Biological studies of *Puccinia lantanae,* a potential biocontrol agent of "Lippia" (*Phyla nodiflora* var. *minor*)

Estudios biológicos en *Puccinia lantanae*, posible agente de biocontrol de"Lippia" (*Phyla nodiflora* var. *minor*)

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ABSTRACT

Phyla nodiflora var. *minor* (syn. *P. canescens* (Kunth) Greene) known *as* "lippia" is an invasive weed with considerable impact on agricultural systems and conservation areas in Australia. The rust fungus *Puccinia lantanae* Farl. has been proposed as a potential biocontrol agent of *Lantana camara*. As it was previously found in *Lippia* s.l. in Argentina, we aim to study: (i) its geographical distribution in Argentina, Bolivia, and Chile; (ii) teliospore germination and basidiospore formation under different incubation temperatures; (iii) the effect of teliospore age on germination capacity; (iv) the effect of heat shock on teliospore germination and basidiospore formation; and (v) the pathogenicity of the rust fungus on *P. nodiflora*. Field surveys were conducted in Argentina, Bolivia, and Chile. *In vitro* experimental assays of germination and pathogenicity were performed. The rust was found in four provinces of Argentina (Jujuy, Salta, Formosa, and Entre Rios) and was not found in Bolivia and Chile. *Puccinia lantanae* showed the maximum values of teliospore germination and basidiospore formation at 20°C. The effect of aging and heat shock treatments significantly reduced teliospore germination. Pathogenicity tests showed that *P. nodiflora* var. *minor*, *reptans*, and *nodiflora* were infected with the "Formosa" isolate. The isolates "Salta" and "Entre Rios" infected var. *minor* and *reptans,* being potential candidates for biocontrol.

Key words: spores, biological control, invasive species, specificity.

RESUMEN

Phyla nodiflora var. *minor* (syn. *P. canescens* (Kunth) Greene), conocida comúnmente como "lippia" es una maleza invasora que genera un grave impacto en los sistemas agrícolas y áreas protegidas en Australia. La roya, *Puccinia lantanae* Farl., ha sido propuesta como un potencial agente de biocontrol de *Lantana camara*. Este hongo fue encontrado en *Lippia* s.l. en Argentina; por esta razón, proponemos estudiar (i) su distribución geográfica en Argentina, Bolivia y Chile; (ii) la germinación de teliosporas y la formación de basidiosporas bajo diferentes temperaturas de incubación; (iii) el efecto de la edad de las teliosporas sobre la capacidad de germinación; (iv) el efecto del choque térmico sobre la germinación de teliosporas y la formación de basidiosporas; (v) la patogenicidad de la roya sobre *P. nodiflora*. Se realizaron ensayos *in vitro* de germinación y de patogenicidad. La roya se encontró en cuatro provincias de Argentina (Jujuy, Salta, Formosa y Entre Ríos), y no se encontró en Bolivia y Chile. La germinación de las teliosporas y la formación de basidiosporas fueron máximas a 20°C. El efecto de la edad de las teliosporas y los tratamientos de choque térmico redujeron significativamente la germinación de estas. *P. nodiflora* var. *minor*, *reptans* y *nodiflora* fueron infectadas con el aislado "Formosa". Los aislados "Salta" y "Entre Rios", infectaron a la var. *minor* y *reptans* siendo candidatos potenciales de biocontrol.

Palabras clave: esporas, control biológico, especies invasoras, especificidad.

Introduction

Phyla nodiflora var. *minor* (synonym *P. canescens* (Kunth) Greene) (Verbenaceae), commonly known as "lippia" in Australia, is a notorious weed of riparian and floodplain production and conservation areas with considerable impact on rural production, land values, and ecosystem services. It is a perennial plant with a prostrate habit and creeping stems that can root at each node, which favors spreading and increasing in density. This invasive plant was commercially introduced in Australia as an ornamental species during the second half of the XIX century and has invaded 5.3 million ha of the "Murray-Darling" basin floodplain. The greatest invasions and impacts occur in the Murray-Darling Basin´s northern catchment, but also throughout the basin and elsewhere in Australia. In the

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Murray-Darling Basin, the weed costs the grazing industry an estimated Australian dollar AUD $38 million per year and the environment AUD $1.8 billion per year (Earl, 2003).

Current short term and unsustainable control methods include the use of herbicides, cultivation, and grazing management (Julien *et al*., 2012). The use of herbicides is restricted by the presence of susceptible crops along waterways. *Phyla nodiflora* var. *minor* may be managed by cultivation, however, in many areas the practice is not sustainable as there is a significant risk of soil loss associated with cultivation of areas adjacent to waterways (Earl, 2003). Biological control was proposed as part of the weed management in reserve areas, woodlands, forests, and along stream banks.

Originally, two species were recognized in Australia, *Phyla nodiflora* and *Phyla canescens* (Munir, 1993). Although *Phyla nodiflora* has a worldwide distribution, the condition of being native to Australia (Gross *et al*., 2017) has implications for the selection of biological control agents. The South American genus *Phyla* Lour (Verbenaceae), originally included in the genus *Lippia* L., currently comprises five species (O'Leary & Múlgura, 2012). Following the taxonomic revision of the genus *Phyla* Lour provided by O'Leary & Múlgura (2012), three varieties of *P. nodiflora* are recognized: *P. nodiflora* (L.) Greene var. *nodiflora*, *P. nodiflora* var. *minor* (Hook.) O'Leary & Múlgura, and *P. nodiflora* var. *reptans* (Kunth) Moldenke. According to Sosa *et al*. (2017), the variety *minor* is an invasive weed in Australia, while the variety *nodiflora* is the native Australian form (formerly known as *P. nodiflora*).

The *P. nodiflora* plant is native to South America with the center of origin probably in Central and Northern Argentina (Julien *et al*., 2012). It is likely that many specific antagonists can be found in this region, and they can be proposed as potential biological control agents against this weed. Systematic surveys on the three varieties of *P. nodiflora* are scarce, and only a few arthropods (Cabrera *et al*., 2016) and pathogens are mentioned as natural enemies (Viégas, 1961; Ellis, 1976; Farr *et al*., 1989).

