Fungal pathogens on Scotch broom (*Cytisus scoparius*) and French broom (*Genista monspessulana*) from Chile

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ABSTRACT

Scotch broom (*Cytisus scoparius*) and French broom (*Genista monspessulana*) are two woody legumes native to Europe and North Africa that have become invasive in different countries around the world. In Chile they are among the most serious invasive plants of the central southern regions, where they cause severe negative impacts to natural environments, and have become a serious problem in plantation forestry. Numerous studies have sought biological control agents for both species, however, this approach has not been explored in Chile. The aim of this study was to prospect for pathogens and diseases occurring on these weeds in invaded areas of Chile, and explore these as potential biocontrol agents. Surveys were conducted in 13 invaded areas between the Biobio and Araucanía regions, and diseased plants of both species were collected for isolations. The putative pathogens isolated from each broom species were inoculated into healthy plants, and isolates producing symptoms identified by DNA sequencing. These isolates were used in pathogenicity tests on both weed species and on *Pinus radiata* and *Eucalyptus globulus* plants. The most aggressive pathogens were *Fusarium sambucinum*, *F. solani* and *Phytophthora multivora* on *C. scoparius*; *Neofusicoccum parvum* and *Chondrostereum purpureum* on both weeds, and *F. tricinctum s.l.* on *G. monspessulana*. Except for *F. trincinctum s.l.*, all isolates caused lesions on both weeds, and except for *P. multivora*, all isolates caused lesions on *E. globulus* and/or *P. radiata*. Most pathogens on *C. scoparius* had been reported previously on this host elsewhere, while most pathogens on *G. monspessulana* represent novel associations.

1. INTRODUCTION

French broom (*Genista monspessulana*; syn. *Teline monspessulana*) and scotch broom (*Cytisus scoparius*) are two woody legumes native to Europe and Northern Africa that have become invasive in different parts of the world (Parsons and Cuthberson 2001; Prasad 2005; Brandes et al. 2019). In countries where they are invasive, these legumes have been responsible for substantial economic losses in plantation forests (Peterson and Prasad 1998; Fowler and Syrett 2000; Isaacson 2000) and invasions of natural ecosystems (Bravo 1985). The consequences of their invasion include competition with natural regeneration (Bravo 1985; Bellingham and Coomes 2003; Wearne and Morgan 2004), becoming intermediate hosts and/or inoculum reservoirs for plant pathogens (Bishop 1978; Purcell and Saunders 1999), altering fire regimes (Pauchard et al. 2008) and altering soil properties including biochemistry, nutrient availability (Caldwell 2006; Slesak et al. 2016), and/or microenvironment (Carter et al. 2019). In Chile, *C. scoparius* and *G. monspessulana* were reported the first time in 1847 (Matthei et al. 1995) and are currently intermittently present from the Valparaíso to the Magallanes regions (Matthei et al. 1995; Fuentes et al. 2013). Similar to their effect in many other countries, they have negatively impacted plantation forests and natural ecosystems (García et al. 2007; Pauchard et al. 2008).

Classically, woody legumes such as *C. scoparius* and *G. monspessulana* have been controlled through manual, mechanical and chemical methods (Talbot 2000; Ketchum and Rose 2003; Oneto et al. 2010). However, these practices can be costly (commonly only possible for commercial forestry companies), non-specific, detrimental to the soil and/or limited by environmental regulations (Talbot 2000). In addition, for weeds with good resprouting capacity and that accumulate large seed banks in the ground, as it is the case for *C. scoparius* and *G. monspessulana* (García et al. 2007; Pauchard et al. 2008; Oneto et al. 2010), these control methods need continuous application, increasing the associated costs. As a result, both weed species have been targeted in numerous studies seeking alternative control strategies, including biological control.

Both *C. scoparius* and *G. monspessulana* have been extensively studied as targets for biological control . Several insect herbivores, and less commonly pathogenic fungi, have been found on both weeds in their native ranges (Syrett et al. 1999; Sheppard and Thomann 2004; Cullen et al. 2012). Furthermore, in numerous cases these pests/pathogens have been introduced in countries as biocontrol agents (e.g., 'classical' biological control), including New Zealand, USA and Australia (Syrett et al. 1999; Winston et al. 2014). *Cytisus scoparius* has also been studied using the 'inundative' biological control approach (e.g., bioherbicides or mycoherbicides) in New Zealand (Johnston and Parkes 1994; Johnston et al. 1995) and Canada (Prasad 2005). Two fungal pathogens, *Fusarium tumidum* and *Chondrostereum purpureum*, have been extensively studied for their use as mycoherbicides (Fröhlich et al. 2000; Morin et al. 2000; Barton et al. 2003; Prasad 2005; Bourdôt et al. 2006; Ramsfield 2006; Yamoah et al. 2008). However, attempts to produce consistent levels of disease in the field were unsuccessful (Fröhlich et al. 2000; Prasad 2005).

There are no formal reports of pathogens on *C. scoparius* or *G. monspessulana* in Chile or records of surveys for biological control agents. Furthermore, reports of fungal pathogens on *G. monspessulana* are scarce at a global scale. The aim of this study was to prospect for fungal pathogens on *C. scoparius* and *G. monspessulana* in invaded areas in Chile, and to conduct a preliminarily assessment of their host range.

