Endophytes associated with *Cirsium arvense* – a step toward understanding their role in the success/failure of *Sclerotinia sclerotiorum* as a bioherbicide

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Summary Endophytic microbial populations in *Cirsium arvense* were assessed as a first step towards testing the endophyte-enemy release hypothesis (E-ERH) of Evans (2008). Culturing and molecular methods complemented each other, increasing the diversity of the endophytic population detected. The leaves showed the greatest endophytic population diversity within the plant.

Keywords Endophytes, weed biocontrol, pathogens, DGGE, Californian thistle, creeping thistle, Canada thistle.

INTRODUCTION

The fungal plant pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary has been investigated as a potential biocontrol agent for the pasture weed Californian thistle (*Cirsium arvense* (L.) Scop.) in New Zealand. However, its biocontrol activity was found to be inconsistent. Three reactions have been observed when inoculum of the pathogen is applied: the pathogen (1) killed the plant, (2) only killed aerial tissues and the plant resprouted, or (3) had no effect on the plant (G.W. Bourdôt pers. comm.).

In 2008 Evans proposed the endophyte-enemy release hypothesis (E-ERH) whereby the presence or absence of co-evolved host plant resident microbes (endophytes) is predicted to make the plant either more resistant or more susceptible to attack by a pathogen. It was therefore hypothesised that the observed inconsistency of *Sclerotinia* to control *C. arvense* could be attributed to variation in the presence/absence of key endophytic populations.

To test this, we first had to determine which endophytes were typically present in *C. arvense* plants in New Zealand. Both culturing and molecular methods were used to achieve this. Results of this work are presented here as a first step towards testing the E-ERH.

MATERIALS AND METHODS

Cirsium arvense plants were collected from five different field locations. At each location at least five replicate plants <10 m apart were collected. At one of these locations replicate plants >20 m apart were also collected for comparison. In addition, five replicates of different plant tissues (leaves, seeds, pappus and root tissues) were sampled from each of the <10 m apart plants. Each replicate sample consisted of 3–5 mm^2 healthy tissue. Plant tissues were surface sterilised sequentially with 96% ethanol for 1 min, 6% sodium hypochlorite for 2–5 min, and 96% ethanol for 30 s followed by air drying. Surface sterilised tissues were plated immediately, while those for DNA extraction were stored in tubes with silica crystals until analysis.

The following culturing and molecular methods were employed to identify endophyte populations.

Culturing method Samples were plated directly onto 2% malt extract agar (MEA) and incubated at 20°C. Fungal and bacterial colonies that grew onto the medium were recorded and representative colonies were sub-cultured onto fresh MEA. DNA was extracted from representative pure cultures. ITS and 16S ribosomal gene sequences were generated for fungi and bacteria, respectively, using a standard PCR protocol with the fungal-specific primers ITS1F and ITS4 (Gardes and Bruns 1993), or bacterial primers 27f and 1492r (Lane 1991). Sequences obtained were subjected to a GenBank BLASTn search to determine the closest sequence-based match. Species identifications were confirmed using spore or cultural morphology where possible.

Molecular method The denaturing gradient gel electrophoresis (DGGE) technique was used to compare and identify fungal populations associated with the plant tissues. Total DNA was extracted from the plant material. Fungal group ribosomal ITS gene regions were amplified from the resulting DNA using standard touchdown PCR protocols. Primers specific for Ascomycota (ITS4A) (Viebahn *et al.* 2005) and Basidiomycota (ITS4B) were both paired with the universal primer ITS5 (White *et al.* 1990) and trialled for their ability to amplify DNA of the fungal populations present. A GC clamp was added to the ITS5 primer to aid DGGE analysis. Resulting PCR products were run through an 8% polyacrylamide gel containing a 20–60% DNA denaturing gradient (100% being 7M urea) at 90 V for 16 h (CBS DGGE system). Bands

were visualised by silver staining (Sanguinetty *et al.* 1994). To identify the microbe represented by each band position, DNA was recovered from a number of representative bands, sequenced, and the resulting sequence submitted to a GenBank BLASTn search for comparison and identification.

RESULTS AND DISCUSSION

In total, 61 genetically unique endophytic microbes were detected in this study (Table 1). Of these, 40 belonged to Ascomycota, 20 to Basiomycota and one was a bacterium.

Of the Ascomycota, only eight were detected by both culturing and molecular methods, 17 by culturing alone and 15 by DGGE alone. This result shows the two methods complement each other in the detection of overall Ascomycota diversity.