Puccinia lantanae Farl. (Pucciniales, Basidiomycota) was described as infecting *P. nodiflora* var. *minor* (formerly *Lippia canescens*=*P. canescens*) in Argentina (Lindquist, 1982). Julien *et al*. (2012) registered this rust damaging *P. nodiflora* under natural conditions in the field in Argentina. *Puccinia lantanae* is an autoecius (completes the life cycle on a single host) microcyclic rust, for which only the teleutosporic stage is known (Cummins & Hiratsuka, 2003). This rust has been reported on *Lippia* spp. in South America (*fide* Jackson, 1932; Lindquist, 1982). Particularly, in Argentina, the geographic distribution range of *P. lantanae* in *Lantana* spp. and *Lippia* spp. includes Buenos Aires, Entre Ríos, Corrientes, Misiones, Tucumán and Salta provinces (Hernández & Hennen, 2002). Previous studies suggest its potential use as a biocontrol agent of *Lantana camara* L., considered a weed with serious impact (Barreto *et al*., 1995).

Rusts are considered very attractive biocontrol agents due to their high specificity and the impressive biocontrol successes in some species (Morin *et al*., 2006), with different pathotypes specialized on different plant species and even in some cases on different genotypes within the same plant species. Additionally, there is evidence of distinct races of *P. lantanae* that attack only one species and are even specific to biotypes within that species (Rentería & Ellison, 2004).

Field surveys in the native range and knowledge of the biology of putative biological agents are essential components of a biocontrol program (Fourie & Wood, 2018). Extensive collecting and research on this rustwere performed in Brazil and Peru (Barreto *et al*., 1995; Ellison & Cortat, 2011), but similar studies do not exist for Argentina or bordering countries. Temperature is one of the environmental limitations of the disease development in the field (Agrios, 2005); its effect on teliospore germination and basidiospore production is crucial in the epidemiology of microcyclic rust disease.

In this research, we aim to study: i) the geographic distribution of *P. lantanae* on *P. nodiflora* in Argentina and two bordering countries (Bolivia and Chile), (ii) the effect of different incubation temperatures, aging and heat shock on teliospore germination and basidiospore formation, and (iii) the pathogenicity of the rust fungus on *P. nodiflora* var. *minor*, *P. nodiflora* var. *reptans*, and *P. nodiflora* var. *nodiflora*.

Materials and methods

Field surveys and collection of rust isolates

Field trips were performed to study the geographic distribution of *Puccinia lantanae* in Argentina, Bolivia, and Chile. Both rust and *P. nodiflora* (var. *minor*, *reptans*, and *nodiflora*) plant material was collected from different locations. Roadside surveys were conducted in Argentina from December 2004 to January 2011 (41°S northwards). As *P. nodiflora* var. *minor* is a small and prostrate plant and often grows in the understory, it is difficult to observe from a moving vehicle. Therefore, inspection sites were assigned according to the following criteria: the first site was randomly selected within the first 50 km away from Buenos Aires along main routes and subsequent inspections were systematically placed every 50, 100 and 180 km, giving a total of 217 visited sites during 6 years of field survey (Fig. 1). Plant specimens from all locations were collected and dried for further identification. Whenever a rust infected plant population was found, material was collected and placed between pieces of newspaper in a plant press for further study in the laboratory (Tab. 1). Each collection of the rust is termed "isolate". A GPS reading was taken to record the site location. Some sites were visited more than once in different seasons.

In the laboratory, field collected samples of infected plants with rust were processed. Freehand razor blade cuts were performed on infected leaves. The cuts were placed in water or potassium hydroxide in an aqueous solution 5%. A coverslip was placed over the cuts (Waller *et al*., 2002). Fifty teliospores were measured (width and length) (Anikster *et al*., 2004) using a compound microscope (Olympus CX 31).

Source and maintenance of plants

New *P. nodiflora* individuals of each variety were obtained to establish rust cultures with isolates of different locations and to conduct pathogenicity tests. Healthy stem cuts with three to four nodes (with leaves) were buried in 10 cm-diameter plastic pots containing steam sterilized soil and organic substrate mixture (1:1, w/w) leaving two nodes over the soil surface. *Phyla nodiflora* propagules were grown for 15 d at 25ºC and 100% relative humidity (RH) (humid chamber), and thereafter at 25ºC and 60% RH until the four-five leaf stage.

Rust inoculation methodology

The leaf disc inoculation method proposed by Morin *et al.* (1993) was used for all inoculation experiments. The host plants (3 months old) from which leaf discs were collected were *P. nodiflora* var. *nodiflora* (350), *P. nodiflora* var. *reptans* (264), and *P. nodiflora* var. *reptans* (205). Each leaf disc was covered by a mass of spores (telia) of the rust 5 mm in diameter. Ten circular discs of leaf with telia (3-4 weeks old) were placed on the surface of 2% water agar (WA) in a 9 cm diameter Petri dish base, with the telia on the side

