Distribution of *Puccinia punctiformis* in above-ground tissue of *Cirsium arvense* (Californian thistle)

Caitlin Henderson[1], Michael Cripps[2] and Seona Casonato[1,*]

[1]*Department of Pest-management and Conservation, Lincoln University, PO Box 5084, Lincoln 7647, New Zealand*
[2]*AgResearch, Lincoln Research Centre, Lincoln, New Zealand*
** Corresponding author: seona.casonato@lincoln.ac.nz*

Abstract *Cirsium arvense* (Californian thistle) is a problematic weed in agricultural systems throughout New Zealand and the rust fungus *Puccinia punctiformis* is a potential biological control agent for this weed. *Puccinia punctiformis* can systemically infect thistles but the movement of the pathogen *in planta* is not fully understood. This research determined the level of infection *in planta* caused by *P. punctiformis* at a single time point. The concentration of *P. punctiformis* DNA *in planta* was determined to ascertain the location of the fungus within naturally field-infected *C. arvense*. Quantitative polymerase chain reaction was undertaken on above-ground symptomatic and asymptomatic *C. arvense* tissue at various locations within leaves (top, middle and bottom) and the main stem. All *C. arvense* shoots had detectable amounts of *P. punctiformis* but the concentration was 100× greater in symptomatic compared with asymptomatic shoots. In general, the concentration of fungus progressed up the leaves with a significant effect between locations ($P<0.001$). *Puccinia punctiformis* was found *in planta* but broadscale disease of *C. arvense* does not occur and the reason for this is unknown.

Keywords asymptomatic infection, symptomatic, rust fungus, Californian thistle

INTRODUCTION

Cirsium arvense, commonly known as Californian thistle, is a problematic weed in agricultural systems throughout New Zealand and is invasive in temperate regions around the world. *Puccinia punctiformis* is a highly host-specific rust fungus that only infects *C. arvense*, and completes its entire lifecycle on this one host plant. The rust was inadvertently introduced to New Zealand, likely along with its host plant, sometime prior to 1881 (Cripps et al. 2011). Therefore, *P. punctiformis* is a potential biological control agent and can systemically infect *C. arvense. Puccinia punctiformis* overwinters in the roots of its host but it is not completely understood whether the fungus directly kills them (Berner et al. 2013). In spring, some shoots emerge that are systemically infected, which results in the pathogen killing the shoots. This process subsequently reduces root biomass and potentially diminishes the abundance of thistle shoots (French & Lightfield 1990; Berner et al. 2013).

The movement of *P. punctiformis in planta* is unknown; however other *Puccinia* spp. have been studied. *Puccinia thlaspeos* grows down the leaves of its host and into the petiole into new leaves of the plant where it moves through the meristematic area, and overwinters (Kropp et al. 1996). Understanding how *P. punctiformis* moves *in planta* and determining where the pathogen is concentrated will give a better understanding of the host–pathogen interaction, and potentially lead to methods for manipulation of this pathogen to optimise biocontrol of *C. arvense.*

MATERIALS AND METHODS

DNA extraction

Nine individual thistle shoots were collected from a dairy farm located near Springston Canterbury (longitude 172.40076, latitude -43.679973, elevation 10 m) at the end of March 2017, where *P. punctiformis* was present. Six plants visibly systemically infected with *P. punctiformis* (etiolated shoots and leaves bearing uredinia) and three plants not visibly infected were collected. Samples of *P. punctiformis* only were also collected for positive controls. For each plant, 1-cm sections were cut from the main stem at the top, middle and bottom. One leaf attached to the main stem from each of the locations, top, middle and bottom, was processed. Each leaf was cut into three sections approximately 10 mm × 20 mm (Fig. 1). Three subsamples of each section were taken. The sections of the plant were surface sterilised in 10% aqueous household bleach solution for 30 s, then 2 × 30 s in water, to remove any external source of the rust. DNA was extracted using a 10% suspension of anion exchange resin (Chelex® 100; BioRad, CA, USA) in water. The plant material was ground in liquid nitrogen and placed into 1.7-mL tube containing 300 µL of 10% Chelex suspension. Tubes were vortexed 3 times for 10 s and incubated at 100°C for 10 min on a heating block; this was repeated twice. Tubes were removed and centrifuged for 10 min at 13,000 g and the supernatant collected. The DNA concentration was measured using a NanoDrop Lite spectrophotometer (Thermo Scientific). Samples were diluted with sterile Millipore water to 30–50 ng/µL.