Interestingly DGGE was the only method that detected fungi belonging to Basiomycota. This is consistent with findings of other culturing based studies where Basiomycota fungi are rarely detected compared with Ascomycota (Vandenkoornhuyse *et al.* 2002), thus suggesting molecular techniques are more sensitive in detecting these fungi.

The only bacterium detected was via culturing. However, bacteria were not targeted with the DGGE technique in the current study.

The biology of the endophytic populations indicated a mix of saprobes and plant pathogens present inside the tissues. The known *C. arvense* pathogens *Sclerotinia* and *Verticillium* were both detected from healthy tissues. A number of rust fungi not previously recorded on *C. arvense*, but known to be pathogens of other host plants, were also detected. However, the known *C. arvense* rust pathogen *Puccinia punctiformis* (F.Strauss) Rohl., was not detected. Future investigations will assess if these rust fungi were actually inside the plant or attached to the surface.

Twelve fungi detected in this study have not previously been recorded in NZ (Table 1). As these were all primarily identified using sequence data and the identification was based on closest sequence similarity to those in GenBank, they would need further investigation before they could be confirmed as new identifications to New Zealand.

Of the different plant tissues, the leaf seemed to contain the greatest diversity of endophytic populations with 25 Ascomycota, 8 Basidiomycota and 1 bacterium. This was followed by the root (18:8:1), seed (12:4:0) and pappus (6:2:0).

Data collected in this study will now be analysed statistically to assess the frequency of each microbe detected, and how much the microbial populations varied between plant organs and between plants at varying distances. Candidate endophytes will be identified from this analysis, selected, and subsequently tested in glasshouse trials to determine if they have a significant impact on the pathogenic activity of *Sclerotinia* on *C. arvense*.

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REFERENCES

Evans, H.C. (2008). The endophyte-enemy release hypothesis: implications for classical biological control and plant invasions. Proceedings of the XII International Symposium on Biological Control of Weeds, eds M.H. Julien, R. Sforza, M.C. Bon, H.C. Evans, P.E. Hatcher, H.L. Hinz and B.G. Rector, pp. 20-5. (CAB International, Wallingford, UK).

Gardes, M. and Bruns, T.D. (1993). ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* 2, 113-18.

Lane, D.J. (1991). 16S/23S rRNA sequencing. *In* 'Nucleic acid techniques in bacterial systematics', eds E. Stackebrandt and M. Goodfellow, pp. 115-75. (Wiley, Chichester, UK).

Sanguinetty, C.J., Dias Neto, E. and Simpson, A.J.G. (1994). Rapid silver staining and recovery of PCR products on acrylamide gels. *Biotechniques* 17, 915-19.

Vandenkoornhuyse, P., Baldauf, S.L., Leyval, C., Straczek, J. and Young, J.P.W. (2002). Extensive fungal diversity in plant roots. *Science* 295, 2051.

Viebahn, M., Veenman, C., Wernars, K., van Loon, L.C., Smit, E. and Bakker, P. (2005). Assessment of differences in ascomycete communities in the rhizosphere of field-grown wheat and potato. *FEMS Microbiology Ecology* 53, 245-53.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In* 'PCR protocols: a guide to methods and applications', eds M.A. Innis, D.H. Golfland, J.J. Sninsky and T.J. White, pp. 315-22. (Academic Press, New York, USA).

Table 1. Endophytic microbes detected in the different tissues of *Cirsium arvense* using either culturing or the DGGE molecular technique.

