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Research Article

Screening for potential mycoherbicides within the endophyte community of *Phelipanche ramosa* parasitizing tobacco

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One sentence summary: A collection of 374 fungal isolates, tested for its ability to control the development of *Phelipanche ramosa*, a parasite of many crops, revealed the potential mycoherbicide effect of *Fusarium venenatum*.

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Abstract

Branched broomrape (*Phelipanche ramosa* (L.) Pomel) is an achlorophyllous root parasitic plant with a wide host range. Its complex management is leading to the abandonment of tobacco or oilseed rape cultivation in the most affected regions in France. Among broomrape regulation factors, soil microorganisms such as fungi seem to be a relevant biocontrol lever. The aim of this work was to detect potential mycoherbicides among fungal endophytic colonizers of *P. ramosa* parasitizing tobacco. Our hypothesis was that both the inhibitory of broomrape seed germination and the necrotic activities are characteristic of the fungal isolates whatever their taxonomic position. To test this hypothesis, we analysed the taxonomic and functional diversity of fungal isolates of symptomatic *P. ramosa* collected from infested tobacco-growing regions in France in order to identify one or more fungal strains for future biocontrol. The fungal isolates were characterized using morphological and molecular identification tools and tested for their ability to inhibit the germination of *P. ramosa* seeds, their necrotic activity on the stems of the pest and their non-pathogenicity to the host plant. We highlighted the specific richness of fungal colonizers associated with symptomatic *P. ramosa*. Among the 374 collected isolates, nearly 80% belonged to 19 *Fusarium* species. Eighty-seven isolates representative of this diversity also showed functional diversity by inhibiting seed germination of the parasite. The 20 best-performing isolates showed differences in germination inhibition of *P. ramosa* at the intraspecific level. Among these 20 fungal isolates, a set of 15 randomly selected isolates was tested for their necrotic activity on the parasite stems. *Fusarium venenatum* isolates showed dual competence, i.e. germination inhibition and necrotic activity, and were non-pathogenic to tobacco. This led us to discuss the potential mycoherbicidal effect of this fungal species on *P. ramosa*.

Keywords: biocontrol, branched broomrape pathovars, *Brassica napus*, *Fusarium* spp., germination inhibition, necrotic activity, *Nicotiana tabacum*, parasitic plant

Introduction

Parasitic plants are angiosperms that resort to a plant parasite strategy to obtain a competitive advantage from neighbouring host plants (Heide-Jørgensen 2013). Broomrapes (*Orobanche* spp. and *Phelipanche* spp.) belong to the Orobanchaceae family and constitute a large group of achlorophyllous root-parasitic plants mainly observed in the south-east of Europe and in west Asia. They inflict heavy damage to several economically important arable crops (Parker 2009). Branched broomrape (*Phelipanche ramosa* (L.) Pomel) is essentially autogamous and has a very broad potential host range consisting of various annual crops and annual weeds. *Solanaceae*, *Brassicaceae* and *Fabaceae* are prominent among them (Parker 2009).

Phelipanche ramosa has been associated with tobacco and hemp in western France since the beginning of the 20th century. However, oilseed rape has recently become its favourite host, and this has promoted its massive expansion in arable fields in several French farming areas (Gibot-Leclerc et al. 2003, 2012). Host-associated genetic divergence is known for at least three distinct *P. ramosa* pathovars functionally identified based on their respective preferences for tobacco, hemp and oilseed rape. They present distinct life cycles, which makes weed management quite complicated in severely infected fields and may lead to the abandonment of tobacco or oilseed rape cultivation in the most affected western and eastern regions in France (Benharrat et al. 2005, Brault et al. 2007, Gibot-Leclerc et al. 2013, Le Corre et al. 2014, Stojanova et al. 2019).

In broad outline, the cycle of Orobanchaceae and in particular that of *P. ramosa* includes two phases, i.e. an underground one and then an aerial one (Gibot-Leclerc et al. 2012). The underground phase is cryptic: it includes many mechanisms, some of which are not understood to date. Thus, the germination of broomrape seeds is dependent on phytohormones such as strigolactones or alkyl isothiocyanates produced by the host plant in its rhizosphere (Auger et al. 2012, Brun et al. 2018). As soon as it has germinated, the seed must quickly attach itself to the roots of the host plant, produce a haustorium to reach the plant's vascular vessels, and then, after several physiological and

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morphological stages such as tubercle formation, produce an underground stem towards the soil surface (Westwood 2013). The emergence of this stem corresponds to the beginning of the aerial phase. Besides flowering, the most spectacular part of that phase is the release of several hundreds of thousands of tiny seeds per flower scape (Gibot-Leclerc et al. 2012). Most of these seeds return to the soil and continuously increment the seed bank. One of the most notable characteristics of these seeds is their longevity for several decades in the soil, which partly explains why it is so difficult to control the development and expansion of broomrape (Fernández-Aparicio et al. 2016). A recent review has addressed broomrape regulation mechanisms (Cartry et al. 2021). Among the few tools available for broomrape control, the authors concluded that one of the relevant levers would be the use of soil microorganisms to inhibit seed germination and strongly necrotize the first stages of the underground development of the broomrapes having bypassed germination inhibition; the aim is to control broomrape development before their parasitic activity seriously affects the development of the host plant. This proposal was supported by the fact that bacterial strains belonging to the genera *Bacillus*, *Pseudomonas* and *Azospirillum* inhibited radicle elongation of germinating seeds of *Orobanche cernua* Loefl., *Phelipanche aegyptiaca* (Pers.) Pomel and *Striga hermonthica* (Delile) Benth. (another parasitic Orobanchaceae), respectively (Miché et al. 2000, Barghouthi and Salman 2010). *Streptomyces* bacterial strains reduced the number of *O. cernua* tubercles by almost 50% (Chen et al. 2016). In the same line, several fungal strains of the genus *Aspergillus* (Aybeke et al. 2015), but especially of the genus *Fusarium* (Amsellem et al. 2001a,b, Boari and Vurro 2004, Dor and Hershenhorn 2009, Abbas 2014), have been isolated and characterized for their deleterious activity towards Orobanchaceae parasitic plants. The deleterious or pathogenic activity relied on the inhibition of seed germination through the production of fusaric acid or trichothecenes, the necrotizing of tubercles and the reduction of the growth of young underground stems (Boari and Vurro 2004, Dor et al. 2007). Under controlled conditions in the soil, this resulted in a reduced number of tubercles being formed, a lower number and biomass of emerging stems, and in host plant development similar to that of the control (Thomas et al. 1999, Boari and Vurro 2004, Müller-Stöver et al. 2009).

Many of the studies cited focused on the genus *Fusarium* and more particularly on the species *F. oxysporum*, while apparently there is no specificity of the *Fusarium* parasitic Orobanchaceae interaction except in very particular cases but this depends on the strain more than on the species. One *F. oxysporum* strain has been described as *P. ramosa* specific (Müller-Stöver et al. 2009). Two other *Fusarium* strains have been found pathogenic on *O. cernua*, *P. aegyptiaca* and *P. ramosa*, but not on *O. cumana* (Amsellem et al. 2001b). A *F. oxysporum* strain isolated from symptomatic broomrape and described as a *forma specialis* called f. sp. *orthoceras* was pathogenic on *O. cumana* and *O. cernua* but not on *P. aegyptica* (Thomas et al. 1999). On the other hand, strains of *F. oxysporum* f. sp. *strigae* have been characterized for their host specificity against *Striga* species (Elzein et al. 2008), and a strain of *F. oxysporum* f. sp. *strigae* was selected for the successful implementation of *Striga* biocontrol in the field in Kenya (Nzioki et al. 2016). However, it can be hypothesized that fungal strains, pathogens of broomrape and more efficient than those described within the species *F. oxysporum*, may exist in other taxonomic groups.

To test this hypothesis, we analysed the taxonomic and functional diversity of fungal isolates taken from symptomatic *P. ramosa* collected in all tobacco-producing regions in France and infested with *P. ramosa*. Indeed, *P. ramosa* constitutes a real threat for the tobacco producers who must definitively give up the culture of this plant in fields too heavily infested by the parasitic plant. Our objective was to identify one or more fungal isolates whose potential could be explored for use in *P. ramosa* biocontrol. For this purpose, the fungal isolates were characterized using morphological and molecular identification tools and tested for (i) their ability to inhibit seed germination of *P. ramosa*, (ii) their necrotic activity on broomrape stems and (iii) their harmlessness to the host plants. The ideal candidate will have to have the double competence.

Materials and methods

Sampling of symptomatic *P. ramosa* plants

An extensive survey was carried out from July to October 2017 in geographical areas with high occurrence of *P. ramosa* in tobacco (*Nicotiana tabacum* L.) fields in thirteen French departments forming a circular arc spanning from the southwest to the northeast of France. (Table 1). Symptomatic *P. ramosa* plants presenting necrosis or wilting were recovered from 23 tobacco fields in these areas. Four to 13 symptomatic plants per field were collected. This campaign resulted in the collection of 148 symptomatic *P. ramosa* plants.

Phelipanche ramosa seed material

Seeds of two populations of *P. ramosa* were used to test germination inhibition by fungal isolates: one from *P. ramosa* parasitizing tobacco and one from *P. ramosa* parasitizing oilseed rape (*Brassica napus* L.). Both were collected from arable fields located in western France, in agricultural regions characterized by the highest levels of infestation for tobacco (T population) at Aigre (45°53′38.908″N, 0°0′36.803″E) in 2017 or for oilseed rape (R population) at Saint-Jean-d'Angély (45°56′41.363″N, 0°31′3.947″W) in 2012. The populations were well differentiated genetically. Indeed, the genotyping of a hundred broomrape populations of various origins using microsatellite markers has identified three genetic groups (genetic groups 1, 2a and 2b) associated with distinct pathovars (Stojanova et al. 2019). More recently, a new genetic group has been identified (genetic group x) with which populations able to infest tobacco and tomato are associated (Le Corre et al. personal communication). Thereby, the T population belonged to this 'genetic group x'. and the R population belonged to 'genetic group 1' comprising populations able to infest various crops, notably winter oilseed rape (Stojanova et al. 2019). The seeds of tobacco used in the pathogenicity tests were provided by L. Gatard (Coopérative Tabac Feuilles de France, Strasbourg, France). Once the seeds were collected, they were all kept in watertight glass containers at ~20°C until the start of experiments.

Isolation and morphological identification of fungi

For each *P. ramosa* sample, one to five 1-cm fragments were excised from symptomatic areas. Plant fragments were disinfected in 70% ethanol for 30 s, rinsed three times in sterile distilled water for 30 s, dried on sterilized absorbent paper, and plated on malt extract agar (MEA: 10 g L^{-1} malt, 15 g L^{-1} agar) supplemented with antibiotics (100 mg L^{-1} streptomycin and 50 mg L^{-1} chlortetracycline) and 250 mg L^{-1} citric acid. After 3–4 days of incubation at 25°C, the fungal colonies developing from the plant material were transferred onto potato dextrose agar (PDA, 39 g L^{-1}) for a first microscopy observation. The fungi that did not form spores were purified by transferring a single hyphal tip onto a new plate containing MEA amended with the same antibiotics as above.

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Table 1. Endophytic fungal isolates collected from symptomatic *P. ramosa*.

