Eur J Plant Pathol (2008) 121:451–461
DOI 10.1007/s10658-007-9263-0

Production of phytotoxic metabolites by five species of Botryosphaeriaceae causing decline on grapevines, with special interest in the species *Neofusicoccum luteum* and *N. parvum*

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Received: 15 October 2007 / Accepted: 14 December 2007 / Published online: 22 January 2008

Abstract In recent years an increasing number of species of Botryosphaeriaceae have been associated with grapevine decline worldwide. Five species isolated from declining grapevines in Spain (*Botryosphaeria dothidea*, *Diplodia seriata*, *Dothiorella viticola*, *Neofusicoccum luteum* and *N. parvum*) were checked for toxin production in liquid cultures. Cultural conditions for all fungi were adjusted to obtain optimal production of phytotoxic culture filtrates, by growing the fungi in steady liquid cultures of Czapek–Dox broth for different time intervals. Phytotoxicity of *D. seriata* and *N. parvum* reached a maximum after 14 days while the remaining species showed the highest phytotoxicity levels after 21 days in culture. All fungi produced hydrophilic high-molecular weight compounds with phytotoxic properties. In addition, *N. luteum* and *N. parvum* produced lipophilic low-molecular weight phytotoxins, not detected consistently among the remaining species. This led to a more exhaustive study on the phytotoxicity of *N. luteum* and *N. parvum*. Culture filtrates and corresponding extracts of both species were consistently highly phytotoxic in different assays. The gas-chromatography analysis of the acetylated *O*-methyl glycosides of the phytotoxic exopolysaccharides produced by *N. parvum* showed these substances to be composed mainly of glucose, mannose and galactose. Results suggest that phytotoxic metabolites could be involved in the virulence of both species *in planta*.

Keywords *Botryosphaeria* · Grapevine decline · Phytopathogenic fungi · Polysaccharides · *Vitis vinifera*

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Introduction

Species of *Botryosphaeria* and related genera in the Botryosphaeriaceae are known as cosmopolitan pathogens, saprophytes and endophytes on a wide range of woody hosts (Arx 1987; Barr 1987; Denman et al. 2000). Since teleomorphs of these fungi are rarely found in nature and their characters show few differences across species, the identification of these

fungi has been based on the anamorph morphology for several years (Denman et al. 2000; Crous et al. 2006). Anamorphs of Botryosphaeriaceae species occurring on grapevines are currently placed in the genera *Diplodia*, *Dothiorella*, *Fusicoccum*, *Neofusicoccum*, and *Lasiodiplodia* (Denman et al. 2000; Phillips et al. 2005; Crous et al. 2006). These five genera are clearly separated on their morphology and their phylogenetic relationships shown by the analysis of sequence data of the 28S rDNA region (Crous et al. 2006).

Thirteen species of botryosphaeriaceous fungi causing diseases on grapevines have been reported to date (Lehoczky 1974; Larignon and Dubos 2001; Phillips 2002; Phillips et al. 2002; Van Niekerk et al. 2004, 2006; Úrbez-Torres et al. 2006, 2007b), the symptoms of which on plants include delayed bud burst, bud mortality, dieback of canes and shoots, stunted growth, leaf chlorosis, wood cankers, and bunch rot (Van Niekerk et al. 2004, 2006; Taylor et al. 2005). Symptoms and their respective causal agents can overlap, thus leading to complex diseases with different sets of symptoms, and their causal fungi occurring in different grapevine-growing regions and on different cultivars (Phillips 1998; Larignon and Dubos 2001; Van Niekerk et al. 2006; Úrbez-Torres et al. 2007b; Martin and Cobos 2007). Thus, different names have been given for the same disease in different countries (Van Niekerk et al. 2006). Additionally, field symptoms of some diseases have often been confused with those of other pathogens, mainly *Eutypa lata* and *Phomopsis viticola*, therefore making the diagnosis of the diseases difficult (Phillips 1998; Castillo-Pando et al. 2001; Van Niekerk et al. 2006). Pathogenicity of these botryosphaeriaceous species has also been a matter of controversy. For example '*Botryosphaeria*' *obtusa* (anamorph: *Diplodia seriata*; see Phillips et al. 2007) has been reported as pathogenic in Italy, Chile, Australia (New South Wales), South Africa, USA (California) and France (Cristinzio 1978; Castillo-Pando et al. 2001; Larignon et al. 2001; Auger et al. 2004; Van Niekerk et al. 2004), but considered only as weakly pathogenic in Portugal (Phillips 2002). In addition, Taylor et al. (2005) did not observe significant lesions of grapevine cuttings inoculated with *D. seriata* in Australia. Comparable data on the pathogenicity of other Botryosphaeriaceae species are reported elsewhere, thus showing the great variability observed on the virulence of those species (Phillips 1998; Larignon et al. 2001; Van Niekerk et al. 2004; Taylor et al. 2005; Úrbez-Torres et al. 2006).

