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Phytoprotection potential of *Fusarium proliferatum* for control of Botryosphaeria dieback pathogens in grapevine

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Summary. The economic impact of grapevine trunk diseases (GTDs) is increasing worldwide, due to the lack of efficient and simple control protocols for these disease complexes. Possible and efficient GTD management strategies must consider the complexity of host physiological alterations affecting metabolism and defense responses determined by GTD pathogens, and linked to disease expression. In this complexity, the use of biocontrol agents could give advantages in GTD control methods. The effect of the potential biocontrol agent (BCA) *Fusarium proliferatum* was evaluated using *in vitro* tests and in dual inoculation with the Botryosphaeria dieback agent *Neofusicoccum parvum in planta*. Artificial inoculations were performed in greenhouse and vineyard experiments at three key vine growth stages, the onset of G (separated clusters), I (flowering) or M (veraison) stages. The biocontrol potential was assessed using pathogenicity tests and transcriptomic analyses. Results showed that the *F. proliferatum* has potential for phytoprotection, with disease control efficiency related to host plant growth stage. Flowering was confirmed as the growth stage when disease control was least, and efficiency of activated defense responses against pathogen infection was minimum.

Keywords. Botryosphaeria dieback, *Diplodia seriata*, *Neofusicoccum parvum*, defense responses, biological control.

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INTRODUCTION

Grapevine trunk diseases (GTDs) are recognized as one of the most destructive and important problem of grapevine worldwide (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016a; Guerin-Dubrana *et al*., 2019). GTDs are caused by several unrelated fungi which are able to live and exclusively colonize the wood of grapevine perennial organs, causing wood necrosis and/or discolouration, vascular infections and decays (Mugnai *et al.,* 1999; Bertsch *et*

al., 2013). Externally, diseased vines show general and progressive dieback, often associated with specific foliar symptoms affected by different diseases and host cultivars. These diseases can initially cause loss of productivity and eventually death of affected vines. Their potential is increased by long latent asymptomatic periods, and by the capability of pathogens to infect vines through pruning wounds (Bertsch *et al.,* 2013) which may remain susceptible to GTD infections up to 4 months (Eskalen *et al.,* 2007). Among GTDs, Botryosphaeria dieback, caused by species in the Botryosphaeriaceae (Chethana *et al.,* 2016) is one of the most widespread GTDs in grape growing regions (Úrbez-Torres, 2011; Spagnolo *et al.,* 2014a; Fontaine *et al.,* 2016a). Esca disease and Eutypa dieback are also important grapevine diseases (Bertsch *et al*., 2013).

After the banning of some disease management active ingredients, such as sodium arsenite, benomyl and carbendazim-based products (Bertsch *et al.,* 2013; Fontaine *et al.,* 2016a; Gramaje *et al.,* 2018; Songy *et al*., 2019), which gave good control of some important GTDs but may have negative effects on humans and environment, a range of fungicides, natural molecules and biological control agents (BCAs) has been tested to set up efficient control strategies towards the increasing incidence of GTDs (Mondello *et al.,* 2018). To date, beyond *Trichoderma* species, other fungal and bacterial agents have been reported to be effective against GTD pathogens, although some have only been tested either *in vitro* or in nurseries (Gramaje *et al.,* 2018; Mondello *et al.,* 2018). Among these agents, several studies have shown how they could efficiently contribute in GTD management strategies. Beside other specific advantages (broad-spectrum activity and long-lasting effect as wound protectants), an interesting potential of BCAs is their effects on host/pathogen relationships, such as systemic induced resistance (SIR) responses (Handlesman and Stabb, 1996; Pal *et al.,* 2006; Berg, 2009). In this way, BCAs could reduce disease incidence by improving host plant resistance to abiotic and biotic stresses, especially for those provoked by GTDs (Sosnowski *et al.,* 2011; van Niekerk *et al.,* 2011; Spagnolo *et al.,* 2014, 2017; Pinto *et al.,* 2018). BCAs could also have positive effects on GTDs through induced changes of host plant metabolism determined by plant defense responses, and disorders caused by fungal toxins (Abou-Mansour *et al.,* 2015; Christen *et al.,* 2015; Burruano *et al.,* 2016; Fontaine *et al.,* 2016a; Trotel-Aziz *et al.,* 2019).

Previous studies focused on physiological changes occurring in GTD-affected grapevines have highlighted that: i) the primary host metabolism is involved, with carbohydrate metabolism variations also related to the activation of host defense responses (Fontaine *et al.,* 2016b); ii) defense responses are activated at least at transcriptomic levels with different induction levels for the same gene according to the host growth stage, especially for Botryosphaeria dieback (Spagnolo *et al.,* 2014, 2017); and iii) these defense responses are probably not enough to avoid the pathogen colonization and disease development, especially during flowering. At flowering, grapevines showed the greatest gene induction levels but also the greatest weakness towards the two Botryosphaeria dieback pathogens, *Diplodia seriata* and *Neofusicoccum parvum* (Spagnolo *et al.,* 2014, 2017; Fontaine *et al.,* 2016b). Furthermore, these pathogens are able to disperse their inoculum at flowering, aggravating the possibility of infections at this stage (Kuntzmann *et al*., 2009; Amponsah *et al*., 2009; Valencia *et al*., 2015).

The aim of the present study was to expand knowledge of physiological changes occurring in green stems of grapevines cv. Mourvèdre that were artificially infected with GTD pathogens at different grapevine growth stages. Furthermore, because of the close relationship between host plant physiology and GTD development, the effects were also assessed of a potential fungal BCA to limit Botryosphaeriaceae *in vitro* and *in planta* through dual inoculation tests. Assessed effects were of: i) artificial inoculation with the pathogen *N. parvum*; and ii) dual inoculation with *N. parvum* and the potential BCA *Fusarium proliferatum*, in greenhouse experiments 2 months post-inoculation and in the field at different grapevine growth stages. *In vitro* biocontrol evaluation tests, pathogenicity tests and transcriptomic analyses were carried out in this study.

MATERIALS AND METHODS

In vitro *evaluation of biocontrol ability of* Fusarium proliferatum

The *F. proliferatum* strain ("Fus") used in this study was frequently isolated epiphytically and endophytically by Larignon *et al.* (2013), from tissues of symptomless, greenhouse-trained young grapevines.

