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Puccinia araujiae, a promising classical biocontrol agent for moth plant in New Zealand: Biology, host range and hyperparasitism by *Cladosporium uredinicola*

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HIGHLIGHTS

- The life cycle and biology of *Puccinia araujiae* was investigated.
- This rust produces galling on *Araujia hortorum*, a problematic weed in New Zealand.
- It is sufficiently specific to be used as a biocontrol agent.
- A protocol was developed for the long term preservation of its teliospores.
- *Puccinia araujiae* is a new host for *Cladosporium uredinicola*.

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ABSTRACT

The rust fungus *Puccinia araujiae* is proposed as a biological control agent for moth plant (*Araujia hortorum*) in New Zealand. This pathogen completes its life cycle on this host, it has the capacity of damaging it by producing premature foliage senescence and defoliation, and, it is only known from members of the Oxypetalinae (Apocynaceae). *P. araujiae* was found to be heavily hyperparasitised by the fungus *Cladosporium uredinicola* in the field in Argentina. The mode of action of this hyperparasite was investigated and efforts are currently being made to completely eliminate it from a culture of the rust through a combination of superficial disinfection and multiple sequential inoculations. A protocol was developed for long term storage of teliospores of the rust at very low temperatures. Stored spores were shown to maintain their ability to germinate and produce infective basidiospores for up to 12 months. The possible effect of the hyperparasite on the performance of the rust as a biological control agent, should it be introduced into New Zealand, is discussed.

1. Introduction

The scientific name of the climbing vine known in New Zealand as "moth plant", and as "tasi" in Argentina, is controversial among botanists. Most floristic works in Argentina refer to it as *Araujia hortorum* E. Fourn., a species different from *A. sericifera* Brot. (Villamil and Barton, 2009) and this is how it will be considered here. *A. hortorum* is native to South America where it is greatly appreciated for its medicinal properties, for its edible fruit, and as an ornamental species (Bayón and Arambarri, 1999). It was first recorded in New Zealand in 1888 (Webb et al., 1988), where it was originally introduced as an ornamental. Here, it is known as "moth plant", in reference to the fact that insects, particularly moths and butterflies, can be trapped within the flowers. Due to its capacity to climb over and smother other vegetation such as shrubs and small trees, moth plant has the potential to cause substantial environmental damage and has been targeted for biological control (Waipara et al., 2006).

Previous studies identified the rust *Puccinia araujiae* Lév. (Ann. Sci. Nat., Bot., **3**: 69. 1845, as "*araujae*") as the most promising pathogen to be considered for introduction as a biological control agent (Delhey et al., 2011; Kiehr et al., 2011; Waipara et al., 2006). Preliminary investigations had shown that *P. araujiae* could

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infect *A. hortorum* from all four populations in Argentina and all three populations from New Zealand that were tested (Delhey et al., 2009). From the literature the rust is known to cause disease on other species of Apocynaceae, all belonging to the same subtribe (Oxypetalinae) as the target host (Lindquist, 1982).

During the course of our studies, the rust was found to be heavily hyperparasitised by another fungus in the field. In this paper, additional information is provided on the biology, host range and experimental manipulation of both the pathogen and its mycoparasitic *Cladosporium* species.

2. Materials and methods

2.1. Field surveys and collection of rust isolates

Field surveys to locate and collect isolates of the rust *P. araujiae* from *A. hortorum* populations were conducted between autumn 2012 and spring 2013, covering a large area of the geographical distribution of the plant species in Argentina. Locations from which the rust had been collected previously were visited (Delhey et al., 2011; Lindquist, 1982; Waipara et al., 2006). Whenever a rust-infected plant population was encountered, infected material was collected and placed between pieces of newspaper in a plant press for further study in the laboratory. A GPS reading was taken to record the site location. A couple of sites were visited more than once in different seasons. Whenever possible, fruits of *A. hortorum* were also collected and placed in paper bags as a source of seeds from which to grow plants for future experiments.

2.2. Sourcing and maintenance of plants

A. hortorum plants from several different locations in Argentina were grown from seed collected during field trips. Seeds were superficially disinfected by immersion in aqueous Sodium hypochlorite solution (3:10) for 2 min followed by a rinse in distilled water. To promote germination, seeds were then placed in Petri dishes lined with wet cotton wool and filter paper and left on a bench in the laboratory at room temperature for 7–10 days. At hypocotyl emergence, seedlings were planted individually into potting mix in plastic seedling trays. At the four-leaf stage, plantlets were transferred to 10-cm-diameter plastic pots containing a 1:1 mixture of potting mix and local soil. Plants were kept until needed on a glasshouse bench (temperature range 16–26 °C).