Several isolates of botryosphaeriaceous fungi obtained from non-grapevine hosts are known for producing bio-active toxic metabolites, which are probably involved in the diseases they cause (Venkatasubbaiah and Chilton 1990; Venkatasubbaiah et al. 1991; Barbosa et al. 2003; Crognale et al. 2003; Selbmann et al. 2003). Other grapevine wood pathogens such as *E. lata*, *Fomitiporia mediterranea*, *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* also produce phytotoxic metabolites in culture (Fallot et al. 1997; Evidente et al. 2000; Tabacchi et al. 2000).

No data are available about production of bioactive metabolites by grapevine isolates of botryosphaeriaceous fungi. The aim of this study was to evaluate the phytotoxicity of metabolites produced by five species of Botryosphaeriaceae isolated from declining grapevines, namely *Fusicoccum aesculi*, *Diplodia seriata*, *Dothiorella viticola*, *Neofusicoccum luteum* and *N. parvum*. We also report the optimization of the *in vitro* culture conditions for the production of toxins by these species and the preliminary results on the chemical and biological characterization of the toxic metabolites.

Materials and methods

Fungal isolates and cultural conditions

The five isolates used in this study were obtained from diseased grapevines sampled in Catalonia, north-east Spain (Table 1). Representative cultures of these fungi were deposited at the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands). The isolates were grown in stationary conditions in 1 l Roux flasks containing 150 ml of modified Czapeck–Dox medium with 0.5% yeast and 0.5% malt extract (pH 6.8). About 10 to 15 mycelium plugs from 1 week-old colonies cultivated on potato dextrose agar were used for seeding liquid cultures. Liquid cultures were incubated at 25°C in darkness. In order to determine the optimum incubation period for each fungus to achieve the maximal toxicity for its culture filtrates (CF), sets of cultures of each species were incubated for 7, 14 and 21 days. After those

Table 1 Isolates of the Botryosphaeriaceae species used in this study

Culture no.	Teleomorph	Anamorph	Host (*Vitis vinifera* cv.)	Location (Spain)
JL353	*Botryosphaeria dothidea*	*Fusicoccum aesculi*	Merlot	Caldes de Montui
JL398	"*Botryosphaeria*" *obtusa*	*Diplodia seriata*	Cabernet-Sauvignon	Pacs del Penedès
CBS 117009	"*Botryosphaeria*" *viticola*	*Dothiorella viticola*	Red Grenache	Vimbodí
JL519	"*Botryosphaeria*" *lutea*	*Neofusicoccum luteum*	Macabeo	Gandesa
JL396	"*Botryosphaeria*" *parva*	*Neofusicoccum parvum*	Parellada	L'Arboç del Penedès

CBS Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands).

periods, mycelial mats were removed by filtration trough four-folded filter paper and kept at −20°C until further processing. Phytoxicity of these culture filtrates was assayed on tobacco plants as explained below. Incubation periods for each species were then established for further phytotoxicity assays and the chemical studies on toxic metabolites.

General procedures for chemical analyses

Analytical thin layer chromatography (TLC) was performed on Kieselgel 60 $F_{254,}$ 0.25 mm, silica gel plates (Merck, Darmstadt, Germany), using chloroform/*iso*-propanol (8:2 or 9:1, *v*/*v*) and ethyl acetate/*n*-hexane (6:4, *v*/*v*) as eluents. The spots on the TLC plates were visualized using different techniques: (1) exposing plates to UV light (254 or 360 nm); (2) spraying the plates with 10% H_2SO_4 in methanol and then with 5% phosphomolybdic acid in ethanol, and then followed by heating at 110°C for 10 min; (3) spraying the plates with 0.5% ninhydrin in acetone, and then followed by heating at 110°C for 10 min; (4) exposure of plates to iodine vapours. Dialysis was carried out using the molecular porous membrane tubing Spectra/Por, with a 3,500 Da cut-off (Spectrum Medical Industries, Houston, TX, USA). Column chromatography was performed with Kieselgel 60, 0.063–0.200 mm, silica gel (Merck, Darmstadt, Germany).

Extraction of toxins from fungal culture filtrates

Extractions were carried out at three different pHs: (1) non-modified pH of culture filtrates (pH ranged from 6.6 to 8.7 among the species tested); (2) pH 2, by acidification with 1M formic acid and (3) pH 10, by alcalinization with 1M NH_3. For each fungal species, three samples of 20 ml were taken from the culture filtrate and their pH modified according to the above procedures. The organic phase was extracted with 60 ml ethyl acetate in three consecutive extractions (20 ml each). Organic phases corresponding to the same pH value were then combined, dried over Na_2SO_4, filtered, and evaporated under reduced pressure. The combined organic extracts obtained from each isolate and pH value, and their corresponding aqueous phases, were tested for phytotoxicity as explained below.