This fungus is a common pathogen of maize but can also colonize trees, vegetables and other cereals (Cendoya *et al.,* 2017). A preliminary *in vitro* evaluation of the biocontrol ability of the Fus strain towards Botryosphaeriaceae included the pathogens *Diplodia seriata* (two strains: 98-1 and 99-7, described in Reis *et al.,* 2016) and *Neofusicoccum parvum* (three strains: Bourgogne (Np Bour) (Ramirez-Suero *et al.,* 2014), Np Sainte Victoire (NpSV) (Larignon *et al.,* 2015) and Np bt67 (Trotel-Aziz *et al.,* 2019).

Two different assays were carried out. The first assessed direct antagonistic effects of the potential BCA against the pathogens, and the second was to verify any antibiotic activity of the BCA's secondary metabolites. The direct antagonistic effects of the potential BCA were evaluated by dual culture assays following the adapted protocol of Bézert *et al.* (1996). A 3 mm diam. agar culture plug of the potential BCA and a plug of the respective pathogen were placed along the same diameter in a Petri dish (9 cm diam.) containing potato dextrose agar (PDA). Controls consisted in Petri dishes each inoculated with the pathogen and with a sterile PDA plug. Each experimental treatment was replicated five times. The plates were then maintained in the dark at 24 ± 1°C. The direct biocontrol activity of *F. proliferatum* was evaluated by measuring the area of mycelial growth (expressed in cm^2), recorded each 24 h until the pathogen covered the entire Petri dish agar surface in the experimental controls. Colony area was determined by measuring the colony margins, and was calculated using the ImageJ software (http://imagej.nih.gov/ij).

The antibiotic activity of *F. proliferatum* secondary metabolites was evaluated by growing the different pathogens on PDA enriched with cultural filtrate of *F. proliferatum*. Three-d-old agar plugs of *F. proliferatum* were put into flasks (one plug per flask) containing 100 mL of sterile potato dextrose broth (PDB), and was then maintained in agitation at 100 rpm for 14 d. The cultural filtrate was collected eliminating the mycelium pellets by non-sterile filtration, and was then sterilized by filtration (0.45 and 0.22 µm pore size filters in sequence), and was then added to autoclaved liquid PDA (55°C) to obtain PDA medium with concentrations of 0 (control), 25 or 50% of culture filtrate. These plates were then each inoculated with a 3 mm plug of a pathogen in the centre of the plate. Each experimental treatment was replicated three times. The antibiotic activity of the *F. proliferatum* secondary metabolites was evaluated by daily measuring of the surface of the pathogen colony up to 9 d post-inoculation (dpi). The colony surface areas were calculated using the ImageJ free software (http://imagej.nih.gov/ij).

Greenhouse assays: plant material, fungus strains and inoculations

To evaluate the *F. proliferatum* control capability *in planta*, greenhouse co-inoculation tests with *F. proliferatum* and Botryosphaeriaceae fungi were carried out. *Neofusicoccum parvum*, due to its high aggressiveness towards *Vitis vinifera*, was preferred to *D. seriata*. Also because of the *in vitro* results, the Fus biocontrol activity was evaluated towards *N. parvum* strain NpSV, whose growth was reduced in dual cultural assay and greatly inhibited by Fus culture filtrate. Four-month-old potted plants of grapevine cv. Mourvèdre were each inoculated with *F. proliferatum* strain Fus using a PDA plug from a 7-d-old colony. One (FN+7d) and two (FN+14d) weeks post-inoculation with Fus, the plants were each co-inoculated at the same internode with the *N. parvum* strain NpSV, about 2 cm above the Fus inoculation point. Control plants were inoculated, respectively, with sterile PDA, Fus alone or NpSV alone. Five plants per experimental treatment were used. Two months after pathogen inoculation, the plants were cut, and the portion of each inoculated internode was observed and internal necrosis was measure. The presence of the potential BCA and pathogen were also verified (Figure 1). Collected data

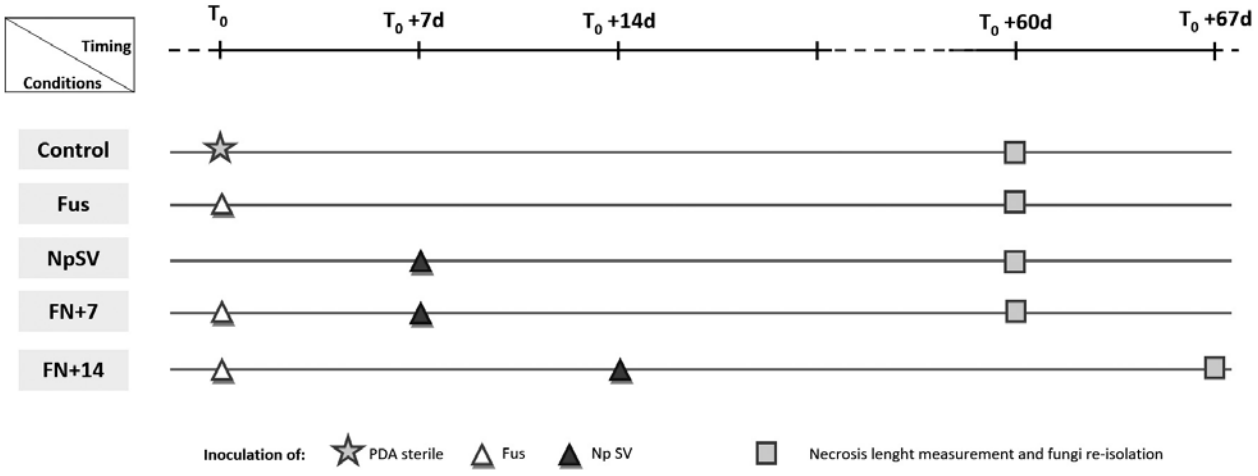

Figure 1. The co-inoculation test scheme, indicating the different conditions assayed and the timing of the inoculations BCA –pathogen, as performed in the greenhouse test.

were submitted to statistical analyses (Kruskal–Wallis and Dunn's multiple comparison tests).