2. MATERIALS AND METHODS

2.1. Sample collection and isolation of fungi

Field surveys were conducted between 2013 and 2014 in areas invaded by *C. scoparius* or *G. monspessulana* in the Biobío and Araucanía regions, Chile (Figure 1). Visited sites included (i) private plantation forests (*Pinus radiata* and *Eucalyptus* spp.) in the Arauco and Malleco provinces for *C. scoparius* and (ii) open and private landholdings adjacent to the main highways of the Concepción province for *G. monspessulana*. Diseased plants or plant tissues of both species were collected, favouring plants without obvious signs of abiotic damage (e.g., mechanical or chemical). Sampled tissues included stems, roots, branches, whole plants and seed pods, and were processed in the laboratory within 48 hrs of collection.

Species/ site	GPS Coordinates
Genista monspessulana	
❶ Itata (a)	-36.744476 , -72.921573
❷ Itata (b)	-36.734197 , -72.758903
❸ Itata (c)	-36.701441 , -72.666389
❹ Cabrero (a)	-36.862182 , -72.859841
❺ Cabrero (b)	-36.938407 , -72.739563
❻ Santa Juana (a)	-36.975798 , -73.025212
❼ Santa Juana (b)	-36.981162 , -72.988328
❽ Santa Juana (c)	-37.06287 , -72.994056
Cytisus scoparius	
◆1 Tranguilboro	-37.993263 , -73.367246
◆2 Lautaro	-38.03927 , -73.375141
◆3 Vegas de Chanquin	-38.25449 , -73.18859
◆4 Licura Bajo	-38.343453 , -73.350663
◆5 Calcoy	-38.355148 , -73.27686

Map source: 1. Free vector maps. Available at https://freevectormaps.com/chile/CL-EPS-01-0001?ref=atr.
2. Instituto Geográfico Militar. Curriculum en línea (Modified). Available at: https://www.curriculumnacional.cl/portal/Educacion-General/Historia-Geografia-y-Ciencias-Sociales-1-basico/HI01-OA-09/132548:Mapa-region-de-Biobio-mudo.

Figure 1. Geographical locations and GPS coordinates of the sampling sites for symptomatic plants of *Cytisus scoparius* and *Genista monspessulana* in Chile.

All samples were first examined with a stereomicroscope for fungal fruiting structures on or around symptomatic tissues. Where these were present, fungal materials were transferred with a sterile needle to Petri dishes containing ½ strength potato dextrose agar (½PDA: 19.5 g/L PDA [Difco], 10 g/L agar). Attempts to stimulate the development of fruiting structures were undertaken by incubating samples in moisture chambers. Plant tissues were placed in glass containers, lined with wet paper towel to mantain humidity, and placed on sterile plastic mesh to avoid direct contact of samples with the water. Samples were incubated for up to 14 days at room temperature (~25 °C) on a bench top away from direct sunlight and inspected routinely. In addition, isolations from lesion margins were performed. Outer tissue layers were removed with a scalpel and small segments (0.5–1 cm^2) were excised and surface disinfested by immersion in 70% ethanol for 30 s, followed by 3 min in 5% sodium hypochlorite and rinsing in sterile distilled water. Subsequently, plant tissues were plated in Petri dishes containing ½PDA amended with streptomycin sulphate (0.5 g/L). In cases where the presence of *Phytophthora* spp. was suspected, additional segments were dissected from the samples for isolations onto NAR-CMA (corn meal agar [CMA; Difco], amended with 0.3 g/L ampicillin, 0.015 g/L rifampicin and 0.015 g/L natamycin). For these tissues, surface disinfestation methods were similar but excluded the sodium hypochlorite step. Isolations made on ½PDA were incubated at 25 °C with a 12 h artificial photoperiod for up to 14 days, and those made in NAR-CMA were incubated at 16 °C under continuous white light for up to seven days. Primary isolation plates were inspected regularly, and any mycelial outgrowths were transferred to fresh ½PDA in Petri dishes. Copies of all isolates were made and stored at 4 °C in glass test tubes containing ½PDA and in glass vials containing sterile saline solution (NaCl 8g/L).

2.2. Fungal identification

2.2.1. Morphotype grouping and preliminary identification

Fungal isolates obtained from each sample were grouped based on colony morphology and microscopic features. Isolates were inspected using a stereomicroscope and a ZEISS light microscope for preliminary identification to genus level, where possible. For fungal colonies lacking fruiting structures, sterile pieces of stems and roots of the host were placed on 7-day old cultures grown on WA (WA: 20 g/L agar). These were incubated at room temperature for up to two months with regular inspection.

2.2.2. **DNA Extractions, PCR amplification and Sequencing**

Fourteen isolates were selected for identification using DNA sequencing, based on preliminary identifications and/or consistency of isolation from specific symptoms. DNA was extracted from colonies cultured for up to 14 days on ½ PDA using the AxyPrep™ Multisource Genomic DNA Miniprep kit (Axygen Scientific; Union City, CA, USA). PCR amplification was prepared for three gene regions, (i) the internal transcribed spacer (ITS) with primers ITS1/ITS4 (White et al. 1990), (ii) the partial β-tubulin gene region (*BT2*) with primers BTubF1/BTubR1 (Armson et al. 1999), and (iii) the partial translation elongation factor 1α (*TEF1*) with primers EF1AF/EF1AR (Olson et al. 2011). PCRs were performed in 12 µL reactions containing 1.25 µL 10X buffer, 0.4 µL of 50mM $MgCl_2$, 0.25 µL of 10 mM dNTPs, 0.375 µL of 10 mM of each primer, 0.1 µL of DNA Taq Polymerase, 2 µL of template DNA and 7.25 of sterile distilled water. PCR conditions included an initial denaturation step of 2 min at 95 °C, then 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C; and a last step of 7 min at 72 °C (Takamatsu and Kano 2001). Sequencing was carried out in both directions (forward and reverse) for each sample at Macrogen (Seoul, South Korea). The forward and reverse sequences obtained for each isolate were visualized and assembled into consensus sequences with CLC Main Workbench v.21.0.3 (Qiagen, Hilden, Germany).