Fungi	MIAE accession number[a]	Isolate (original code)	Geographic origin[b]	Sampling date	Molecular marker(s)[c]	GenBank accession number(s)	Germination rate of *P. ramosa* seeds (CI)[d]
Alternaria infectoria	MIAE02036	85M	Aigre (16)	17/07/17	ITS	OL840545	0.68 (0.62–0.73)
Alternaria sp.	MIAE02852	221M1	Griesheim sur Souffel (67)	23/08/17	ITS	OL840546	0.44 (0.38–0.5)
	MIAE02858	242M	Westhouse (67)	24/08/17	ITS	OL840547	0.56 (0.5–0.62)
	MIAE02880	435H5	Isse (51)	31/08/17	ITS	OL840548	0.77 (0.71–0.82)
	MIAE02888**	562H2	Chabournay (86)	10/10/17	ITS	OL840549	0.12 (0.08–0.16)
	MIAE02894	566M1	Chabournay (86)	10/10/17	ITS	OL840550	0.61 (0.54–0.68)
Botrytis sp.	MIAE02831	185M2	Taizé-Aizie (16)	23/08/17	ITS	OL840551	0.93 (0.89–0.96)
	MIAE02837	198H1	Messé (79)	23/08/17	ITS	OL840552	0.65 (0.59–0.7)
	MIAE02863	257M2	Stutzheim (67)	25/08/17	ITS	OL840553	0.88 (0.84–0.91)
	MIAE02869	280H2	Ayet (47)	28/08/17	ITS	OL840554	0.76 (0.71–0.81)
	MIAE02892	565M	Chabournay (86)	10/10/17	ITS	OL840555	0.76 (0.7–0.81)
Cladosporium sp.	MIAE02868	278B3	Ayet (47)	28/08/17	ITS	OL840556	0.86 (0.81–0.9)
	MIAE02878	434H	Isse (51)	31/08/17	ITS	OL840557	0.92 (0.88–0.95)
	MIAE02883	444M	Hindisheim (67)	06/09/17	ITS	OL840558	0.85 (0.8–0.89)
	MIAE02896	566H	Chabournay (86)	10/10/17	ITS	OL840559	0.74 (0.67–0.81)
Epicoccum nigrum	MIAE02818	88M1	Aigre (16)	17/07/17	ITS	OL840560	0.77 (0.72–0.82)
	MIAE02851	219H3	Griesheim sur Souffel (67)	23/08/17	ITS	OL840561	0.90 (0.86–0.93)
	MIAE02885	461H1	Pfettisheim (67)	12/09/17	ITS	OL840562	0.32 (0.26–0.38)
	MIAE02886	470M3	Griesheim sur Souffel (67)	13/09/17	ITS	OL840563	0.32 (0.26–0.38)
	MIAE02891	564H3	Chabournay (86)	10/10/17	ITS	OL840564	0.91 (0.87–0.95)
Fusarium avenaceum	MIAE02853**	221H3	Griesheim sur Souffel (67)	23/08/17	ITS, *RPB1*	OL840565, OL828292	001 (0–0.03)
F. brachygibbosum	MIAE02827**	175M1	Aussac-Vadalle-Ravaud (16)	18/08/17	ITS, *TEF1*, *RPB1*	OL840566, OL828295, OL828293	0.04 (0.02–0.07)
F. cerealis	MIAE02848	215H1	Griesheim sur Souffel (67)	23/08/17	*TEF1*	OL828296	0.89 (0.84–0.92)
F. culmorum	MIAE02840	200M1	Messé (79)	23/08/17	*TEF1*	OL828297	0.55 (0.49–0.61)
	MIAE02890**	563M2	Chabournay (86)	10/10/17	*TEF1*	OL828298	0.08 (0.04–0.13)
F. equiseti	MIAE02847*	215M	Griesheim sur Souffel (67)	23/08/17	*TEF1*	OL828299	0.22 (0.18–0.27)
F. incarnatum	MIAE02845	210H1	Schnersheim (67)	21/08/17	*TEF1*	OL828300	0.68 (0.63–0.73)
F. incarnatum–equiseti species complex	MIAE02037	57M2	Blaison-Gohier (49)	11/07/17	ITS	OL840567	0.62 (0.56–0.67)
	MIAE02053	145B2	Stutzheim (67)	18/07/17	ITS	OL840568	0.64 (0.59–0.7)
	MIAE02833	188M	Taizé-Aizie (16)	23/08/17	ITS	OL840569	0.73 (0.69–0.78)
	MIAE02834	195B	Messé (79)	23/08/17	ITS	OL840570	0.80 (0.75–0.84)
	MIAE02835	197B	Messé (79)	23/08/17	ITS	OL840571	0.95 (0.92–0.98)
	MIAE02838	198H2	Messé (79)	23/08/17	ITS	OL840572	0.58 (0.53–0.64)
	MIAE02850	216H2	Griesheim sur Souffel (67)	23/08/17	ITS	OL840573	0.32 (0.27–0.38)
	MIAE02855	233H1	Nordhouse (67)	24/08/17	ITS	OL840574	0.68 (0.63–0.73)
	MIAE02887	562H1	Chabournay (86)	10/10/17	ITS	OL840575	0.32 (0.26–0.38)
F. graminearum	MIAE02054	145B3	Stutzheim (67)	18/07/17	*TEF1*	OL828301	0.54 (0.48–0.61)
	MIAE02829	183B1	Taizé-Aizie (16)	23/08/17	*TEF1*	OL828302	0.29 (0.22–0.35)
	MIAE02867	277M2	Ayet (47)	28/08/17	*TEF1*	OL828303	0.91 (0.87–0.94)
F. oxysporum	MIAE02019	87B2	Aigre (16)	17/07/17	*TEF1*	OL828304	0.74 (0.69–0.79)
	MIAE02028**	53B	Blaison-Gohier (49)	11/07/17	*TEF1*	OL828305	0.16 (0.12–0.2)
	MIAE02031	81B2	Pliboux (79)	12/07/17	*TEF1*	OL828306	0.76 (0.71–0.81)
	MIAE02067	160M1	Pliboux (79)	25/07/17	*TEF1*	OL828307	0.35 (0.3–0.4)
	MIAE02856	234H1	Nordhouse (67)	24/08/17	*TEF1*	OL828308	0.41 (0.35–0.47)
	MIAE02862	256B1	Stutzheim (67)	25/08/17	*TEF1*	OL828309	0.26 (0.21–0.31)
	MIAE02865	276M1	Ayet (47)	28/08/17	*TEF1*	OL828310	0.51 (0.45–0.58)
	MIAE02870	306M2	Mazeuil (86)	28/08/17	*TEF1*	OL828311	0.27 (0.22–0.33)

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Table 1. Continued

Fungi	MIAE accession number[a]	Isolate (original code)	Geographic origin[b]	Sampling date	Molecular marker(s)[c]	GenBank accession number(s)	Germination rate of *P. ramosa* seeds (CI)[d]
F. redolens	MIAE02046	141M1	Stutzheim (67)	18/07/17	*TEF1*	OL828312	0.90 (0.87–0.93)
	MIAE02823	172B3	Aussac-Vadalle-Ravaud (16)	18/08/17	*TEF1*	OL828313	0.69 (0.63–0.75)
F. sambucinum	MIAE02864	266H1	Limersheim (67)	24/08/17	*TEF1*	OL828314	0.35 (0.29–0.4)
	MIAE02876**	433H	Isse (51)	31/08/17	*TEF1*	OL828315	0.00 (0–0.01)
F. solani	MIAE01983	52B	Blaison-Gohier (49)	11/07/17	*TEF1*	OL828316	0.46 (0.4–0.53)
	MIAE02042	144B2	Stutzheim (67)	18/07/17	*TEF1*	OL828317	0.30 (0.25–0.35)
	MIAE02106	148B2	Linazay (86)	25/07/17	*TEF1*	OL828318	0.93 (0.89–0.95)
	MIAE02854	231B4	Nordhouse (67)	24/08/17	*TEF1*	OL828319	0.87 (0.82–0.91)
	MIAE02866	276M3	Ayet (47)	28/08/17	*TEF1*	OL828320	0.71 (0.65–0.76)
	MIAE02871	307H	Mazeuil (86)	28/08/17	*TEF1*	OL828321	0.66 (0.61–0.72)
	MIAE02872	310H1	Mazeuil (86)	28/08/17	*TEF1*	OL828322	0.65 (0.59–0.7)
	MIAE02874	432H1	Isse (51)	31/08/17	*TEF1*	OL828323	0.71 (0.64–0.77)
	MIAE02024*	66M1	Pliboux (79)	12/07/17	*TEF1*	OL828324	0.15 (0.11–0.19)
	MIAE02062*	163B	Pliboux (79)	25/07/17	*TEF1*	OL828325	0.20 (0.16–0.25)
	MIAE02826*	174H1	Aussac-Vadalle-Ravaud (16)	18/08/17	*TEF1*	OL828326	0.20 (0.16–0.25)
F. sporotrichioides	MIAE02832**	186H	Taizé-Aizie (16)	23/08/17	*TEF1*	OL828327	0.00 (0–0.02)
	MIAE02839**	199M2	Messé (79)	23/08/17	*TEF1*	OL828328	0.00 (0–0.01)
	MIAE02846**	211H	Schnersheim (67)	21/08/17	*TEF1*	OL828329	0.00 (0–0.01)
	MIAE02859**	254B2	Stutzheim (67)	25/08/17	*TEF1*	OL828330	0.00 (0–0.01)
	MIAE02893**	565B3	Chabournay (86)	10/10/17	*TEF1*, *RPB1*	OL828331, OL828294	0.00 (0–0.02)
F. tricinctum	MIAE02895	566M2	Chabournay (86)	10/10/17	*TEF1*	OL828332	0.91 (0.86–0.94)
F. venenatum	MIAE02825**	174M2	Aussac-Vadalle-Ravaud (16)	18/08/17	*TEF1*	OL828333	0.02 (0.01–0.04)
	MIAE02836**	198B3	Messé (79)	23/08/17	ITS, *TEF1*	OL840576, OL828334	0.00 (0–0.01)
	MIAE02849**	216M3	Griesheim sur Souffel (67)	23/08/17	*TEF1*	OL828335	0.00 (0–0.01)
	MIAE02879*	435H3	Isse (51)	31/08/17	*TEF1*	OL828336	0.00 (0–001)
F. verticillioides	MIAE02843	205H3	Schnersheim (67)	21/08/17	*TEF1*	OL828337	0.83 (0.79–0.87)
Penicillium sp.	MIAE02841	202H1	Schnersheim (67)	21/08/17	ITS	OL840577	0.77 (0.73–0.81)
	MIAE02844	208M2	Schnersheim (67)	21/08/17	ITS	OL840578	0.94 (0.91–0.97)
Phomopsis sp.	MIAE02819	88M2	Aigre (16)	17/07/17	ITS	OL840579	0.88 (0.84–0.92)
Pithomyces chartarum	MIAE02889	562H3	Chabournay (86)	10/10/17	ITS	OL840580	0.32 (0.26–0.39)
Plectosphaerella ramiseptata	MIAE02875**	433M2	Isse (51)	31/08/17	ITS	OL840581	0.08 (0.05–0.13)
Plectosphaerellaceae sp.	MIAE02877	434M	Isse (51)	31/08/17	ITS	OL840582	0.79 (0.73–0.84)
	MIAE02882	437H	Isse (51)	31/08/17	ITS	OL840583	0.30 (0.24–0.35)
Pleosporineae sp.	MIAE02861	255M2	Stutzheim (67)	25/08/17	ITS	OL840584	0.51 (0.45–0.57)
Rhizoctonia solani	MIAE02881	436M	Isse (51)	31/08/17	ITS	OL840585	0.87 (0.83–0.91)
	MIAE07525	149M	Linazay (86)	25/07/17	ITS	OL840586	0.90 (0.87–0.93)
Rhizoctonia sp. AG-A	MIAE02817	67M2	Pliboux (79)	12/07/17	ITS	OL840587	0.92 (0.88–0.95)
Sarocladium strictum	MIAE02873	321B1	Mussig (67)	28/08/17	ITS	OL840588	0.54 (0.47–0.61)
Sclerotinia sp.	MIAE02830	185B2	Taizé-Aizie (16)	23/08/17	ITS	OL840589	0.93 (0.89–0.95)
Stereum sp.	MIAE02824	172M1	Aussac-Vadalle-Ravaud (16)	18/08/17	ITS	OL840590	0.84 (0.8–0.88)

[a] Microorganisms of Interest for Agriculture and Environment (MIAE) collection, INRAE Dijon, France. The 20 isolates most efficient in inhibiting seed germination of *P. ramosa* are marked with one asterisk. The 15 isolates also tested for their necrotic activity on *P. ramosa* stems are marked with two asterisks.
[b] Numbers in brackets indicate French departments, located in eastern (51, 67) or western France (16, 47, 49, 79, 86).
[c] ITS, internal transcribed spacer; *TEF1*, translation elongation factor 1-α; *RPB1*, DNA-directed RNA polymerase II largest subunit.
[d] 95% confidence interval.