Dialysis of fungal culture filtrates

Samples of culture filtrates (3 ml) corresponding to each fungal species were mixed with 3 ml ultrapure Milli-Q water and dialysed. Tubes were immersed in 10 ml distilled water for 48 h at 10°C. Water was renewed every 6 h. The remaining tube content (IN) and the outer aqueous phases (OUT) were recovered and lyophilized for further phytotoxicity analyses.

Chromatographic fractionation of the organic extracts from *Neofusicoccum luteum* and *N. parvum*

The organic phases resulting from the acid extraction of culture filtrates from *N. luteum* and *N. parvum* were chromatographed separately through a silica gel column (80 cm high, 4 cm i.d.) using chloroform/*iso*-propanol (8:2, *v*/*v*) as eluent. Fractions of 7 ml were collected and monitored by TLC analysis using the same eluent. Those fractions representing a homogenous group in the TLC analyses were combined and evaporated under reduced pressure. The most polar compounds were recovered from the chromatography column by adding 1 l methanol and collecting them in a unique fraction. Residues from each combined group and the residual methanolic fraction were tested for phytotoxicity.

Analyses of exopolysaccharides from *Neofusicoccum luteum* and *N. parvum*

The aqueous phase resulting from the acid extraction of the culture filtrates of *N. luteum* and *N. parvum* was fractionated with cold ethanol in order to obtain the extracellular polysaccharide fraction (EPS) as follows. After lyophilizing, the aqueous phase residues were dissolved in 80 ml ultrapure Milli-Q water, cooled at 4°C, mixed with 4 volumes of absolute cold ethanol (320 ml) and left overnight at −20°C. The precipitate was separated by centrifugation at 7,000 rpm for 45 min at 4°C. The ethanolic phase was evaporated under reduced pressure, yielding a homogenous viscous oil. The precipitate was dissolved in 70 ml ultrapure Milli-Q water, and re-precipitated with 350 ml cold absolute ethanol as described above. The resulting precipitates were dissolved in 50 ml ultrapure Milli-Q water and dialysed as described above. The IN and OUT crude EPS fractions together with the ethanolic fractions obtained during the precipitation process were tested for phytotoxicity.

Small amount of the EPS fraction obtained from the culture filtrate of *N. parvum* (200–500 μg) were utilised to obtain the sugar content by performing a methanolysis. The sample was methanolised with MeOH/HCl 1 M at 85°C for 18 h. Monosaccharides derivatized as *O*-methyl glycosides were acetylated with acetic anhydride and pyridine at 85°C for 30 min. Acetylated *O*-methyl glycosides obtained were extracted with $CHCl_3/H_2O$ and analysed via GC-MS. GC-MS analysis was performed using an Agilent 5973 instrument (Santa Clara, CA, USA) with a SPB-5 capillary column (Supelco, 30 m 0.25 i.d.; flow rate, 0.8 ml min^{-1}; He as carrier gas). The temperature programme was as follows: 150°C for 5 min, 150 → 300°C at 5.0°C min^{-1}, 300°C for 15 min. Mass spectra were recorded at an ionisation energy of 70 eV and an ionising current of 0.2 mA (Vinograd et al. 1992; Holst 2000).

Phytotoxicity assays

A preliminary test was carried on tobacco plants to detect the phytotoxic activity in all tested species of Botryosphaeriaceae and therefore to optimize the incubation period for achieving the maximal toxicity. For each fungal species and incubation period (7, 14 and 21 days), single healthy leaves from tobacco plants were chosen randomly, and injected with 500 μl of each culture filtrate into the foliar mesophyll using an insulin syringe. Four leaves per fungus and incubation period were used as replicates. Equal volumes of sterile Czapeck–Dox medium and sterile distilled water (SDW) were used as controls following the same injection procedure. Phytotoxic activity was expressed as the percentage of leaves showing necrotic lesions on the leaf surface 48 h after infiltrations.

Phytotoxicity of *N. parvum* culture filtrate was also tested on mature leaves of *Vitis vinifera* cv. Tempranillo. Previous studies confirmed this species as one highly virulent on grapevine (Van Niekerk et al. 2004; Luque and Martos, unpublished data). The culture filtrate of this species was tested at four different dilutions (100, 50, 10 and 1%) using SDW as solvent. Five grapevine leaves were used as replicates for each tested dilution. Fully developed, asymptomatic leaves from adult plants were chosen randomly and detached from the plant. The petiole of each leaf was immersed in a vial containing 3 ml of the filtrate dilution for 20 h. Leaves were then transferred to a new vial with 3 ml SDW and maintained for an additional 28 h period until the end of the assay. Leaves were kept in a growth chamber with 12 h light /12 h darkness period at 28°C during the assay. Czapeck–Dox medium and distilled water were used as controls. Leaves were checked for symptoms at the end of the trial using a 0–3 scale: (0) no symptoms; (1) slight withering; (2) presence of necrotic areas; and (3) severely necrosed leaf. The phytotoxic activity was calculated by standardizing the mean value to a 0–100% range.