Field tests: plant material, fungus strains and inoculations

To further evaluate the biocontrol activity of the Fus strain in natural conditions, and the role of host plant growth stage in disease development, a co-inoculation test was also performed in the field, as described in Reis *et al.* (2019). The experimental site was a vineyard of cv. Mourvèdre/3309, planted in 1997, and located at Rodilhan (Costières de Nîmes, France). The vineyard was owned by the Lycée agricole Marie-Durand of Rodilhan. Similar to the greenhouse essay, the Botryosphaeria dieback agent *N. parvum* strain NpSV and *F. proliferatum* strain Fus were used. These fungi were inoculated into green stems of standing vines, either alone (NpSV or Fus) or in combination (FN), using the method described by Spagnolo *et al.* (2017). In the case of the dual inoculations (FN), *N. parvum* was inoculated 7 d after Fus. The inoculations were performed at the onset of the grapevine Baggiolini phenological stages G (separated clusters), I (flowering) or M (veraison) (Baggiolini, 1952). Individual plants received the respective experimental treatments, and eight repetitions per treatment were applied. For each growth stage, non-wounded and non-inoculated green stems (C1), or stems inoculated with sterile malt agar (C2), were the experimental controls.

Observation of lesion development and re-isolation tests (for five of the 8 repetitions of each treatment) were performed at the end of vegetative season for samples from the three growth stages, as described by Larignon and Dubos (1997).

TRANSCRIPT ANALYSES

To study the effects of presence of the pathogens and the BCA on host plant physiology, inoculated stems in the vineyard were collected and submitted to transcriptomic analyses. Except for C1 stems, all the treatments were considered for transcript analysis. Samples for RNA extraction (three of 8 repetitions) were collected 20 dpi, and each consisted of the portion of the inoculated internode. For co-inoculated stems, each sample consisted of the portion of internode inoculated with both NpSV and Fus. Samples were collected, stored and processed to obtain a fine powder, according to the protocol outlined by Spagnolo *et al.* (2017).

RNA extraction

The Plant RNA Purification Reagent (Thermo Fischer Scientific Inc.) was used to extract total RNA from 2 x 50 mg of powdered green stem tissues from each assayed plant, and was DNase treated. The quality of RNA was checked by agarose gel electrophoresis, and the quantity was determined by measuring the absorbance at 260 nm.

Real-time RT-PCR analysis of gene expression

Reverse transcription was performed on 150 ng of total RNA, using the Verso cDNA synthesis kit (Thermo Fischer Scientific Inc.). Real-time PCR was performed with Absolute Blue QPCR SYBR Green (Thermo Fischer Scientific Inc.), using a CFX96 thermocycler system (Bio-Rad).

The thermal profile used was: 15s at 95°C (denaturation) and then 40 cycles each of 1 min at 60°C (annealing/extension). Melting curve assays were performed from 65–95°C at $0.5°C \cdot s^{-1}$. Melting peaks were visualized to check the specificity of each amplification. Results were expressed as the values of relative expression ($\Delta\Delta Ct$), corresponding to the mean of three independent experiments. The genes analyzed were considered significantly up- or down-regulated when changes in their expression were, respectively, >2-fold or <0.5-fold. The specific primers for the 16 targeted genes are listed in Table 1.

RESULTS

Evaluation of the biocontrol ability of Fusarium proliferatum

In dual culture tests, *F. proliferatum* strain Fus limited the growth of *D. seriata* and *N. parvum* strains, forming clear limits between the two touching colonies in each Petri plate after 4-7 d of co-culture, even where the differences with the controls were not statistically significant. The one exception was that of the strain Np bt67 (Figure 2, A to E). A slight decrease (7-20%) was recorded in growth of the *N. parvum* strains in dual culture with Fus, 1 d before the contact between both colonies in each plate. This effect was not recorded for the tested *D. seriata* strains (data not shown). The contact zones were often characterized by the contemporary presence of intertwining hyphae of Fus and the pathogens (Figure 2, A' and B'). Fus was also able to grow over most of the pathogens, starting at 10 dpi (Figure 2C).

Stronger Fus biocontrol activity was observed for the Fus culture filtrate (Fus CF), with differences related to

Table 1. Primers of genes analyzed by real-time reverse-transcription polymerase chain reaction.

Function	Gene	Primer Sequences	GenBank or TC TIGR* Accession Number
Housekeeping genes	*EF1* (EF1-α elongation factor)	5'-GAACTGGGTGCTTGATAGGC-3'	GU585871
		5'-AACCAAAATATCCGGAGTAAAAGA-3'	
	60SRP (60S ribosomal protein L18)	5'-ATCTACCTCAAGCTCCTAGTC-3'	XM_002270599
		5'-CAATCTTGTCCTCCTTTCCT-3'	
Phenylpropanoid metabolism	*CHI* (Chalcone isomerase)	5'-GCAGAAGCCAAAGCCATTGA-3'	NM_001281104
		5'-GCCGATGATGGACTCCAGTAC-3'	
	PAL (Phenylalanine ammonia lyase)	5'-TCCTCCCGGAAAACAGCTG-3'	X75967
		5'-TCCTCCAAATGCCTCAAATCA-3'	
	POX4 (Peroxidase-like 4)	5'-AACATCCCCCCTCCCACTT-3'	XM_002269882
		5'-TGCATCTCGCTTGGCCTATT-3'	
	STS (Stilbene synthase)	5'-AGGAAGCAGCATTGAAGGCTC-3'	FJ851185
		5'-TGCACCAGGCATTTCTACACC-3'	
Defense protein	*CHV5* (Chitinase class V)	5'-CTACAACTATGGCGCTGCTG-3'	AF532966
		5'-CCAAAACCATAATGCGGTCT-3'	
	GLUC (β-1,3 glucanase)	5'-TCAATGGCTGCAATGGTGC-3'	DQ267748
		5'-CGGTCGATGTTGCGAGATTTA-3'	
	PPO (Polyphenol oxidase)	5'-TGGTCTTGCTGATAAGCCTAGTGA-3'	XM_002727606
		5'-TCCACATCCGATCGACATTG-3'	
	PR6 (Serine-protease inhibitor 6)	5'-AGGGAACAATCGTTACCCAAG-3'	AY156047
		5'-CCGATGGTAGGGACACTGAT-3'	
	SAMS (S-adenosylmethionine synthetase)	5'-CCTGAAATCAAAGTTCTCCTTCACA-3'	XM_002266322
		5'-CCGGGCCTGAAATCAAAGTT-3'	
	TL (Thaumatin-like)	5'-CCTAACACCTTAGCCGAATTCGC-3'	AF532965
		5'-GGCCATAGGCACATTAAATCCATC-3'	
Detoxification and Stress tolerance	*epoxH2* (Epoxide hydrolase 2)	5'-TCTGGATTCCGAACTGCATTG-3'	XM_002270484
		5'-ACCCATGATTAGCAGCATTGG-3'	
	GST5 (Glutathione s-transferase 5)	5'-GCAGAAGCTGCCAGTGAAATT-3'	XM_002277883
		5'-GGCAAGCCATGAAAGTGACA-3'	
	HSP (alpha crystalline heat shock protein)	5'-TCGGTGGAGGATGACTTGCT-3'	XM_002272382
		5'-CGTGTGCTGTACGAGCTGAAG-3'	
	SOD (Superoxide dismutase)	5'-GTGGACCTAATGCAGTGATTGGA-3'	AF056622
		5'-TGCCAGTGGTAAGGCTAAGTTCA-3'	
Primary metabolism	*PglyDH* (Phosphoglycerate dehydrogenase)	5'-CGTCGAAGATGCTCAATGATGA-3'	XM_002285322
		5'-CCCCCACGAGCAACATTAATT-3'	
Water stress	*TIP1* (Tonoplast intrinsic protein)	5'-ATCACCAACCTCATTCATATGC-3'	AF271661
		5'-GTTGTTGTCTCAACCCATTTCC-3'	

