with 100 % BP, confirming their *Cochliobolus*/*Bipolaris* identity. Isolates C19-144 [98 % identity with *Cochliobolus* sp. (JQ754043.1) in BLAST analysis] and B14-69 [99 % identity with *Cochliobolus* sp. (JQ936204.1) in BLAST analysis] also grouped with *Cochliobolus sativus* in

Table 1 Identification of 28 endophytic fungi of *Vitis labrusca* based on rRNA sequencing and inhibition percentages (IP) and competitive interactions (CI) with three phytopathogenic fungi in dual culture

Endophytic fungi	Closely related fungal sequences	Maximal identity	Phytopathogenic fungi					
			Alternaria		*Sphaceloma*		*Glomerella*	
			IP*	CI**	IP	CI	IP	CI
B14-69	Uncultured fungus (GU053859.1) *Cochliobolus sativus* (JQ936204.1)	99 % 99 %	22.80 f	A	27.64 d	A	44.44 c	A
B15-03	Fungal endophyte (JX155973.1) *Phomopsis* sp. (KF159989.1)	99 % 98 %	37.42 c	A	31.82 c	A	37.94 d	A
B17-49	Sordariomycetes (JX174146.1)	99 %	39.34 c	A	42.79 b	A	63.95 a	A
B27-116	Sordariomycetes (JX174146.1)	98 %	22.94 f	A	11.44 f	A	15.19 f	A
B28-47	*Phomopsis* sp. (JN153054.1)	99 %	25.93 e	B	26.33 d	B	32.33 e	A
B30-122	Fungal endophyte (KF435373.1) *Phomopsis* sp. (GQ461582.1)	99 % 99 %	31.46 d	A	32.60 c	A	47.40 c	A
B31-38	*Phoma herbarum* (KJ767079.1)	99 %	37.63 c	C_{A1}	17.45 e	A	36.47 d	A
B33-61	*Bipolaris* sp. (GU017499.1)	98 %	21.03 g	A	25.55 d	A	37.65 d	A
B35-107	Sordariomycetes (JX174146.1)	99 %	20.39 g	A	33.65 c	A	37.65 d	A
B40-119	Fungal endophyte (KF435617.1) *Diaporthe* sp. (EF423554.2)	97 % 98 %	23.37 f	A	34.69 c	A	30.85 e	A
B43-48	*Phomopsis* sp. (GQ461582.1)	99 %	25.93 e	A	38.61 b	A	45.92 c	A
B45-62	*Phomopsis* sp. (JN153062.1)	99 %	35.29 c	A	14.05 f	A	15.48 f	A
B46-95	*Phoma* sp. (KJ572232.1)	100 %	47.85 b	A	33.65 c	C_{A1}	53.01 b	A
B60-132	*Bipolaris* sp. (GU017499.1)	99 %	22.73 f	A	33.13 c	A	42.97 c	A
B61-72	Fungal endophyte (JN163857.1) *Phoma herbarum* (JX867222.1)	97 % 99 %	24.01 f	C_{A1}	12.79 f	A	45.33 c	A
B63-34	Sordariomycetes (JX174146.1)	97 %	35.72 c	C_{A1}	29.21 d	A	65.72 a	A
B64-46	Sordariomycetes (JX174146.1)	100 %	31.67 d	A	43.57 b	A	47.70 c	A
C03-45	*Fusarium culmorum* (KC311482.1)	99 %	26.78 c	C_{A1}	44.36 b	C_{A1}	60.70 b	C_{A1}
C07-137	*Phoma exigua* (AY531684.1)	99 %	21.88 e	B	10.66 d	A	37.06 e	A
C16-83	Uncultured soil fungus (EU479850.1) *Alternaria* sp. (KJ935016.1)	98 % 97 %	18.69 e	C_{A1}	16.93 d	A	37.94 e	A
C18-40	*Phoma herbarum* (KJ767079.1)	99 %	21.03 e	C_{A1}	9.61 d	A	49.47 d	A
C19-144	Uncultured fungus (GU053874.1) *Cochliobolus* sp. (JQ754043.1)	99 % 98 %	19.75 e	C_{A1}	14.31 d	A	39.12 e	A
C21-69	*Alternaria alternata* (KJ410038.1)	98 %	19.54 e	A	11.71 d	A	37.95 e	A
C22-22	*Fusarium oxysporum* (FJ867936.1)	99 %	24.44 d	C_{A1}	12.23 d	A	53.01 c	A
C25-19	*Diaporthe longicolla* (JQ753971.1)	99 %	31.20 b	A	17.4 n	A	52.13 c	A
C27-07	Sordariomycetes (JX174146.1)	98 %	30.82 b	A	33.65 c	C_{A1}	63.65 b	A
C40-59	Sordariomycetes (JX174146.1)	99 %	26.56 c	A	39.92 b	C_{A1}	58.33 b	A
C53-134	Sordariomycetes (JX174146.1)	99 %	27.20 c	C_{A1}	25.55 c	A	36.17 e	A