The remaining experiments were carried out using tomato cuttings of cv. Incas. The substances tested for phytotoxicity were: (1) for all isolates, the aqueous phase and organic extract obtained at three different pHs; (2) the fractions obtained by column chromatography from the acid organic extract of *N. luteum* and *N. parvum* culture filtrates; and (3) the crude EPS obtained by precipitation of *N. luteum* and *N. parvum* culture filtrates. Lipophilic samples were diluted in 100 μl methanol, and the volume was later adjusted to 6 ml with distilled water. Hydrophilic samples were diluted in 6 ml distilled water. The pH of acid and basic extractions, both lipophilic and hydrophilic, was adjusted to 7 either with 1% NaOH (*w*/*v*) or 1% HCl

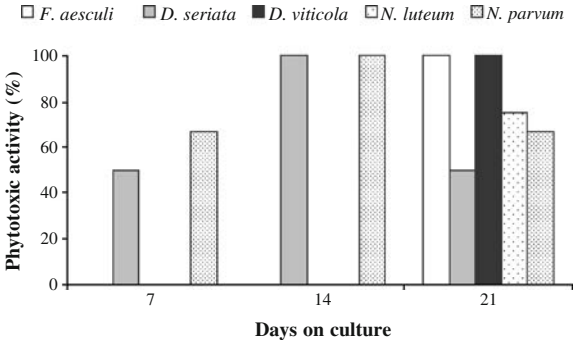

Fig. 1 Phytotoxic activity on tobacco leaves of culture filtrates obtained from different aged cultures of five Botryosphaeriaceae species isolated from grapevine. Control substances (Czapeck–Dox medium and distilled water) did not cause any necrosis and hence are not depicted

(*v*/*v*) before the toxicity assays. The phytotoxic activities of all CF were determined with no pH adjustment. Czapeck–Dox medium (pH 6.7) and distilled water were used as control solutions. Three replicates were used for each tested sample. The stem of a 2 week-old rootless tomato plant was immersed in 2 ml of each dilution for 12 h. Plants were transferred to distilled water and maintained at the same conditions of light and temperature as above. Lesion symptoms were evaluated 48 h after immersion in distilled water using a 0–4 scale: (0) no symptoms; (1) slight withering in one leaf; (2) presence of necrotic spots and withering on some leaves; (3) severe withering on leaves and (4) completely wilted plant. The percentage of phytotoxic activity was calculated as explained above.

Results

Phytotoxic activity of culture filtrates

Culture filtrates from all the studied species showed phytotoxic activity on tobacco leaves but at different culture periods. Phytotoxicity of *D. seriata* and *N. parvum* reached the maximum level after 14 days in culture, but decreased thereafter, while the remaining species showed the highest phytotoxicity levels after 21 days in culture (Fig. 1). Thus the optimum incubation period for each species was determined after these results. Tobacco leaves of both control groups (infiltrated either with Czapek–Dox or distilled water) showed no lesions at any time. Additionally, the toxicity of the culture filtrate of *N. parvum* after 14 days in culture was also confirmed on its host plant. Fourteen day-old culture filtrates of *N. parvum* caused either discoloured or necrotic spots, to total withering in all grapevine leaves (Figs. 2 and 3). Phytotoxic activity of the *N. parvum* culture filtrate was reduced as the dilution factor of filtrate increased (Fig. 3). Grapevine leaves of both control groups showed no lesions on their surface.

Metabolite extraction and phytotoxicity

The highest amounts of residues were found in the aqueous phases as compared to those of the organic phases (Table 2). Additionally, the residues of the aqueous phases were maximized in the basic extraction (pH 10) for almost all species. For all isolates studied the organic extracts obtained at acid pH were significantly higher than those obtained at the other two pH treatments.

The highest levels of phytotoxic activity were detected among the extracts obtained from the aqueous phases (Fig. 4). All fungal species showed phytotoxic activities ranging 88–100% among the products extracted at pH 10. At lower pH values, phytotoxic activity for *D. seriata*, *D. viticola* and *F. aesculi* was reduced, while phytotoxicity of *N. luteum* and *N. parvum* remained consistently over 90%. The highest phytoxicity of organic extracts was detected

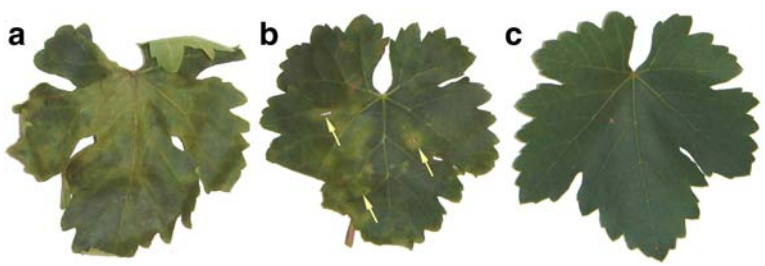

Fig. 2 Symptoms caused by 14 day-old culture filtrates of *Neofusicoccum parvum* on grapevine leaves cv. Tempranillo: **a** severe withering; **b** partial withering with necrotic spots (*arrows*); **c** symptomless leaf (control immersed in distilled water)