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Spore-forming fungi were purified by single-spore isolation. For this purpose, sterile water was added to the plates, and the fungal cultures were scraped with a sterile cone tip to suspend the propagules. The propagule suspensions were filtered through sintered glass filters (100 μm porosity) to obtain spore suspensions. Serial dilutions of the spore suspensions were plated on MEA amended with 250 mg L^{-1} citric acid and 3 g L^{-1} Triton X100 (Merck, Darmstadt, Germany). After a few days of incubation at 25°C, a randomly selected colony grown from a single spore was transferred onto PDA. A total of 374 fungal isolates were recovered (Table S1, Supporting Information) and stored on PDA at room temperature and by cryopreservation. To this end, isolates were cultured on PDA, and spore suspensions were prepared by adding 750 μL of potato dextrose broth (24 g L^{-1}) and mixing. Around 500 μL of the suspension were added to 600 μL of one-fourth glycerol in a cryotube. Isolates were stored at −80°C in the 'Microorganisms of Interest for Agriculture and Environment' (MIAE, INRAE Dijon, France) collection. Fungal isolates were first identified from morphological characters using a light microscope, according to illustrated identification keys (Nelson et al. 1983, Barnett and Hunter 1998). The flow chart of the procedure used for the screening of potential mycoherbicides is described in Fig. 1.

Molecular identification of the fungal isolates

A subcollection of 87 isolates representative of the morphological diversity and geographical origin of the whole collection was selected (Table 1). This selection ruled out the risk of duplicates in the collection because several fragments per plant sample were used for fungal isolation. The subcollection was further characterized by sequencing the internal transcribed spacer (ITS) region and/or part of the translation elongation factor 1-α (*TEF1*) gene, and/or part of the DNA-directed RNA polymerase II largest subunit (*RPB1)* gene. The ITS region was used to identify undetermined fungi and fungi supposed to belong to genera other than *Fusarium*. The fungal isolates identified as *Fusarium* spp. by microscopy observations were further identified by *TEF1* or *RPB1* sequencing (O'Donnell et al. 2015). Several markers were sequenced for a few *Fusarium* isolates (Table 1).

Fungal DNA was extracted from PDA cultures using a rapid mini-preparation procedure (Edel et al. 2001). The ITS region was amplified by PCR using primers ITS1-F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) in a final volume of 25 μL containing 1 μL of DNA, 0.3 μM of each primer, 150 μM dNTP, 1 U of *Taq* DNA polymerase (MP Biomedicals, Illkirch, France) and polymerase chain reaction (PCR) buffer. Amplifications were conducted in a Mastercycler (Eppendorf) with an initial denaturation step of 3 min at 94°C, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 50°C, 1 min extension at 72°C and a final extension step of 10 min at 72°C. The presence of PCR products was confirmed by gel electrophoresis. The PCR products were sequenced using the two PCR primers as sequencing primers. For each PCR product, sequences from the two strands were assembled using SEQMAN 6.0 (DNASTAR Lasergene, GATC Biotech). Sequence identities were determined by BLAST analysis against the *Fusarium* MLST database (O'Donnell et al. 2015) or the National Center for Biotechnology Information (NCBI) available online.

Part of the *TEF1* gene was amplified by PCR using primers EF1 and EF2 (O'Donnell et al. 1998a) in a final volume of 25 μL containing 1 μL of DNA, 0.1 μM of each primer, 150 μM dNTP, 1 U of *Taq* DNA polymerase and PCR reaction buffer. Amplifications were conducted with an initial denaturation step of 7 min at 95°C, followed by 38 cycles of 1 min denaturation at 95°C, 1 min 15 s annealing at 57°C, 1 min extension at 72°C and a final extension step of 10 min at 72°C. The PCR products were sequenced and analysed as above.

Part of the *RPBI* gene was amplified by PCR using primers Fa and G2R (O'Donnell et al. 2010) in a final volume of 50 μL containing 1 μL of DNA, 0.2 μM of each primer, 100 μM dNTP, 2 U of *Taq* DNA polymerase and PCR reaction buffer. Amplifications were conducted with an initial denaturation step of 3 min at 95°C, followed by 35 cycles of 1 min denaturation at 95°C, 1 min annealing at 50°C, 1 min 30 s extension at 72°C and a final extension step of 5 min at 72°C. The PCR products were sequenced using primers Fa, F6, F7, F8, R8 and G2R (O'Donnell et al. 2010). Sequences were assembled and identified as above. All the sequences were deposited in GenBank (Table 1).

Effects of the fungal isolates on *in vitro* germination of seeds of T and R populations of *P. ramosa*

The procedure used to test *P. ramosa* seed germination was adapted from Müller-Stöver et al. (2009) with slight modifications. Seeds of *P. ramosa* were conditioned to make them susceptible to germination stimulants as follows. They were surface-disinfected under a laminar flow hood by a 5-min immersion in ethanol 70%, followed by a 5-min immersion in a solution of $Ca(OCl)_2$ 3% (p/v) and Tween 20 (0.1%) to limit fungal spread (Gibot-Leclerc et al. 2012). Then, they were rinsed five times with sterile double-distilled water. After disinfection, 30–40 seeds of *P. ramosa* were placed on 1 cm × 1 cm paper squares (Whatman®, Glass microfiber filters GF/A) using a pipette. Four paper squares were placed on a Whatman® GF/A paper sheet (Ø 90 mm) at the bottom of a plate (Ø 90 mm) and then hydrated with 3 mL of sterile distilled water. The plates were sealed with Parafilm® (American Can Company), wrapped in aluminium foil and placed in a growth chamber at 20°C in the dark for at least 14 days.

Fungal isolates were cultivated on PDA for 7 days. Five mL of minimal medium (MM) were added to the plates. The minimal medium was composed of glucose 5 g L^{-1}, $NaNO_3$ 2 g L^{-1}, KH_2PO_4 1 g L^{-1}, $MgSO_4$-$7H_2O$ 0.5 g L^{-1}, KCl 0.5 g L^{-1} and trace element solution 2 mL. The trace element solution contained citric acid 5 g L^{-1}, $ZnSO_4$-$7H_2O$ 5 g L^{-1}, $FeSO_4$-$7H_2O$ 4.75 g L^{-1}, Fe $(NH_4)_2$-$(SO_4)_2$–$6H_2O$ 1 g L^{-1}, $CuSO_4$-$5H_2O$ 0.25 g L^{-1}, $MnSO_4$-H_2O 50 mg L^{-1}, H_3BO_4 50 mg L^{-1} and $NaMoO_4$-$2H_2O$ 50 mg L^{-1}. The surface of the culture was scraped with a sterile cone tip to suspend the fungal propagules, and the fungal suspension was mixed with 15 mL of MM.

After seed conditioning, each Whatman® paper square was transferred onto a Whatman® GF/A paper sheet (Ø 90 mm) at the bottom of a plate (Ø 90 mm) and moistened with 3 mL of fungal suspension. Four dishes each containing four paper squares were prepared per fungal isolate. A plate with MM instead of the fungal suspension was prepared as a control. The plates were sealed and wrapped as mentioned above and placed in a growth chamber at 20°C in the dark for 7 days. After incubation, the paper squares were transferred again onto a Whatman® GF/A paper sheet (Ø 90 mm) at the bottom of a plate and then moistened with the strigol analogue growth regulator GR24 (3 mL at 0.1 mg L^{-1}. This concentration—10 times lower than that conventionally used to certainly stimulate the germination of broomrape seeds—was chosen in order to assess either an inhibition or a stimulation of germination by fungi (Gibot-Leclerc et al. 2004). Two plates were prepared per fungal isolate, and two with sterile distilled water (3 mL). The synthetic stimulant GR24 was chosen rather than host root exudates because it is more stable, whereas the

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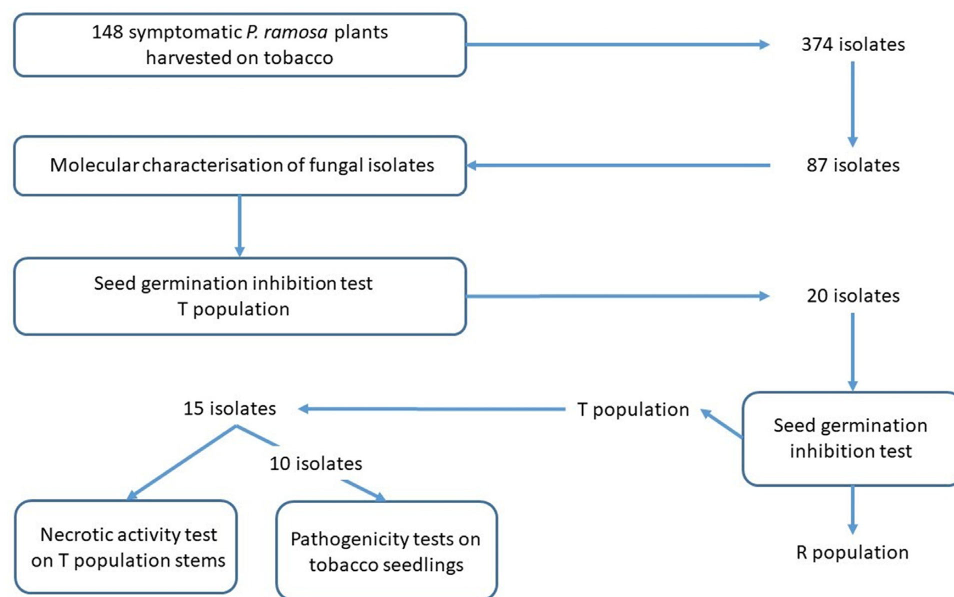

Figure 1. Flow chart of the procedure leading to the selection of promising candidates for the biocontrol of *P. ramosa*.

stimulatory activity of root exudates varies among species and may be affected by environmental conditions such as light (Yoneyama et al. 2013). All plates were sealed with Parafilm®, wrapped in aluminium foil and placed at 20°C in darkness. The number of germinated seeds was counted after 7 days of GR24 stimulation using a stereo microscope (1.95–250×). A seed was considered to have germinated when the radicle had pierced through the seed coat.

The 87 fungal isolates were tested for their ability to inhibit seed germination of *P. ramosa* taken from tobacco (T population) in successive experiments. Each experiment included seven isolates, one negative control (i.e. MM fungus-free) and one isolate previously described as pathogenic to *P. ramosa* (*F. oxysporum* FT2, MIAE02108; Boari and Vurro 2004). The experiment was replicated three times with the 20 isolates most effective in inhibiting seed germination. These 20 isolates were also tested three times for their capacity to inhibit seed germination of *P. ramosa* taken from oilseed rape (R population) using the same protocol as above.

Determination of *P. ramosa* seed viability

The viability of the seeds from both populations that did not germinate was assessed by adding 3 mL of 2,3,5-triphenyl tetrazolium chloride (TTC, Sigma) at 1% in each plate. The plates were wrapped in aluminium foil and placed in a drying oven at 40°C for 48 h. Then, the seeds were placed on a new Whatman® GF/A paper sheet (Ø 90 mm) imbibed with saline solution (NaOCl 1%). Viability was assessed after 20 min. Viable and non-viable seeds were counted under a stereo microscope (1.95–250×). The seeds with an embryo exhibiting no carmine red staining were scored as non-viable (Gibot-Leclerc et al. 2004).

Evaluation of the necrotic activity of the fungal isolates on the stems of the T population of *P. ramosa*

For practical reasons, i.e. the number of *P. ramosa* stems available, 15 isolates were taken at random out of the 20 isolates previously selected for their ability to strongly inhibit *P. ramosa* seed germination, and tested for their capacity to induce necrosis on *P. ramosa* stems. The stems used were produced from tobacco cultivated in pots in the greenhouse, in a soil previously autoclaved and mixed with *P. ramosa* seeds (T population), as previously described (Gibot-Leclerc et al. 2013). *Phelipanche ramosa* stems emerging by ∼60–120 mm were cut and wrapped in previously moistened blotting paper to preserve their turgor. Each stem was surface-disinfected by rapid bathing (10 s) in NaOCl 3%, rinsed in three successive baths of sterile distilled water, dried on sterile absorbent paper and deposited on a sheet of absorbent paper placed on a plastic rack inside a crystal box containing sterile water. The ends of the absorbent paper were immersed in water so that the paper was constantly soaked. Seventeen *P. ramosa* stems were placed in each crystal box, on the paper, perpendicular to a line previously drawn on the paper. For each isolate, a drop of fungal suspension prepared as described above was deposited on each of 14 *P. ramosa* stems at the crossing point with the line, while a drop of sterile water was deposited on the three remaining stems. A 20-mm-long plastic standard was also placed in each box between the inoculated stems and the controls to serve as a reference scale when taking photographs. Fifteen crystal boxes, were prepared—one per fungal isolate tested. The boxes were incubated in the dark at 20°C and checked for the appearance and evolution of symptoms every 24 h for 3 days. Notations were made in axenic conditions, by removing the lid of the crystal box to take a photograph of all 17 stems without touching them so as not to disturb the *P. ramosa*–fungus interaction. The lengths of the necrotic spots were measured and averaged for the 14 *P. ramosa* stems per isolate.