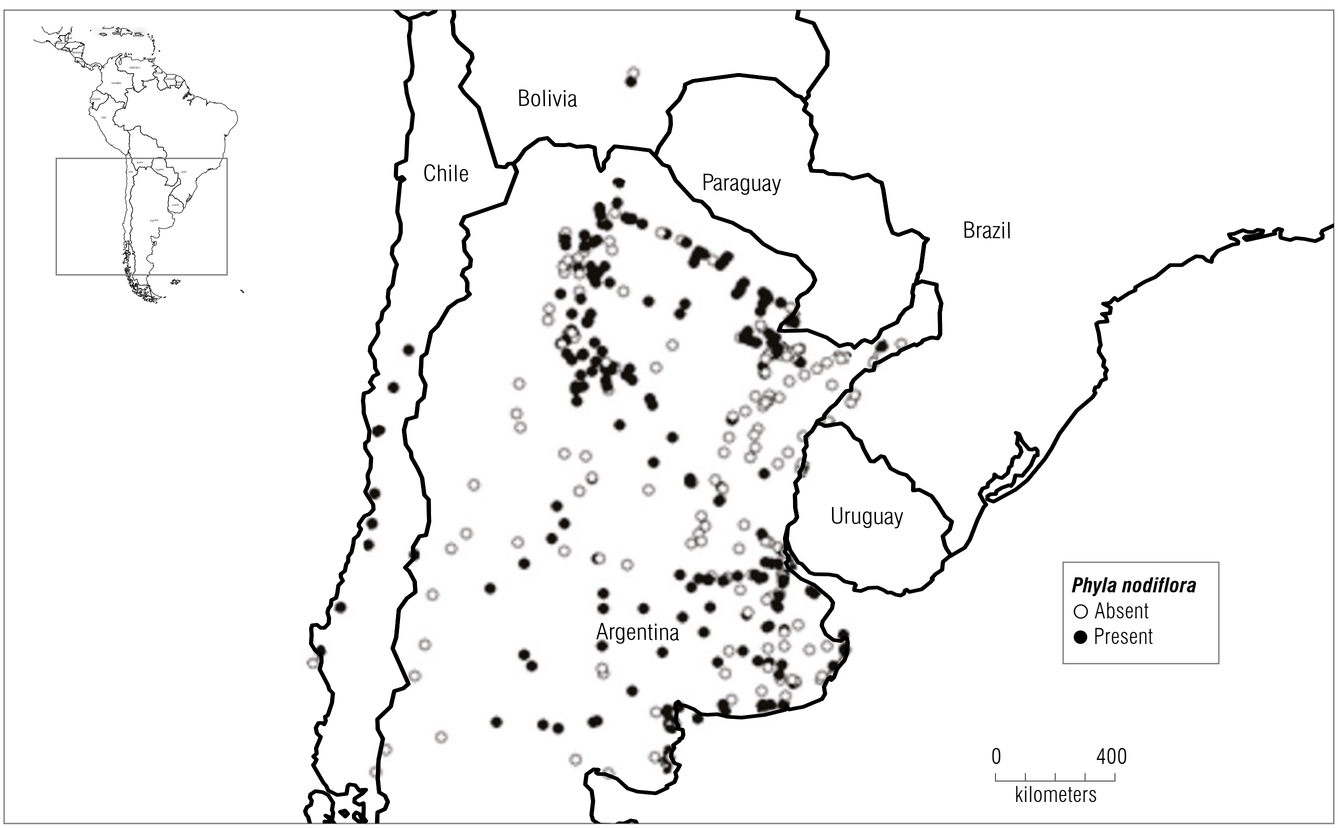

FIGURE 1. Distribution of *Phyla nodiflora* var. *minor, reptans*, and *nodiflora* in southern South America. Circles indicated sites visited to sample the plants. Solid circles: plant present, empty circles: plant absent. Data from Chile were obtained by A. Sosa.

away from the water agar. The base of the dish was inverted and fixed to the top of a plastic pot with holes. Three young, healthy plants of *P. nodiflora* were placed in a plant pot. The pot with the plants was sealed to the pot containing the telia inverted with masking tape and incubated at 20ºC for 48 h in the dark. The inside of the pots as well as the plants was sprayed with a fine mist of sterile distilled water. The pot chambers were then placed inside an inoculation chamber. This consisted of a cube shaped polyethylene boxes with the floor covered with water-soaked newspaper to provide around 100% RH.

After 48 h, the plants were transferred to the glasshouse (24±2ºC, 12-h dark/12-h light (fluorescent, 1400 Lux) regime, at around 75% RH), until telia started to develop. For conservation purposes, infected leaves collected from both field surveys and pathogenicity tests were preserved inside paper bags at 3±0.5ºC with silica gel to avoid the germination of teliospores.

Isolation and maintenance of rust isolates

Rust cultures were established from a single telium (Thomas *et al*., 2021) from rust-infected material collected at sites 205 (Salta province), 264 (Entre Rios province) and 350 (Formosa province) (Supplementary material). A single telium isolate was obtained using the leaf disc method. The single telium isolate was used to inoculate a plant of the same origin of the telium (site where the rust was collected). Healthy *P. nodiflora* plants were inoculated monthly with *P. lantanae* to ensure a continuous supply of mature telia throughout the experiments.

Biology of *P. lantanae*

Morphological characterization and microscopy

For light microscope observations, a drop of water was placed on a slide, the sori were scraped with a sterile needle and the teliospores were transferred into the drop of water. A coverslip was placed over it.

An Olympus SP350 camera was attached to the microscope eyepieces and photos were taken to illustrate the morphology of the telia and teliospores and characterize the pre-infection development (*i.e*., from teliospore to basidiospore production) of the rust fungus.

For scanning electron microscope observations, a 2.5% glutaraldehyde fixation was performed in 0.067 M phosphate buffer (pH 7.2) and then washed with phosphate buffer (15 min, 3 times). Gradual dehydration was conducted with alcohol and acetone and then critical point drying was done (E3000, Polaron) using CO_2. The samples were mounted in stubs. Finally, gold was evaporated (300 Å) using an Argon plasma metal evaporator (91000 Model 3, Pelco). Microphotographs were taken with a LEO EVO 40 scanning electron microscope at 7.0 kV potential (Mercer & Birbeck, 1972).

Germination studies

Effect of temperature on teliospore germination and basidiospore formation

Twenty days after teliospores had developed on plants, they were scraped from leaves bearing mature telia and further suspended in 5 ml of sterile distilled water (SDW). Aliquots of 0.10 ml were transferred to 9 cm diameter Petri dishes containing water agar (WA) medium were incubated at 10, 15, 20, 25, and 30ºC in several growth chambers, one for each temperature. The Petri dishes were wrapped in aluminum foil to exclude light and sealed with adhesive tape. Teliospore germination was examined after 4, 7, 11, 20, 24, and 48 h of incubation. A completely randomized factorial design was used (N=90, n=3). The Petri dish was placed under the compound microscope (Olympus CX 31) at (16x and 40x of lenses eyepiece and objective) 640x, and germination percentages were recorded by randomly selecting 50 teliospores per replicate. A teliospore was considered germinated when the germ tube was observed. Basidiospore formation was determined as the percentage of teliospores producing basidiospores (*i. e*., that has at least one basidiospore in the metabasidium or next to it) (Morin *et al*., 1992b) at 10, 15, 20, 25, and 30ºC after 24 h of incubation. Pictures of the Petri dishes were taken using a camera (Olympus SP 350) attached to the Olympus CX 31 microscope. Dishes were sampled destructively.

Effect of heat shock on teliospore germination and basidiospore formation

An experiment was performed in order to test the hypothesis that a heat shock during incubation (*i.e*., high temperature exposure) would influence the capacity of teliospore germination and/or basidiospore formation. Spores were exposed to three different incubation temperature regimes (3 h at 30ºC + 21 h at 20ºC; 6 h at 30ºC + 18 h at 20ºC; 24 h at 20ºC). Teliospore germination and basidiospore formation were registered after 4, 7, 11, 20, and 24 h of incubation using the same procedure described above. A completely randomized factorial design was used (n=3) (n: number of replicates).