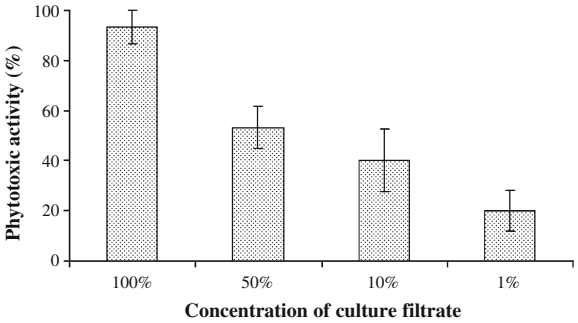

Fig. 3 Phytotoxic activity of 14 day-old culture filtrates of *Neofusicoccum parvum* on grapevine leaves at different concentrations. Control substances did not cause any symptoms and hence are not depicted. *Error bars* indicate standard error of the mean

for the residues obtained at pH 2 from *N. luteum* and *N. parvum* cultures. Lower phytoxicity values were obtained as the pH of extraction increased; no phytotoxicity was detected for organic extracts of *N. luteum* at a higher pH. Some phytotoxic activity was detected for the *D. viticola* extract obtained at the culture filtrate pH (Fig. 4).

Partial purification and characterization of metabolites

The residues of the aqueous phases obtained through dialysis of culture filtrates showed a higher amount (about two to three times) of low-molecular weight compounds collected outside the tubes (Table 3). Highest values were recorded for *N. luteum* (IN, 5.7 mg) and *F. aesculi* (OUT, 21.4 mg) while the lower values corresponded to *D. viticola* for both phases (IN, 2.8 mg; OUT, 5.0 mg). The dialyses of the EPS precipitates obtained from *N. luteum* and *N. parvum* yielded IN fractions of 268 and 138.7 mg, respectively. The OUT fractions obtained from the EPS precipitation of *N. luteum* and *N. parvum* liquid culture were higher than those of the IN fractions although the respective OUT/IN proportions were not as high as those observed in the dialyses of the culture filtrates (Table 3). The highest amounts from the EPS precipitation were obtained in the ethanolic phase for both species.

The IN fractions from CF dialysis showed variable phytotoxicity activities for all tested species, as shown in Fig. 5a. The highest phytoxicity value was observed for *N. luteum* (88%) while the lowest corresponded to *D. seriata* (25%). The OUT fractions showed activity only for *F. aesculi*, *N. luteum* and *N. parvum*, although in lower levels than respective IN fractions. Dialysed EPS fractions obtained from *N. luteum* showed similar phytotoxic activities in both IN and OUT fractions, while in *N. parvum* the IN fraction only proved to be phytotoxic (Fig. 5b). The residual ethanolic phases corresponding to the EPS precipitation showed a low phytotoxicity for both *Neofusicoccum* species. The GC profile and the later MS analysis of the acetylated *O*-methyl glycosides from the IN EPS fraction of *N. parvum* culture showed the presence of the monosaccharides mannose, glucose and galactose in the approximate molar ratio 2:1:1.

Eleven groups of homogeneous fractions were obtained from the column chromatography of the acid organic extract of *N. luteum*: 1–2 (1.5 mg), 3–4 (1.6 mg), 5–7 (3.3 mg), 8 (2.8 mg), 9–11 (18.6 mg), 12–18 (30.1 mg), 19–26 (13.6 mg), 27–51 (22.6 mg), 52–80 (8.2 mg), 81-End (10.3 mg) and methanol (MeOH; 3.0 mg). A total weight of 115 mg was obtained in the chromatographic process, starting from the acid organic phase (572 mg) which was extracted from 800 ml culture filtrate. The fraction-

Table 2 Yields of crude organic extracts and aqueous phases (in mg) obtained from stationary liquid cultures of five Botryosphaeriaceae species isolated from grapevine

Species	Final pH of CF	CF pH		pH 2		pH 10	
		Organic	Aqueous	Organic	Aqueous	Organic	Aqueous
Fusicoccum aesculi	6.6	0.5	104.8	5.2	119.3	0.8	110.5
Diplodia seriata	7.0	0.4	165.9	3.1	136.3	0.5	195.1
Dothiorella viticola	7.5	0.8	46.1	3.8	54.0	0.9	99.7
Neofusicoccum luteum	6.7	1.2	165.2	8.7	167.8	1.0	206.0
Neofusicoccum parvum	8.7	0.7	125.1	4.1	106.3	0.6	145.8