2.3. General rust inoculation methodology

An adaptation of the "leaf disc method" (Morin et al., 1993) was used for all inoculation experiments. Small (*ca.* 2.5 × 2.5 mm) square discs bearing mature telia (4–6 weeks after inoculation) were cut with a scalpel from diseased *A. hortorum* plants and placed onto the surface of 1% water agar (WA) in 9 cm diameter Petri dishes. Dishes containing 35–40 telial discs were inverted (after the lid was removed) over young, healthy, recently pruned plants, at a distance of *ca.* 5 cm from the uppermost leaves, by placing them on a wire framework attached to the roof of the inoculation chambers. Inoculation chambers consisted of cube-shaped polyethylene boxes with the floor lined with water-soaked newspaper to provide around 100% relative humidity (RH). Inoculated plants were sprayed with a fine mist of water before placing in the chamber. After 48 h plants were removed and kept in controlled environment cabinets at around 75% RH, 18–20 °C and a 12-h dark/12-h light (fluorescent, 1400 l) regime. This inoculation procedure was used in all experiments unless otherwise stated.

2.4. Isolation and maintenance of rust isolates

A rust culture was established from the only isolate found to have viable spores, that from Laguna de Gómez, close to the city of Junín, Buenos Aires province, Argentina (Fig. 1). However, it was observed that telia collected from the field, and indeed those obtained from artificial inoculations, were heavily hyperparasitised. Several procedures were investigated to eliminate the hyperparasite and obtain a clean culture. Of these, the following was selected: plant material bearing mature telia was immersed in aqueous Sodium hypochlorite solution (3:10) for two minutes and dried on sterile absorbent paper. Material was then cut with a scalpel into *ca.* 2.5 × 2.5 mm square sections and placed onto WA in Petri dishes. These were immediately inverted over healthy vigorously growing *A. hortorum* plants in inoculation chambers as explained above. In order to have a source of spores for other experiments, monthly sub-culturing onto fresh plants was undertaken.

The above described procedure allowed for a decrease in the level of hyperparasite infection but did not eliminate it from the system. With the purpose of achieving complete elimination, pustules were collected from artificially inoculated plants, treated as above, and incubated at room temperature for 24 h to promote germination. Then, 12 clean, fully germinated pustules were selected and placed in four Petri dishes containing WA (three pustules in each) and each dish inverted over a single *A. hortorum* plant for 24 h. After this period, the dishes were removed and the plants left in the humid chamber for another 24 h. After that they were incubated until the development of rust pustules, as explained in the general methodology section. These "first generation" plants were thereafter kept in a separate, disinfected cabinet. The dishes that had been inverted over these plants were monitored for a week. If the hyperparasite was seen to develop during that time, the plant over which the corresponding dish had been inverted was removed from the cabinet. After a month this process was repeated using pustules from remaining plants to produce "second", "third" and "fourth" generation plants. As after four generations the culture was still getting contaminated by the hyperparasite, the process was continued to obtain "eighth generation"

Fig. 1. Location of all sites visited during surveys (white dots) and of the two (black squares) at which the rust was found and collected in Buenos Aires province, Argentina.

plants. The latter were expected to be free from the hyperparasite and used as a source to start and build up a new, clean culture.

Artificially inoculated leaves of *A. hortorum* bearing telia of the rust are deposited in the Herbarium of the Universidad Nacional del Sur (1502 BBB).

2.5. Isolation and Identification of the hyperparasite

The hyperparasite was isolated on potato dextrose agar (PDA) by transfer of fructifications found growing over the rust pustules with a fine sterile needle. Fructifications were mounted in water for microscopic observation and measurements.

An isolate of this fungus is deposited in the Herbarium of the Universidad Nacional del Sur (1503 BBB).

2.6. Determining the hyperparasite's mode of action

In order to investigate the mode of action of the hyperparasite, hyperparasitised rust pustules were sectioned by hand using a razor blade, and the resulting slices were mounted in water on microscope slides. Some of these sections were stained with Phloxine B, and all were examined under a compound microscope. In addition, samples from the same pustules were taken with a fine needle, placed in water in single concave slides and kept at room temperature overnight before microscopic examination.

2.7. Short and long-term storage of the rust

Preliminary experiments found that teliospores could be stored at low temperatures in both the fridge and the freezer for a short period of time without losing their viability and pathogenicity. In order to investigate this further the following procedure was followed: Square discs (2 × 2 mm) were cut from telia-bearing leaf blades with a sharp razor blade, trying to eliminate as much underlying plant material as possible. Ten discs were separated and used to estimate the germination percentage at the starting point of the experiment as explained below. The other discs were then placed on a filter paper in a small plastic container with silica gel and left on a laboratory bench for 48 h at room temperature. After this procedure, they were subjected to one of the following cold treatments: (1) refrigerator (8 °C): twelve plastic containers with ten telial discs each and (2) freezer (−70 °C): twelve plastic containers with ten telial discs each. One container from the freezer and one from the fridge were recovered monthly for twelve months. Germination of teliospores (regarded as an indication of viability) was estimated at the beginning of the experiment from freshly collected material and later at the end of each storage period. To promote germination, telial discs were placed on WA contained in Petri dishes. Dishes were kept at room temperature on a laboratory bench for 24 h after which the germination of each disc was estimated according to the following subjective visual scale: **0**: no germination; **1**: 1–20% germination; **2**: 21–60% germination; **3**: >60% germination. Finally, the average rating score of all the telial discs per dish was calculated. The pathogenicity of the recovered spores was determined by inverting the same Petri dishes used for the scoring process over healthy *A. hortorum* plants following the procedure given in Section 2.3. This was done at monthly intervals. Inoculated plants were inspected weekly for pustule development. If any developed, then the spores were regarded as still being infective after the corresponding storage period.