2.2.3. **Phylogenetic analyses**

Consensus sequences for all isolates and all gene regions were aligned to NCBI's GenBank database (NCBI, http://www.ncbi.nlm.nih.gov) utilizing the BLAST utility. For each isolate a database was constructed with sequences for the ITS, *TUB1* and *TEF1* gene regions from reference strains for species representing the best matches and closely related taxa (Table S1). Datasets for each gene region were aligned using MAFFT (Katoh et al. 2017), edited using MEGA 7.0 (Kumar et al. 2016) and concatenated using MEGA XI (Tamura et al. 2021). Maximum-likelihood trees were built for the individual and concatenated datasets using the IQ-TREE Web server (Trifinopoulos et al. 2016). The nucleotide substitution model was selected using ModelFinder (Kalyaanamoorthy et al. 2017) based on the Bayesian information criterion (using partitioned data in case of concatenated datasets), and statistical support was calculated using Ultrafast Bootstrap analysis (Minh et al. 2013). Phylogenetic trees were visualized in FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) and edited using Affinity Designer v.1.10.5.1342 (Serif (Europe) Ltd, Nottingham, UK).

2.3. Pathogenicity trials

Two pathogenicity trials were carried out. First, all potentially pathogenic fungi were inoculated into the same host species from which they were isolated, to test Koch's postulates. Subsequentially, all the isolates that caused lesions in the initial trial were selected for a second pathogenicity trial, which included the two weed species, *C. scoparius* and *G. monspessulana*, as well as *P. radiata* and *E. globulus* seedlings.

2.3.1. Koch´s postulates

Thirty-five isolates were selected for pathogenicity tests based on identifications and consistency of isolation from a specific symptom (Table S2). Of these isolates, 19 were obtained from *C. scoparius* and 16 from *G. monspessulana*.

For the production of *C. scoparius* and *G. monspessulana* seedlings, mature seed pods were collected during field visits and dried in the laboratory to stimulate opening. Seeds were sown in polystyrene trays with pine mulch in a glasshouse and grown for two months, before transferring to individual plastic bags. By the time of inoculation, *C. scoparius* seedlings were ~6 months old and had reached 87 ± 17 cm in height and 5.6 ± 1.2 mm diameter at the collar; *G. monspessulana* plants were 130 ± 12 cm in height and 7 ± 0.8 mm diameter at the collar. For inoculation, stems were wounded using a cork borer 2–5 cm above the collar to make a small incision. Mycelial discs (5 mm diam.) were taken from the margins of 7–14 day old cultures of each isolate using a similar size cork borer and placed on wounds. Inoculations were covered with Parafilm® for seven days before removal. Five seedlings of each species were inoculated with each isolate, and five additional seedlings were inoculated with sterile ½ PDA plugs as a control treatment. Seedlings were arranged randomly in the same glasshouse where the plants were grown and watered daily until the end of the trial. After 30 days, lesions were examined by removing the outer bark layers, lesion length was measured, and lesioned stem segments were collected for re-isolations. These stem segments were plated onto ½ PDA, except for seedlings inoculated with one *Phytophthora* isolate for which NAR-CMA was used. Similar surface disinfestation methods as described earlier were used, with 3–5 dissected pieces of lesion margin plated per stem sample. Lesion length data were analysed statistically by performing Kruskal-Wallis and Wilcoxon rank sum tests in RStudio v. 2022.12.0+353 (Posit Software, PBC).

2.3.2. Pathogenicity trial on multiple hosts

Nine isolates were selected for simultaneous inoculations on four hosts: *C. scoparius*, *G. monspessulana*, *E. globulus* and *P. radiata*. Five of these isolates were obtained from *C. scoparius* and four from *G. monspessulana*. *Cytisus scoparius* and *G. monspessulana* seedlings were grown following the same methods described above), while seedlings of *P. radiata* and *E. globulus* were provided by the Carlos Douglas nursery (Forestal Mininco SA). Seedling height and diameter at the collar at the time of inoculation for *C. scoparius*, *G. monspessulana*, *E. globulus* and *P. radiata* were 117 ± 12.1 cm × 6.1 ± 0.6 mm, 140 ± 15.7 cm × 5.95 ± 0.4 mm, 68 ± 5.7 cm × 6.1 ± 0.7 mm, and 48.3 ± 2.9 cm × 7.0 ± 0.7 mm, respectively. For the inoculation, wounds were made on the main stem of each seedling using a cork borer (7.5 mm diam.), and equivalent-sized mycelium plugs obtained from 7–14-day old cultures on ½ PDA were placed on wounds and covered with Parafilm® for seven days. Five seedlings were inoculated with each isolate, and five additional seedlings were inoculated with sterile ½ PDA plugs as controls. The plants were arranged randomly within four blocks (host species) in the greenhouse and watered daily until the end of the trial. After 21 days, lesions were examined, measured, analysed and re-isolations were made following the methods described above.