* see http://www.jcvi.org/cms/research/projects/tdb/overview/

the Botryosphaeriaceae species and the strain (Figure 3, A and B). *Diplodia seriata* strains showed similar reactions to the Fus CF, with a severe growth reduction even from the lowest aliquot of filtrate. Growth of the Ds99-7 strain was inhibited until 3 dpi on 25% Fus CF and 7 dpi on 50% filtrate. This strain then had abnormal and slow growth with sparse hyphae (Figure 4). Different from *D. seriata*, *N. parvum* growth in Fus CF-amended media varied according to the strain. Fus CF showed the greatest activity against NpSV growth compared to the control, with statistically significant reductions at both 25 and 50% filtrate. In contrast, there was no statisti-

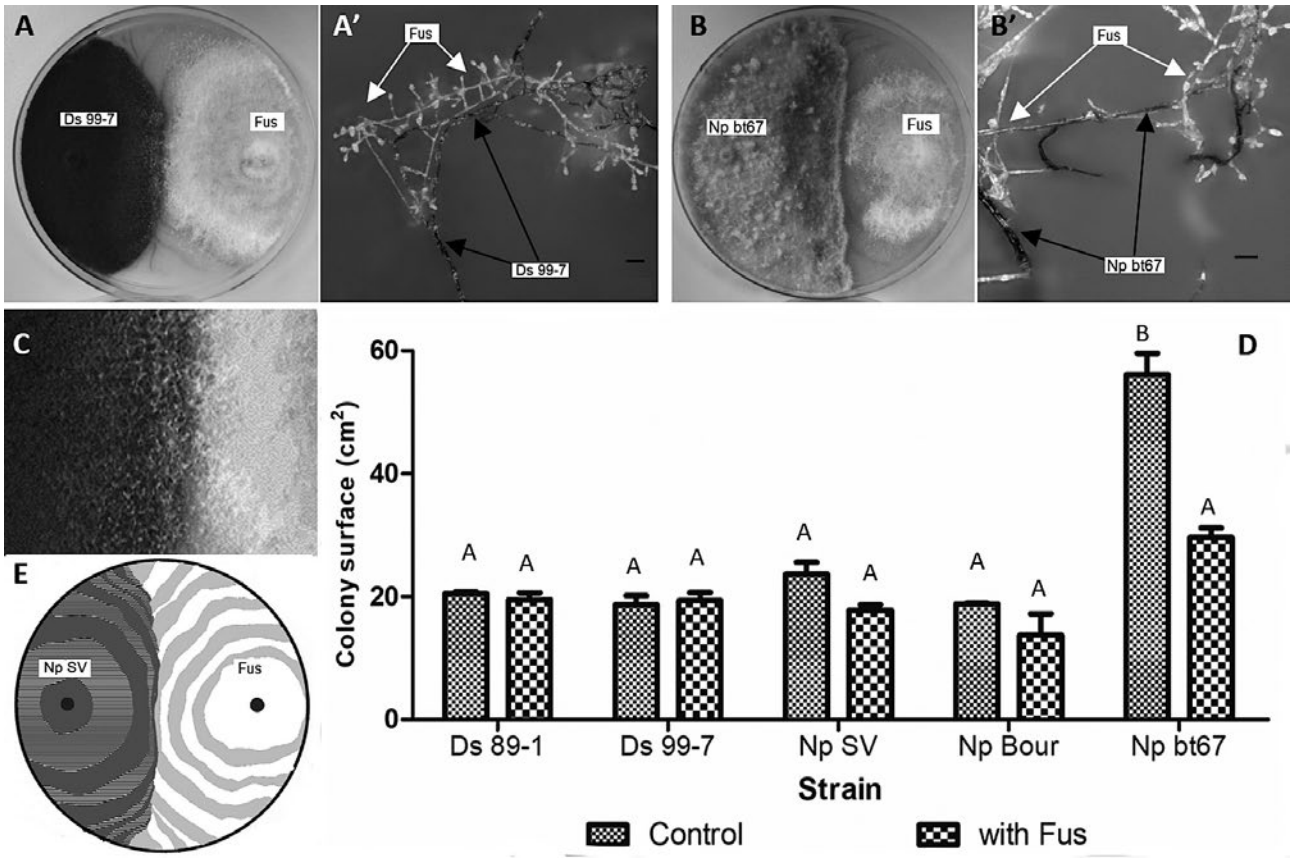

Figure 2. *In vitro* biocontrol activity of *Fusarium proliferatum* strain Fus on the tested Botryosphaeriaceae in dual culture tests. In A and B the limited growth of Ds99-7 and Np bt67 strains was noted after 4 - 7 d of dual inoculation with *F. proliferatum*. In A' and B', the respective zones of confrontation, observed with a microscope, often showed intertwining hyphae of the two strains (indicated by arrows). In C, the Fus strain are growing as white flocks over the Botryosphaeriaceae, as observed at 10 dpi. In D, the mean colony surface (in cm^2 ± SE; two-way ANOVA + Bonferroli test), of the five strains compared to the controls and recorded on the day when the colonies touched, as calculated using a graphical representation of the co-cultures (E). Scale bars = 20μm. dpi = day post inoculation. Columns accompanied by the same letter are not significant different ($P \leq 0.05$).

cally significant difference the Np Bour growth values, although Fus CF growth was stimulated growth at 25% of filtrate but inhibited growth at 50%. Intermediated response was recorded for the Np Bt67 strain, which was significantly inhibited only when growing on plates with the 50% of Fus CF.