2.8. Biology of P. araujiae

2.8.1. Teliospore germination

To promote teliospore germination, discs containing telia cut from diseased tissue with a scalpel were placed on WA and kept at room temperature on a laboratory bench for 24 h. Germinating teliospores were mounted in water, some samples stained with Phloxine B, and all examined using a compound microscope.

2.8.2. Basidiospore release

The initiation and duration of basidiospore release from teliospores at different temperatures was estimated as follows. Two Petri dishes containing WA and four telial discs each, were inverted (after the lid had been removed) over the bottom parts of Petri dishes of the same size lined with WA, kept together in place with a paper tape, and incubated at 10, 15, 20, 25 and 30 °C. Each bottom dish was divided into four sections with a marker pen, and a circle was drawn to show the surface of each section directly under the corresponding telial disc, on which the greater number of basidiospores was expected to fall. The initiation of germination was determined by hourly inspection of telial discs under the stereomicroscope while the initiation of basidiospore release was investigated by hourly microscopic observation (at a 10× magnification) of the areas within the circles on the bottom dishes. After the first basidiospores were observed to fall, bottom dishes were replaced by fresh ones which were after that removed and replaced by new ones at 24 h intervals for as long as basidiospores were observed to fall within the marked circles under the microscope.

2.8.3. Life-cycle studies

The infection process and disease development on moth plant were investigated both macro- and microscopically. For these observations plants were inoculated following the *General rust inoculation methodology*. For microscopic observations, samples of inoculated leaves collected at 6 h, 24 h and 10 days after inoculation were cleared and stained following Bruzzese and Hasan (1983).

2.8.4. Host specificity testing

The plant list compiled for host specificity testing was prepared, in accordance with international best practice for weed biocontrol host-range testing (Briese, 2005; Sheppard et al., 2005; Wapshere, 1974) and contains 12 species of Apocynaceae. One plant on the proposed list, a minor ornamental in New Zealand, *Oxypetalum caeruleum*, could not be tested as two attempts to propagate it failed. *Mandevilla sanderi* was not originally included in the list but as two plants of this species were purchased under the name of *M. laxa*, it was decided to include them anyway. The number of plants tested per species is given in Table 1. Each species was tested on at least two separate occasions. At least two *A. hortorum* plants were included in each test as positive controls. Inoculation methodology was as explained earlier. Most species were inoculated by inverting the Petri dish containing the germinating teliospores over the top of vigorously growing plants, in which the youngest (uppermost) leaves were the most closely (5 cm) exposed to the basidiospore "shower". The plants of the species within the Oxypetalinae and Asclepiadinae were all grown from seed and inoculated when their size was appropriate. All the others were purchased at local nurseries as fully grown plants, and although the smallest available individuals were chosen, some were quite big. In the case of the taller plants, such as those of *Nerium oleander* and *Mandevilla laxa*, two dishes were inverted over each plant, one approximately 5 cm over the upper leaves and the other at approximately half the total height of the plants, to allow the lower leaves to also be exposed to the inoculum. Plants were monitored for five weeks for macroscopic evidence of disease, i.e. pustule development. At the end of each test, leaves directly exposed to the inoculum were cut off and inspected under a stereoscopic microscope to check for any sign of pustule development that might have escaped inspection with the naked eye. The infectivity

Table 1
Host specificity tests: plants tested and results.