*Means of triplicate. Means in the same column followed by different letters indicate that intervals of IP are significantly different according to the Scott-Knott test ($P<0.05$)

**Classification by Badalyan scale (Badalyan et al. 2002): A=deadlock with mycelial contact; B=deadlock at a distance; CA1=partial replacement after initial deadlock

the second sub-clade with 99 % BP, showing their *C. sativus* identity. Isolates C16-83 [97 % identity with *Alternaria* sp. (KJ935016.1) in BLAST analysis] and C21-69 [98 % identity with *Alternaria alternata* (KJ410038.1) in BLAST analysis] were grouped in the third sub-clade of *Alternaria* sp. with 100 % BP.

In vitro evaluation of antagonism of endophytic fungi against pathogenic fungi

According to the Badalyan scale, competitive interactions between endophytes and pathogens were defined as follws: A= deadlock with mycelial contact; B=deadlock at a distance and

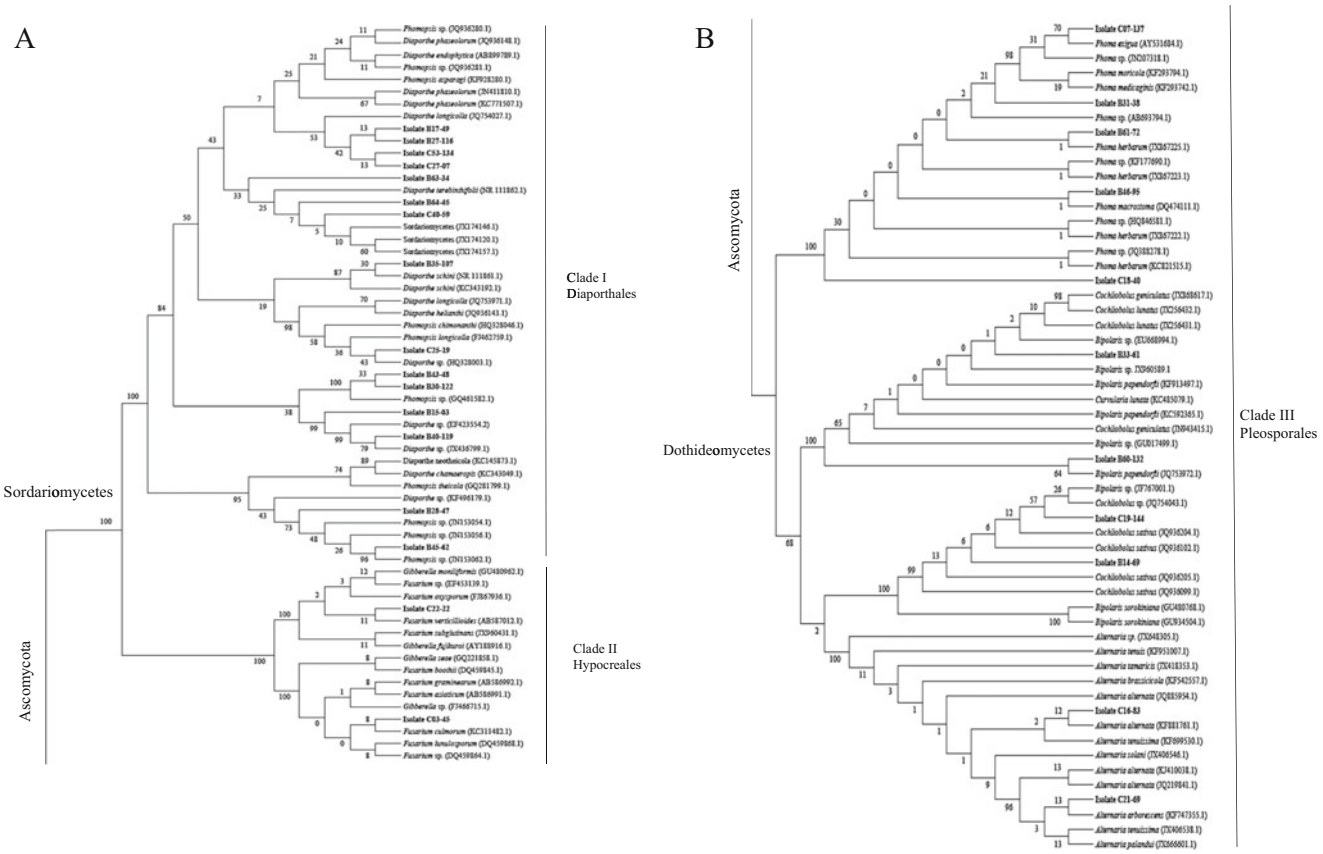

Fig. 1 Phylogenetic tree constructed with endophytic sequences from *Vitis labrusca* L. (in *black*) and sequences from GenBank (indicated by database code), using the neighbor-joining (NJ) method and the p-distance matrix for nucleotides, with the pairwise gap deletion option. The numbers above and beneath each knot indicate the frequency (%) of each branch in bootstrap analyses of 10,000 repetitions. All clades comprise fungi from the phylum Ascomycota. Clade I includes the Sordariomycetes class and Diaporthales order. Clade II comprises the Sordariomycetes class and Hipocreales order. Clade III contains the Dothideomycetes class and Pleosporales order