Effect of teliospore age on germination capacity

Final germination was assessed on WA after 48 h of incubation at 20°C after different periods of storage: 2, 5, 7, 12, and 18 months. Storage time was defined as the time between the harvest of the teliospores until the end of storage. The leaves with telia collected from inoculated plants were kept inside paper bags with silica gel in a refrigerator at 3.0±0.5ºC. A completely randomized factorial design was used (n=3).

Pathogenicity tests

Pathogenicity tests determine the ability of a pathogen to provoke diseases. These tests are essential studies when a rust is proposed as a potential biocontrol of a weed (Fourie & Wood, 2018). The tests were conducted to confirm if *P. lantanae* could infect the three varieties of *P. nodiflora*. Also, the level of *in vitro* damage was determined.

Telia collected from fields and obtained from the laboratory plants were used for inoculations. Three experiments were designed. Telia taken from plants of *P. nodiflora* var. *nodiflora* (site 350) and from *P. nodiflora* var. *reptans* (from sites 264 and 205) (see Supplementary material) were inoculated on plants of the three varieties (plants from sites 205, 264, 345, 350). Those plant specimens inoculated with telia of the same origin, were considered as positive controls for the pathogenicity test.

Pathogenicity tests were performed using the leaf disc method (Morin *et al*., 1993). Infection was checked daily and ranked using categories proposed by Ellison *et al*. (2008): 0 - without macroscopic symptoms; 1 - chlorosis; 2 - between 1-5 telia on leaves and stems, localized infection; 3 - more than 5 telia, until semi-systemic infection.

Statistical analysis

Germination percentages and basidiospore formation data were analyzed with ANOVA followed by a mean comparison test (protected LSD Fisher, $P<0.05$). Analyses of variance were performed using InfoStat software (Di Rienzo *et al*., 2013).

Results and discussion

Field surveys and collection of rust isolates

Puccinia lantanae was found only in locations from Argentina (Entre Rios, Formosa, Jujuy, and Salta). None of the populations of *P. nodiflora* in Bolivia or Chile showed symptoms of rust infection. To give a definitive conclusion on the distribution of the rust infecting *Phyla* in South America, it would be necessary to make more extensive collecting. The rust was observed only in five sites. Its prevalence was low (2%). Mostly it was collected on plants belonging to the var. *reptans* (four sites) and it was found infecting *P. nodiflora* var. *nodiflora* in only one site of the several visited. The prevalence of the rust was 2%.

TABLE 1. Rust collection sites and dates. Pnr: *Phyla nodiflora* var. *reptans.* Pnn: *Phyla nodiflora* var. *nodiflora*.

Site	Host	Date
205. Rt 34.11 km S Pichanal. Salta province	Pnr	12 December 2005
		20 June 2007
		26 January 2008
		23 July 2008
		30 March 2009
		13 November 2009
		22 July 2010
		13 January 2011
		28 August 2011
264. Parque Nacional Predelta, Entre Ríos province	Pnr	1 June 2006
		24 February 2009
		10 November 2009
		10 January 2011
292. 7 km N Gral. San Martin. Parque Nacional Calilegua. Jujuy province	Pnr	28 August 2006
		11 November 2006
		11 February 2007
		20 June 2007
		27 January 2008
300. Dique Itiyuro. 3 km S Salvador Mazza. Salta province	Pnr	21 June 2007
350. Rt 81, Km 1682, 60 km NO Guillermo Juarez. Formosa province	Pnn	24 July 2008
		22 February 2009
		12 November 2009
		17 February 2010
		22 July 2010
		12 January 2011

Telia were observed in pustules of 5 mm of diameter. On leaves, telia were mostly hypophyllous, rounded, isolated, or arranged in concentric circles causing early defoliation of infected plants (Fig. 2A). Symptoms on leaves were characterized by chlorotic spots on the adaxial side corresponding with telia on the abaxial side. Under high humidity conditions, germination of basidiospores was evidenced as a hyaline layer covering the telia (Fig. 2B).

Biology of *Puccinia lantanae*

Morphological characterization and microscopy

The rust produced one- and two-celled golden-brown teliospores (Fig. 2C). The one-celled were ovoid to ellipsoidal, 17-28.5 x 13.5-23 µm. The two-celled spores were ellipsoidal to clavate, constricted at the septum, 23-36 x 12-23 µm, with thick walls, 1.5-2 µm laterally, and 5-7 µm at the apices. Two-celled spores with an oblique septum were rarely observed. In all the teliospores, the pedicel was persistent, sometimes eccentrically inserted (Fig. 2D). A germ pore was often clear on the distal end of the cells (Fig. 2F). When germinating, two-celled teliospores formed one or two germ tubes as observed in other *Puccinia* species (Alexopoulos *et al*., 1996) (Fig. 2G). Occasionally, germ tubes ramified, but these did not develop metabasidia (Fig. 2H). Basidiospores were hyaline, ellipsoidal to shortly oval (Fig. 2E).

The commonest teliospores (about 75-80% of the population) were one-celled. Thomas *et al*. (2021) also considered this type as the dominant one on *Lantana camara*, recording abundances of up to 96%. The one-celled spores showed the same mean size as the teliospores formed by the same rust species on *L. camara*, but the two-celled spores were narrower than those measured on that host (Thomas *et al*., 2021). The constriction at the septum was reported by Barreto *et al*. (1995) also in teliospores developing on *L. camara*. Therefore, the rust species of the present research was confirmed through morphological studies.

Germination studies

Effect of temperature on teliospore germination and basidiospore formation

Two processes were observed, (i) germination of teliospores with germ tube and (ii) four-celled metabasidia (Fig. 2I), and formation of basidiospore. Not all the teliospores germinated and not all the germ tubes developed a metabasidium with basidiospores.