Determination of the pathogenicity of the selected isolates to tobacco

Pathogenicity tests were performed to ensure that the isolates selected for their pathogenic activity towards *P. ramosa* were not pathogenic to tobacco.

Tobacco seeds of the ITB683 variety were placed in a small tube and surface-disinfected by soaking in ethanol 70% for 2 min, and then in a $Ca(ClO)_2$ 3% solution for 3 min. The seeds were rinsed five times with sterile osmotic water. At each of these steps, the tube containing the seeds was shaken manually to ensure the dis-

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infection of the entire seed surface. Then, the seeds were soaked in sterile osmotic water for 2 h to facilitate their germination, quickly transferred onto sterile absorbent paper and placed in a Petri dish on MEA medium supplemented with antibiotics and citric acid, as previously described. Each Petri dish was sealed with Parafilm®, wrapped in aluminium foil and incubated at 25°C for 5 days.

At the end of incubation, the germinated tobacco seeds were gently transferred to square Petri dishes (120 mm × 120 mm × 15 mm) on Murashige and Skoog basal medium (M5519; Sigma) at the rate of five germinated seeds per dish. They were distributed along a virtual line located 5 cm from the bottom of the Petri dish. The Petri dishes were sealed with Parafilm®, and their lower part was wrapped in aluminium foil to keep the seedling roots in the dark. The Petri dishes were positioned on a rack in an oblique vertical position, and incubated in a climatic chamber at 25°C during the day (16 h) and 20°C at night (8 h) for 10 days.

The fungal isolates were grown in Petri dishes on PDA medium for 7 days. For each isolate, 1 mL of liquid malt extract medium was dropped on the surface of the colony, which was scraped. The suspension of propagules thus produced was transferred to an Erlenmeyer flask containing 100 mL of liquid malt extract medium. The flask was incubated at 25°C and 125 rpm. After 5 days of incubation, the cultures were filtered to remove the mycelial mat, centrifuged at 6000 × *g* for 10 min, and each spore pellet was suspended in sterile osmotic water, centrifuged again, resuspended in osmotic water and quantified by counting the spores using a Malassez counting chamber. For each isolate, the concentration of the suspension was adjusted to 10^5 spores mL^{-1}.

Three Petri dishes (15 seedlings) were used for each fungal isolate. Ten microlitres of the suspension were added to the crown of each of the 15 tobacco seedlings two successive times and slowly flowed down the root system. The Petri dishes were positioned horizontally for 30 min to maintain the inoculum near the roots, and then they were put back on the rack in an oblique vertical position in the climatic chamber under the same conditions as before. Sterile osmotic water was used as a negative pathogenicity control on 15 seedlings. A strain of *Phytophthora parasitica* (Pp329, Sophia-Antipolis collection, INRAE, France) grown like the isolates but neither filtered or centrifuged was used as a positive pathogenicity control. After 7 and 12 days of incubation, the pathogenicity of the isolates was estimated by assigning a score to each seedling according to the following scale: [0] healthy roots; [1] less growth than the control but no apparent necrosis; [2] minor necrosis <25% of the root surface; [3] true necrosis between 25% and 50% of the root surface; [4] necrosis between 50% and 75% of the root surface; and [5] dead seedling. An average value per isolate was calculated.

All the measurements were carried out with more than three repetitions per isolate and all these measurements were duplicated two or three times as specified for each of them. They are all provided with a mean value and a 95% confidence interval allowing a classification to be established between the isolates.

Results

Identification of fungi associated with symptomatic *P. ramosa*

A total of 374 fungal isolates were collected from the 148 symptomatic *P. ramosa* plants harvested on tobacco (Table S1, Supporting Information). They represented 14 different genera and potentially more, since two isolates were only identified at the family level (*Plectosphaerellaceae*) and one at the suborder level (*Pleosporineae*). The most represented genus was *Fusarium* (78.9% of the isolates), followed by *Alternaria* (6.7%), *Epicoccum* (3.2%), *Cladosporium* (2.9%) and *Rhizoctonia* (2.9%) (Fig. 2). The group of species *F. oxysporum/F. redolens* represented more than one-third of the 295 *Fusarium* isolates. These isolates were first identified morphologically to be *F. oxysporum*, but sequencing of part of the *TEF1* gene of ten of them revealed that eight were actually *F. oxysporum* whereas the other two were *F. redolens* (Table 1). Consequently, the 93 isolates for which no sequencing was performed were designated as *F. oxysporum/F. redolens*. The other most abundant *Fusarium* species or group of species were *F. solani* (31.5%) and the *F. incarnatum–equiseti* species complex (FIESC) (7.5%) (Fig. 2). Finally, 10 other *Fusarium* species were collected from symptomatic *P. ramosa* plants, and each of them was represented by one to five isolates (Fig. 2).

Inhibition of seed germination of the T population of *P. ramosa* by fungal isolates

Eighty-seven isolates representative of the diversity and the geographical origin of the collection were tested for their ability to inhibit seed germination of the T population of *P. ramosa* (Fig. 3, Table 1). The germination rates varied from 0 to almost 1 in the form of a continuum, depending on the fungal isolate. In the absence of GR 24 (water only), the germination rate was zero regardless of the isolate (data not shown). The germination rates of the control isolate remained stable throughout the replicates: in the absence of fungus (C-NoF), the seeds had an average germination rate of 0.91 ([0.83–0.95], [min–max]). The control isolate MIAE02108 inhibited seed germination of *P. ramosa* by ~68% (average germination rate of 0.32 [0.30–0.34]), and the results were relatively constant across replicates. Some fungal isolates showed lower germination inhibitory activity than the control isolate MIAE02108 and for a number of them the seed germination rate was close to that of the fungus-free control (C-NoF), while ~20 isolates strongly inhibited the germination of *P. ramosa* seeds. In addition, there was a diversity of actions within the same fungal taxon. For example, very different germination rates were observed in the presence of isolates MIAE02106 and MIAE02024 identified as *F. solani* (0.92 vs 0.15). On the other hand, similar germination rates were observed in the presence of isolates belonging to different fungal species like *Fusarium sporotrichioides* and *Fusarium venenatum*. Among the 87 studied isolates, 25 inhibited germination better than the control MIAE02108 did. The 20 most promising isolates inhibited germination by >75%. Apart from two taxa (*Alternaria* sp., *Plectosphaerella ramiseptata*), all others belonged to the genus *Fusarium* and to nine different *Fusarium* species, among which *F. sporotrichioides* (five isolates), *F. venenatum* (four isolates) and *F. solani* (three isolates) were the most abundant ones (Fig. 3). All *F. sporotrichioides* isolates and all but one *F. venenatum* isolate led to 100% of germination inhibition, while the fourth *F. venenatum* isolate led to 98% of germination inhibition (Table 1). Three isolates belonging to three other *Fusarium* species also inhibited seed germination of *P. ramosa*: isolate MIAE02876 of *F. sambucinum*, isolate MIAE02853 of *F. avenaceum* and isolate MIAE02827 of *F. brachygibbosum* inhibited germination by 100%, 99% and 96%, respectively. The single isolate representing the species *F. oxysporum* in this 'top 20' inhibited germination by 84%. For these 20 isolates strongly inhibiting seed germination, the TTC measurement of the viability of the inhibited seeds revealed a high variability in the lethal nature of this inhibition. Thus, the inhibitory activity of the four isolates of *F. sporotrichioides* only affected the viability of 25% (MIAE02893) to 50% (MIAE02839) of the inhibited seeds

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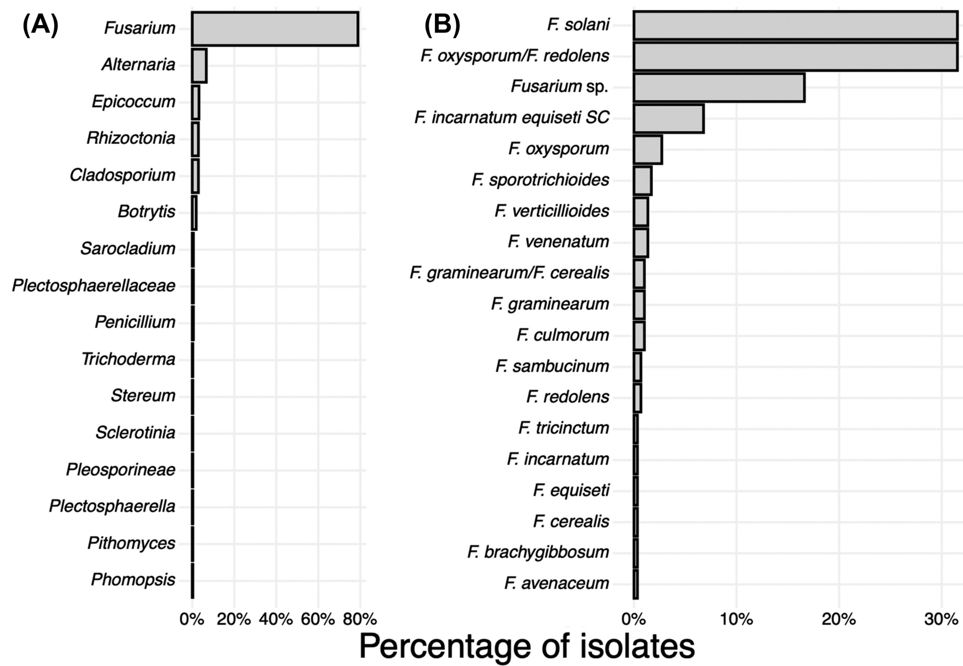

Figure 2. Percentage of fungal isolates per **(A)** genus or family for the whole collection; **(B)** species, pair of species or species complex (SC) for the *Fusarium* genus.

and that of the three isolates of *F. solani* was lethal for 63–74% of the inhibited seeds. The lethal nature of the inhibitory activity of *F. venenatum* also varied according to the isolates. MIAE02879, MIAE02849 and MIAE02825 affected viability of 67%, 70% and 78% of inhibited seeds respectively while MIAE02836 only affected viability of 45% of inhibited seeds. On the other hand, this activity was lethal for 97%, 91% and 88% of the seeds inhibited by *F. avenaceum* (MIAE02853), *F. sambucinum* (MIAE02876) and *F. brachygibbosum* (MIAE02827), respectively. The control MIAE02801 affected seed viability by 60–64%.

Stability of seed germination inhibition of the T and R populations of *P. ramosa* by fungal isolates

The >75% inhibitory effect of the 20 isolates on seed germination of the T population of *P. ramosa* was tested twice again, and was also tested in triplicate on the R population following the protocol described previously. A few isolates such as *Alternaria* sp. MIAE02888 and *F. solani* MIAE02024 showed heterogeneous results among the three replicates, but most of them yielded reproducible results (Fig. 4). These isolates, for which a greater variability was observed, were also those with the weakest germination inhibition activity, whatever the seed population. It is therefore possible that the signal molecules exchanged between these isolates and the seeds were less concentrated or less specific than those exchanged between strongly inhibitory isolates and seeds. The comparison of the results obtained on the T and R populations highlighted a significant difference in the rate of germination inhibition between the two pathovars, mainly in the case of isolates inhibiting germination by <95% (Fig. 4). Fungi isolated from symptomatic *P. ramosa* plants harvested from tobacco inhibited seed germination of the T population more than seed germination of the R population. These isolates would therefore have a specific action on the seeds of each of the two *P. ramosa* genotypes. On the other hand, among the 10 most promising isolates that exhibited a germination inhibition percentage of nearly 100% on T population seeds, 9 isolates also exhibited a germination inhibition percentage of nearly 100% on R population seeds; they belonged to the species *F. sporotrichioides*, *F. venenatum* and *F. sambucinum*.