CF Culture filtrate

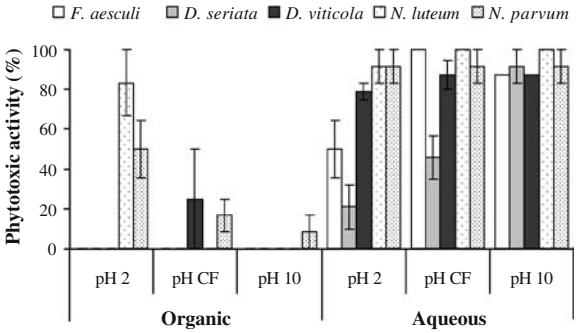

Fig. 4 Phytotoxic activity on tomato cuttings of organic and aqueous phases from the extraction of liquid cultures of five Botryosphaeriaceae species isolated from grapevine. Extractions were performed at three different pHs. Control substances did not cause any symptoms and hence are not depicted. *Error bars* indicate standard error of the mean. (*CF* culture filtrate, pH not modified)

ation procedure carried out on the acid organic extract of *N. parvum* yielded seven groups of homogeneous fractions: 1–8 (14.0 mg), 9–14 (7.4 mg), 15–30 (14.3 mg), 31–61 (10.0 mg), 62–80 (1.4 mg), 81-End (15.0 mg) and MeOH (102 mg). Thus, 122 mg were obtained through the chromatography of the acid organic phase (173 mg) extracted from 800 ml culture filtrate. Recovered fractions from the liquid chromatography accounted for 20.1% (*N. luteum*) and 70.5% (*N. parvum*) of the starting acid organic extracts. A polar yellow pigment was observed on top of the silica gel column for the chromatography of the *N. luteum* acid organic extract. This could explain the low percentage of recovered fractions in this species.

The residues of combined fractions corresponding to *N. luteum* chromatography showed a toxicity ranging from low to moderate (≤50%) in all fractions except for the third one, which was non-phytotoxic (Fig. 6a). The first fraction (1–2) showed the highest activity (50%) while variable values were observed in the remaining fractions, moderate phytotoxicity values in the second and the three last fractions, and lower values in intermediate fractions. The highest phytotoxic activity for *N. parvum* was exhibited by less polar metabolites contained in the first two fraction groups 1–8 and 9–14, with phytotoxicity values of 92 and 75%, respectively (Fig. 6b). The three final fractions collected (62–80, 81-End and MeOH) were almost inactive, containing very polar metabolites which correspond to a conspicuous part (62%) of the total extract separated by chromatography.

Discussion

To date, several phytotoxic metabolites are known to be produced by different fungi causing grapevine decline. Scytalone and isosclerone, two phytotoxic naphthalenone pentaketides, were isolated from culture filtrates of *Phaeoacremonium aleophilum* (Evidente et al. 2000). Tabacchi et al. (2000) identified different phytotoxic metabolites in liquid culture filtrates of four esca-related fungi (*Fomitiporia mediterranea*, *Pm. aleophilum*, *Phaeomoniella chlamydospora* and *Stereum hirsutum*) and showed that each fungal species produces at least one phytotoxin in culture. Eutypine, eutypinol, methyl eutypinol, eulatachromene, 2-*iso*-propenyl-5-formylbenzofuran and eulatinol are known to be produced by *Eutypa lata*, the causal agent of the Eutypa dieback of grapevines (Fallot et al. 1997;

Table 3 Yields of dialyses and fractionated precipitation of exopolysaccharides (EPS) obtained from stationary liquid cultures of five Botryosphaeriaceae species isolated from grapevine

Species	Dialysis of culture filtrate[a]		EPS precipitation[b]		
	IN (mg)	OUT (mg)	IN (mg)	OUT (mg)	Ethanol (g)
Fusicoccum aesculi	5.4	21.4	–	–	–
Diplodia seriata	3.1	12.8	–	–	–
Dothiorella viticola	2.8	5.0	–	–	–
Neofusicoccum luteum	5.7	12.8	268.0	307.0	5.014
Neofusicoccum parvum	4.4	15.5	138.7	171.1	5.333