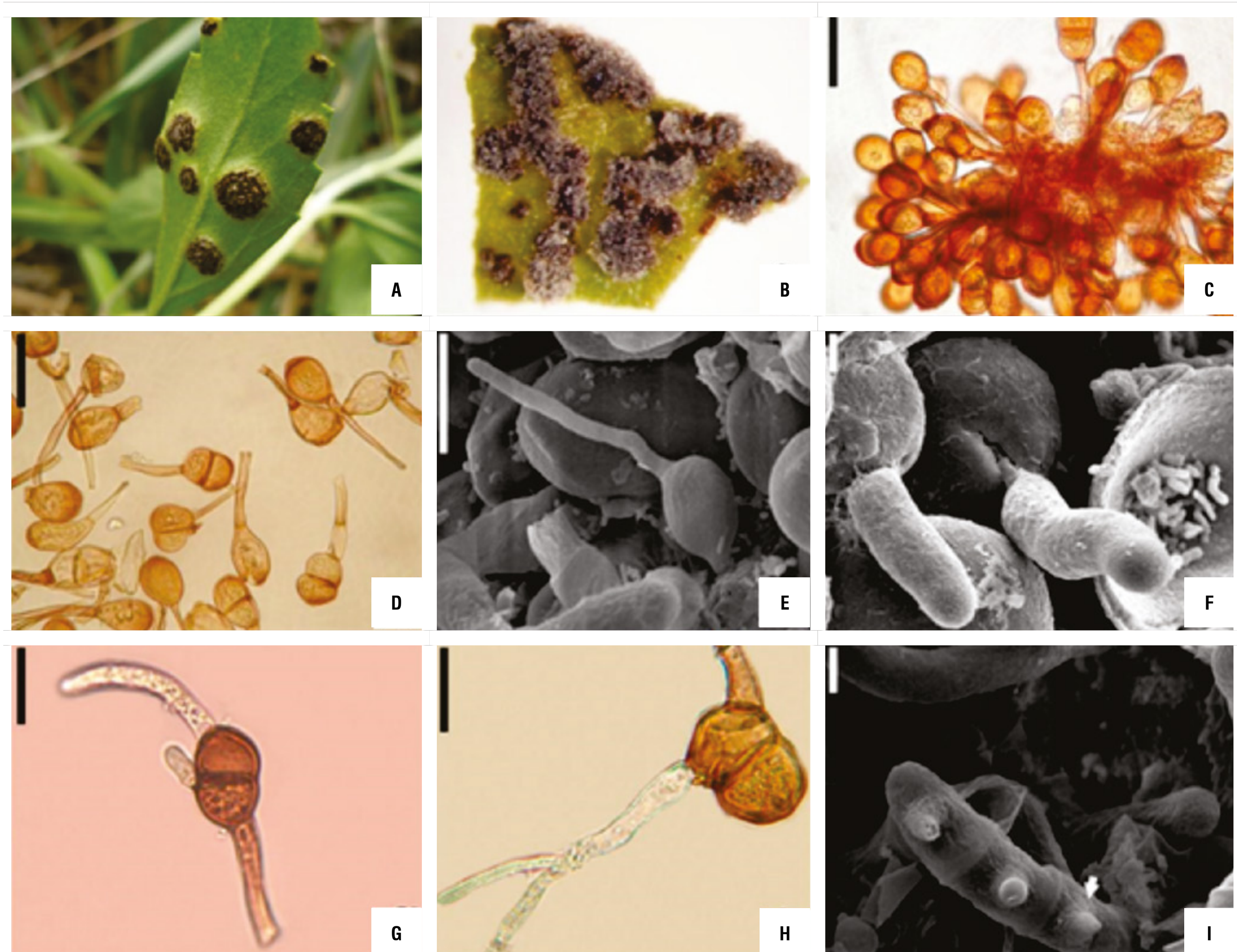

FIGURE 2. Telia of *Puccinia lantanae.* A) Infection symptoms in the field, B) Hyaline formation over telia of an infected leaf, C) One-celled and two-celled teliospores, D) Pedicel eccentrically inserted, E) Germinated basidiospore, F) Germ pore, G) Two-celled teliospores form two germ tubes, H) Ramification of germ tube, I) Metabasidia with conical sterigma. Bars: C and D: 30 μm, E: 10 μm, F: 2 μm, G: 20 μm, H: 15 μm, I: 3 μm.

Teliospore germination was influenced predominantly by temperature. As observed in Figure 3, the highest germination values were registered at 20°C at each incubation time ($P<0.05$). The same result was obtained by Thomas *et al.* (2021), working with an Amazonian pathotype of *P. lantanae* on *L. camara*. According to Thomas *et al.* (2021), the maximum infection on *L. camara* was obtained at 20°C. In the present study, the lowest germination figures were obtained at 30°C, while no infection was found on *L. camara* at 30°C (Thomas *et al.*, 2021). The germination started over a period ranging between four and 7 h of incubation (Fig. 3). Koutsidou (personal communication, August 21, 2020) obtained similar germination rates working with the Amazonian isolate of *P. lantanae* from *L.camara*.

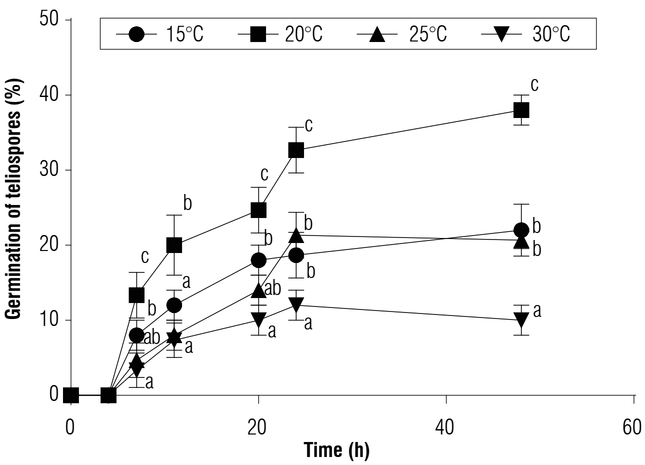

FIGURE 3. Germination of teliospores of *Puccinia lantanae* at different constant temperature regimes as a function of incubation time. Different letters indicate statistical differences among means (protected LSD Fisher test, $P<0.05$) at each incubation time. No teliospore germination was registered at 10°C. Bars show standard error.

The percentage of teliospores that produced basidiospores was determined at 10, 15, 20, 25, and 30°C (Fig. 4). No basidiospores formed at 10°C. At 20°C most of the teliospores formed metabasidia and produced basidiospores. Basidiospore development was inhibited or prevented by extreme temperatures. As observed in Figure 4, the optimum temperature for basidiospore formation was 20°C. Wide metabasidia with long sterigmata and long germ tubes did not form basidiospores. Similarly, Morin *et al.* (1992a) observed the branching of the germ tube, long germ tubes and wide basidia while working with *Puccinia xanthii* Schw. Abnormal germination can occur when telia are exposed to either sub or supraoptimal temperatures. However, the nuclear state of the mycelium which is formed from the long germ tubes needs to be addressed to establish its effective infection capacity.