Quantitative PCR

This work was done using quantitative polymerase chain reaction (qPCR) to determine the concentration of *P. punctiformis* DNA in different areas of the plant (Dworkin et al. 2002; Peirson & Butler 2007). All qPCR cycles were undertaken in a Rotor-Gene Q (QIAGEN) machine. Reactions were undertaken in 10-µL volumes.

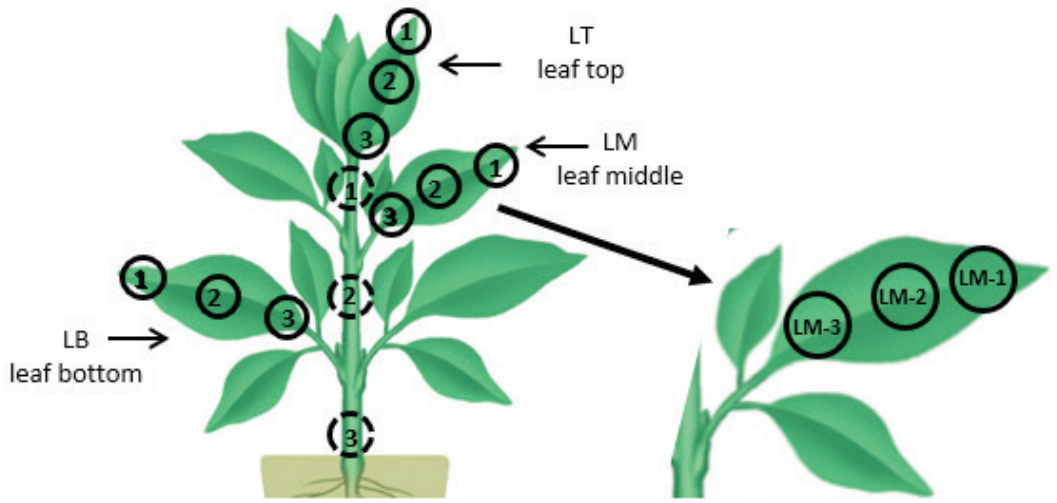

Figure 1 Sections of a *Cirsium arvense* plant (Californian thistle) for qPCR. Solid circles indicate leaf samples; dashed circle, stem samples. Numbers 1–3 indicate the position of the samples on the shoot or leaf: 1 = tip of the leaf; 2 = middle of leaf; and 3 = end of the leaf closest to stem. For the shoot: 1 = top of the stem; 2 = middle; and 3 = bottom. For each plant, there were 12 sections in total, 3 for each of the 3 leaves and 3 for the shoot.

Each tube contained 10–20 ng DNA, 5 µL SYBR Green (QIAGEN), 0.13 µM of forward primer PuncF (5' ACCCCTAACACTTGTTTGTG 3') and reverse primers, PuncR (5' GCACTAAAGGTATTGGCAAG 3') (Berner et al. 2015a) and PCR H_2O. Four standards of the rust fungus were used with a known concentration of 1.1 ng, 11 ng, 36.25 ng and 72.5 ng for each reaction. In addition, each reaction included two negative standards, one with water and one with *C. arvense* DNA containing no rust. The qPCR cycle was, one cycle at 94°C for 10 min, 35 cycles of 94°C for 10 s, 58°C for 20 s and 72°C for 30 s, and 1 cycle of final extension 72°C for 2 min. The melting curve of each sample was checked to validate amplicon specificity. All melt curves were analysed and gave a single consistent peak at 80.5°C. Amplicon efficiency was determined by analysis of the standard curve which was always >0.9 (Taylor et al. 2019). Standard curve validation occurred for each individual reaction and those reactions that did not conform to a standard curve were not included in the results. In addition, if there were any ambiguities, gel electrophoresis on the products were ran to ascertain if there was a single band, 1% agarose (BioRad) run at 90 V for 45 minutes in Tris-acetate-EDTA buffer then placed in ethidium bromide for 15 minutes and rinsed in H_2O for 10 minutes and visualised under ultraviolet light to confirm *P. punctiformis* amplification.