3. RESULTS

3.1. Sample collection and isolation of fungi

A total of 47 samples of potentially diseased materials were collected during the field visits. Of these, 31 samples were collected from *C. scoparius* and 16 were from *G. monspessulana*. The most common source of damage observed for both species, at all sites visited, was mechanical, due to control practices or animal tramping. Among the symptoms of biotic origin, the most common and severe on *C. scoparius* were stem cankers (Figure 2b-c) and collar rots (Figure 2d-e) associated with wilting of the crowns (Figure 2a). Most collar rots on *C. scoparius* extended through the main stems as large cankers and had fruiting structures on them, such as pinkish sporodochia or orange/black perithecia (Figure 2f-g). White to pink or carmine red colonies of *Fusarium* species (Figure 2j) were consistently recovered from most of these samples (28 isolates from 17 samples), either from fruiting structures or from lesion margins. Less frequently, colonies typical of the Botryosphaeriaceae (Figure 2j) were recovered from stem cankers (two isolates from two samples). *Phytophthora* isolates were recovered from the roots and collars of three plants from Lautaro (isolates L-13, L-15 and L-16), associated with basal cankers and root rot. In two sites (Lautaro and Calcoy) wilting plants with pink/purple basidiocarps (Figure 2h) and vascular discolouration (Figure 2i) on the main stem were discovered. White colonies typical of Basidiomycota (Figure 2j) were consistently recovered from the fruiting structures and discoloured vascular tissues (two isolates from two plants). A range of less severe and potentially secondary symptoms and signs were found less regularly, including necrotic spots on leaves and young stems, and/or fruiting structures on decaying samples. Isolates obtained from these samples included fungi in the genera *Alternaria*, *Diaporthe* (*Phomopsis* anamorphs), *Epicoccum*, *Pleiochaeta, Pestalotiopsis*; the *Nectriaceae* (*Cylindrocarpon*-like anamorphs), and several unidentified colonies in low frequencies.

Figure 2. Main symptoms, signs and fungal isolates obtained from *Cytisus scoparius*. **a.** Yellowing of the crowns (wilting). **b-c.** Stem cankers. **d-e.** Collar rots. **f-g.** Common fruiting structures found on collar rots, **f**: orange perithecia, **g**: pinkish sporodochia. **h.** Collar with basidiocarps. **i.** Vascular discolouration. **j.** Colonies of commonly isolated fungi/oomycetes grown on ½ PDA for 7-14 days; from left to right FB033 (*Fusarium solani*), FB100 (*Fusarium sambucinum*), FB073 (*Neofusicoccum parvum*), FB016 (*Chondrostereum purpureum*) and L-16 (*Phytophthora multivora*).

Figure 3. Main symptoms, signs and fungal isolates obtained from *Genista monspessulana*. **a-c.** Defoliation, shoot blight and dieback. **d-g.** Collar rot, d-f: collar rot, g: pink perithecia on rotten collar. **g.** Vascular discolouration. **h.** Stem cankers. **i.** Colonies of commonly isolated fungi grown on ½ PDA for 7-14 days; from left to right FB132 (*Neofusicoccum parvum*), FB137 (*Chondrostereum purpureum*), FB177 (*Fusarium tricinctum s.l.*).

The most prevalent symptoms of biotic origin on *G. monspessulana* throughout all visited sites were defoliation and dieback (Figure 3a-c). Isolations from these symptoms consistently yielded fungal colonies typical of the Botryosphaeriaceae (Figure 3i; seven isolates from six samples) and Diaporthales with *Phomopsis* anamorphs (five isolates from five samples). In some cases, dieback extended through the main stem causing more severe cankers (Figure 3h), from which similar Botryosphaeriaceae colonies were obtained. A few plants with collar rot (Figure 3d-e), in some cases bearing pinkish perithecia (Figure 2f), and vascular discolouration (Figure 2g) were found in sites near the Cabrero and Santa Juana highways. Pink/violet colonies of *Fusarium* (four isolates from four samples) and unidentified white Basidiomycota (two isolates from two samples) colonies were most consistently recovered from this type of samples (Figure 3i). Similar to *C. scoparius*, isolates of *Alternaria*, *Diaporthe* (*Phomopsis* anamorphs), *Epicoccum*, *Sordaria* and several unidentified species were recovered from secondary or minor symptoms (including leaf spots and minor stem discolouration) or decaying samples. Additionally, the uredinial phase of an unidentified rust fungus was commonly observed on leaves of *G. monspessulana* across all sites. However, infection was limited to lower and older leaves and caused negligible damage to the plants, therefore, no efforts were made to include this rust in further work.

3.2. Identification of fungal strains and phylogenetic analyses

Phylogenetic analyses with the ITS, *BT* and/or *TEF1* gene regions resulted in the identification of nine isolates representing five species of plant pathogens (Figure 4). The most common *Fusarium* morphotype recovered from collar rots and cankers on *C. scoparius* was identified as *Fusarium sambucinum* based on two isolates (FB040 and FB100; Figure 4a). One isolate (FB033), representing the second most common *Fusarium* morphotype obtained from *C. scoparius*, was resolved inside the *F. solani s.str.* clade (Figure 4b). Two isolates (L-13 and L-16) identified preliminary as *Phytophthora* sp. resulted in identical (or almost identical) sequences for all gene regions, and both were resolved consistently along *P. multivora* (Figure 4c). Two isolates with typical characteristics of the *Botryosphaeriaceae*, one from *C. scoparius* (FB073) and one from *G. monspessulana* (FB132), were resolved by multi-gene phylogenetic analyses inside the *Neofusicoccum parvum s.str.* clade (Figure 4d). Two isolates were identified as *Chondrostereum purpureum* (Figure 4e), from which, one (FB016) was isolated from basidiocarps on stems of *C. scoparius*, and the second (FB137), from discoloured vascular tissues of *G. monspessulana*. A range of other isolates were incompletely identified by sequencing of ITS (Table S2), which included a range of species in *Fusarium*, *Epicoccum* and *Diaporthe*. These were not studied further based on their results on pathogenicity tests (see section 3.3 below).