Greenhouse pathogenicity tests

The Fus and NpSV strains were re-isolated from the edges of internal green stem lesions at 60 dpi. No fungi were isolated from vines that received sterile PDA plugs. Both the strains induced lesions starting from the inoculation points, while no lesions occurred in the control plants. The presence of *F. proliferatum* strain Fus in FN+7d co-inoculations gave a significant reduction ($P \leq 0.05$) in the lesion length caused by NpSV. In contract, NpSV gave increased of lesion lengths in co-inoculated vines when the pathogen was inoculated 14 d after inoculation with Fus (FN+14d), although the difference was statistically significant compared to control (Figure 5)

Field pathogenicity tests

The pathogen *N. parvum* and the potential BCA *F. proliferatum* were always re-isolated from the edges of the lesions associated with their inoculations, so the postulates associating disease and effects were fulfilled. No fungi were isolated from the lesions of control stems C2, indicating the lesions developed as a consequence of the wound in inoculated conditions. Mean lesion sizes associated with *F. proliferatum* in the single (Fus) and dual

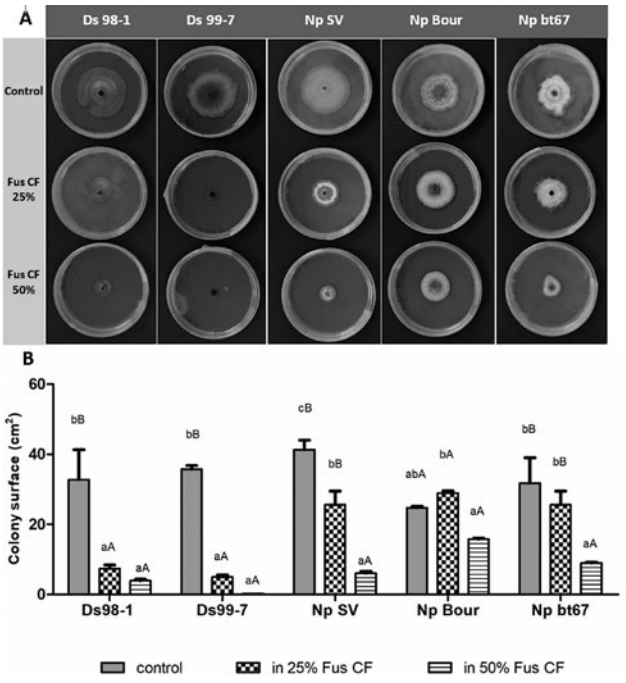

Figure 3. Biological control activity of the two different culture filtrate aliquots of *F. proliferatum* strain Fus (Fus CF) against growth of Botryosphaeriaceae fungi at 7 dpi. In B), the ANOVA results (two-way + Bonferroli test) on mean colony areas (cm^2) indicated a different Fus CF effect, for species and strain. Columns accompanied by the same letters are not significantly different (lowercase, $P \leq 0.05$, uppercase $P \leq 0.01$).

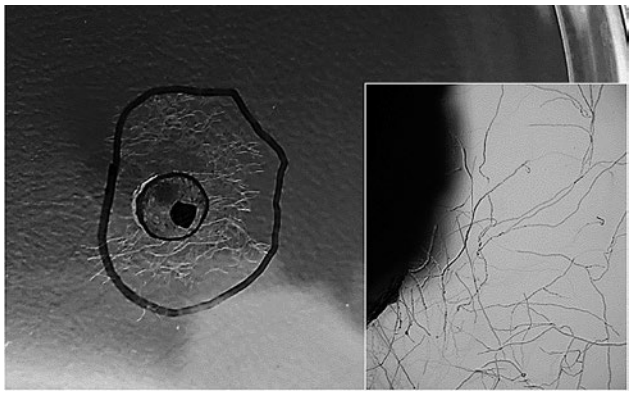

Figure 4. Detail of inhibited growth of strain Ds 99-7 on PDA plates amended with 50% of Fus cultural filtrate (Fus CF), observed with a microscope at 8 dpi. The mycelium rarely formed and had sparse hyphae.

inoculations with the pathogen (FN) were never statistically different from the controls (C2). Statistical differences were recorded for *N. parvum* in all the three vine phenological stages for single inoculation, and at the I and M stages for the in dual inoculations. At G stage, the longest lesions were recorded for Np condition (mean = 10.8 mm (± 1.3 mm)). At stages I (flowering) and M (veraison) longer lesions were associated with the pathogen from single (NpSV) and dual (FN) inoculations. At flowering, *N. parvum* produced longer lesions in the presence of Fus (mean = 24.0 mm (± 3.0 mm))

