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## Studies to assess the suitability of *Uromyces pencanus* as a biological control agent for *Nassella neesiana* (Poaceae) in Australia and New Zealand

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**Abstract.** *Nassella neesiana* (Chilean needle grass), a South American species, is an intractable weed invading managed pastures and natural grasslands that has become a target for biological control in Australia and New Zealand. Studies have been carried out to assess the potential of three rusts naturally infecting this grass species in Argentina: *Uromyces pencanus*, *Puccinia graminella* and *P. nassellae*, as biocontrol agents. Of the three, *U. pencanus* was recognised as the most promising candidate. It causes significant damage to its host in the field and there is an isolate that can infect most Australian populations of the weed tested. Herein are described methods for: maintaining the rust in the glasshouse; storing urediniospores over 12 months; and, for inoculating urediniospores in order to test the host-specificity of selected isolates of the rust. Evidence from the literature, and a preliminary host range test indicates that *U. pencanus* is sufficiently host-specific for use as a classical biocontrol agent. Attempts at elucidating the life cycle of *U. pencanus* were unsuccessful as teliospores did not germinate. It appears that these have become redundant and the rust cycles as urediniospores on its grass host.

**Additional keywords:** field surveys, propagation, spore germination, spore storage.

### Introduction

*Nassella neesiana* (Chilean needle grass) is a stipoid grass from South America that causes serious problems in Australia and New Zealand (Fig. 1e–h). It is unpalatable to stock when mature, and forms dense stands in pasture that are difficult to manage and lead to losses in production. The sharp seeds, which give the plant its common name, contaminate wool and can blind livestock. The seeds can also penetrate the animals' skin and move into body muscles causing abscesses and the downgrading of pelts and carcasses (Bell 2006). *N. neesiana* also invades native grasslands, particularly in Australia where it threatens to infest extensive areas rich in indigenous species (McLaren et al. 1998). Consequently, *N. neesiana* has been included in the list of 'Weeds of National Significance' in Australia (Thorp and Lynch 2000). In New Zealand, the status of the weed varies geographically: it is a 'containment pest' in Marlborough where the largest and worst infestations occur; a 'restricted pest' in Canterbury where the weed is scattered and the focus is on reducing its spread; it is subject to 'surveillance' in Manawatu; and, a target for 'total control/eradication' in the Auckland, Waikato and Hawke's Bay regions where there are only a few small infestations (MAF 2009).

*N. neesiana* infestations are difficult to eradicate with herbicide because the weed is only readily identifiable when

producing aerial inflorescences, at which point seed is often already present. In addition to the panicle seed, the grass also produces hidden seeds (cleistogenes) that develop under the leaf sheath at each stem node and at the base of the stem (Connor et al. 1993). Herbicide will kill the vegetative parts of the grass but when applied at practical rates it will not kill all of the cleistogenes (Pritchard 2004). The seedlings that grow from cleistogenes can replace dead parent plants to produce a thicker sward of grass (Bell 2006). The grass spreads slowly, probably because seeds are not wind dispersed. Seeds fall to the ground around the parent plant and small numbers are picked up and dispersed from there by animals and/or farm machinery (Bourdôt 1995). As a result, in most areas containment of *N. neesiana* is perceived to be a less costly option for management than eradication (Bourdôt 1995).

In 1999, a biological control project was initiated to investigate pathogens for control of *Nassella trichotoma* (serrated or nassella tussock) and *N. neesiana* (Anderson et al. 2002). For the first 3 years, investigations were mainly focussed on *N. trichotoma*. Several pathogens were identified for this species: a rust; a smut; a crown rot Corticiaceae species; a *Rhizoctonia* species; four leaf spot Coelomycetes; and, one bacterial species (Briese and Evans 1998; Briese et al. 2000; F. E. Anderson, unpubl. data). Of these, the one showing the