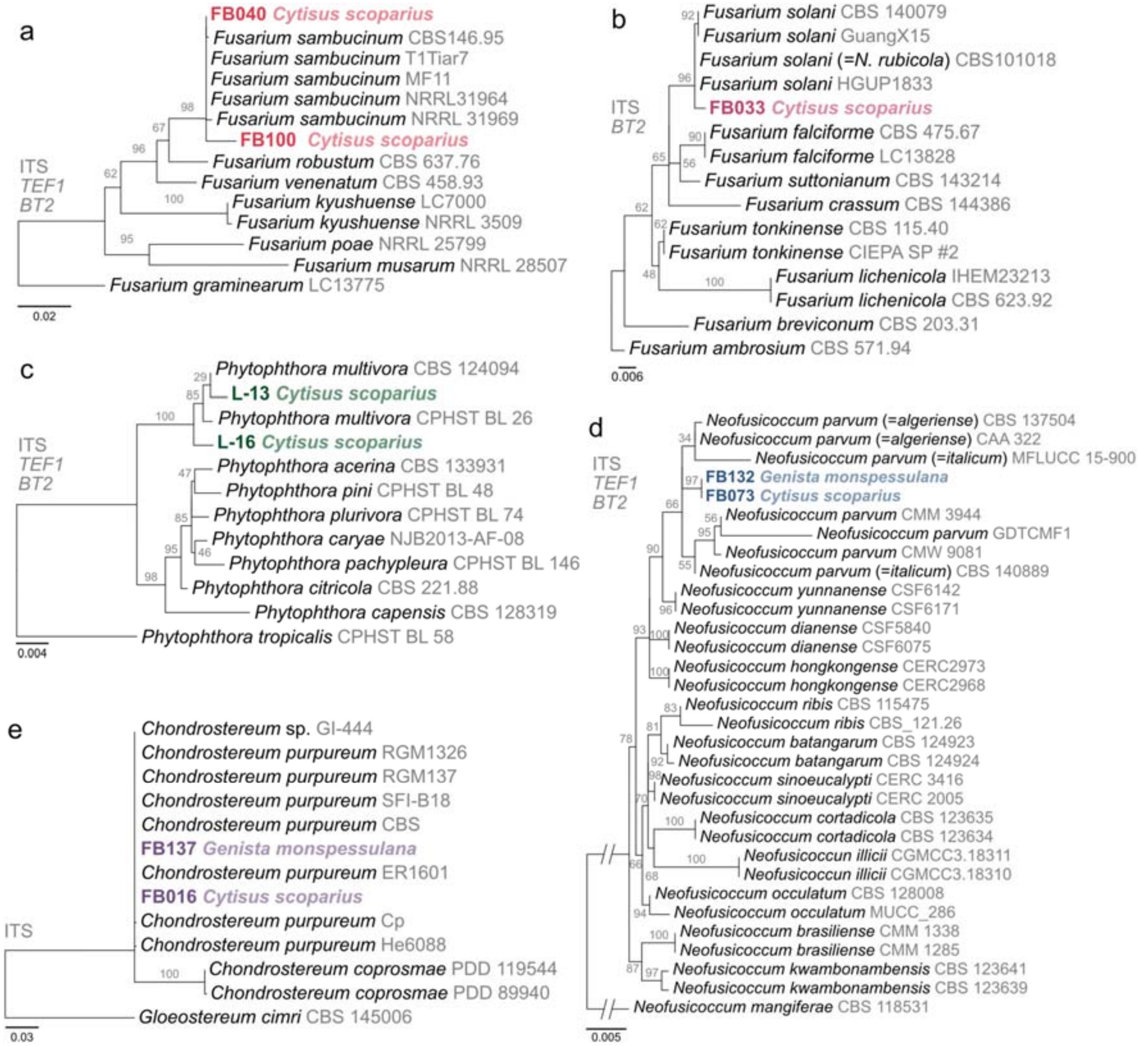

Figure 4. Maximum likelihood phylogenetic trees for main pathogenic isolates obtained from symptomatic plants of *Cytisus scoparius* and *Genista monspessulana*. **a.** ML tree for the concatenated ITS, *TEF1* and *BT* gene regions for two isolates (in bold red) residing in the *Fusarium sambucinum* species complex. **b.** ML tree for the concatenated ITS and *BT* gene regions for one isolate (in bold pink) residing in the *Fusarium solani* species complex. **c.** ML tree for the concatenated ITS, *TEF1* and *BT* gene regions for two isolates (in bold green) residing in *Phytophthora* clade 2c. **d.** ML tree for the concatenated ITS, *TEF1* and *BT* gene regions for two isolates (in bold blue) residing in *Neofusicoccum*. **e.** ML tree for the ITS gene region for two isolates (in bold purple) residing in *Chondrostereum*. Isolation hosts are indicated for each isolate in shaded colours. Scale bars indicate substitutions per site and numbers on branches indicate Superfast Bootstrap values (n=1000).