Necrotic activity of the fungal isolates on stems of the T population of *P. ramosa*

The necrotic activity of the fungal isolates was tested on a set of 15 isolates randomly taken from those exhibiting the strongest germination inhibition of *P. ramosa* seeds among the 87 tested isolates (Fig. 3). This group included nine taxa, seven of which belonged to the genus *Fusarium*, one to the genus *Plectosphaerella* and one to the genus *Alternaria* (Table 1). As they were all isolated from surface-disinfected symptomatic plants, they were expected to be highly aggressive. Actually, some isolates really induced strong necrosis >50 mm long after 72 h of incubation, but others induced only very little necrosis <4 mm long (Table 1). The isolate with the highest necrotic activity belonged to the species *F. sporotrichioides* (Fig. 5). This species was also the most represented one within this set of isolates (5 isolates among 15). However, one of these five *F. sporotrichioides* isolates—isolate MIAE02846—had lower necrotic activity (25.9 mm) than the other four (>45 mm). Such intraspecific diversity was also true for the species *F. venenatum* that included four representatives among the fifteen isolates. One of them—isolate MIAE02825—had very little necrotic activity (3.6 mm), while the other two—MIAE02836 and MIAE02849—were quite aggressive and caused very similar necrosis (43.5 and 41.9 mm, respectively). With five and three representatives, respectively, these two species constituted more than half of all the tested isolates; the other seven isolates each belonged to a different species. Among them, *F. sambucinum* isolate MIAE02876 caused necrosis >45 mm long and thus ranked among the most aggressive isolates, like the *F. sporotrichioides* isolates (Fig. 6). In addition, *F. sporotrichioides* isolates exhibited abundant mycelium on the necrotic surface of the *P. ramosa* stems, while no mycelial development was visible on the necrosis caused by the equally aggres-

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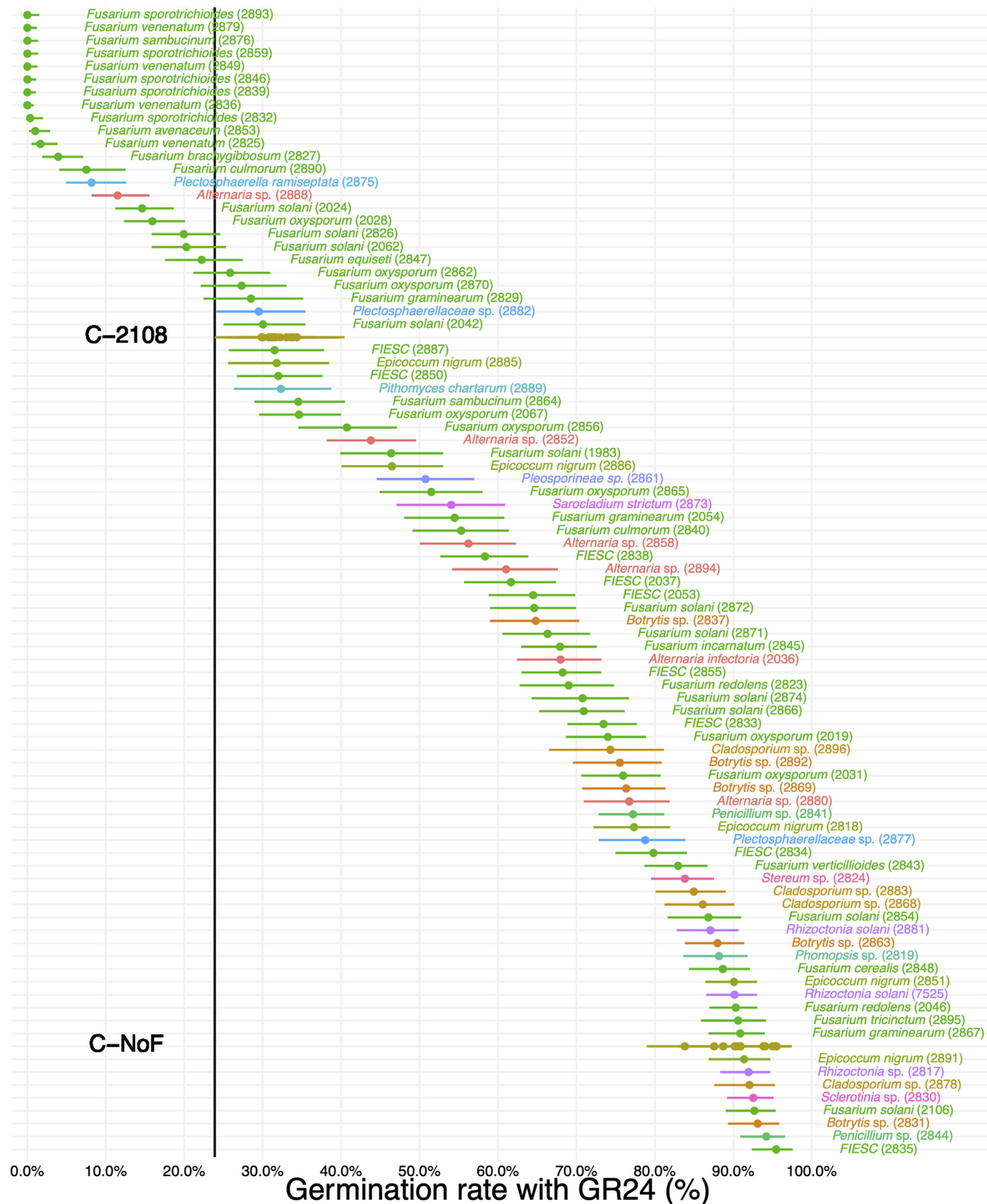

Figure 3. Inhibition of seed germination of the T population of *P. ramosa* by fungal isolates. Fungal genera or species complexes are differentiated by colours (FIESC, *Fusarium incarnatum–equiseti* species complex). The isolates are described in Table 1. C-NoF, negative control (fungus-free seeds); C-2028, positive control. MIAE numbers are indicated in parentheses. Error bars correspond to 95% confidence intervals.

sive isolate MIAE02876 of *F. sambucinum* (Fig. 6). Isolate MIAE02836 of *F. venenatum* behaved like the *F. sporotrichioides* isolates, while isolate MIAE02849 and the less aggressive isolate MIAE02825 of the same *F. venenatum* species behaved like the *F. sambucinum* isolate, and like all remaining isolates tested (necrosis <20 mm long) (Fig. 5). One isolate (MIAE02028) represented the species *F. oxysporum*; it ranked 13th, with relatively low necrotic activity: necrosis was 7.5 mm long. The eight isolates with the highest necrotic activity also inhibited seed germination of the two R and T populations by 100%, except isolate MIAE02839 that inhibited seed germination by 100% on the T population versus 90% on the R population.

Fungal pathogenicity to tobacco host plants

Ten isolates—MIAE02825, MIAE02827, MIAE02836, MIAE02846, MIAE02849, MIAE02853, MIAE02859, MIAE02876, MIAE02879 and MIAE02893—belonging to the species *F. avenaceum*, *F. brachygibbosum*, *F. sambucinum*, *F. sporotrichioides* and *F. venenatum* were tested for their pathogenicity to tobacco (Table 2). The negative control (sterile water) did not cause any symptom on the tobacco seedlings and scored 0 on the pathogenicity scale, while the positive control *P. parasitica* (Pp329) caused seedling death 12 days post-inoculation and scored 5 on the pathogenicity scale 12 days post-inoculation. No isolate scored zero 7 days post-inoculation.

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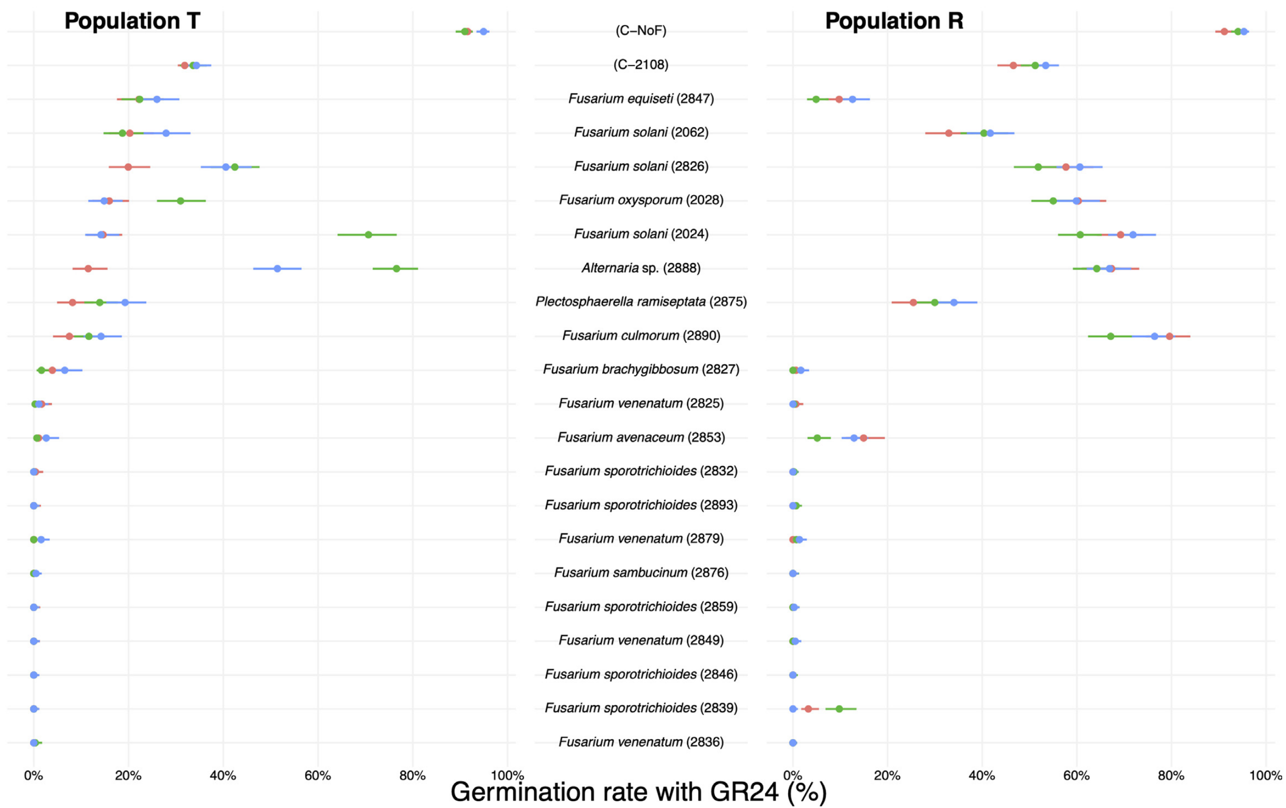

Figure 4. Germination inhibition of seeds of (left) the T population and (right) the R population of *P. ramosa* by fungal isolates. Twenty isolates with a germination inhibition percentage greater than 75% in the first test were tested three times on the two populations of *P. ramosa*. C-NoF, negative control (fungus-free seeds); C-2028, positive control. Colours correspond to the three technical replicates. MIAE numbers are indicated in parentheses. Error bars correspond to 95% confidence intervals.