CA1=partial growth of the antagonist after deadlock with mycelial contact (Fig. 2). We analyzed 68 of the endophytes from the Bordô cultivar. Interactions with *Alternaria* sp. (CNPUV 674) were A (58 of endophytes), B (6) and CA1 (4). The same interactions were observed regarding *Sphaceloma* sp. (CNPUV 102), representing 62, 3 and 3 endophytes, respectively. Most endophytes (67) from the Bordô cultivar showed inhibition per mycelial contact (type A) with *Glomerella* sp. (378 CNPUV), while one isolate presented interaction deadlock at a distance (type B).

A sample of 62 endophytes from the Concord cultivar was analyzed. Interactions with *Alternaria* sp. (CNPUV 674) were classified as A (45 of the endophytes), B (6) and CA1 (11). Type A and CA1 interactions were observed with *Sphaceloma* sp. (CNPUV 102) (57 and 5 endophytes, respectively) and with *Glomerella* sp. (CNPUV 378) (61 and 1, respectively).

Figure 3 shows the statistical groups distribution based on the inhibition percentage (IP) of each phytopathogen by endophytes, i.e., the number of endophytes that belongs to a specific range of IP. So, the IP of mycelial growth of the three pathogens analyzed in relation to the 68 Bordô cultivar isolates and 62 Concord cultivar isolates showed variations, but all endophytic fungi demonstrated some antagonistic activity. In Table 1, we can see the IP value of diverse endophytes. Endophytes B55-50 associated with the Bordô cultivar were the most efficient in the control of *Alternaria* sp. (CNPUV 674) with an IP of 58.71 %. The endophyte B45-62, identified molecularly as *Phomopsis* sp., exhibited an IP of 35.29 % against this pathogen. The isolates B63-34 (Table 1; Fig. 4b), B25-86 and B17-49 showed IPs between 65.72 % and 63.95 % in relation to the pathogen *Glomerella* sp. (CNPUV 378). Endophytes B46-95 (*Phoma* sp.) and B43-48 (*Phomopsis* sp.) presented IPs of 53.01 and 45.92 %, respectively, against *Glomerella* sp. (CNPUV 378). Isolate B25-86, which was promising in the control of *Glomerella* sp. (CNPUV 378), was the best antagonist to *Sphaceloma* sp. (CNPUV 102) showing an IP of 52.98 %.