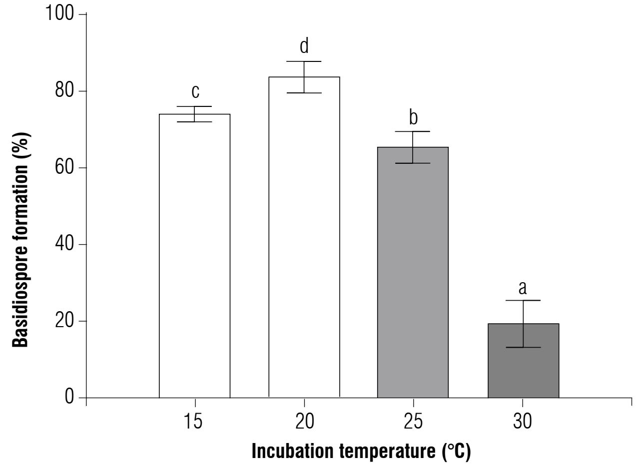

FIGURE 4. Basidiospore development of *Puccinia lantanae* at different constant temperature regimes after 24 h of incubation. Different letters indicate statistical differences among means (protected LSD Fisher test, $P<0.05$). Bars show standard error (n=3).

After 24 h of incubation at 20°C, four conical sterigma developed (Fig. 2I). Most of the basidiospores germinated while still joined to the sterigma or close to it as a common cytological phenomenon in WA.

Effect of heat shock on teliospore germination and basidiospore formation

The effect of high temperatures on the germination of teliospores was evaluated. Teliospore germination and basidiospore formation were influenced by the interaction between temperature and incubation time. Both heat shock treatments significantly reduced teliospore germination (75% in average) and basidiospore formation (64% in average) compared to the control (Figs. 5-6). Basidiospores

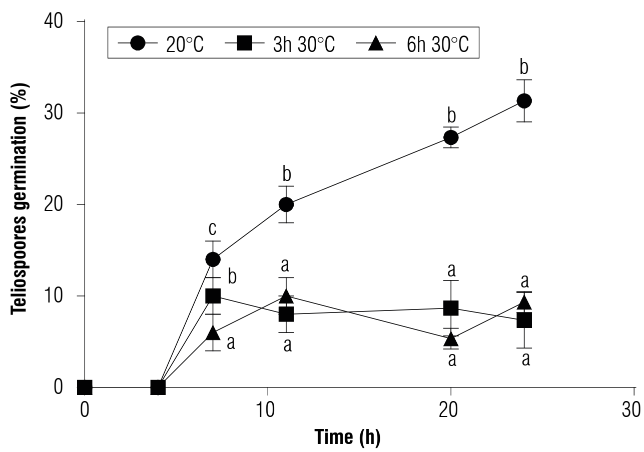

FIGURE 5. Effect of heat shock on germination of teliospores of *Puccinia lantanae*. Teliospores were incubated at 20ºC for 24 h (control), 3 h at 30ºC + 21 h at 20°C, or 6 h at 30ºC + 18 h at 20°C. Different letters indicate statistical differences among means (protected LSD Fisher test, $P<0.05$) at each incubation time. Bars show standard error (n=3).

formed in a sequential manner under high humidity conditions (Fig. 6) as well as in *P. araujiae*, thus, increasing the period over which the inoculum is produced and is available for new infections to take place (Anderson *et al.*, 2016). Similar observations were reported by Morin *et al.* (1993) in *P. xanthii*.

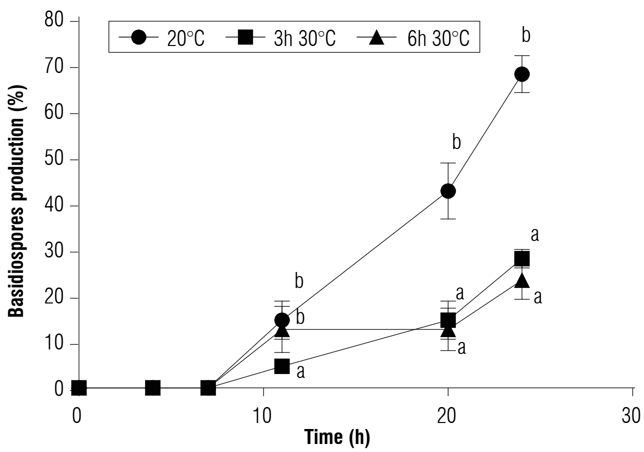

FIGURE 6. Effect of heat shock on formation of basidiospores of *Puccinia lantanae*. Teliospores were incubated at 20ºC for 24 h (control), 3 h at 30ºC + 21 h at 20ºC, or 6 h at 30ºC + 18 h at 20ºC. Different letters indicate statistical differences among means (protected LSD Fisher test, $P<0.05$) at each incubation time. Bars show standard error (n=3).

Effect of teliospores storage on germination

Storage on teliospore germination showed a negative exponential pattern (Fig. 7). A 50% reduction on germination was recorded after 7.2 months of storage at 3.0±0.5ºC.

Pathogenicity tests

The first symptoms of *P. lantanae* on *P. nodiflora* plants were visible after 15-20 d. Sporulation occurred mainly on the lower surface of the leaves. The rust caused significant damage to *P. nodiflora*, infecting leaf, petiole, and stem tissues, resulting in cankering and stem die-back as premature leaf drop.

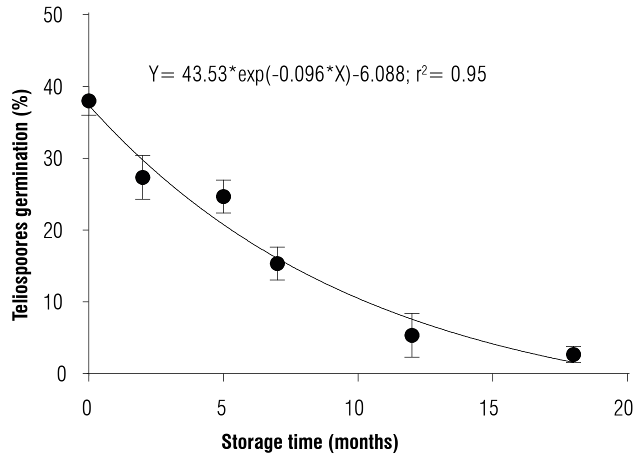

FIGURE 7. Effect of teliospore age of *Puccinia lantanae* on germination after 48 h of incubation at 20ºC. Different letters indicate statistical differences among means (protected LSD Fisher test, $P<0.05$). Bars show standard error (n=3).