3.3. Pathogenicity trials: Koch's postulates

Thirty days after inoculation, six isolates out of the 20 tested on *C. scoparius* and four out of the 16 inoculated on *G. monspessulana* had developed significantly larger lesions than their respective controls (based on Wilcoxon rank sum test, α=0.01; Figure 5, Figure S1). The most severe symptoms on *C. scoparius* were caused by *P. multivora* (L-16, 7±5 cm lesions and wilting) and *F. sambucinum* isolates (FB100: 11.6±2.7 cm lesions and wilting; FB040: 7.4±4.2 cm lesions). In addition, *F. solani* (FB033) and an isolate of *N. parvum* (FB073) also produced evident lesions (6.1±5.2 cm and 5±5 cm, respectively). Among the isolates recovered from symptoms originally considered secondary (see 3.2), only two caused discolouration that spread from the inoculation point (Figure S1), namely isolates FB065 (*Fusarium* cf. *lateritium*) and FB025 (*Diaporthe foeniculina*). However, the size of the lesions caused by these isolates did not have significant differences with the control treatment and were therefore not considered aggressive enough and were excluded from further experiments.

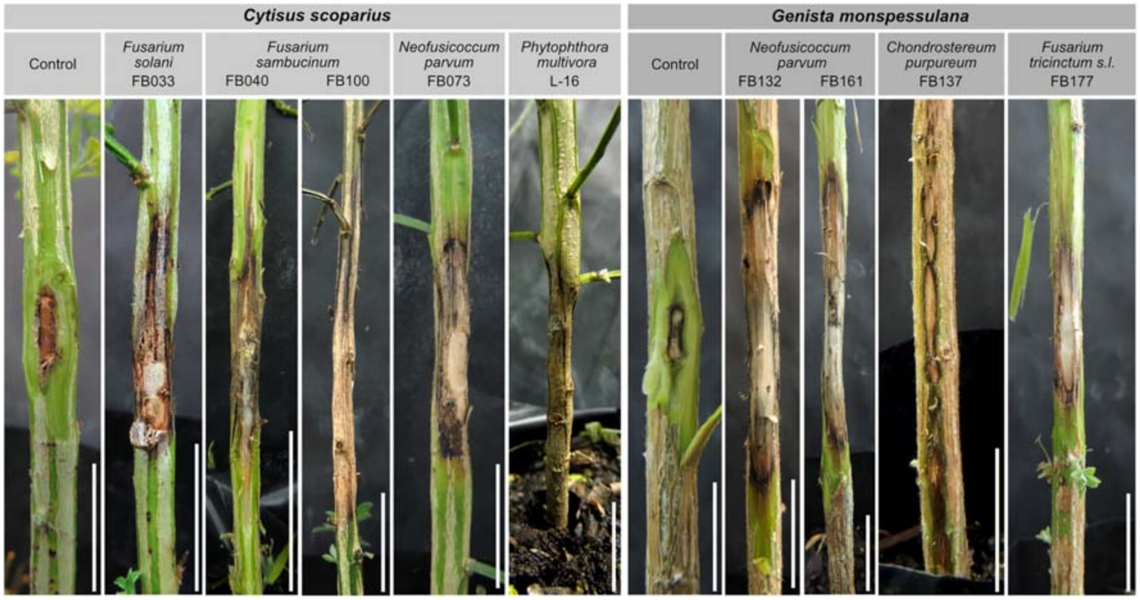

Figure 5. Lesions developed 30 days after inoculation with fungal/oomycete isolates and a control treatment on *Cytisus scoparius* (left) and *Genista monspessulana* (right). Scale bars (white): 2 cm.

On *G. monspessulana*, the most severe symptoms were caused by *N. parvum* isolates (FB132 and FB161) which caused large lesions on the stems (9.7±3,7 cm and 6.2±1.8 cm, respectively). One isolate of *Fusarium tricintum s.l.* (FB177) caused conspicuous but smaller stem lesions (3.8±1.1 cm) and seedlings inoculated with *Ch. purpureum* (FB137) resulted in severe vascular discolouration (Figure 5) with early signs of wilting. Data for isolate FB137 was excluded from statistical analyses, as it produced a different symptom (vascular discolouration throughout the stems) conflicting with the measurement strategy. Additionally, three isolates, FB166 (*Diaporthe* sp. with *Phomopsis* anamorph); and FB182 and FB187 (both identified morphologically as *F. oxysporum*), caused conspicuous small lesions on the stems. These lesions were significantly larger than the controls based on statistical analyses (Figure S1), however, considerably smaller compared to the other pathogenic isolates. Considering the symptoms from which these isolates were recovered (i.e., minor spots on leaves and stems), they were considered to represent less aggressive and/or opportunistic pathogens and were therefore excluded from further investigations.

All isolates that caused lesions were reisolated from 100% of the inoculated plants. Isolations from the control treatments yielded unidentified fungi not matching the colony morphology of any of the inoculated isolates.

3.4. Host range testing

After 21 days all isolates, except FB177 (*F. tricinctum s.l.*), reproduced the symptoms they caused in the first pathogenicity tests on their isolation host. In addition, all isolates that produced lesions on the weed species from which they were obtained, also did so on seedlings of the second weed (Figure 6). The extent of symptoms varied considerably between the different host/pathogen combinations. The most severe symptoms were caused by *P. multivora* (L-16) and *F. sambucinum* (FB100) on *C. scoparius*, causing large stem lesions (9.5±2.7 cm and 7.4±0.4 cm, respectively) and the death of plants after 8–12 days (n=3) and 10–16 days (n=2), respectively. The same *P. multivora* isolate (L-16) caused evident but considerable smaller lesions in *G. monspessulana* (2.3±0.4 cm), and no symptoms developed on *E. globulus* and *P. radiata* plants.