Subfamily	Tribe	Subtribe	Plants species	Plants tested	Developed pustules	Affected organs	Other symptoms	Susceptibility
Asclepiadoideae	*Asclepiadeae*	Oxypetalinae	*Araujia hortorum* (positive controls)	51	96%	Leaves, petioles, stems	No	Susceptible
			A. angustifolia (Hook. & Arn.) Steud.	6	100%	Leaves, petioles, stems	No	Susceptible
			Morrenia odorata Hort. ex Kunze	7	86%	Leaves, petioles	No	Susceptible
			M. brachystephana Griseb.	6	100%	Leaves, petioles	No	Susceptible
		Asclepiadinae	*Asclepias curassavica* L.	12	0	–	No	Immune
			Gomphocarpus physocarpus E. Mey.	16	0	–	Chlorotic specks (on three leaves)	Immune**
	Marsdenieae		*Hoya carnosa* (L.f.) R. Br.	9	0	–	No	Immune
Apocynoideae	*Nerieae*		*Nerium oleander* L.	8	0	–	Chlorotic specks (on two leaves)	Immune**
	Mesechiteae		*Mandevilla laxa* (Ruiz & Pav.) Woodson	8	0	–	No	Immune
			M. sanderi (Hemsl.) Woodson	2	0	–	No	Immune
	Echiteae		*Parsonsia heterophylla* A. Cunn.*	5	0	–	No	Immune
Rauvolfioideae	*Vinceae*		*Vinca major* L.	8	0	–	No	Immune

* Indigenous to New Zealand.
** see comments in Section 4.

of spores recovered from pustules developed on non-target species was tested by re-inoculating them on healthy *A. hortorum* plants.

3. Results

3.1. Field surveys and collection of rust isolates

Most of the known geographical distribution of the host plant in Argentina was surveyed (Fig. 1). The rust was found and collected at only one of the sites at which it had been recorded earlier (Delhey et al., 2011; Lindquist, 1982; Waipara et al., 2006), in the outskirts of the city of La Plata, and at a new one, located in Laguna de Gómez, near the city of Junín, both in the province of Buenos Aires (Fig. 1, black squares). Teliospores collected from the La Plata site did not germinate to produce basidiospores under any of the treatments applied. Telia from this site were observed to have a dark brown colouring and to be heavily hyperparasitised by *Cladosporium* sp. In contrast, telia collected at Laguna de Gómez germinated readily to produce basidiospores when subjected to high RH, despite the fact that *Cladosporium* sp. was also found to be growing on telia, albeit less frequently.

Pale to dark reddish brown telia were found on stems, petioles and leaf blades of *A. hortorum* (Fig. 2a–d), often accompanied by hypertrophy of the affected tissues. On stems and especially on petioles, telia can coalesce to cover large sections of these organs, sometimes leading to premature leaf shed and defoliation. On leaf blades telia are mostly hypophylous but sometimes amphigenous, rounded, isolated or disposed in concentric circles. The affected section of leaf blade tissue is often swollen and distorted, with chlorotic sunken spots on the adaxial side of blades (Fig. 2c) corresponding with swollen protruding tissue bearing telia on the abaxial side (Fig. 2d).

Teliospores golden brown, ellipsoidal, smooth, somewhat constricted at the septum, 34–47 × 17–24 µm ($\bar{x}$ = 40 × 20, n = 25), pedicellate, pedicel colourless, as long as or longer than the spore body. A single germ pore observed in each cell, apical in the upper cell and septal in the lower one (Fig. 2e). Telia reddish brown, darkening with age.

3.2. Establishment of a pure culture

It was not possible to completely eliminate the *Cladosporium* hyperparasite from telia of *P. araujiae.* This process is currently being repeated to build up a clean culture.

3.3. Identification of the hyperparasite

The hyperparasite was identified by its morphology as *Cladosporium uredinicola* Speg. based on the descriptions given by Bensch et al. (2012) and Heuchert et al. (2005).

Colonies of *C. uredinicola* were found growing on rust pustules and surrounding host tissue (Fig. 2f), caespitose, initially hyaline to whitish, later becoming dark greenish grey. Mycelium tangled with that of the rust, hyphae branched, septate, subhyaline, up to 8 µm wide, stroma absent. Conidiophores solitary, erect, straight, sometimes branched, without basal thickening of the walls, 3–8 septate, not constricted, smooth, brown, paler towards the apex, 102–405 × 3–5 µm ($\bar{x}$ = 203.6 × 4.3 µm, n = 6). Conidiogenous cells terminal and intercalary, mostly cylindrical, apical zone sometimes protuberant because of the presence of scars, polyblastic, with 1–4 lateral and apical scars, proliferation sympodial, 8–35 × 3–5 µm ($\bar{x}$ = 19.2 × 3.5 µm, n = 15). Ramoconidia present, limoniform to oblong cylindrical, with numerous scars, 0–1 septate, smooth, pale brown, 4–35 × 2–5 ($\bar{x}$ = 11.6 × 3.2 µm, n = 50). Conidia in chains (sometimes branched), ellipsoid, oblong ellipsoid, limoniform or ovoid, finely verruculose, subhyaline to pale brown, olivaceous brown when in groups, 3–11 × 2–4 µm ($\bar{x}$ = 4.9 × 2.9 µm, n = 30).

3.4. Hyperparasite's mode of action

Microscopic examination revealed that spores of the hyperparasite germinate and penetrate the teliospores of the rust through both the apical and lateral germ pores. Destruction of the spore cytoplasm and intracellular growth of *Cladosporium* hyphae was observed (Figs. 2g and h). Directional growth of the hyperparasite towards the rust was sometimes observed (Fig. 2i), but no coiling of its hyphae around the pathogen's structures was ever observed.