The different symptoms that the three varieties of *Phyla nodiflora* plants developed after being inoculated with teliospores from different sample sites are shown in Table 2. Inoculation was considered successful when plants exhibited low or high levels of infection (Reaction 2 and 3). Only the *Puccinia lantanae* isolate R350n ("Formosa isolate") produced infection on all the varieties of *P. nodiflora*. *Phyla nodiflora* var. *nodiflora* was the variety that exhibited most resistance to infection. The rust isolates R264r ("Entre Rios isolate") and R205r ("Salta isolate"), both from *P. nodiflora* var. *reptans,* were successfully inoculated on *P.*

TABLE 2. Results of the inoculations with *Puccinia lantanae* on *Phyla nodiflora* (leaf disc method).

Experiment Nº	Inoculum source (site of origin)	Inoculated on	Reaction				Plants with symptoms	Plants inoculated (N: total number)
			0[1]	1	2	3		
1	R350n[2]	Pnr205[3]	0	4	12	2	16	18
		Pnm345	1	0	10	7	17	18
		Pnn350	4	6	8	0	14	18
2	R264r	Pnm345	0	3	4	11	18	18
		Pnn350	13	5	0	0	5	18
		Pnr264	2	5	0	11	16	18
3	R205r	Pnr205	0	3	3	12	18	18
		Pnm345	0	2	10	6	18	18
		Pnn350	11	7	0	0	7	18

[1] Reaction: (0) plants without symptoms, (1) chlorotic spots, (2) low level of sporulation (localized infection, 1-5 pustules) and (3) high level of disease (more than 5 pustules until semisistemic infection).

[2] Strain of the fungus. For example: R350n means that the strain of the rust (R) was originally found in site 350 on var. *nodiflora* plants.

[3] Inoculated plants, for example: Pnr205 means that *Phyla nodiflora* var. *reptans* was obtained from site 205.

N: 162

nodiflora var. *minor* and *P. nodiflora* var. *reptans*. These rust isolates would be potential candidates for biocontrol of the target weed.

Conclusions

The rust *Puccinia lantanae* exhibited pathogenicity on three varieties of *Phyla nodiflora,* including *P. nodiflora* var. *minor*, which is a weed with serious impact in Australia. However, some features of the fungal biology could alter its effectivity. The rust showed a mesophilic behavior; higher temperatures negatively influenced both the germination of teliospores as the development of basidiospores. In addition, aging also affected teliospores, diminishing their capacity of germination after five months of storage. Of the three isolates of *P. lantanae* studied, two are promising candidates for biocontrol of the weed but one should be disregarded as it infects *P. nodiflora* var. *nodiflora*, which is native to Australia. This study demonstrates that there are differences between isolates of the same pathogen, which is why in-depth genetical studies of potential agents are necessary as part of a biocontrol study.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

GT and GC designed the study. GT developed research within six-year field surveys and wrote the manuscript. AS performed field surveys with GT. GC and VB performed statistical analyses and in collaboration with AS contributed to manuscript content and revisions. All authors reviewed the final version of the manuscript.

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Supplementary material

TABLE S1. Sites visited to sample *P. nodiflora* var. *minor*, *P. nodiflora* var. *reptans* and *P. nodiflora* var. *nodiflora* and *P. lantanae* in southern South America. ND stands for no data available.

GPS	Code	Latitude (South)	Longitude (West)	Country	GPS	Code	Latitude (South)	Longitude (West)	Country
10	1	25.51	64.98	Argentina	203	32	24.27	64.85	Argentina
11R	2	ND	ND	Argentina	204	33	24.00	64.55	Argentina
18	3	27.51	64.89	Argentina	205	34	23.40	64.30	Argentina
33	4	37.17	59.42	Argentina	206	35	23.93	64.04	Argentina
34	5	36.85	59.89	Argentina	207	36	24.99	64.59	Argentina
53	6	29.14	59.24	Argentina	208	37	25.03	64.58	Argentina
81	7	27.43	58.85	Argentina	209	38	24.91	64.42	Argentina
92	8	37.19	59.05	Argentina	210	39	25.50	63.60	Argentina
126	9	35.01	58.75	Argentina	211	40	25.82	62.82	Argentina
128	10	32.08	60.59	Argentina	212	41	26.22	61.86	Argentina
144	11	34.60	60.96	Argentina	213	42	27.22	62.10	Argentina
150	12	27.94	63.41	Argentina	214	43	27.93	65.59	Argentina
180	13	22.87	64.36	Argentina	215	44	31.55	61.44	Argentina
184	14	29.61	57.22	Argentina	216	45	28.31	63.36	Argentina
186	15	34.46	60.04	Argentina	217	46	35.45	58.79	Argentina
187	16	34.45	61.87	Argentina	218	47	40.17	62.65	Argentina
188	17	34.13	63.40	Argentina	219	48	40.71	64.07	Argentina
189	18	33.60	65.30	Argentina	220	49	40.26	65.11	Argentina
190	19	33.30	65.88	Argentina	221	50	39.17	66.13	Argentina
191	20	32.85	65.47	Argentina	222	51	39.09	67.57	Argentina
192	21	32.29	65.69	Argentina	223	52	39.55	69.31	Argentina