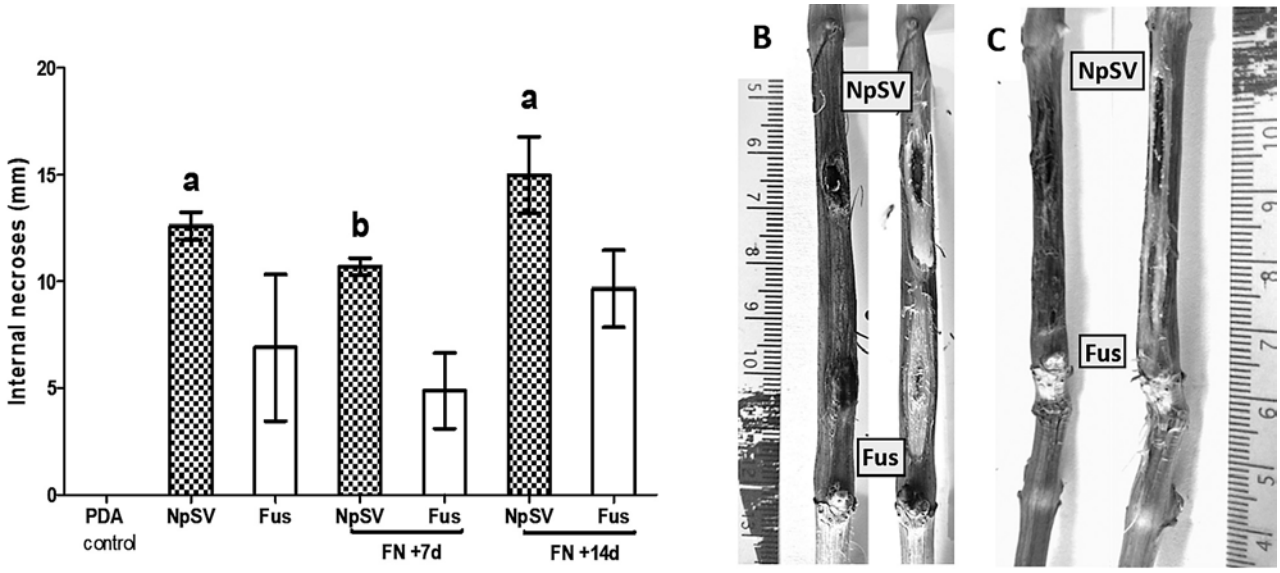

Figure 5. In A, mean lesion lengths (± SE) on rooted grapevine cv. Mourvèdre plants after inoculation with *N. parvum* strain NpSV and *F. proliferatum* strain Fus, at 60 dpi for single (NpSV, Fus) or dual inoculations (carried out in the greenhouse. Differences among the means were evaluated by the Mann-Whitney non-parametric test. Bars accompanied by the same letter are not statistically significant different ($P \leq 0.05$). In B and C, external and internal lesions produced by Fus and NpSV in FN+7d and in FN+14d co-inoculated plants.

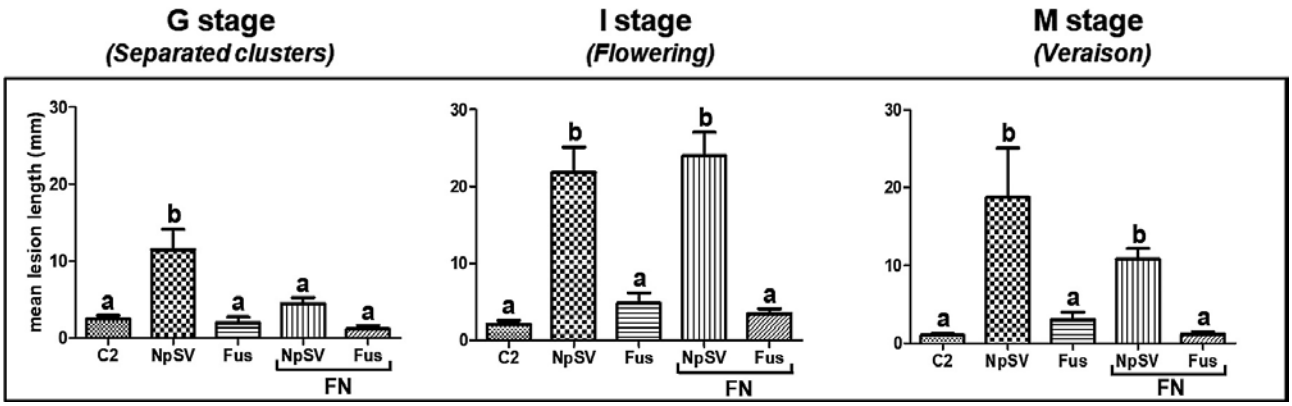

Figure 6. Mean lesion lengths (± SEs) on green grapevine stems after inoculation with *N. parvum* strain Saint Victoire and *F. proliferatum* strain Fus at the onset of the G, I and M stages, after single (NpSV, Fus) or dual (Inoculation. Control stems (C2) were wounded and inoculated with sterile malt agar. Differences among the means were evaluated by the Dunn's Multiple Comparison Test, following which the null hypothesis (equal means) was rejected in the Kruskal–Wallis test, assuming significance of $P \leq 0.05$. Columns accompanied by the same letters are not significantly different ($P \leq 0.05$).

than when inoculated alone (mean = 21.8 (± 3.3 mm)), although this difference was not statistically significant ($P > 0.05$). In contrast, at M stage (veraison), lesion lengths were greater from the *N. parvum* single inoculation (mean = 18.7 mm (± 3.4 mm)) than from the dual inoculations (mean = 10.8 mm (± 1.3 mm)) (Figure 6).

Characterization on targeted plant responses

The transcript analyses were used to evaluate the influence of the potential BCA *F. proliferatum* on host plant defense responses towards the Botryosphaeria dieback pathogen *N. parvum*. The genes were selected based on similar previous studies (Spagnolo *et al.,* 2014, 2017; Magnin-Robert *et al.,* 2011; 2014; 2016). A total of 16 genes encoding components of the phenylpropanoid pathway (genes *CHI, PAL, POX4, STS*), Pathogenesis-related (PR) and other defense proteins (*CHV5, GLUC, PPO, PR6, SAMS, TL*), proteins involved in detoxification processes (*epoxH2, GTS5, HSP, SOD*) and in primary metabolism (*PglyDH*) or water stress (*TIP1*), were chosen measure a profile of the grapevine response.

As already observed in previous studies (Spagnolo *et al.,* 2014; 2017), most of changes in the expression levels concerned the phenylpropanoid pathway and *PR*-genes, especially at the G and I vine growth stages. At these stages maximum levels of gene induction were detected in stems co-inoculated with *N. parvum* and *F. proliferatum* (FN) (Figure 7). For example, the relative expression of gene *STS* increased by up to 34-fold in FN at the G stage, and expression of *PR6* was increased almost 33-fold in FN at I stage. At flowering, a more homogenous background of upregulation was observed, determined by increased induction from both the Np and Fus inoculations, and a simultaneous decrease in some genes from the FN treatments. A general decrease in gene induction occurred at veraison (M stage), with slight upregulations occurring mostly in the co-inoculated stems (FN). Among the stress tolerance and detoxification genes, only *GST5* at the G stage and *HSP* at the I stage were slightly induced, especially in presence of the BCA either alone or in presence of the pathogen. No genes were down regulated by any of the treatments.