3.5. Rust short and long term storage

Results of the storage experiment are shown in Fig. 3. Storage at very low temperatures (−70 °C) proved the best way to preserve the rust. An average germination rating score of 1.2 was obtained at 12 months after storage (Fig. 3). Infectivity and pathogenicity of such basidiospores (i.e. that had been stored for 12 months at −70 °C) were also tested and they were still able to infect *A. hortorum* plants and produce pustules. Storage in a fridge is adequate for shorter periods. Spores recovered after six months storage at 8 °C showed some germination and produced

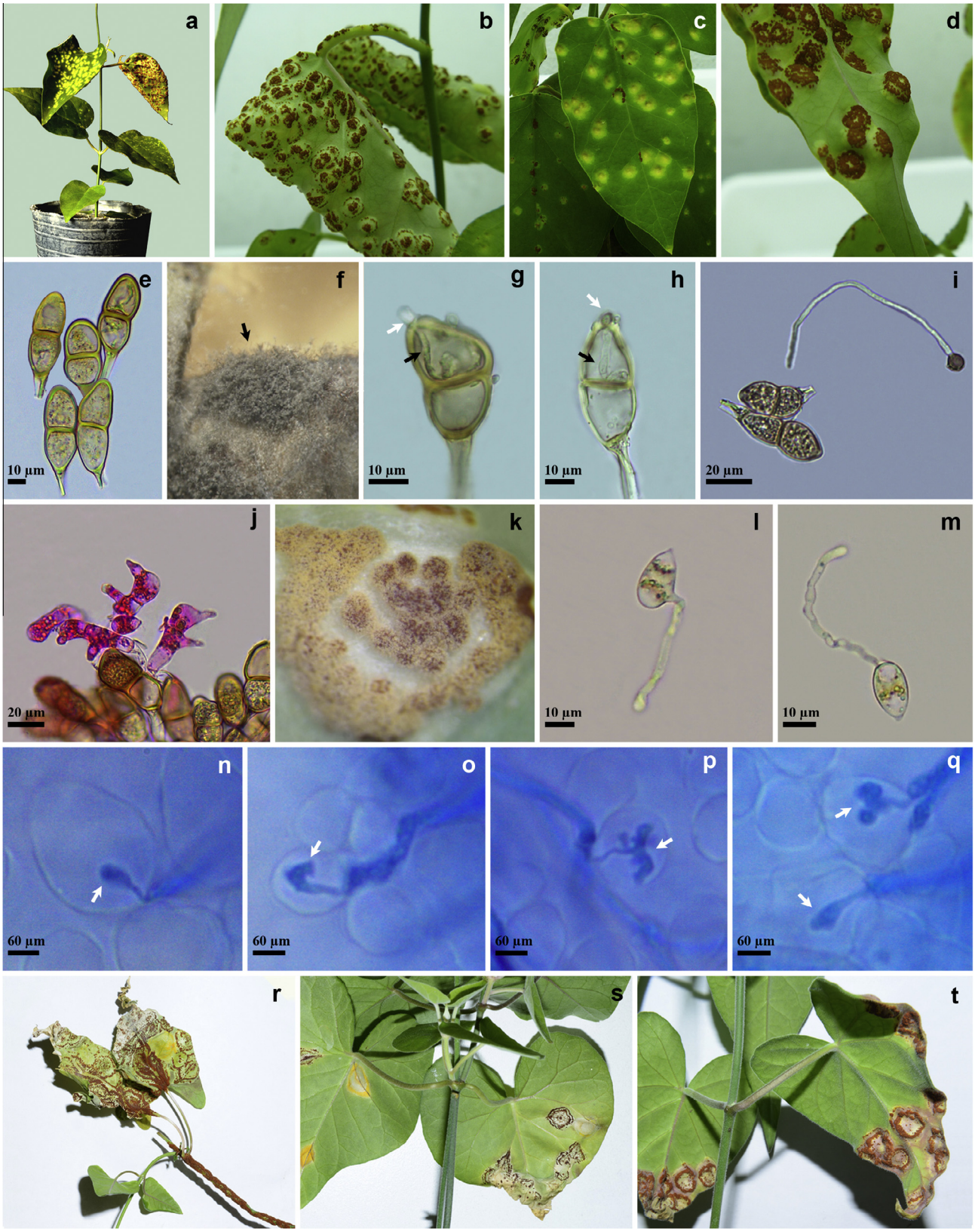

Fig. 2. **(a–d)** Telia of *Puccinia araujiae* on moth plant. Note chlorotic sunken spots on the adaxial side of blades **(c)** corresponding with swollen protruding tissue bearing telia on the abaxial side **(d)**. **(e)** Teliospores. **(f)** Colony of *Cladosporium uredinicola* growing on a rust pustule and surrounding host tissue (arrow). **(g** and **h)** Destruction of teliospore cytoplasm and intracellular growth of *Cladosporium* hyphae (black arrows). Note spores of the hyperparasite located on telial pores (white arrows). **(i)** Directional growth of the hyperparasite towards the rust. **(j)** Two-celled metabasidia with a prominent sterigma on each cell. **(k)** Germinated telia. **(l)** Lateral basidiospore germination. **(m)** Apical basidiospore germination. **(n–q)** M-haustoria in parenchymatic cells of moth plant (arrows). **(r–t)** Pustules of the rust on non-target species, *Araujia angustifolia* **(r)**, *Morrenia brachystephana* **(s)** and *M. odorata* **(t)**.

basidiospores which were able to infect *A. hortorum* plants and produce pustules. There was no germination of spores stored for seven months or longer at 8 °C.

C. uredinicola was observed to develop on pustules placed in Petri dishes with water agar to promote germination after all treatments.

3.6. Biology of *P. araujiae*

3.6.1. Teliospore germination

Teliospores germinated readily without dormancy when subjected to high RH to produce hyaline, smooth, generally two-celled (rarely four-celled) metabasidia with a prominent sterigma on each cell, on which basidiospores were formed (Fig. 2j). In some cases, after 24 h such germination was visible under the stereomicroscope and indeed to the naked eye, as a thick, whitish "coating" (a bloom of basidia and basidiospores *sensu* Ellison et al., 2008) covering the entire surface of telia (Fig. 2k). This level of germination corresponds to level 3 (>60%) in our subjective visual scale of assessment. The basidiospores produced were hyaline, smooth, obovoid-ellipsoidal, with a prominent hilar appendix, 14–19 (−20) × 10–12 (−13) µm, and also germinated readily when discharged onto the dish lined with WA. When teliospores were germinated in water, they usually produced long germ tubes that did not differentiate to produce sterigmata and basidiospores.