Isolates C13-98, C52-63 and C11-65 from the Concord cultivar displayed the best inhibition percentages against *Alternaria* sp. (CNPUV 674), varying between 51.04 % and 45.93 %. The C13-98 isolate also showed the best results in antagonism tests against *Glomerella* sp. (CNPUV 378) and *Sphaceloma* sp. (CNPUV 102) with IPs of 75.18 % and 57.94 %, respectively. Isolate C27-07 isolate also

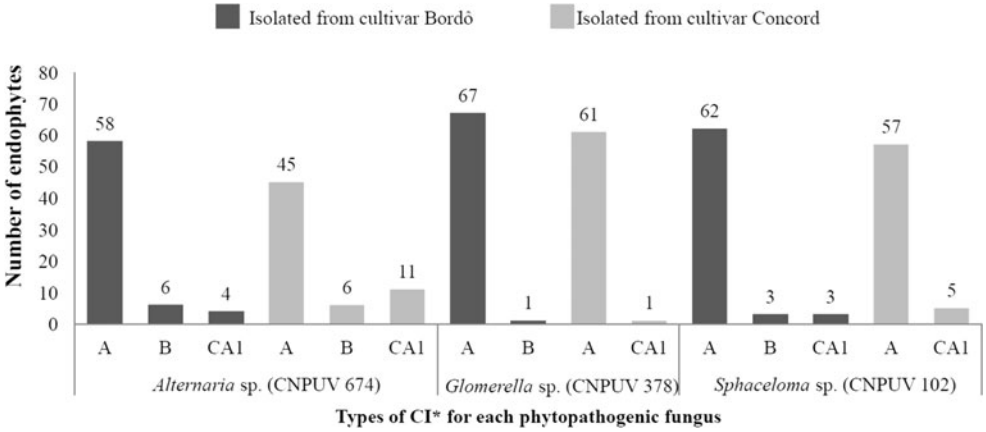

Fig. 2 Competitive interactions (CI) between endophytes of *V. labrusca* and phytopathogenic fungi in dual culture. Badalyan rating scale (Badalyan et al. 2002): A=deadlock with mycelial contact; B=deadlock at a distance; CA1=partial replacement after initial deadlock

demonstrated promising in vitro control of *Glomerella* sp. (CNPUV 378) with an IP of 63.65 % (Table 1; Fig. 4c). Endophyte isolate C03-45 identified as *Fusarium culmorum* demonstrated an IP of 60.70 % in relation to *Glomerella* sp. (CNPUV 378) and an IP of 44.36 % to *Sphaceloma* sp. (CNPUV 102).

Scanning electron microscopy

Fungal hyphae intensely colonizing the leaf mesophyll (Fig. 5a) were visualized by SEM. A hypha enveloping the leaf mesophyll cell (Fig. 5b) was also observed, indicating the possibility of intercellular colonization of leaves by endophytic fungi.

Discussion

The composition of the endophytic community associated with plants may be influenced both by the identity of the host (Arnold et al. 2007) and by environmental factors such as temperature and annual precipitation (Arnold and Lutzoni 2007). This was observed by Burruano et al. (2008) who verified that *Acremonium byssoides* fungus—an isolated endophyte of *V. vinifera* cv. Regina Bianca—was found regularly in samples collected in the fall. In summer, with dry weather and high temperatures, the physiological state of the vineyards was affected and, consequently, the colonization levels of this fungus were reduced. In our study, the samples were collected during mild spring temperatures, and the frequencies of fungal colonization of the Bordô and Concord cultivars were 74 % and 64 %, respectively.

Organic cultivars probably maintain endophytic communities better than cultivars treated with phytosanitary products. According to Azevedo et al. (2000), the use of insecticides and fungicides to control pests and phytopathogens also eliminates important species such as endophytes. This was verified by Gonzáles and Tello (2011) for endophytic fungi isolated from grapevine cultivars (*V. vinifera*), where less diverse samples were obtained from a cultivar planted in an experimental farm that was subjected to phytosanitary treatments.