Statistical analyses and calculations

Genstat 18th Edition statistical software (VSNI, Hemel Hempstead, UK) was used to analyse the data. Prior to analyses, data were transformed (ln (x+1)) to conform with the assumption of normality. The concentration of fungal DNA at the different sampled locations and sublocations were examined using two-way analysis of variance (ANOVA), comparing the main effects of location and sublocation, and their interaction. Separate two-way ANOVAs were carried out for symptomatic and asymptomatic plants. Individual replicate plants were included as a blocking factor (n=6 for symptomatic, and n=3 for asymptomatic). Differences between the mean values for locations and sublocations were subsequently compared using Fisher's least significant difference (LSD, α=0.05) test.

RESULTS

Symptomatic shoots

There was a significant effect of location (P<0.001) indicating that the concentration of the rust fungus progressively increased in the leaves from the bottom to the top of the thistle shoot. The concentration of the fungus was not significantly different between sublocations (within leaves or stem) on the thistle shoot (P=0.695). There was a significant interaction between location and sublocation (P=0.002). At the top of the shoot, the concentration decreased from the tip (highest concentration) to the base (lowest concentration) of the leaves, whereas the opposite occurred at the middle of the shoot, where the concentration increased from the tip (lowest concentration) to the base (highest concentration) of the leaves (Fig. 2).

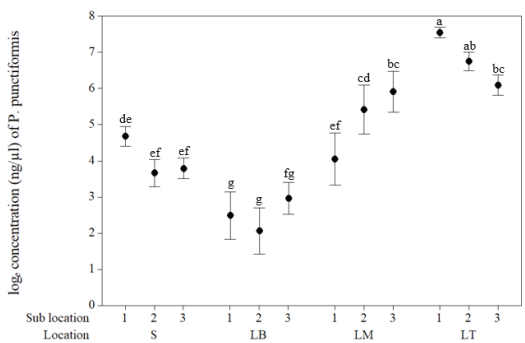

Figure 2 Average (±SEM) *Puccinia punctiformis* concentration (ng/µL) *in planta* across six symptomatic shoots of *Cirisium arvense* collected from a site in Canterbury, New Zealand. Locations: LB = bottom of leaf; LM = middle of leaf; LT = tip of leaf; S = stem. Sublocations 1–3 are sections within the location. 1 = top; 2 = middle; 3 = base of stem. Different letters above error bars indicate values that are significantly different according to Fisher's Least Significant Difference (LSD, α=0.05).

Asymptomatic shoots

The pattern of the rust fungus concentration was similar for asymptomatic plants (Fig. 3), with a significant effect of location (P<0.001), no effect of sublocation (P=0.785), and a significant location by sublocation interaction (P=0.006). However, the highest concentration was in the middle leaves, followed by the basal leaves, and then the top leaves (Fig. 3). At the middle of the shoot, the concentration decreased from the tip (highest concentration) to the base (lowest concentration) of the leaves, whereas the opposite occurred at the bottom of the shoot where the concentration increased from the tip (lowest concentration) to the base (highest concentration) of the leaves (Fig. 3). While the pattern of rust fungus concentration was similar for symptomatic and asymptomatic shoots, on average the concentration of *P. punctiformis* was 100× greater in symptomatic compared to asymptomatic shoots.

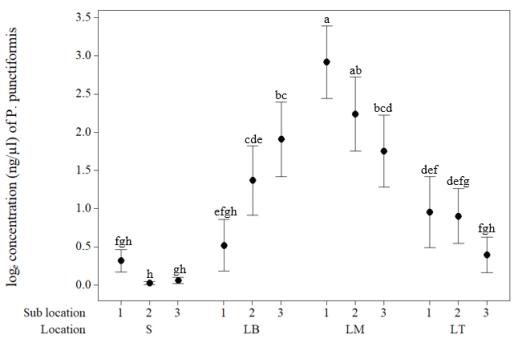

Figure 3 Average (±SEM) *Puccinia punctiformis* concentration (ng/µL) *in planta* across three asymptomatic shoots of *Cirisium arvense* collected from a site in Canterbury. Locations: LB = bottom of leaf; LM = middle of leaf; LT = tip of leaf; S = stem. Sublocations 1–3 are sections within the location. 1 = tip; 2 = middle; 3 = base of stem. Different letters above error bars indicate values that are significantly different according to Fisher's Least Significant Difference (LSD, α=0.05).