3.6.2. Basidiospore release

The time taken for germination and basidiospore release to start varied with the incubation temperature, as did the length of time during which the release process continued (Table 2). At high temperatures (30 °C) germination was observed to occur through the formation of germ tubes but no basidiospore formation was observed. At milder temperatures (25 °C) basidiospores were formed but it took longer for the release process to start (between 12 and 24 h) and then it lasted for only 48 h. The longest release period occurred at 10 °C, the lowest temperature tested.

3.6.3. Life-cycle studies

Only telia of the rust were ever observed in the field. This observation was confirmed in artificial inoculation experiments. Teliospores germinated without a resting period to produce basidiospores which were able to infect all vegetative parts of *A. hortorum* plants, giving rise to new telia and teliospores, thus confirming the microcyclic nature of the life cycle.

3.6.4. Infection process

Basidiospores germinated on the leaf surface (and the agar lined dishes) by producing a single germ tube. Germination usually happened laterally (Fig. 2l), although germ tubes were also observed less frequently to be formed from the apical (opposite to the apiculus) pole (Fig. 2m). Penetration was observed to occur at the junctions between host epidermal cells, over which appresoria were formed. It could not be discerned whether the developing hyphae penetrated through or between epidermal cells, or both. No intra-epidermal vesicles could be observed. Primary hyphae were seen to grow and branch towards the parenchyma where hyphae were mostly intercellular. Terminal intracellular hyphal structures (M-haustoria) were formed in parenchymatic (both spongy and palisade) cells and were observed to be highly variable in shape from vermiform or hyphal-shaped to highly lobed (Figs. 2n–q).

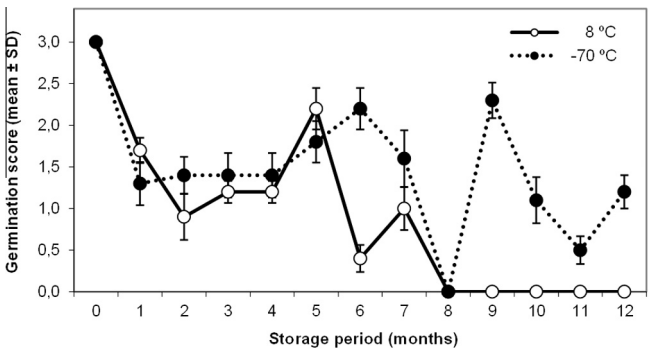

Fig. 3. Storage experiment. Germination score of telial discs after storage at low temperatures for up to twelve months. Germination score: **0**: no germination; **1**: 1–20% germination; **2**: 21–60% germination; **3**: >60% germination. The average scores of 10 telial discs examined per month are presented.

Table 2
Basidiopore release experiment. Periods after which germination started, and, basidiospore release started and ended, at each incubation temperature.