Pancher et al. (2012) carried out an extensive comparison of communities of endophytic fungi between Merlot and

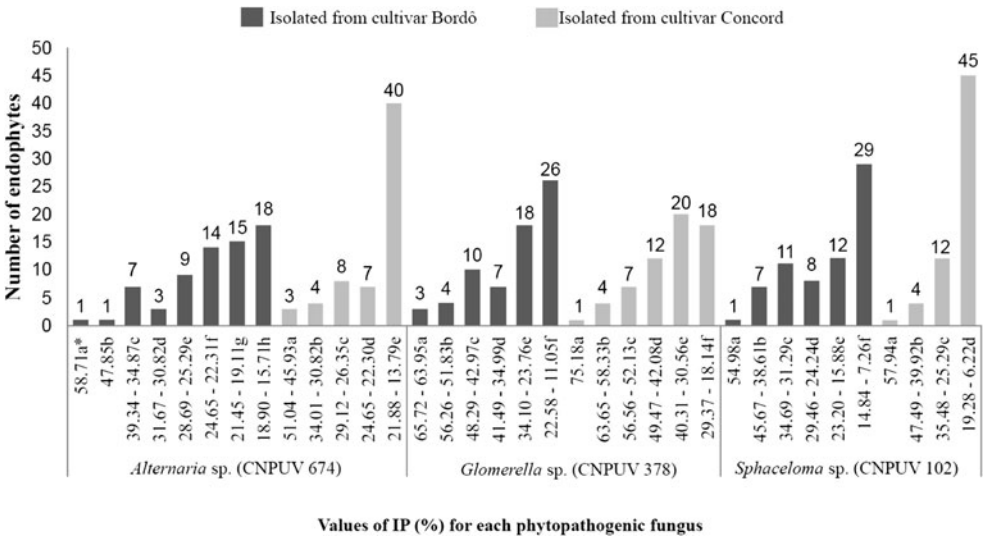

Fig. 3 Inhibition percentage (IP) between 130 endophytic fungi of *Vitis labrusca* and pathogenic fungi in dual culture. IP indicates the reduction (%) in growth of mycelia of the pathogen. *Means of triplicate compared by the Scott-Knott test ($P<0.05$), in which different letters indicate that IP intervals are significantly different

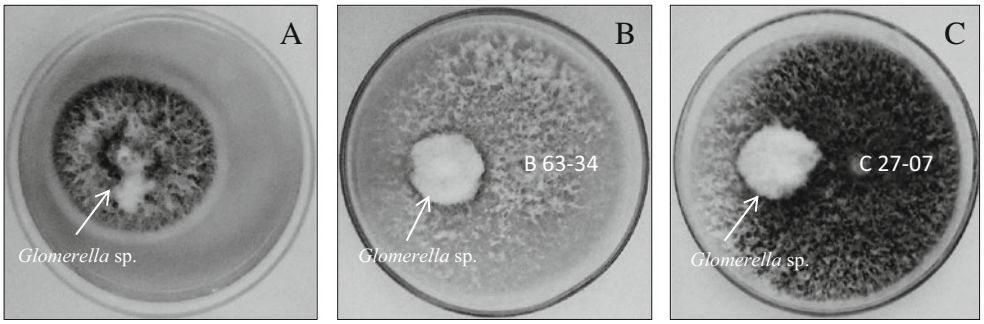

Fig. 4 Inhibition of pathogen *Glomerella* sp. (CNPUV 378) growth. **a** Control plate with only the pathogen. **b**, **c** Antagonism mediated by *V. labrusca* endophytes [both show the interaction deadlock with mycelia contact (type A)]: **b** fungal isolate B63-34 from Bordô cv. (IP= 65.72 %), **c** fungal isolate C27-07 from Concord cv. (IP=63.65 %)

Chardonnay cultivars (*V. vinifera*) in vineyards under integrated pest management and organic management. Their results indicated that mycota present in grapevines of organic farms form significantly different communities than those in grapevine farms under integrated pest management.

Traditional approaches to identifying endophytic fungi involve the microscopic analysis of morphological characteristics. However, significant portions of the isolated endophyte consist of sterile mycelium and consequently could not be identified by this method (Rivera-Orduña et al. 2011). Data on the ITS region available in databases increase the chances of accurately identifying taxa because of the possibility of obtaining a taxonomically correct correspondence (Albrectsen et al. 2010).