DISCUSSION

All sampled shoots, symptomatic and asymptomatic, had detectable amounts of *P. punctiformis*, although the concentration of the fungus was on average 100× greater for symptomatic shoots. In symptomatic shoots, the highest concentration of *P. punctiformis* was at the top of the stem, and progressively decreased towards the shoot base. The lower amounts of *P. punctiformis* in the bottom leaves could be due to the lower leaves being older and beginning to senesce (Green & Bailey 2000). *Puccinia punctiformis* is a biotrophic fungus, requiring live plant tissue to survive and grow. The greater concentration of the fungus at the growing tips of the shoot and leaves is likely a function of the fungus tracking plant growth and accumulating in the tissues with greatest nutritional value.

The fungus is primarily concentrated in the bundle sheath cells surrounding the vascular tissue during the early stages of pycnial development. Then, at the later uredinial stage of development, the fungus enters the vascular tissues, including both the xylem and phloem (Baka & Lösel 1992). In this study, we examined the concentration of *P. punctiformis*, at the later uredinial stage where the fungus would have been throughout the vascular tissues, allowing for efficient access to the most nutrient rich tissue of the actively growing stem and leaves. If we had examined the fungus at the earlier pycnial growth stage possibly it would have exhibited a more uniform concentration throughout the shoot.

In asymptomatic shoots, the highest concentration was found in the middle leaves. It is unclear why the fungus would have greater concentration in this portion of the shoot, but it seems reasonable that the plant has defensive mechanisms limiting or regulating the concentration and movement of the pathogen. Frantzen and van der Zweerde (1994) reported varying degrees of quantitative resistance to adventitious shoot bud infection by *P. punctiformis*, but otherwise there is little information on possible resistance mechanisms used by the plant against this pathogen. Genetic diversity of *C. arvense* is known to be high, even

within populations (Bodo Slotta et al. 2010), and it is likely that most or all of the individual shoots collected for this study were different genotypes. Genotypes may vary with respect to morphological or physiological mechanisms that restrict fungal growth and movement *in planta*.

The timing of sampling may have some influence on the results reported here, particularly with regard to the detection of asymptomatic disease. For the symptomatic shoots, it is clear that they follow the known disease cycle, where systemically infected shoots arise from infected roots at any time during the growing season (Berner et al. 2013). However, for the asymptomatic shoots, it is unclear if the fungus entered the shoots via shoot to shoot spread of spores, or they arose asymptomatic from infected roots. Based on observations from previous experimental work, we think it is likely that the presence of asymptomatic disease is the result of infection from germinating teliospores on plant tissue (i.e. shoot to shoot spread of spores). Previously, it was demonstrated that infection is rarely (if ever) inoculum limited, but rather timing of infection is important, and that cooler autumn temperatures promote heavy dew that provide necessary moisture for the germination of teliospores. During these previous experimental inoculations, disease was never evident on the inoculated shoots, but rather disease was observed on new shoots arising during spring, in the vicinity of the autumn inoculations (Cripps et al. 2014). Thus, it is possible that the asymptomatic disease detected here represent a cohort of shoots with latent infection that is more likely to occur in autumn, with potential disease expression in spring. In our study, only three asymptomatic shoots were collected, and all tested positive for *P. punctiformis*. The frequency of asymptomatically diseased shoots in New Zealand populations of *C. arvense* is uncertain, and the importance of asymptomatic disease for the survival and spread of the pathogen is uncertain. Furthermore, the effect of asymptomatic infection on the plant is also unclear. Berner et al. (2015b) reported up to 60% of asymptomatic shoots were positive for *P. punctiformis*, and suggested that asymptomatic disease may have contributed to the decline in thistle shoot population that was observed. The importance of asymptomatic infection for the survival of the fungus, and effects on the host plant remain to be determined, and warrant further study.