Temperature (°C)	Germination (h)	Basidiospore release	
		Beginning	End
30	6	–	–
25	2	12–24 h*	48 h
20	2	5 h	7 days
15	4	6 h	10 days
10	5	6 h	11 days

* Release process started at some point during this period.

3.6.5. Host specificity testing

Only plants that were very closely related to the target weed, in the two tested *Oxypetalinae* genera (*Araujia* and *Morrenia*), developed pustules (Table 1, Figs. 2r–t). Affected plants showed chlorotic specks on the adaxial side of leaves around seven to ten days after inoculation. Specks enlarged with time and often became sunken; at the same time that on the other side tissues became swollen and protruding. Telia were first apparent around 12–15 days after inoculation. Telia collected from the three non-target species that developed pustules were inoculated onto healthy *A. hortorum* plants. These *A. hortorum* plants became infected and more pustules developed on them, an indication that the teliospores produced on the non-target species were viable and infective. None of the individuals belonging to the other eight non-target species tested were susceptible. Almost no macrosymptoms developed on any of the tested plants outside the *Oxypetalinae*. All but two of the 51 positive control plants developed disease symptoms. The two plants that did not develop symptoms were not in the same batch and every batch had at least some *A. hortorum* plants that showed disease symptoms.

4. Discussion and conclusions

P. araujiae was found at only two sites in the areas surveyed in Argentina, at both of which a mycoparasite was common to abundant. This compromised the establishment of a pure culture in the laboratory, and prevented gaining insight into the full effect of the rust on its host in the field. Despite high levels of hyperparasite infection, some premature necrosis of foliage and defoliation of the host plants was observed. The hyperparasite was identified as *C. uredinicola* (Bensch et al., 2012; Heuchert et al., 2005). This is the first report of *P. araujiae* as a host for this hyperparasite (Heuchert et al., 2005; Moricca et al., 2005).

The hyperparasite directly penetrates the teliospores of the rust, a process known as invasive necrotrophy (Jeffries, 1995; Traquair et al., 1984). This is consistent with observations by Srivastava et al. (1985), who found that teliospores of microcyclic rusts were readily invaded by *C. uredinicola* through the germ pores. These authors also found that teliospores that germinate immediately, such as those of *P. araujiae*, are more easily penetrated than those

that take longer, and speculated that these differences could be due to differences in the chemical composition of spores, or, to the fact that germination of teliospores may be necessary for penetration to occur.

Necrotrophic parasitism results in the death of the host, and the mycoparasite then uses the dead remains as a source of nutrients (Jeffries, 1995). Necrotrophic mycoparasites tend to have a broad range of host fungi (Jeffries, 1995), and there is no evidence of differences in specificity at the infraspecific level (Dolińska and Schollenberger, 2012; Dolińska et al., 2011). It is expected that the effect of the rust on its host would be greater if freed from the hyperparasite since hyperparasites may reduce infection and inoculum production by their host (Barros et al., 1999). Efforts are being made to establish a hyperparasite-free culture in the laboratory. *C. uredinicola* has been recorded in New Zealand on *Puccinia coprosmae* on *Coprosma macrocarpa* and on *Melampsora laricis-populina* on *Populus* sp. (Bensch et al., 2012; Heuchert et al., 2005), so there is a possibility that these strains could infect the teliospores of *P. araujiae* at some stage should it be introduced to New Zealand, and this could reduce the potential of the pathogen as a biocontrol agent. That said, the pathogen can cause damage and defoliation in the field in Argentina in the presence of the hyperparasite (FEA pers. obs.), so it is expected to still have a significant impact on moth plant in New Zealand.

C. uredinicola survived freezing at −70 °C for twelve months, a procedure that had proved effective in killing spores of *Simplicillium* sp., a hyperparasite of *Uromyces pencanus* (Dietel & Negel) Arthur & Holw. in previous studies (Anderson et al., 2010), leading to the need to design and perform a laborious and time consuming process to establish a pure culture.

Non-culturable microorganisms, especially microcyclic rust fungi, as is the case of *P. araujiae*, are notoriously difficult to cryopreserve. Ryan and Ellison (2003) were able to successfully preserve teliospores of *P. spegazzinii* in liquid nitrogen, but found that the basidiospores produced when recovered from storage, had lost their pathogenicity. Here a protocol has been developed that allows for the preservation of teliospores of *P. araujiae* at very low temperatures for up to a year without losing the ability to infect and produce disease. At −70 °C it was found that sufficient spores stored for 12 months were still able to germinate to produce infective basidiospores. In addition, it was shown that more moderate low temperatures are suitable for storage over shorter periods.