Our genomic sequence analysis of ITS1-5.8S-ITS2 regions verified the higher general frequency of *Phoma* and *Phomopsis* fungi, as well as the presence of isolates of the genera *Cochliobolus*, *Bipolaris*, *Fusarium*, *Alternaria* and *Diaporthe*. Most of these fungi have also been identified in previous research into endophytic microbiota in grapevines. The genera *Alternaria*, *Fusarium* and *Phoma* were among the isolates of endophytic fungi of *V. vinifera* most frequently found by Gonzáles and Tello (2011) and Musetti et al. (2006).

Alternaria was the dominant genus in studies of grapevines reported by Mostert et al. (2000) and Pancher et al. (2012); these authors also reported the presence of *Phoma* sp. In an additional study, *Alternaria* sp. and *Fusarium* sp. were reported as dominant in five sampled grapevine cultivars along with the less frequent *Diaporthe* sp., *Phoma* sp. and *Phomopsis viticola* (Casieri et al. 2009). The latter species were also reported in studies by Mostert et al. (2000) and Gonzáles and Tello (2011). In the isolation of endophytes of *V. labrusca*, Brum et al. (2012) observed the presence of the general fungi *Diaporthe* and *Fusarium*.

Fungi belonging to the genera *Cochliobolus* and *Bipolaris* identified in our study were not observed previously in grapevines; however, they and other genera identified in our study are present in several other plants. In the isolation of endophytic fungi of *Taxus globosa* by Rivera-Orduña et al. (2011), *Cochliobolus* and *Alternaria* were among the genera most frequently identified.

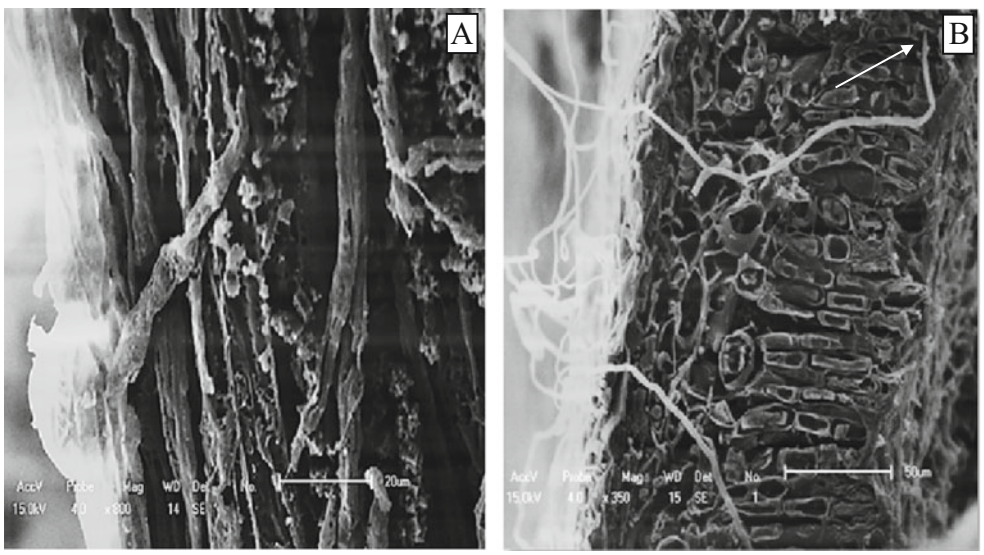

Fig. 5a,b Scanning electron microscopy (SEM) image of leaves of Concord cv. (*V. labrusca*) incubated for 3 days. **a** Hyphae intensely colonizing the leaf mesophyll. **b** Leaf mesophyll with *arrow* indicating fungal hyphae enveloping mesophyll cell

Isolates of Bordô and Concord cultivars identified in this study were grouped into three clades belonging to the phylum Ascomycota: Clade I belongs to the Sordariomycetes class, Diaporthales order; Clade II also belongs to the Sordariomycetes class, Hypocreales order; and Clade III belongs to the class Dothideomycetes and Pleosporales order. This predominance of endophytic fungi belonging to the phylum Ascomycota was also reported in many other recent studies (Albrectsen et al. 2010; Vega et al. 2010; Rocha et al. 2011; Rhoden et al. 2012; Garcia et al. 2012; Orlandelli et al. 2012; Aharwal et al. 2014).