– Not analysed

[a] From 3 ml culture filtrate

[b] From 800 ml culture filtrate

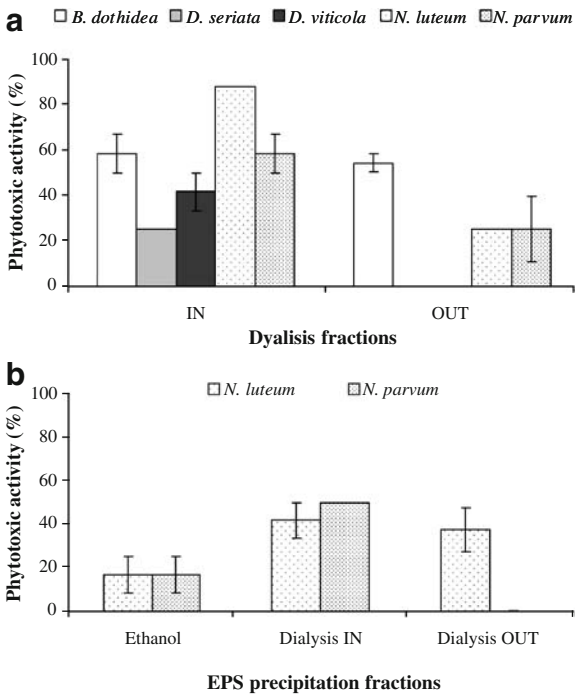

Fig. 5 Phytotoxic activity on tomato cuttings of **a** dialysed culture filtrates from liquid cultures of the five studied Botryosphaeriaceae isolates and **b** dialysed EPS fraction obtained from *Neofusicoccum luteum* and *Neofusicoccum parvum*. Control substances did not cause any symptoms and hence are not depicted. *Error bars* indicate standard error of the mean

Mahoney et al. 2005; Lardner et al. 2006). Research has recently started on toxic metabolites produced by *Phomopsis* species on grapevine (Abou-Mansour et al. 2007). However, little is known on the phytotoxic metabolites produced by the Botryosphaeriaceae species associated with grapevine diseases. Some toxic substances have been studied from botryosphaeriaceous fungi that occur on grapevines, but on isolates obtained from non-grapevine hosts. Venkatasubbaiah and Chilton (1990) identified the toxins mellein, tyrosol, 4-hydroxymellein, 5-hydroximellein and 4-hydroxybenzaldehyde in the culture filtrate of '*B.*' *obtusa*, the causal agent of the black rot fruit and frogeye leaf spot of apple. *Lasiodiplodia theobromae* isolate DABAC-P82 was found to produce the β-glucan (Selbmann et al. 2003). To the best of our knowledge, the present work deals for the first time with the production of phytotoxic metabolites by species of Botryosphaeriaceae causing decline on grapevines, namely *Fusicoccum aesculi*, *Diplodia seriata*, *Dothiorella viticola*, *Neofusicoccum luteum* and *N. parvum*.

The results obtained in this study indicate that all the botryosphaeriaceous fungi studied exhibited phytotoxic activity, although the tested isolates showed variable levels. Moreover, culture filtrates and their respective organic extracts and aqueous phases corresponding to *N. luteum* and *N. parvum* consistently showed high phytotoxic activity in different assays, therefore suggesting that phytotoxic metabolites could be involved in the virulence of both species *in planta*. While virulence of *N. luteum* JL519 was determined in a previous experiment (Luque and Martos, unpublished data), the high virulence of *N. parvum* on grapevine was known from previous work (Van Niekerk et al. 2004). Significant damage was shown on grapevine leaves immersed in *N. parvum* culture filtrate, including leaf withering and necrotic and discoloured spots. These

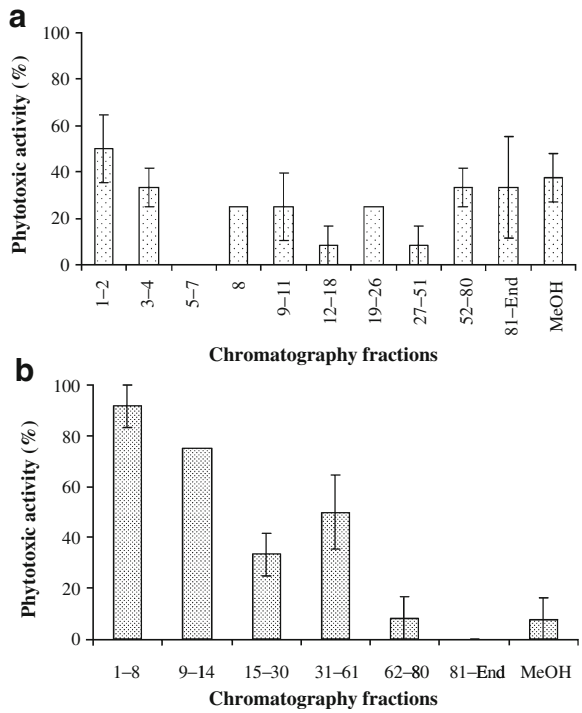

Fig. 6 Phytotoxic activity on tomato cuttings of chromatographic fractions obtained from the acid organic extracts of **a** *Neofusicoccum luteum* and **b** *Neofusicoccum parvum*. Control substances did not cause any symptoms and hence are not depicted. *Error bars* indicate standard error of the mean

foliar symptoms looked similar to those caused by culture filtrates, EPS, pullulans and organic extracts obtained from *Pm. aleophilum* and *Pa. chlamydospora* (Sparapano et al. 2000). The relationships between phytotoxic activities and dilution factors for culture filtrates of *N. parvum* reported here were not linear: low concentrations of culture filtrates (i.e. 1 and 10%) showed a higher activity than that expected for those concentrations.