Mature teliospores germinated without dormancy while still attached to telia in accordance with the findings of other authors for similar microcyclic rusts (Morin et al., 1992; Ellison et al., 2008). Rust teliospores usually germinate by producing four-celled basidia which in turn produce four basidiospores, but the occurrence of two-celled basidia, as observed here, is not uncommon (Hiratsuka and Sato, 1982; Ono, 2002). Ellison et al. (2008) found that basidiospore release started after *ca.* 2 h under high humidity conditions for the microcyclic rust *Puccinia spegazzinii* De Toni. In the present study it was found that it took longer for this to happen at all tested temperatures. Between 10 °C and 20 °C the process started after a 5–6 h incubation period and continued for at least a week, indicating that teliospores from mature telia do not germinate all at once when subjected to ideal conditions, but rather in a sequential manner, thus lengthening the period over which inoculum is produced and available for new infections to take place.

Most basidiomycete fungi actively eject their spores. The process begins with the condensation of a water droplet at the base of the spore known as Buller's drop. The fusion of the droplet onto the spore creates a momentum that propels the spore forward (Noblin et al., 2009). Buller's drop is generated by condensation of water from the humid air surrounding the spore (Webster et al., 1984). Although the exact moment at which the release process started at 25 °C could not be established, it was found to take much longer than at the other lower temperatures tested. This delay might be explained by a lower RH within the Petri dishes subjected to 25 °C than that within those subjected to lower ones. Temperatures over 30 °C seem to inhibit basidiospore formation. It should be noted though that the cabinet subjected to this temperature showed a greater fluctuation than those at lower temperatures, reaching peaks at 35 °C. Northern New Zealand where moth plant flourishes is a sub-tropical climate zone, with warm humid summers and mild winters. Typical summer daytime maximum air temperatures range from 22 °C to 26 °C, but seldom exceed 30 °C. Winter daytime maximum air temperatures range from 12 °C to 17 °C (NIWA, 2015). Temperatures during the night, when dew is likely to be present to aid teliospore germination, would typically be about 6 °C lower than during the day. Nonetheless, there should be plenty of wet winter days and warm summer nights when conditions are suitable for basidiospores of *P. araujiae* to discharge and germinate.

It was not possible to observe the whole infection process with the whole leaf clearing–staining technique used, but the part that was observed was similar to that recorded for other similar microcyclic rusts (Morin et al., 1992; Zhang et al., 2011).

The Apocynaceae comprise five subfamilies and twenty-two tribes (Nazar et al., 2013). Of these, members of three subfamilies and six tribes were tested. The taxa included on the test list were chosen because they occur in New Zealand, and therefore are relevant to assessing the safety of introducing the rust into New Zealand, or because they were closely related to the target weed and were readily obtainable in Argentina (see Table 1). The target weed *A. hortorum* belongs to subfamily Asclepiadoideae, tribe Asclepiadeae, subtribe Oxypetalinae. The rust has proved to be reasonably specific, causing disease only to species within the same subtribe as *A. hortorum*. These results are consistent with the host range of the fungus reported in the literature and previous experimental results (Kiehr et al., 2011). There are no members of the Oxypetalinae native to New Zealand. The only three indigenous taxa within the Apocynaceae are three species of *Parsonsia* (native jasmine) which belong in a different subfamily (Apocynoideae) (New Zealand Plants, 2015). Five individuals of one of these species, *P. heterophylla*, were tested and all of them showed an immune reaction. No symptoms or signs of infection were observed on any of the remaining plant species outside of the Oxypetalinae. The few chlorotic specks that were observed on a couple of leaves of *Gomphocarpus* and *Nerium* plants occurred in only one of the inoculated batches and could not be unequivocally attributed to infection by the rust. *O. caeruleum*, commonly called tweedia, belongs in the Oxypetalinae and is an ornamental species grown in New Zealand gardens. Although this species was not tested, it would almost certainly be susceptible to the rust because it belongs to the same subtribe as the other susceptible plant species. If the rust were released in New Zealand, and tweedia became infected (as expected), home gardeners could mitigate damage by applying fungicide. Tribe Asclepiadeae to which the target species belongs, includes six more subtribes apart from the Oxypetalinae and Asclepiadinae tested here (Rapini, 2012). Although no members of those taxa were included in the specificity test list because they were not considered relevant to the introduction of the rust in New Zealand, it is acknowledged some should be tested to fully delimit the host range of *P. araujiae*, especially representatives of the Metastelmatinae and Cynanchinae, known to be hosts to closely related rusts (França et al., 2010; Hennen et al., 2005; Lindquist, 1982). Observations of plants infected in the laboratory, and of the few infected plants found in the field, indicate the rust is able to negatively affect the growth of its host *A. hortorum*. It is not known to what extent the presence of the hyperparasite *C. uredinicola* in Argentina

limits this impact, or if the presence of this hyperparasite in New Zealand might also reduce its effect there. Nevertheless, the evidence gathered to date indicates that the rust could be a very good biological control agent against moth plant in New Zealand, and permission to import and release it there has recently been granted.

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