Fusarium sambucinum isolates FB040 and FB100 caused largest lesions on *C. scoparius* (10±3.1 cm and 7.4±0.4 cm, respectively) among the tested hosts, followed by *G. monspessulana* (5.7±1.4 cm and 5±1.4 cm, respectively). Both isolates also caused lesions in *E. globulus* and *P. radiata* (1.0–3.2 cm), and although considerably smaller than in the weeds, these were among the largest observed among all isolates in the case of *P. radiata*. *Fusarium solani* (FB033) caused lesions on all hosts, being the largest produced on both weed species (4.2±06 cm on *C. scoparius* and 3.3±0.6 cm on *G. monspessulana*), however, differences with *E. globulus* (2.7±0.7 cm) and *P. radiata* (1.9±0.3 cm) were only minor.

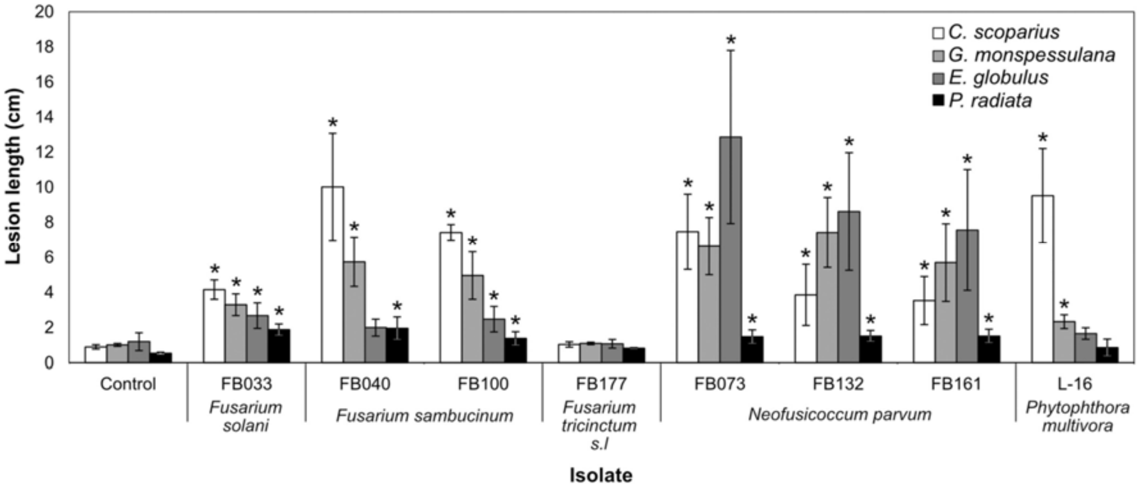

Figure 6. Lesion lengths 21 days after stem inoculation with eight isolates and a control on of seedlings of *Cytisus scoparius*, *Genista monspessulana*, *Eucalyptus globulus* and *Pinus radiata*. Bars represent standard deviation and asterisks above bars indicate significant differences ($p \leq 0.05$) with the control treatment for each respective species.

Neofusicoccum parvum isolates FB073, FB132 and FB161 reproduced the symptoms observed in the previous tests in their respective original hosts, causing large lesions on stems (6.2–7 cm in *C. scoparius* and 5–10 cm in *G. monspessulana*). All isolates caused similar yet slightly smaller lesions in the second weed species. However, the most severe symptoms (largest lesions) for all isolates were observed in *E. globulus* (FB073=12.9±4.9; FB132=8.6±3.4; FB161=7.5±3.4). Additionally, all isolates caused small yet distinctive lesions on *P. radiata* (FB073=1.5±0.4; FB132=1.5±0.3; FB161=1.5±0.4).

Chondrostereum purpureum (FB137; not presented in Figure 6) produced a similar symptom on *G. monspessulana* as observed in the previous tests, causing vascular discolouration throughout the stem and wilting. Only *E. globulus* seedlings developed a similar type of symptom, however, the discolouration only extended through a section of the stem (15–20 cm) and there were no signs of wilting. *Cytisus scoparius* seedlings inoculated with the same isolate developed small lesions (5.3±2.7 cm) on the stems, while no signs of discolouration were observed in *P. radiata*.

4. **DISCUSSION**

This study reports the occurrence of five fungal pathogens on *C. scoparius* and three on *G. monspessulana* in Chile, where both species are long-known invasives. Although all the pathogens discovered have previously been reported from Chile (Kunstmann et al. 1986; Montealegre et al. 2003; Díaz et al. 2011; González-Teuber et al. 2017; Rojo et al. 2017; Gálvez et al. 2018), none have been reported from these hosts in the country. Four of the pathogen-host combinations discovered are novel (*N. parvum* on both weed species, *F. solani* on *C. scoparius* and *F. tricinctum s.l.* on *G. monspessulana*), and two of the previously known associations had not been confirmed through pathogenicity tests (*Ch. purpureum* on *G. monspessulana* and *P. multivora* on *C. scoparius*) (Bishop 1978; Badalà 2014). In addition, pathogenicity tests showed that *F. solani*, *F. sambucinum* and *P. multivora* are capable of infecting *G. monspessulana*, even though they were not isolated from this species.