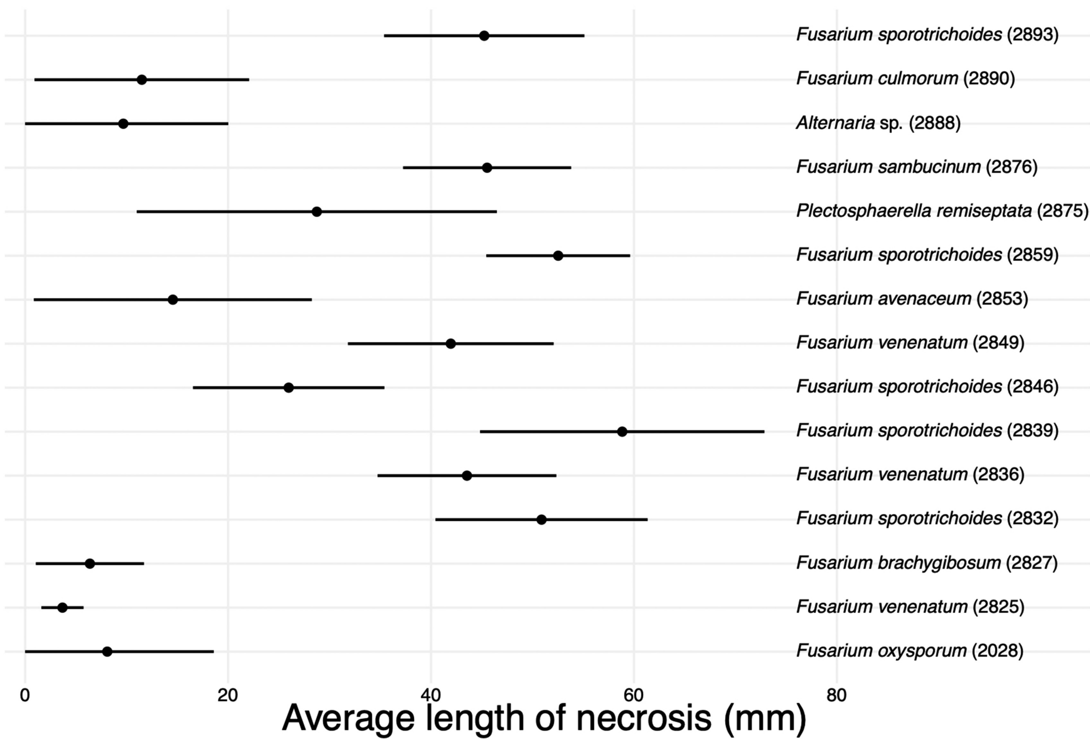

Figure 5. Necrotic activity estimated by the average length of the necrosis measured on 14 stems of the T population of *P. ramosa* 72 h post-inoculation for 15 of the 20 isolates with a germination inhibition percentage greater than 75%. The MIAE numbers of the isolates are indicated in parentheses. Error bars correspond to 95% confidence intervals.

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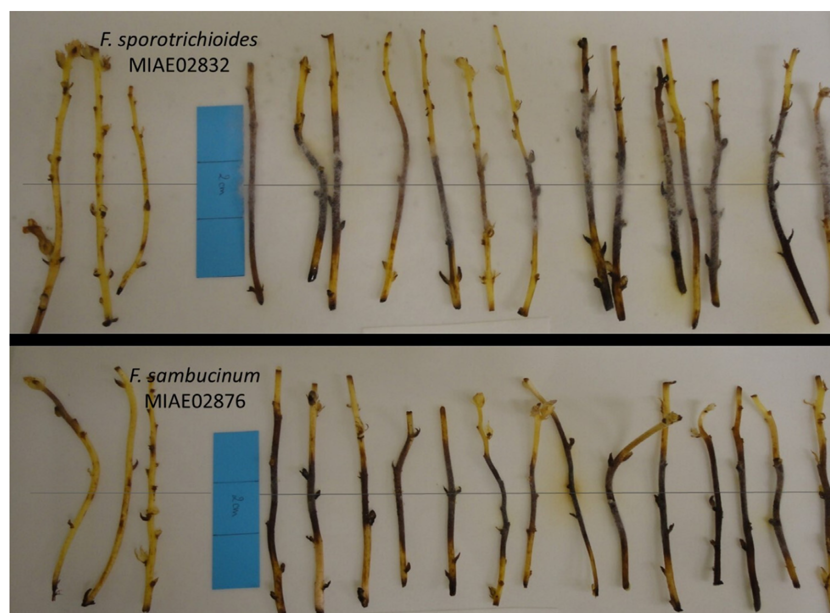

Figure 6. Necrotic activity of two aggressive isolates belonging to the species *F. sporotrichioides* (top) and *F. sambucinum* (bottom) 72 h post-inoculation. The 14 stems of the T population of *P. ramosa* on the right of the blue internal standard were inoculated with a drop of the fungal suspension of the tested isolate at the crossing point with the horizontal line, while the three *P. ramosa* stems on the left of the internal standard were the controls inoculated with a drop of sterile water. The necrotic activity of isolate MIAE02832 (*F. sporotrichioides*) was associated with the production of an aerial mycelium, but the necrotic activity of isolate MIAE02876 (*F. sambucinum*) was not. The dark spot observed on one of the control stems in the lower photograph is at the upper end of the stem and corresponds to a physiological disorder independent of inoculation. The blue tag on each of the photographs indicates the scale, namely 2 cm between the two horizontal lines.

Table 2. Means and standard deviations of the disease scores of fungal pathogenicity to tobacco plants.

		7 days post-inoculation		12 days post-inoculation	
Fungal species	**Isolate**	**Mean disease score[a]**	**Standard deviation**	**Mean disease score**	**Standard deviation**
Phytophthora parasitica	Pp329	2.13	1.30	5.00	0.00
Fusarium avenaceum	MIAE02853	1.00	0.00	2.40	0.51
F. brachygibbosum	MIAE02827	1.00	0.00	1.00	0.00
F. sambucinum	MIAE02876	0.53	1.41	0.53	1.41
F. sporotrichioides	MIAE02846	2.00	1.60	2.07	1.58
F. sporotrichioides	MIAE02859	1.33	0.82	1.33	0.82
F. sporotrichioides	MIAE02893	1.20	0.41	2.00	0.00
F. venenatum	MIAE02836	1.40	1.06	1.40	1.06
F. venenatum	MIAE02849	1.47	0.99	1.40	0.83
F. venenatum	MIAE02879	1.07	0.26	1.07	0.26
F. venenatum	MIAE02825	1.00	0.00	1.00	0.00
No fungus (negative control)		0.00	0.00	0.00	0.00

[a]The pathogenicity of the isolates was estimated by assigning a score to each seedling according to the following scale: [0] healthy roots; [1] less growth than the control but no apparent necrosis; [2] minor necrosis <25% of the root surface; [3] true necrosis between 25% and 50% of the root surface; [4] necrosis between 50% and 75% of the root surface; and [5] dead seedling. An average value per isolate was calculated.

They all affected seedling growth but without causing necrosis at this stage. The lowest score 7 days post-inoculation was 0.53 for isolate MIAE02876 (*F. sambucinum*), and the highest one was 2.00 for isolate MIAE02846 (*F. sporotrichioides*). The other isolates scored around 1. The scores of most isolates remained unchanged between 7 and 12 days post-inoculation or remained below 1.5, apart from isolates MIAE02893 (*F. sporotrichioides*), MIAE02846 (*F. sporotrichioides*) and MIAE02853 (*F. avenaceum*) that scored 2.00, 2.07 and 2.40, respectively.

Discussion

Species richness of fungi associated with symptomatic *P. ramosa*

The present study was conducted without any preconceived idea about the taxa that would be isolated from symptomatic *P. ramosa* plants taken from different tobacco-producing regions in France. The dominant genus was *Fusarium*, consistent with other studies based on collections of symptomatic *Orobanche* spp. among which this genus was most frequently isolated (Amsellem et al. 2001b, Boari and Vurro 2004, Müller-Stöver et al. 2009, Abbas 2014). The study carried out in Germany also focused on the search for a local agent for the biological control of *P. ramosa* parasitizing tobacco (Müller-Stöver et al. 2009). In the three other studies on *P. ramosa*, and one on *P. aegyptiaca* also highlighting the prevalence of *Fusarium* in their isolates, symptomatic *P. ramosa* were collected from fields where host plants were different crops in the same region. The originality of our study lies in the multiplicity of the sampling sites all located in one country, with tobacco as the only host plant taken into account. In addition to the 16 *Fusarium* species, among which *F. oxysporum* and *F. solani* were abundantly represented but not the most aggressive isolates as in previous studies, isolates belonging to the genera *Alternaria* and *Plectosphaerella*—which harbour plant pathogenic species—were also identified among the endophytes of *P. ramosa*. However, in our case, all taxa came from symptomatic plants belonging to the single species *P. ramosa* parasitizing tobacco. Moreover, despite the abundance and diversity of these pathogens, the number of parasitic infections of tobacco

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by *P. ramosa* still remains so high and damaging that tobacco cultivation has to be abandoned in certain places.

Most of the *Fusarium* species highlighted by the various studies can produce many spores and theoretically spread from a symptomatic broomrape to a healthy one, as in the case of many fusarioses affecting cultivated plants like Panama disease (Ploetz 2015). It appears that the r strategy adopted by parasitic Orobanchaceae is particularly effective: thanks to the hundreds of thousands of seeds released by each flower scape, broomrapes—and more particularly *P. ramosa*—can bypass all the natural regulation mechanisms of animal, plant or microbial origin (Pianka 1970, Cartry et al. 2021). Under such conditions, it is very likely that a single biocontrol agent will not prove sufficient to maintain the population density of broomrape seeds below a tolerable threshold for producing foodstuffs. This is why it is necessary to assess the infectious potential of the diversity of *P. ramosa* pathogens to later consider their stimulation in an agroecological context.

Highlighting seed germination inhibition and necrotic activity by endophytic fungal isolates of *P. ramosa*

All the isolates were isolated from surface-disinfected *P. ramosa* stems; therefore, they belonged to the endophyte community of *P. ramosa*. These isolates represented a significant diversity strongly dominated by the genus *Fusarium*, but also a significant diversity in their pathogenic activity, including a large number of isolates whose inhibitory effect on seed germination was lower than that of the control isolate. Nevertheless, ∼25 isolates were more efficient than the control isolate, and a dozen totally inhibited seed germination. This functional trait characterized the species *F. sporotrichioides* and *F. venenatum*: all their representatives strongly inhibited broomrape seed germination, whereas inhibition by the other *Fusarium* species varied. In the same way, the lethal character of the inhibition was not expressed in the same way according to the isolates and according to the taxa. The resulting loss of seed viability confers a definitive character to the control of the broomrape, while an inhibition that does not permanently affect the seed viability does not guarantee such definitive control. Under these conditions, isolates *F. avenaceum* (MIAE02853), *F. sambucinum* (MIAE02876) and *F. brachygibbosum* could appear as the most promising. However, it is uncertain whether seeds still viable after fungal inhibition were still able to germinate and infest a host plant. The fact that for most of the fungi tested, a variable proportion of the seed viability was lost suggests that the lethal activity was ongoing and that a longer interaction between the seeds and the fungi would have resulted in a complete loss of seed viability. Different mechanisms depending on the isolates and/or the taxa and involving fungal enzymatic activities and defence reactions of the broomrape seeds must have operated during the fungus–seed interaction as for the interactions between fungi and *Striga* seeds, or other weed seeds in soils (Neondo et al. 2017, Fuerst et al. 2018, Pollard 2018). It is however difficult to distinguish two well-defined groups based on a clear functional trait—lethal/non-lethal inhibition/absence of inhibition—along with a clear taxonomic position. Our study rather highlights a continuum, i.e. a wide range of aptitudes to inhibit seed germination.

The second test was innovative. It was set up to characterize the necrotic activity of the isolates. Unlike the first test, we expected all isolates to respond positively to this test because they had been isolated from symptomatic stems. Yet, it is likely that some of the isolates were non-aggressive endophytes and were not involved in the symptoms that prevailed for their isolation. *Fusarium sporotrichioides* and *F. venenatum*, with five and three representatives, respectively, represented more than half of all the tested isolates. As these two species displayed the highest necrotic activity, this suggests that necrotic activity was a taxon-dependent functional trait. However, the intensity of this activity varied according to the isolate within a given species, suggesting that the expression of necrotic activity came from intraspecific diversity. Necrotic activity and its intensity could indeed be a characteristic of a fungal species, but also a characteristic of an isolate within a species. A single isolate (MIAE02028) represented the species *F. oxysporum* and ranked 13th, with relatively low necrotic activity (7.5 mm length). The literature lists isolates of *F. oxysporum* as candidates for biocontrol (Boari and Vurro 2004, Dor et al. 2007, Müller-Stöver et al. 2009), but only one of the tested *F. oxysporum* isolates ranked in the top 20 of double skills—germination inhibition and necrotic activity—and it was among the least effective ones.