The remaining three species, namely *F. aesculi*, *D. seriata* and *D. viticola*, showed a lower phytotoxicity in comparison with *N. luteum* and *N. parvum*, although variable among the different phytotoxicity assays. We were not able to establish a clear relationship between phytotoxic activities of these three species and previously reported virulence for these fungi (Van Niekerk et al. 2004; Taylor et al. 2005; Úrbez-Torres et al. 2007a).

Different incubation periods were established for each fungal species to develop maximum phytotoxicity in the culture filtrates; 14 days for *D. seriata* and *N. parvum*, and 21 days for the remaining species. In general, higher amounts of toxic organic compounds were obtained from acid extractions, whereas toxic hydrophilic compounds were obtained mainly from alkaline extractions.

Preliminary research on the culture filtrates of *N. luteum* and *N. parvum* showed that both species produced lipophilic, acid low-molecular weight phytototoxins. The TLC analyses carried out under different eluting conditions for the organic extracts from these two species revealed the presence of metabolites with similar chromatographic patterns in all toxic fractions.

All Botryosphaeriaceae species tested produced hydrophilic, high-molecular weight phytotoxic compounds. The GC-MS analysis of the acetylated *O*-methyl glycosides of the EPS produced by *N. parvum* showed these substances to be composed mainly of glucose, mannose and galactose, which are different from the botryosphaerans. Botryosphaerans are branched (1→3; 1→6)-β-D-glucans known to be produced by *B. rhodina* (Corradi da Silva et al. 2005) and an unidentified ligninolytic *Botryosphaeria* species (Barbosa et al. 2003). The involvement of EPS in bacterial and fungal disease has been reported by Hogdson et al. (1949), Harborne (1983) and Denny (1995), but their effective action as phytotoxins has still to be clarified (Van Alfen 1989; Denny 1995). The production of polysaccharides that induce phytotoxic effects on plants has been reported for some specific fungal pathogens such as *Cephalosporium* (Spalding et al. 1961), *Ceratocystis fagacearum* (McWain and Gregory 1972), *Ophiostoma ulmi* (Strobel et al. 1978), *Fusarium solani* (Thomas 1949), and several species of *Phytophthora*, including *P. cinnamomi*, *P. megasperma* var. *sojae* and *P. palmivora* (Keen et al. 1975). Moreover, the EPS from the culture filtrates of *P. cinnamomi*, *P. cryptogea* and *P. nicotianae* induced severe wilting on several hosts (Woodward et al. 1980). It is supposed that these macromolecules interfere with water movement in plant tissues by plugging the vessels, which leads to wilt symptoms (Barbosa et al. 2003). This phenomenon seems to be related to the size of the molecules and their viscosity rather than to their structure (Harborne 1983) although some results on host specificity suggest a possible different behaviour. In fact, a certain relationship between toxicity and host specificity has been suggested for some bacterial leaf spot diseases (Rudolph et al. 1989). However, the recent isolation of EPS from *Cryphonectria parasitica* (Corsaro et al. 1998b), *Phomopsis foeniculi* (Corsaro et al. 1998a), *Pm. aleophilum* and *Pa. chlamydospora* (Sparapano et al. 2000), which showed phytotoxic effects on natural hosts as well as other non-host plants, suggests a non-specific character for these toxic metabolites. The significant activity observed in the outside fraction of the dialysis experiment of *N. luteum* could be due to hydrophilic low-molecular weight metabolites that could be absorbed in the EPS precipitate and could be released in the aqueous washes during the dialysis experiment.

In conclusion, the phytotoxic activity from the five Botryosphaeriaceae species studied here may be due to hydrophilic high-molecular weight compounds, probably exopolysaccharides as was specifically demonstrated for *N. parvum*. Moreover, *N. luteum* and *N. parvum* also produce lipophilic, low-molecular weight phytototoxins; therefore these two species are clearly hydrophilic and lipophilic phytotoxin producers. Future research should address the identification of the chemical structure of these phytotoxic compounds and their production on naturally infected grapevines. Additionally, the detection of these phytotoxic compounds on non-symptomatic plants may provide an early prediction tool for infected declining grapevines.

Acknowledgements This research study was commissioned from ARSIA-Toscana (Regional Agency for Development and Innovation in Agriculture and Forest) by fourteen administrative Regions and one autonomous province, and financed with funds provided by the 'Ministero per le Politiche Agricole e Forestali' (Ministry for Agriculture and Forestry Policy, Italy) to implement the inter-Regional Project: Grapevine esca: research and experiment in the nursery and in the field for prevention and cure. Additional funds were provided by the 'Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria' (INIA) under project RTA03-058-C2-1 (Spain). Soledad Martos was supported by the 'Departament d'Educació i Universitats de la Generalitat de Catalunya' (Regional Government of Catalonia, Spain) and the European Social Fund. The authors would like to thank M. Fiore and F. Peduto for their continuous help in the laboratory work, Dr. Teresa Ieranò for EPS GC-MS analysis and P. Manzano for his valuable suggestions. Contribution DISSPAPA 162.

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