CONCLUSIONS

In this study, the concentration of *P. punctiformis* in different locations within shoots of *C. arvense* was determined using qPCR at a point in time (late March, autumn) that is known to be conducive for infection via teliospores. From this study, we concluded that, in general, there is more *P. punctiformis* present at the top of the plant compared to the bottom and the stems. Shoots that showed no visible symptoms had low concentrations of the rust fungus. Monitoring plants that are asymptomatic and sampling them throughout the life cycle of the plant may indicate whether the plant has a defence mechanism that limits the pathogen growth and movement, or whether symptoms will eventually express once the fungus reaches a critical concentration. More plants should be sampled along with the roots of plants with visual symptoms and plants without symptoms. The reason as to why there are different levels of *P. punctiformis* within a plant warrants further investigation throughout the season. This is a first step towards a better understanding of the *in planta* movement and concentration of the rust that might improve how the potential effect of the pathogen is monitored in to relation to the weed population dynamics.

REFERENCES

Baka Z, Lösel D 1992. Infection of vascular tissues by the autoecious rusts *Puccinia punctiformis* and *Puccinia lagenophorae*: a cytological study. Physiological and Molecular Plant Pathology 40: 411–421.

Berner D, Smallwood E, Cavin C, Lagopodi A, Kashefi J, Kolomiets T, Pankratova L, Mukhina Z, Cripps M, Bourdôt G 2013. Successful establishment of epiphytotics of *Puccinia punctiformis* for biological control of *Cirsium arvense*. Biological Control 67: 350–360.

Berner D, Smallwood E, Vanrenterghem M, Cavin C, Michael J, Shelley B, Kolomiets T, Pankratova L, Bruckart W, Mukhina Z 2015a. Some dynamics of spread and infection by aeciospores of *Puccinia punctiformis*, a biological control pathogen of *Cirsium arvense*. Biological Control 88: 18–25.

Berner DK, Smallwood EL, Cavin CA, McMahon MB, Thomas KM, Luster DG, Lagopodi AL, Kashefi JN, Mukhina Z, Kolomiets T, Pankratova L 2015b. Asymptomatic systemic disease of Canada thistle (*Cirsium arvense*) caused by *Puccinia punctiformis* and changes in shoot density following inoculation. Biological Control 86: 28–35.

Bodo Slotta TA, Foley ME, Chao S, Hufbauer RA, Horvath DP 2010. Assessing genetic diversity of Canada thistle (*Cirsium arvense*) in North America with microsatellites. Weed Science 58: 387–394.

Cripps M, Gassmann A, Fowler SV, Bourdôt GW, McClay AS, Edwards GR 2011. Classical biological control of *Cirsium arvense*: Lessons from the past. Biological Control 57: 165–174.

Cripps M, Bourdôt G, Saville DJ, Berner DK 2014. Success with the rust pathogen, *Puccinia punctiformis*, for biological control of *Cirsium arvense*. Paper presented at the XIV International Symposium on Biological Control of Weeds, 2-7 March, Kruger National Park, South Africa.

Dworkin L, Gibler T, Van Gelder R 2002. Real-time quantitative polymerase chain reaction diagnosis of infectious posterior uveitis. Archives of Ophthalmology 120: 1534–1539.

Frantzen J, van der Zweerde W 1994. Quantitative resistance of *Cirsium arvense* to root bud infection by *Puccinia punctiformis*. Biocontrol Science and Technology 4: 223–228.

French R, Lightfield A 1990. Induction of systemic aecial infection in Canada thistle (*Cirsium arvense*) by teliospores of *Puccinia punctiformis*. Phytopathology 80: 872–877.

Green S, Bailey K 2000. Effects of leaf maturity, infection site, and application rate of *Alternaria cirsinoxia* conidia on infection of Canada thistle (*Cirsium arvense*). Biological Control 19: 167–174.

Kropp B, Hansen D, Flint K, Thomson S 1996. Artificial inoculation and colonization of dyers woad (*Isatis tinctoria*) by the systemic rust fungus *Puccinia thlaspeos*. Phytopathology 86: 891–896.

Peirson SN, Butler JN 2007. Quantitative Polymerase Chain Reaction. In: Rosato, E. ed. Circadian rhythms: methods and protocols. Methods in Molecular Biology Series Vol. 362. pp. 349-362.

Taylor SC, Nadeau K, Abbasi M, Lachance C, Nguyen M, Fenrich J 2019. The ultimate qPCR experiment: producing publication quality, reproducible data the first time. Trends in Biotechnology 37(7): 761–774.