Gonzáles and Tello (2011) obtained 91 % of ascomycetes while investigating the endophytic mycota in nine grapevine cultivars (*V. vinifera*). Seven main orders of this phylum showed a similar distribution among the six cultivars most sampled, and the Hyporeales and Pleosporales orders were the most abundant of all cultivars. In addition, analyzing the fungal communities of five grapevine cultivars (*V. vinifera*) in Switzerland, Casieri et al. (2009) showed that the vast majority of isolates were ascomycetes (87.5 %), including Sordariomycetes as the most represented class comprising 55.1 % of isolates. Brum et al. (2012) examined the diversity of endophytic fungi in leaves from the Niagara Rosada grapevine (*V. labrusca*) and observed that 77 % of the species identified belonged to the phylum Ascomycota.

Biological control is based on the beneficial interactions resulting from competition, antibiotic activity and hyperparasitism of microorganisms against pathogens, insects and weeds (Mathre et al. 1999). It is an important alternative strategy for reducing or eliminating the use of chemicals in agriculture (Azevedo et al. 2000).

Several studies have demonstrated a reduction in the growth of pathogens resulting from interactions between endophytic fungi isolates of various plants and different phytopathogens (Živković et al. 2010; Rehman et al. 2011). Most studies related to the characterization of endophytic communities and applications in viticulture pathogen biocontrol are focused on the cause of the disease known as downy mildew, caused by the fungus *Plasmopara viticola*. Musetti et al. (2006) confirmed the inhibition of sporulation of the pathogen by the endophyte *Alternaria alternata* isolated from grapevine leaves (*V. vinifera*). Burruano et al. (2008) also reported the inhibition of sporulation of the *P. viticola* pathogen by crude extracts of the endophyte *Acremonium byssoides* isolated from grapevine leaves (*V. vinifera*).

However, Brum et al. (2012) tested endophytic fungi isolated from the Niagara Rosada grapevine (*V. labrusca*) in an experiment with a dual culture against the pathogenic grapevine fungus *Fusarium oxysporum*. In this experiment, 52 % of endophytes were able to inhibit pathogen growth, and the largest halo of inhibition was from the isolates identified as *Colletotrichum gloeosporioides* and *Flavodon flavus*. In our study, with fungi isolated from the same plant species and also in a dual culture experiment, all the endophytes showed some degree of inhibition against phytopathogens. Among the fungi identified molecularly, highlighted are B46-95 (*Phoma* sp. KJ572232.1) with 47.85 % inhibition of growth of the phytopathogen *Alternaria* sp., C03-45 (*Fusarium culmorum* KC311482.1) with 44.36 % inhibition of *Sphaceloma* sp. and B63-34 (Sordariomycetes JX174146.1) with 65.72 % inhibition of *Glomerella* sp.

Analyzing 46 endophytic fungi isolated from *Luehea divaricata* in a dual culture experiment against the grapevine phytopathogen *Alternaria alternata*, Bernardi-Wenzel et al. (2013) observed that antagonism rates ranged from 3.7 % to 62.7 %, and competitive interactions classified as B and CA1 were observed. The same interactions were observed in our study, but antagonism rates among our endophytes and this phytopathogen ranged from 13.79 % to 58.71 %.

This is the first study using SEM to demonstrate the colonization of leaf endophytes in *V. labrusca* species. The technique employed was effective enough to view endophytes in leaf tissues incubated for up to 3 days, confirming the presence of endophytic fungi in leaf mesophyll cells. Their intercellular colonization could also be seen as fungal hyphae clearly emerging from inside a cell. According to Stone et al. (2000), the colonization of endophytes may be intracellular and limited to a single cell, intercellular and located in an intra- and intercellular systemic fashion.

The results obtained in this study demonstrate the presence of endophytic fungi in *V. labrusca* as well as the biotechnological potential of this organism to control grapevine pathogens. Future studies should focus on developing efficient techniques for applying these endophytes in biological control strategies against fungal diseases of grapevine.

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