Endophytic microbes from thistle (closest GenBank match)	Culturing[A]	DGGE[A]	Known biology
Fungi – Ascomycota			
Alternaria sp.	L,S	LR	saprobe
Aureobasidium pullulans (De Bary) Arnaud.	S	PS	possible plant pathogen or saprobe
Bionectria sp.	L	–	possible plant pathogen
Botryosphaeria laricina (K. Sawada) Y.Zhong.[B]	–	L	possible secondary pathogen
Cladosporium cladosporioides (Fresen.) de Vries.	–	SR	saprobe
Cladosporium sp.	L,S	–	saprobe
Codinaeopsis sp.	–	R	saprobe
Colletotrichum acutatum Simmonds	L,S,P	L	possible secondary pathogen
Cryptococcus rajasthanensis Saluja & G.S.Prasad[B]	S	–	yeast-like saprobe
Curvularia sp.	L	–	grass pathogen
Cylindrocarpon sp.	–	R	possible plant pathogen
Davidiella tassiana (De Not.) Crous & U.Braun	–	S	saprobe
Epicoccum nigrum Link	L	L,S,R,P	saprobe
Eudarluca caricis (Fr.) O.E.Erikss.	–	L,S	rust mycoparasite
Exophiala sp.	–	R	saprobe
Fusarium cortaderiae O'Donnell, T.Aoki, Kistler & Geiser	L	–	possible plant pathogen
Fusarium oxysporum Schltdl.	R	R	possible plant pathogen
Fusarium solani (Mart.) Sacc.	R	–	possible plant pathogen
Fusarium sp. 1.	L,R	–	possible plant pathogen
Hypocrea/Trichoderma	L	–	possible fungal pathogen
Leptodondium orchidicola Sigler & Currah[B]	L,R	–	saprobe
Lewia infectoria (Fuckel) Barr & Simmons	L	L	saprobe
Neonectria radicola (Gerlach & L.Nilsson) Mantiri &. Samuels[B]	R	–	possible plant pathogen
Nigrospora oryzae (Berk. & Broome) Petch	L	–	saprobe
Phaeococcomyces chersonesos Bogom. & Minter[B]	–	R	saprobe
Phoma exigua Desm.	–	L,R	possible secondary pathogen or saprobe
Phoma sp.	R	–	possible secondary pathogen or saprobe
Phomopsis theicola Curzi[B]	L	–	possible secondary pathogen or saprobe
Phomopsis sp.	L	–	possible secondary pathogen or saprobe
Pichia fermentans Lodder	–	L	yeast
Plectosphaerella cucumerina (Lindf.) Gams	–	R	plant pathogen
Plectosphaerella sp.	–	L	possible plant pathogen
Preussia isomera Cain	L	–	coprophilous saprobe
Preussia sp.	L	L,R,P	coprophilous saprobe
Pyrenochaeta terrestris (Hansen) Gorenz, Walker & Larson	–	R	onion path/soil saprobe
Sclerotinia sp.	L	–	plant pathogen
Stachybotrys echinata (Rivolta) G.Sm.	–	L	saprobe
Stemphylium sp.	–	S	saprobe
Verticillium dahliae Kleb.	S,R	L,P	plant pathogen
Xylariaceae sp.	L,S,P	–	saprobe

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Endophytic microbes from thistle (closest GenBank match)	Culturing[A]	DGGE[A]	Known biology
Fungi – Basidiomycota			
Bovista plumbea Pers.	–	R	saprobe
Ceratobasidium sp.	–	R	possible plant pathogen
Cyathus stercoreus (Schwein.) De Toni	–	S	saprobe
Exidiopsis sp.	–	R	saprobe
Flammulina velutipes (Curtis) P.Karst.	–	R	saprobe
Gloeoporus dichrous (Fr.) Bres.	–	L	saprobe
Kuehneromyces rostratus Singer & A.H.Smith[B]	–	S	saprobe
Langermannia gigantean (Batsch) Rostk.	–	L	saprobe
Limonomyces roseipellis Stalpers & Loer.	–	P	saprobe
Melampsora laricis-populina Kleb.	–	L	plant pathogen (rust)
Mycena sp. / *Nolanea* sp.	–	R	saprobe
Panaeolus sphinctrinus (Fr.) Quél.	–	R	saprobe
Peniophora pini (Fr.) Boidin / *aurantiaca* (Bres.) Höhn. & Litsch.[B]	–	L,P	saprobe
Pleurotopsis longinqua (Berk.) E. Horak	–	S,R	saprobe
Pleurotus pulmonarius (Fr.) Quél.	–	L	possible plant pathogen
Puccinia chrysanthemi Roze / *carduorum* Jacky[B]	–	L	plant pathogen (rust)
Puccinia cnici-oleracei Pers. ex Desm.[B]	–	L	plant pathogen (rust)
Rogersella griseliniae (G. Cunn.) Stalpers / *Hyphodontia crustosa* (Pers.) J.Erikss.	–	R	saprobe
Schizophyllum commune FR.	–	L	saprobe
Tomentellopsis submollis (Svrcek) Hjortstam[B] / *bresadoliana* (Sacc. & Trotter) Julich & Stalpers[B]	–	S	saprobe
Bacteria			
Pantoea sp.	L,R	–	unknown

[A] Letter depicts where this microbe was found in the plant for the method used; L = leaf, R = root, P = pappus and S = seed. - depicts the microbe was not detected anywhere in the plant using this method.
[B] Not recorded in New Zealand.