DISCUSSION

Antagonistic effects of Fusarium proliferatum *against Botryosphaeriaceae species*

Fusarium proliferatum strain Fus showed antagonistic effects towards *N. parvum* and *D. seriata* in the laboratory tests. The *in vitro* dual culture test highlighted the role of the potential BCA in limiting growth of the tested Botryosphaeriaceae pathogens, which were unable to overgrow the antagonist Fus when in contact with its mycelium. The slight inhibition recorded 1 d before confrontation with Fus, observed only for *N. parvum* due to the slower growth rate compared to *D. seriata*, suggested a role of antibiosis. Antibiosis was demonstrated from the results of culture filtrate test. *Fusarium proliferatum* metabolites limited the growth of the pathogens, with greater and more homogeneous inhibition of the *D. seriata* strains than of the *N. parvum* strains. The different strains of *N. parvum* showed different amounts of

Function / Gene		G stage (Separated clusters)			I stage (Flowering)			M stage (Veraison)		
		NpSV	Fus	FN	NpSV	Fus	FN	NpSV	Fus	FN
Phenyl-propanoid metabolism	*CHI*	1,16	0,79	1,69	2,04	2,21	2,24	1,05	0,92	1,26
	PAL	2,22	1,21	3,52	0,66	1,20	0,99	0,96	2,05	1,99
	POX4	1,81	1,07	3,45	2,68	1,48	2,02	1,11	1,37	1,53
	STS	5,71	3,08	34,07	7,13	7,50	9,27	1,06	0,95	2,19
Defense protein	*CHV5*	3,39	3,61	13,78	7,70	7,51	13,21	2,96	1,59	2,43
	GLU	4,69	0,96	23,30	4,70	6,80	14,29	1,93	1,16	2,71
	PPO	1,09	1,47	3,41	3,64	2,22	5,06	2,68	2,51	2,87
	PR6	3,57	2,40	7,35	12,63	25,75	32,73	0,88	0,62	1,06
	SAM	1,16	0,97	1,57	1,21	1,48	1,51	0,95	1,23	1,62
	TL	2,85	3,01	7,39	7,31	4,08	8,40	2,44	4,63	2,13
Detoxification and Stress tolerance	*epoxH2*	1,16	0,84	1,24	1,44	1,13	1,15	0,87	1,13	1,39
	GST5	1,93	1,18	3,60	0,85	1,47	1,26	0,80	1,01	1,24
	HSP	1,19	1,02	1,00	0,60	2,85	3,46	1,09	1,13	1,17
	SOD	1,07	0,88	1,23	1,23	1,31	1,24	0,81	0,98	1,01
Primary metabolism	*PglyDH*	1,56	1,05	1,36	0,62	1,13	1,47	0,82	1,05	1,40
Water stress	*TIP1*	0,94	0,90	1,01	3,04	1,40	1,81	0,73	0,96	0,99

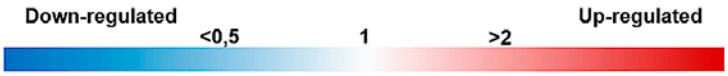

Figure 7. Expression levels of selected genes (determined with quantitative reverse-transcription polymerase chain reactions). The values (each the mean of three technical replicates) represent expression levels (ΔΔCt) of treatments relative to the controls (C2). The expression of each gene was considered up-regulated when the value was > 2-fold compared to the controls, or down-regulated when the value was < 0.5-fold compared to the controls.

growth inhibition, with NpSV as the most susceptible to Fus CF at both tested culture filtrate concentrations. *Fusarium proliferatum* was able to grow over the pathogens at 10 dpi, which also indicated direct antagonism effects, as reported previously for *F. proliferatum* and other *Fusarium* species. For instance, *F. proliferatum* was shown to be effective against the oomycete *Plasmopara viticola*, which causes grapevine downy mildew (Falk *et al.,* 1996). *Fusarium sporotrichioides* inhibited *in vitro* the growth of *Phoma negriana*, responsible of lesions and necrosis on grapevine shoots (Krol, 2008), while *F. lateritium* was shown to metabolize toxins produced by *Eutypa lata*, a cause of Eutypa dieback (Christen *et al.,* 2005). Some of the mechanisms involved in the *Fusarium* spp. biocontrol, such as mycoparasitism (Falk *et al.,*1996), extracellular β-glucosidase and endo-1,4-β-glucanase production (Bakshi *et al.,* 2001) and cyanide degradation (Christen *et al.,* 2005), were also found in other BCAs such as *Trichoderma* spp., *Ampelomyces quisqualis*, *Penicillium purpurogenum*, *Coniothyrium minitans* and *Pseudomonas fluorescens* (Haran *et al.,* 1996; Kubicek *et al.,* 1993; Rotem *et al.,* 1999; Whipps *et al.,* 2008; Winding *et al.,* 2004).

Fusarium proliferatum *reduced the necrosis produced by* N. parvum *strain NpSV* in planta

Under controlled conditions, *F. proliferatum* also showed biocontrol activity, reducing *N. parvum* strain NpSV aggressiveness when inoculated 7 d before the pathogen. In contrast, presence of *F. proliferatum* tended to increase the size of pathogen internal necroses when inoculated 14 d previously, although this effect was not statistically significant. In co-inoculations performed in the field, *F. proliferatum* showed biocontrol activity by reducing lesion sizes associated to *N. parvum* at the G (separated clusters) and, to a lesser extent, at M (veraison) grapevine stages. In contrast, the mean sizes of lesions associated with *N. parvum*, from single and dual inoculations, was greater at flowering, confirming the hypothesis that flowering is the period when the grapevine cv. Mourvèdre is more susceptible to Botryosphaeria dieback pathogens. As reported in previous studies (Spagnolo *et al.,* 2014; 2017), this susceptibility could be determined by high host plant metabolic activity focused towards inflorescence development. This metabolic re-orientation developing reproductive

organs could have also determined the inefficient biocontrol of *F. proliferatum* at I growth stage, which was unexpected considering the relative transcriptomic data. Beside biocontrol capabilities towards *N. parvum*, *F. proliferatum* was also not pathogenic to grapevine cv. Mourvèdre, although in greenhouse inoculations the strain induced longer lesions than in the field. These differences may be explained by the different host types used (cuttings *vs* standing vines), and the less stressing environmental conditions of the greenhouse compared to the field, which may have modulated the *F. proliferatum* behaviour. Greenhouse conditions could have also favored aggressiveness *F. proliferatum* and *N. parvum* in unstressed plants which were less reactive to artificial infections.