The 15 isolates tested for their necrotic activity were selected among the isolates exhibiting the strongest inhibitory activity on germination. As part of an effective biological control of *P. ramosa*, isolates with several modes of action are necessary to act at different stages of the broomrape life cycle. Thus, the isolates of *F. sporotrichioides* and unexpectedly those of *F. venenatum*, better known for its mycoprotein production than for any pathogenic activity (Finningan et al. 2019), had this dual competence: they limited seed germination of broomrape and necrotized the stems of the broomrapes. All the isolates came from symptomatic stems; therefore, it is very likely that some of them had strong necrotic activity but were not selected because of their low inhibitory activity on seed germination. However, this unique competence is not enough for these isolates to be retained for biocontrol. This is why they were not tested for their necrotic activity.

Variable necrotic capacities among fungal isolates associated with symptomatic *P. ramosa*

The differences between the different isolates tested for their necrotic activity were not all significant: a continuum emerged, partly justified by (i) the endophytic origin of the isolates, including non-pathogenic isolates and (ii) the variability of the broomrape stems for which it is difficult to have individuals perfectly synchronized in their development. This functional diversity was comparable to the varying abilities of the isolates to inhibit seed germination. In other words, the intensity of the pathogenic activity varied across species, or even across isolates. This mechanism is quite frequent, and results in the characterization of pathogenic isolates according to their level of aggressiveness (Lecomte et al. 2016). This may be due to the up- and downregulation of pathogenicity genes (Jonkers et al. 2013). This can also correspond to the expression of different genes among isolates, which results in different modes of action of isolates (Jangir et al. 2021). Thus, mycelium production by isolates of the species *F. sporotrichioides* reflected a very efficient internal and external colonization of the broomrape stem. The production of various highly toxic mycotoxins, including fumonisin, T2 and trichothecenes may contribute to the necrotizing activity of *F. sporotrichioides* (Bekele et al. 1991, Yuan et al. 2016, Banati et al. 2017). As for *F. sambucinum* species, they would have only one mode of internal colonization that seems as effective as that of *F. sporotrichioides* isolates. In that case too, the production of phytotoxic trichothecenes by *F. sambucinum* could explain this necrotic success (Ismail et al. 2013, Piacentini et al. 2019). Different modes of action were also notable within the species *F. venenatum*: two isolates—MIAE02836 and MIAE02849–of comparable aggressive-

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ness exhibited two different behavioural strategies, including or not external mycelium to colonize the host plant. Trichothecenes A have indeed been found in very small quantities in rice inoculated with the A3/5 strain of *F. venenatum* (O'Donnell et al. 1998b). Therefore, strains of this species could produce phytotoxic mycotoxins during broomrape colonization, which explains the necrosis observed with isolate MIAE02849 despite the absence of external mycelium during the first 72 h post-inoculation. This ability to produce mycotoxins and/or external mycelium varies from one isolate to another and explains the varying necrotic activities of *F. venenatum* isolates (Miller and MacKenzie 2000). Modes of action based on different isolate-dependent mechanisms within a given species have already been observed, but mainly within *Trichoderma* species (Anees et al. 2010).

Among the weakly aggressive isolates, *F. culmorum* is also known to produce mycotoxins in cereals, but its necrotic activity was low compared with that of the other three species. This result suggests that mycotoxins are not a determining factor in fungal necrotic activity, or that *F. culmorum* does not produce mycotoxins when interacting with broomrape (Castilblanco et al. 2020). Finally, isolates with higher necrotic activity than that observed in the present study may not have been isolated because the broomrape stems died before their emergence and were not collected.

Specific action of fungal isolates at the intraspecific scale of *P. ramosa*

Performances of *P. ramosa* can be suboptimal on most host species, suggesting some host specificity (Schneeweiss 2007). The host specificities of genetically distinct *P. ramosa* populations may also modulate their seed germination rates. Actually, this broad host range seems to coincide with host specificity in *P. ramosa* pathovars. For example, even if they can colonize different hosts, seeds of *P. ramosa* pathovars exposed to exudates from different host species have different germination rates (Gibot-Leclerc et al. 2016, Perronne et al. 2017). Similarly, intraspecific variability of *P. ramosa* aggressiveness has been observed (Gibot-Leclerc et al. 2013), as well as seasonal variation in seed dormancy (Pointurier et al. 2019), and germination rates in response to GR 24 concentrations (Gibot-Leclerc et al. 2021).

In parallel to seed sensitivity to germination stimulation, a difference was found in seed sensitivity to germination inhibition by some fungal isolates. Two populations (T and R) of *P. ramosa* seeds taken from two different host plants—tobacco and oilseed rape, respectively—were tested for their sensitivity to germination inhibition by 20 fungal isolates. The 11 isolates most effective in inhibiting seed germination of the T population also proved to be the most effective ones on R population seeds; should these isolates be retained for biocontrol, this is rather encouraging. The situation was different for the nine isolates that were a little less effective than the eleven ones mentioned above: R seeds were less receptive than T seeds to fungal inhibition molecules. This confirms the host specificity of *P. ramosa* pathovars but also suggests the existence of a mechanism of germination inhibition analogous to that observed with strigolactones. Each plant genotype indeed produces specific strigolactones for its own purpose, but strigolactones also specifically stimulate the seeds of broomrapes that co-evolved with these genotypes to ensure successful parasitism. The specificity of this interaction is based on the presence of receptors specific to one of the strigolactone types and leads to the definition of pathovars within the species *P. ramosa* (Le Corre et al. 2014, Brun et al. 2018, Stojanova et al. 2019). Thus, the pathovar's receptors that are susceptible to the strigolactones of the corresponding host plant would also be most susceptible to the inhibitory molecules produced by fungi associated with this host plant. To verify this hypothesis, germination inhibition by isolates collected from *P. ramosa* oilseed rape should also be tested on the two R and T populations. We demonstrate for the first time that different seed germination rates of two genotypes of *P. ramosa* may be partly due to a specific action of the isolates.

Non-pathogenicity of the selected fungal isolates to tobacco

A certain number of the isolates had characteristics suggesting a potential role in the biological control of *P. ramosa*. However, it was important to ensure that these isolates were not pathogenic to tobacco. Under the very artificial conditions of our pathogenicity test, inoculation affected tobacco seedling growth without causing necrosis for 7 of the 10 isolates, which can be interpreted as a physiological response of tobacco to biotic stress but not as a pathological symptom. Concerning the three isolates, MIAE02893, MIAE02846 (*F. sporotrichioides*) and MIAE02853 (*F. avenaceum*), their necrotic activity on seedling roots was much lower than that of the reference pathogenic strain *P. parasitica* Pp329. Moreover, *F. sporotrichioides* and *F. avenaceum* have never been described as tobacco pathogens. It is more likely that these three isolates were putative endophytes aiming at colonizing tobacco root tissues, which still remains to be demonstrated. Therefore, the selected isolates were not harmful to tobacco.

Fusarium venenatum, a mycoherbicide to truly control *P. ramosa?*

Biological control of *P. ramosa* with mycoherbicides raises quite a dilemma because the available potential relies on fungal genera or species harbouring pathogens of cultivated plants. Even if the broomrape biocontrol agent is not pathogenic to the host plant at time *t*, it is not certain that it is not or will not become pathogenic to other cultivated plants of the rotation. Out of all the isolates of this study, three species—*F. sambucinum*, *F. sporotrichioides* and *F. venenatum*—seem promising based on their activity against broomrape, but each has limitations that should not be overlooked. For instance, isolate MIAE02876 of *F. sambucinum* could be a promising candidate since it has the dual competence of both definitively inhibiting seed germination and inducing necrosis on broomrape stems. However, *F. sambucinum* harbours many pathogenic isolates causing potato dry rot (Aydin 2019), so that using such isolates for the biocontrol of *P. ramosa* represents an unreasonable hazard. *Fusarium sporotrichioides* is a soil-borne fungus often associated with the Fusarium head blight (FHB) complex, which never appears as the major causative agent of fusariosis in cereals even though it produces mycotoxins, some of which are dangerous (Yuan et al. 2016, Banati et al. 2017). It has been reported once as a pathogen of impatiens (*Impatiens* spp.) seeds in Poland (Najberek et al. 2018). It is a litter decomposer whose pathogenicity also comes from the secondary metabolites it produces during its saprotrophic development; these metabolites could be repellent or toxic for the shredder *Gammarus roeselii* (Assmann et al. 2010). It has also been described for its entomopathogenic activity on *Anopheles stephensi* and *Culex quinquefasciatus* larvae (Maurya et al. 2011). Despite its apparently weak role in the FHB complex, its ecological role seems important, and our results are particularly innovative and encouraging as to considering the exploitation of its isolates for the biocontrol of *P. ramosa*. *Fusarium venenatum* is particularly known for its strain A3/5, which is highly productive of mycoproteins used in human nutrition, in

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particular by vegetarian and vegan people (Wiebe 2002, Reihani and Khosravi-Darani 2018). Although this species was previously assimilated to the species *F. graminearum*, a pathogen of cereals, it is now recognized as a soil-borne saprophytic and non-pathogenic species, as confirmed by the recent comparison of its genome to that of *F. graminearum* (O'Donnell et al. 1998b, King et al. 2018). The pathogenicity of *F. venenatum* to *P. ramosa* is revealed here for the first time and deserves to be emphasized. This is why *F. venenatum* isolates (MIAE02825, MIAE02836, MIAE02849 and MIAE02879) were registered as a French Declaration of Invention and Valuable Results (DIRV) with the INRAE and are currently being investigated for further development. The non-pathogenicity of the tested isolates on tobacco, and on all cultivated plants according to the literature, suggests that isolates MIAE02836 and MIAE02849 of *F. venenatum* are potential candidates for the control of *P. ramosa*. In addition, it would be relevant to test the pathogenicity of these isolates on other parasitic plants of the Orobanchaceae family to broaden the spectrum of potential targets. Since strains of *Fusarium* are already used as biocontrol agents in other pathosystems, the acceptability of the isolates retained by our study should not pose a problem for tobacco growers.

Conclusion

The aim of this work was to evaluate the mycoherbicide potential of endophytic fungal colonizers associated with symptomatic *P. ramosa* parasitizing tobacco, knowing that it would be preferable to use a consortium of different, compatible and functionally complementary strains to ensure better biological control of broomrapes. Among the isolates from different *Fusarium* species that we selected, we highlighted the role that the species *F. venenatum* could play in the control of this parasitic plant. This original result was unexpected given the previous knowledge about this *Fusarium* species producing cholesterol-free, protein-rich food items and presenting an interest in the field of human health by improving nutritional digestibility. This species is very promising because it naturally occurs in the soil and its use should not present any environmental risk or a risk to human and animal health.

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Supplementary data

Supplementary data are available at FEMSEC online.

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References

Abbas M. Biological control of *Orobanche ramosa* by *Fusarium solani*. *Int J Adv Biol Biom Res* 2014;**2**:2751–5.

Amsellem Z, Barghouthi S, Cohen B *et al.* Recent advances in the biocontrol of *Orobanche* (broomrape) species. *BioControl* 2001a;**46**:211–28.

Amsellem Z, Kleifeld Y, Kerenyi Z. Isolation, identification, and activity of mycoherbicidal pathogens from juvenile broomrape plants. *Biol Control* 2001b;**21**:274–84.

Anees M, Tronsmo A, Edel-Hermann V *et al.* Characterization of field isolates of *Trichoderma* antagonistic against *Rhizoctonia solani*. *Fungal Biol* 2010;**114**:691–701.

Assmann C, Nechwatal J, Rinke K *et al.* The impact of axenic strains of fungi and oomycetes on the preference of *Gammarus roeselii* for leaf litter. *Fundam Appl Limnol* 2010;**176**:235–48.

Auger B, Pouvreau JB, Pouponneau K *et al.* Germination stimulants of *Phelipanche ramosa* in the rhizosphere of *Brassi ca napus* are derived from the glucosinolate pathway. *Mol Plant Microbe Interact* 2012;**25**:993–1004.

Aybeke M, Sen B, Okten S. Pesta granule trials with *Aspergillus alliaceus* for the biocontrol of *Orobanche* spp. *Biocontrol Sci Technol* 2015;**25**:803–13.

Aydin MH. Evaluation of some *Trichoderma* species in biological control of potato dry rot caused by *Fusarium sambucinum* fuckel isolates. *Appl Ecol Environ Res* 2019;**17**:533–46.

Banati HB, Darvas S, Feher-Toth A *et al.* Determination of mycotoxin production of *Fusarium* species in genetically modified maize varieties by quantitative flow immunocytometry. *Toxins* 2017; **9**:70.