Continued

GPS	Code	Latitude (South)	Longitude (West)	Country	GPS	Code	Latitude (South)	Longitude (West)	Country
193	22	31.80	65.00	Argentina	224	53	40.66	71.35	Argentina
194	23	30.62	65.47	Argentina	225	54	39.93	71.06	Argentina
195	24	29.70	66.80	Argentina	226	55	37.62	70.16	Argentina
196	25	28.45	66.87	Argentina	227	56	36.64	69.83	Argentina
197	26	27.22	66.82	Argentina	228	57	35.08	69.59	Argentina
198	27	26.06	65.95	Argentina	229	58	33.62	69.01	Argentina
199	28	25.58	65.57	Argentina	230	59	33.12	68.53	Argentina
200	29	24.72	65.50	Argentina	231	60	33.43	66.94	Argentina
201	30	23.92	65.46	Argentina	232	61	37.36	59.10	Argentina
202	31	23.68	65.44	Argentina	233	62	37.58	58.74	Argentina
234	63	38.54	58.64	Argentina	270	93	27.87	63.97	Argentina
235	64	38.52	60.51	Argentina	271	94	27.99	64.01	Argentina
236	65	38.71	60.45	Argentina	272	95	22.97	64.37	Argentina
237	66	38.98	61.28	Argentina	273	96	25.25	64.47	Argentina
243	67	30.05	59.53	Argentina	274	97	25.23	64.05	Argentina
244	68	29.02	59.17	Argentina	276	98	24.23	63.98	Argentina
245	69	28.34	58.43	Argentina	277	99	24.73	64.20	Argentina
246	70	27.97	57.58	Argentina	278	100	24.73	64.21	Argentina
247	71	27.63	56.83	Argentina	279	101	24.72	64.64	Argentina
248	72	27.77	55.81	Argentina	280	102	25.91	61.71	Argentina
249	73	27.75	57.25	Argentina	282	103	38.59	61.89	Argentina
250	74	27.55	58.52	Argentina	283	104	37.33	57.06	Argentina
251	75	27.12	58.97	Argentina	284	105	36.85	56.69	Argentina
252	76	26.48	58.28	Argentina	285	106	36.34	56.74	Argentina
253	77	26.48	58.29	Argentina	289	107	33.93	64.44	Argentina
254	78	26.02	58.39	Argentina	291	108	23.87	65.45	Argentina
255	79	25.58	59.23	Argentina	292	109	23.75	64.85	Argentina
256	80	25.23	59.87	Argentina	293	110	25.74	64.94	Argentina
257	81	25.34	59.94	Argentina	294	111	26.51	64.75	Argentina
258	82	25.62	60.08	Argentina	295	112	26.46	64.74	Argentina
259	83	27.07	59.71	Argentina	297	113	36.26	61.69	Argentina
260	84	30.07	58.00	Argentina	298	114	24.10	64.82	Argentina
261	85	30.60	58.47	Argentina	299	115	22.74	63.82	Argentina
262	86	31.24	59.24	Argentina	300	116	22.10	63.73	Argentina
263	87	31.71	60.53	Argentina	301	117	16.19	67.72	Bolivia
264	88	32.11	60.63	Argentina	302	118	16.22	67.68	Bolivia
265	89	32.11	60.63	Argentina	303	119	16.18	67.73	Bolivia
267	90	33.15	59.29	Argentina	304	120	16.21	68.85	Bolivia
268	91	24.73	64.64	Argentina	305	121	18.91	63.39	Bolivia
269	92	30.12	62.10	Argentina	306	122	18.64	63.29	Bolivia
310	123	34.56	59.37	Argentina	342	152	29.71	63.73	Argentina
311	124	34.46	59.51	Argentina	343	153	30.92	62.68	Argentina
312	125	34.63	60.51	Argentina	344	154	31.53	61.54	Argentina
313	126	34.60	61.01	Argentina	345	155	38.70	62.27	Argentina
314	127	34.84	61.50	Argentina	346	156	39.08	64.56	Argentina
315	128	35.51	63.00	Argentina	347	157	23.03	63.90	Argentina
316	129	35.91	62.82	Argentina	350	158	23.70	62.31	Argentina
317	130	36.90	62.39	Argentina	351	159	25.76	59.12	Argentina
318	131	38.01	63.34	Argentina	352	160	26.16	59.35	Argentina

Continued

GPS	Code	Latitude (South)	Longitude (West)	Country	GPS	Code	Latitude (South)	Longitude (West)	Country
321	132	36.70	64.28	Argentina	354	161	26.84	59.05	Argentina
322	133	35.50	64.10	Argentina	355	162	27.10	58.96	Argentina
323	134	35.04	64.26	Argentina	357	163	27.90	59.27	Argentina
324	135	29.88	61.26	Chile	359	164	36.76	56.70	Argentina
325	136	27.37	70.34	Chile	370	165	39.05	64.39	Argentina
326	137	28.56	70.81	Chile	371	166	39.30	65.66	Argentina
327	138	29.94	70.34	Chile	372	167	39.28	65.67	Argentina
328	139	31.87	71.40	Chile	373	168	37.33	66.49	Argentina
329	140	32.83	71.48	Chile	374	169	36.96	66.72	Argentina
330	141	33.52	71.60	Chile	375	170	34.86	67.78	Argentina
331	142	33.82	70.17	Chile	376	171	34.09	66.71	Argentina
332	143	35.46	72.48	Chile	378	172	26.77	59.71	Argentina
333	144	36.84	73.10	Chile	379	173	26.77	59.71	Argentina
334	145	37.25	73.32	Chile	381	174	26.95	59.85	Argentina
336	146	27.71	64.47	Argentina	382	175	25.54	60.02	Argentina
337	147	26.81	65.24	Argentina	383	176	25.49	59.98	Argentina
338	148	23.92	65.46	Argentina	386	177	24.34	61.13	Argentina
339	149	24.06	65.42	Argentina	387	178	27.41	58.84	Argentina
340	150	27.95	64.22	Argentina	388	179	27.33	58.44	Argentina
341	151	28.30	64.18	Argentina	390	180	27.39	64.31	Argentina
391	181	26.21	64.63	Argentina	ND12	200	38.39	62.85	Argentina
392	182	24.54	65.37	Argentina	ND13	201	37.86	63.80	Argentina
401	183	24.78	65.04	Argentina	ND14	202	37.12	64.29	Argentina
403	184	26.23	65.24	Argentina	ND15	203	36.40	64.28	Argentina
404	185	27.68	65.23	Argentina	ND16	204	35.95	64.28	Argentina
405	186	28.62	65.10	Argentina	ND17	205	34.11	64.38	Argentina
406	187	28.51	64.87	Argentina	ND18	206	34.16	64.38	Argentina
407	188	28.63	64.09	Argentina	ND19	207	34.72	64.42	Argentina
ND1	189	ND	ND	Argentina	ND20	208	33.28	64.42	Argentina
ND2	190	ND	ND	Argentina	ND21	209	33.00	64.16	Argentina
ND3	191	ND	ND	Argentina	ND22	210	33.02	63.59	Argentina
ND4	192	34.80	61.97	Argentina	ND23	211	33.12	63.08	Argentina
ND5	193	34.88	62.65	Argentina	ND24	212	33.21	62.59	Argentina
ND6	194	35.06	62.99	Argentina	ND25	213	33.30	62.05	Argentina
ND7	195	ND	ND	Argentina	ND26	214	33.59	61.47	Argentina
ND8	196	36.50	62.63	Argentina	ND27	215	33.84	61.73	Argentina
ND9	197	37.66	62.45	Argentina	ND28	216	34.18	61.51	Argentina
ND10	198	38.03	62.31	Argentina	ND29	217	34.58	61.00	Argentina
ND11	199	38.49	62.38	Argentina					