This is the first study to report pathogens on *G. monspessulana* that including from isolation to pathogenicity trials. Systematic surveys for biological control agents for this weed species that have been carried in the past (Cullen et al. 2012) focused mainly on insect pests. There are few reports of fungal pathogens (e.g., Horst 2013), of which none mention performing pathogenicity trials. These include *Chondrostereum purpureum* (Bishop 1978) and *Eutypa lata* (Cook and Dube 1989; Farr et al. 2021) in Australia and a rust species inconclusively identified as *Uromyces genistae-tinctoriae* in Australia (Cullen et al. 2012) and Argentina (Traversa et al. 2007). In this study *Ch. purpureum* was isolated from two samples with vascular discolouration and Koch's postulates fulfilled. *Eutypa lata* was not recovered from any samples collected during the current study. A rust fungus was observed during the field surveys in this study, however, because of the low severity of symptoms there were no collections made. The identity of this rust fungus remains unclear (Sanfuentes et al. 2003), however, the descriptions of *U. genistae-tinctoriae* from Argentina (Traversa et al. 2007) match well with our observations and it is reasonable to suspect it corresponds to the same species. Two new pathogens were found for *G. monspessulana*, namely *N. parvum* and *F. tricinctum s.l.*, both causing cankers and dieback (*N. parvum* with relatively high prevalence). Nevertheless, the diversity of symptoms and number of pathogens found in the study were relatively low, and additional surveys including less disturbed areas (e.g., chemical and mechanical damage) would likely reveal more.

The diversity of fungi detected on *C. scoparius* in the current study overlapped with previous reports from this host in New Zealand (Johnston et al. 1995). *Fusarium sambucinum*, the species most commonly isolated from *C. scoparius* in this study, was also reported from New Zealand (Johnston and Parkes 1994; Johnston et al. 1995).

However, in contrast to reports of it causing only mild symptoms in studies by Johnston and Parkes (1994), isolates obtained in our study were highly aggressive to *C. scoparius*. Johnston and Parkes (1984) instead focused on *F. tumidum* (not found in this study), that showed high levels of aggressiveness in their pathogenicity tests. However, recent taxonomic revisions on *F. tumidum* isolates from *C. scoparius* and *U. europaeus* from New Zealand (Laraba et al. 2021; isolates NRRL 31964 and NRRL 31969 included in our phylogenetic analyses) showed that these isolates rather represented *F. sambucinum*. These findings suggest that the fungus extensively studied for biological control, considered as *F. tumidum* in previous studies (Fröhlich et al. 2000; Morin et al. 2000; Barton et al. 2003; Prasad 2005; Bourdôt et al. 2006; Ramsfield 2006; Yamoah et al. 2008) was rather *F. sambucinum*, the same species found causing severe symptoms in Chile. A similar situation is possible in the case of *P. multivora* in Italy. Pane et al. (2005) reported *P. citricola* causing severe symptoms and death of plants of *C. scoparius*, which they confirmed with pathogenicity tests. However, these isolates were not sequenced and the study came prior to the description of *P. multivora* (Scott et al. 2009), one of the species that was hidden within the diversity of *P. citricola s.l.* (Jung and Burgess 2009). Furthermore, findings of pathogens in the Botryosphaeriaceae and/or *Fusarium* (Johnston et al. 1995), two groups of fungi with complex taxonomic delineation and considerable morphological overlap between species, can also hide further species overlaps between studies. The lack of DNA sequences for most previous studies hinders the capacity to compare more finely and distinguish which associations are truly novel.

Four of the fungal species pathogenic on the weeds also caused lesions on *E. globulus* and three on *P. radiata*. *Neofusicoccum parvum*, that caused large lesions on *E. globulus* seedlings, has been associated with canker diseases on *Eucalyptus* spp. in numerous studies (Burgess et al. 2005) and in many countries, including Chile (Ahumada 2003; Sakalidis et al. 2013). Similarly, *Ch. purpureum* has been associated with pruning wounds on different *Eucalyptus* spp. (Gadgil and Bawden 1981; Ramsfield 2006). *Fusarium sambucinum* and *F. solani* have been reported from *P. radiata* (Kunstmann et al. 1986; Dick and Dobbie 2002), associated with relatively mild symptoms in nurseries. In contrast, no records could be found for the *Fusarium* species on *E. globulus* or *N. parvum* on *P. radiata*. These hosts were included in pathogenicity trials as they were present at most of our sampling sites and where the broom species negatively affect the growth of these trees (García et al. 2007). However, considering that (i) the fungi obtained in this study caused lesions in one or both commercial species, (ii) that all of them have a wide host range, and (iii) that none of them is likely to be native to the area, their potential as a biological control agent should be addressed with caution. Nevertheless, studying pathogens on common weeds of plantation forests is also relevant in finding potential inoculum sources and reservoirs, as is likely the case for *N. parvum* on both broom species.

The results of this study overlapped considerably with the diversity of pathogens found on *C. scoparius* and *G. monspessulana* in previous studies in New Zealand (Johnston et al. 1995), Australia (Bishop 1978) and Italy (Pane et al. 2005). All the pathogens encountered have wide host ranges, including several crop species (Farr et al. 2021) which are traded internationally which has likely led to their wide geographical distribution. Nevertheless, explorations in this study and those available in the literature have predominantly been carried around forestry and agricultural plantations and nurseries, possibly biasing the pathogen diversity recorded. Future studies of diseases of *C. scoparius* and *G. monspessulana* could rather aim to explore areas where these weeds overlap with natural flora, to reveal additional candidate pathogens to be used as bioherbicides for these plants.

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Conflicts of Interest The authors declare no competing interests.

Data Availability Statement The data that support the findings of this study are openly available in the NCBI GenBank databank at https://www.ncbi.nlm.nih.gov/ (reference numbers provided in Table S2).

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