Changes induced by Fusarium proliferatum *to the grapevine*-Neofusicoccum parvum *interaction*

This also focused on the physiological changes occurring in annual stems in the host/pathogen/BCA tritrophic interaction of grapevine/*N. parvum*/*F. proliferatum,* at three host growth stages.

Fusarium proliferatum strain Fus weakly induced genes at the G stage (genes *STS*, *CHV5*, *PR6, TL*) and the M stage (*PAL*, *PPO, TL*). This indicated host plant tolerance towards *F. proliferatum* and supported the observed in field non-pathogenicity of this strain to *V. vinifera* cv Mourvèdre. In contrast, at the I stage Fus induced genes at levels equivalent to those of the pathogen *N. parvum*. This indicated a possible switch at flowering, from "neutral" to "parasitic" perception of *F. proliferatum* by the host defense system (Kogel *et al*., 2006). The greatest induction recorded for the most of genes in all FN treatments suggests the role of *F. proliferatum* strain Fus in priming the host defense system towards pathogen infections, which resulted in reduced necrosis from *N. parvum* strain NpSV observed at the G stage and to a lesser extent, at the M stage. This priming effect, elicited by several BCAs (*Trichoderma* sp., Perazzolli *et al*., 2011; *Pythium oligandrum*, Yacoub *et al*., 2016) was reported for *Aureobasidium pullulans* strain Fito_278 (Pinto *et al*., 2018) and *Bacillus subtilis* PTA-271 (Aziz *et al*., 2015; Trotel-Aziz *et al*., 2019) towards pathogens associated Botryosphaeria dieback.

Changes in the grapevine-*N. parvum* interaction determined by the potential BCA were characterized by gene expression associated either with plant defense/stress responses (secondary metabolism) or with energy metabolism (glycolysis, Krebs cycle). In agreement with Spagnolo *et al.* (2014), most of the modifications detected in the present study were more related to the host phenological stage than to a particular inoculation tested. Fasoli *et al.* (2012) also highlighted the importance of grapevine growth stage for gene expression. Gene expression in different organs is probably based on the host developmental stage rather than on organ type.

The over-expression of the genes *CHI*, *PAL*, *POX4* and especially *STS* at both the G and I stages on the infected stems from the NpSV and FN treatments could be related to activation of the phenylpropanoid pathway linked to plant defense. Phenylpropanoids are related in plant defense responses, playing preformed or inducible functions, forming physical or chemical barriers to infection, or local and/or systemic signalling for the defense gene induction (Dixon *et al.,* 2002). Similarly, stilbenes may limit development of GTD fungi in wood. *In vitro* tests have shown ability of different phenolic compounds to limit growth of several GTD-associated pathogens in the Botryosphaeriaceae and *Phaeomoniella chlamydospora* (Fontaine *et al.,* 2016b; Lambert *et al.,* 2012; Lima and Dias, 2012). Furthermore, the stilbene resveratrol could also act as a signaling molecule in the activation of defense-related responses in *Vitis* cells (Chang *et al.,* 2011).

The induction of *GLUC* expression, recorded in single or dual infected stem treatments, especially at the G (NpSV and FN) and I (NpSV, Fus and FN) growth stages, could be related to the multiple role of the related proteins. β-1,3-glucanases are abundant in plants, and are involved in cell division, movement of materials through plasmodesmata and in plant resistance towards abiotic stresses. These proteins also protect plants against fungal pathogens, alone or in association with other antifungal proteins such as chitinases or isoenzymes (Balasubramanian *et al.,* 2012). The synergistic effects between β-1,3-glucanases and chitinases towards fungal infections is frequently observed in other crops (including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat), and their presence led to increased disease resistance in plants (Balasubramanian *et al.,* 2012; Saboki *et al.,* 2011; Jach *et al.,* 1995). In the present study, *GLUC* induction was always coupled with increases in chitinase *CHV5* at the G growth stage (NpSv and FN treatments), the I stage (all treatments) and the M stage (FN treatment). At the transcriptomic level, this synergism of *Gluc/CHV5* could be also have occurred in green stems cv. Mourvèdre infected by *F. proliferatum* and Botryosphaeriaceous pathogens, either alone or in combination, as observed here and in the previous study Spagnolo *et al.* (2017). For defense protein genes, the observed high induction of *PR6* genes at flowering (the I stage) from all treatments (NpSV, Fus, and FN) has been previously reported in grapevines naturally affected by

GTD pathogens (Magnin-Robert *et al.,* 2014; Falk *et al.,* 1996; Magnin-Robert *et al.,* 2014; Valtaud *et al.,* 2009; Letousey *et al.,* 2010) and in artificially inoculated plants (Camps *et al.,* 2010), and has been described in grapevine cells in the presence of extracellular compounds produced by Botryosphaeria dieback pathogens (Ramirez-Suero *et al.,* 2014). The strong induction of defense genes (*PR6, PPO, TL, GLU, CHV5*) observed in FN-treated vines at the I growth stage, was either unrelated to the reduction of lesion sizes observed at G and M stages, or not great enough to limit lesion development at flowering. The implication of inflorescences reducing responsiveness to pathogen attack in green stems (Spagnolo *et al.,* 2014; 2017), supports the observation of little biological control effect from *F. proliferatum* at flowering.

CONCLUSIONS

Results from this study agree with previous observations, and confirm the importance of flowering in the defense of grapevine cv. Mourvèdre towards the development of *N. parvum* infections, even in the presence of the potential BCA *F. proliferatum*. This fungus limited the growth of pathogens both through antibiosis and direct antagonism *in vitro*, and development of pathogen-induced necrosis *in planta*, by priming effects enhancing some plant responses to infection. Based on these results, further studies should assess the physiological mechanisms that could influence host metabolism and responses to biotic stress during flowering.

The results observed in the *in planta* dual inoculation assays suggest further analysis of possible use of *F. proliferatum* as BCA is warranted, since the priming of plant defense response is an efficient and low cost way to improve resistance to abiotic and biotic stresses (van Hulten *et al*., 2006). Toxicological studies should also be carried out when considering this fungus as a possible BCA against GTDs, as variable toxigenic potential was detected in *F. proliferatum* populations by Stepien *et al.* (2011). This would reduce the risk that toxic fungal metabolites would enter human or animal food chains. Furthermore, the role of *F. proliferatum* as opportunistic human pathogen should be also considered, since it and other *Fusarium* species are reported to be associated with infections of immunocompromised patient (Summerbell *et al*., 1988; Tulin, 2018).

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