Barghouthi S, Salman M. Bacterial inhibition of *Orobanche aegyptiaca* and *Orobanche cernua* radical elongation. *Biocontrol Sci Technol* 2010;**20**:423–35.

Barnett HL, Hunter BB. *Illustrated Genera of Imperfect Fungi*. 4th edn. St Paul, MN: APS Press, 1998.

Bekele EAA, Rottinghaus GE, Rottinghaus HH *et al.* Two new trichothecenes from *Fusarium sporotrichioides*. *J Nat Prod* 1991;**54**:1303–8.

Benharrat H, Boulet C, Theodet C *et al.* Virulence diversity among branched broomrape (*O. ramosa* L.) populations in France. *Agron Sustain Dev* 2005;**25**:123–8.

Boari A, Vurro M. Evaluation of *Fusarium* spp. and other fungi as biological control agents of broomrape (*Orobanche ramosa*). *Biol Control* 2004;**30**:212–9.

Brault M, Betsou F, Jeune B *et al.* Variability of *Orobanche ramosa* populations in France as revealed by cross infestations and molecular markers. *Environ Exp Bot* 2007;**61**:272–80.

Brun G, Braem L, Thoiron S *et al.* Seed germination in parasitic plants: what insights can we expect from strigolactone research? *J Exp Bot* 2018;**69**:2265–80.

Cartry D, Steinberg C, Gibot-Leclerc S. Main drivers of broomrape regulation. A review. *Agron Sustain Dev* 2021;**41**:17.

Castiblanco VH, Castillo E, Miedaner T. Be flexible and adapt easily: the great role of plasticity relative to genetic variation for aggressiveness of *Fusarium culmorum* isolates. *J Phytopathol* 2020;**168**: 162–74.

Chen J, Xue QH, McErlean CSP *et al.* Biocontrol potential of the antagonistic microorganism *Streptomyces enissocaesilis* against *Orobanche cumana*. *BioControl* 2016;**61**:781–91.

Dor E, Evidente A, Amalfitano C *et al.* The influence of growth conditions on biomass, toxins and pathogenicity of *Fusarium oxysporum* f. sp. *orthoceras*, a potential agent for broomrape biocontrol. *Weed Res* 2007;**47**:345–52.

Dor E, Hershenhorn J. Evaluation of the pathogenicity of microorganisms isolated from egyptian broomrape (*Orobanche aegyptiaca*) in Israel. *Weed Biol Manag* 2009;**9**:200–8.

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Edel V, Steinberg C, Gautheron N *et al.* Genetic diversity of *Fusarium oxysporum* populations isolated from different soils in France. *FEMS Microbiol Ecol* 2001;**36**:61–71.

Elzein A, Kroschel J, Cadisch G. Efficacy of pesta granular formulation of Striga-mycoherbicide *Fusarium oxysporum* f. sp. *strigae* Foxy 2 after 5-year of storage. *J Plant Dis Prot* 2008;**115**:259–62.

Fernández-Aparicio M, Reboud X, Gibot-Leclerc S. Broomrape weeds. Underground mechanisms of parasitism and associated strategies for their control: a review. *Front Plant Sci* 2016;**7**:23.

Finnigan TJA, Wall BT, Wilde PJ *et al.* Mycoprotein: the future of nutritious nonmeat protein, a symposium review. *Curr Dev Nut* 2019;**3**:5.

Fuerst EP, James MS, Pollard AT *et al.* Defense enzyme responses in dormant wild oat and wheat caryopses challenged with a seed decay pathogen. *Front Plant Sci* 2018;**8**:2259.

Gardes M, Bruns TD. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. *Mol Ecol* 1993;**2**:113–8.

Gibot-Leclerc S, Brault M, Pinochet X *et al.* Potential role of winter rape weeds in the extension of broomrape in Poitou-Charentes. *CR Biol* 2003;**326**:645–58.

Gibot-Leclerc S, Connault M, Perronne R. Differences of seed germination response of two populations of *Phelipanche ramosa* (L.) Pomel to a set of GR24 concentrations and durations of stimulation. *Seed Sci Res* 2021;**31**:243–8.

Gibot-Leclerc S, Corbineau F, Sallé G *et al.* Responsiveness of *Orobanche ramosa* l. seeds to GR 24 as related to temperature, oxygen availability and water potential during preconditioning and subsequent germination. *Plant Growth Regul* 2004,**43**:63–71.

Gibot-Leclerc S, Perronne R, Dessaint F *et al.* Assessment of phylogenetic signal in the germination ability of *Phelipanche ramosa* on *brassicaceae* hosts. *Weed Res* 2016;**56**:452–61.

Gibot-Leclerc S, Reibel C, Dessaint F *et al. Phelipanche ramosa* (L.) Pomel populations differ in life-history and infection response to hosts. *Flora* 2013;**208**:247–52.

Gibot-Leclerc S, Sallé G, Reboud X *et al.* What are the traits of *Phelipanche ramosa* (L.) Pomel that contribute to the success of its biological cycle on its host *Brassica napus* L.? *Flora* 2012;**207**: 512–21.

Heide-Jørgensen HS. Introduction: the parasitic syndrome in higher plants. In: Joel DM, Gressel J, Musselman LJ. (eds). *Parasitic Orobanchaceae*. Berlin: Springer, 2013, 1–14.

Ismail Y, McCormick S, Hijri M. The arbuscular mycorrhizal fungus, *Glomus irregulare*, controls the mycotoxin production of *Fusarium sambucinum* in the pathogenesis of potato. *FEMS Microbiol Lett* 2013;**348**:46–51.

Jangir P, Mehra N, Sharma K *et al.* Secreted in xylem genes: drivers of host adaptation in *Fusarium oxysporum*. *Front Plant Sci* 2021;**12**:17.

Jonkers W, Xayamongkhon H, Haas M *et al.* EBR1 genomic expansion and its role in virulence of *Fusarium* species. *Environ Microbiol* 2013;**16**:1982–2003.

King R, Brown NA, Urban M *et al.* Inter-genome comparison of the quorn fungus *Fusarium venenatum* and the closely related plant infecting pathogen *Fusarium graminearum*. *BMC Genomics* 2018;**19**:269.

Le Corre V, Reibel C, Gibot-Leclerc S. Development of microsatellite markers in the branched broomrape *Phelipanche ramosa* l. (Pomel) and evidence for host-associated genetic divergence. *Int J Mol Sci* 2014;**15**:994–1002.

Lecomte C, Edel-Hermann V, Cannesan M-A *et al. Fusarium oxysporum* f. sp. *cyclaminis*: underestimated genetic diversity. *Eur J Plant Pathol* 2016;**145**:421–31.

Maurya P, Mohan L, Sharma P *et al.* Evaluation of larvicidal potential of certain insect pathogenic fungi extracts against *Anopheles stephensi* and *Culex quinquefasciatus*. *Entomol Res* 2011;**41**:211–5.

Miche L, Bouillant ML, Rohr R *et al.* Physiological and cytological studies on the inhibition of *Striga* seed germination by the plant growth-promoting bacterium *Azospirillum brasilense*. *Eur J Plant Pathol* 2000;**106**:347–51.

Miller JD, Mackenzie S. Secondary metabolites of *Fusarium venenatum* strains with deletions in the Tri5 gene encoding trichodiene synthetase. *Mycologia* 2000;**92**:764–71.

Müller-Stöver D, Kohlschmid E, Sauerborn J. A novel strain of *Fusarium oxysporum* from germany and its potential for biocontrol of *Orobanche ramosa*. *Weed Res* 2009;**49**:175–82.

Najberek K, Pusz W, Solarz W *et al.* The seeds of success: release from fungal attack on seeds may influence the invasiveness of alien *Impatien s*. *Plant Ecol* 2018;**219**:1197–207.

Nelson PE, Toussoun TA., Marasas WFO. *Fusarium Species. An Illustrated Manual for Identification*. University Park, PA: Pennsylvania State University Press, 1983.

Neondo JO, Alakonya AE, Kasili RW. Screening for potential *Striga hermonthica* fungal and bacterial biocontrol agents from suppressive soils in western Kenya. *BioControl* 2017;**62**:705–17.

Nzioki HS, Oyosi F, Morris CE *et al. Striga* biocontrol on a toothpick: a readily deployable and inexpensive method for smallholder farmers. *Front Plant Sci* 2016;**7**:8.

O'Donnell K, Cigelnik E, Casper HH. Molecular phylogenetic, morphological, and mycotoxin data support reidentification of the quorn mycoprotein fungus as *Fusarium venenatum*. *Fungal Genet Biol* 1998b;**23**:57–67.

O'Donnell K, Kistler HC, Cigelnik E *et al.* Multiple evolutionary origins of the fungus causing panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proc Natl Acad Sci* 1998a;**95**:2044–9.

O'Donnell K, Sutton DA, Rinaldi MG *et al.* Internet-accessible DNA sequence database for identifying fusaria from human and animal infections. *J Clin Microbiol* 2010;**48**:3708–18.

O'Donnell K, Ward T, Robert V *et al.* DNA sequence-based identification of *Fusarium*: current status and future directions. *Phytoparasitica* 2015;**43**:583–95.

Parker C. Observations on the current status of *Orobanche* and *Striga* problems worldwide. *Pest Manage Sci* 2009;**65**:453–9.

Perronne R, Gibot-Leclerc S, Dessaint F *et al.* Is induction ability of seed germination of *Phelipanche ramosa* phylogenetically structured among hosts? A case study on *Fabaceae* species. *Genetica* 2017;**145**:481–9.

Piacentini KC, Rocha LO, Savi GD *et al.* Assessment of toxigenic *Fusarium* species and their mycotoxins in brewing barley grains. *Toxins* 2019;**11**:1.

Pianka ER. On r- and K-Selection. *Am Nat* 1970;**104**:592–7.

Ploetz RC. Management of Fusarium wilt of banana: a review with special reference to tropical race 4. *Crop Prot* 2015;**73**:7–15.

Pointurier O, Gibot-Leclerc S, Le Corre V *et al.*. Intraspecific seasonal variation of dormancy and mortality of *Phelipanche ramosa* seeds. *Weed Res* 2019;**59**:407–18.

Pollard AT. Seeds vs fungi: an enzymatic battle in the soil seedbank. *Seed Sci Res* 2018;**28**:197–214.

Reihani SFS, Khosravi-Darani K. Mycoprotein production from date waste using *Fusarium venenatum* in a submerged culture. *Appl Food Biotech* 2018;**5**:543–252.

Schneeweiss GM. Correlated evolution of life history and host range in the nonphotosynthetic parasitic flowering plants *Orobanche* and *Phelipanche* (Orobanchaceae). *J Evol Biol* 2007;**20**:471–8.

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Stojanova B, Delourme R, Duffe P *et al*. Genetic differentiation and host preference reveal non-exclusive host races in the generalist parasitic weed *Phelipanche ramosa*. *Weed Res* 2019;**59**:107–18.

Thomas H, Heller A, Sauerborn J *et al*. *Fusarium oxysporum* f. sp. *orthoceras*, a potential mycoherbicide, parasitizes seeds of *Orobanche cumana* (sunflower, broomrape): a cytological study. *Ann Bot* 1999;**83**:453–8.

Westwood JH. The physiology of the established parasite–host association. In: Joel DM, Gressel J, Musselman LJ (eds). *Parasitic Orobanchaceae*. Berlin: Springer, 2013, 87–114.

White TJ, Bruns T, Lee S *et al*. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ *et al*. (eds). *PCR Protocols: A Guide to Methods and Applications*. San Diego, CA: Academic Press, 1990, 315–22.

Wiebe MG. Myco-protein from *Fusarium venenatum*: a well-established product for human consumption. *Appl Microbiol Biotechnol* 2002;**58**:421–7.

Yoneyama K, Ruyter-Spira C, Bouwmeester H. Induction of germination. In: Joel DM, Gressel J, Musselman LJ (eds). *Parasitic Orobanchaceae: Parasitic Mechanisms and Control Strategies*. Berlin, Heidelberg: Springer-Verlag, 2013, 167–94.

Yuan ZH, Matias FB, Yi JE *et al*. T-2 toxin-induced cytotoxicity and damage on TM3 Leydig cells. *Comp Biochem Physiol C Toxicol Pharmacol* 2016;**181-2**:47–54.

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