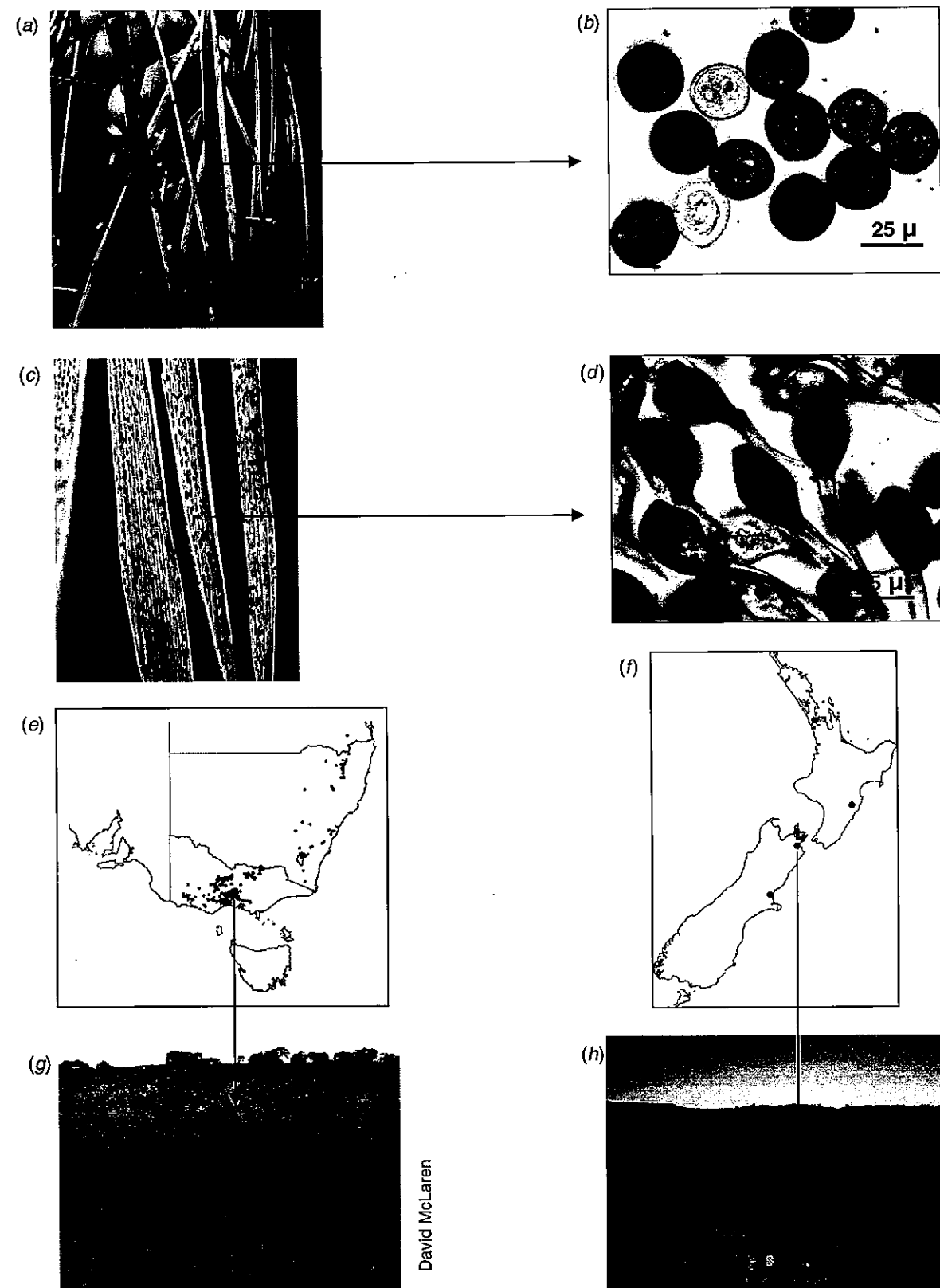


Fig. 1. (a) *Uromyces pencanus* uredinia infecting *Nassella neesiana* in Argentina. (b) Urediniospores. (c) Telia on detached leaves of *U. pencanus* collected from the field. (d) Teliospores. Distribution of *N. neesiana* in (e) Australia and (f) New Zealand. Dense *N. neesiana* infestations in (g) Australia and (h) New Zealand.

greatest potential and, therefore, the most studied was the rust *Puccinia nassellae*. As the project proceeded, it was shown that this pathogen was not as specific as first expected and that it was very difficult to work with. Results of studies on the smut and crown rot species were also disappointing (Anderson et al. 2004). Attention was then refocussed on the pathogens of *N. neesiana*, in the hope that these would be more promising. Five rust species, one smut species, a powdery mildew and a Coelomycete species that causes a leaf spot were identified (Briese et al. 2000; F. E. Anderson, unpubl. data). Of these, the rusts *Uromyces pencanus*, *Puccinia graminella* and *P. nassellae*, all of which naturally infect the grass in Argentina (Lindquist 1982), were selected for further study (Anderson et al. 2004, 2006). Subsequently, *U. pencanus* was recognised as the most promising potential biocontrol agent. This is because the literature suggested it had an autoecious life cycle (Arthur 1925; Cummins 1971; Lindquist 1982) and a narrow host range which appears to be confined to the genus *Nassella* (Arthur 1925; Cummins 1971; Lindquist 1982; Barkworth and Torres 2001). Also, our field observations indicated that heavily infected plants were at a disadvantage compared with sympatric healthy ones (Anderson et al. 2006). The rust can severely inhibit its host, first by robbing it of nutrients and water, and second by damaging the leaf epidermis with its fruiting structures. Heavily infected foliage senesced prematurely in hot and dry weather and plants with high levels of infection tended to produce less seed than healthy ones.

This paper provides information about: the behaviour of *U. pencanus* in the field; the development of methods for its collection and manipulation; conditions necessary for infection; and, efforts to clarify the rust's life cycle. The

information collected was applied while conducting a preliminary experiment on its host range. The suitability of *U. pencanus* as a biological control agent for *N. neesiana* in Australia and New Zealand is discussed.

## Material and methods

### Field surveys

Field surveys to locate and collect isolates of *U. pencanus* from *N. neesiana* populations were initiated in spring 2003, and continued for 5 years. A significant part of the geographical distribution of the grass in Argentina has been explored. All populations of *N. neesiana* encountered while travelling were inspected. Whenever a rust-infected plant population was found, infected material was collected in paper bags and dried in a plant press for further study in the laboratory, and a GPS reading was taken to record the site location. Dicotyledonous plant species that grew in close association with rust-infected *N. neesiana* plants were searched for aecia in case one should prove to be an alternate host of *U. pencanus*. Sites were numbered in the order in which they were recorded. Rust isolates were named with a prefix according to species (i.e. UP for *U. pencanus*), and with the number of the site at which they were collected. Material collected in the field was examined microscopically to identify fungal species and spore types. In addition, site 27 was visited frequently (every few weeks) during 12 months in an attempt to follow the life cycle of *U. pencanus* in the field throughout the year. Figure 2 shows the location of sites referred to in this paper, and the *U. pencanus* spore types encountered at each site.

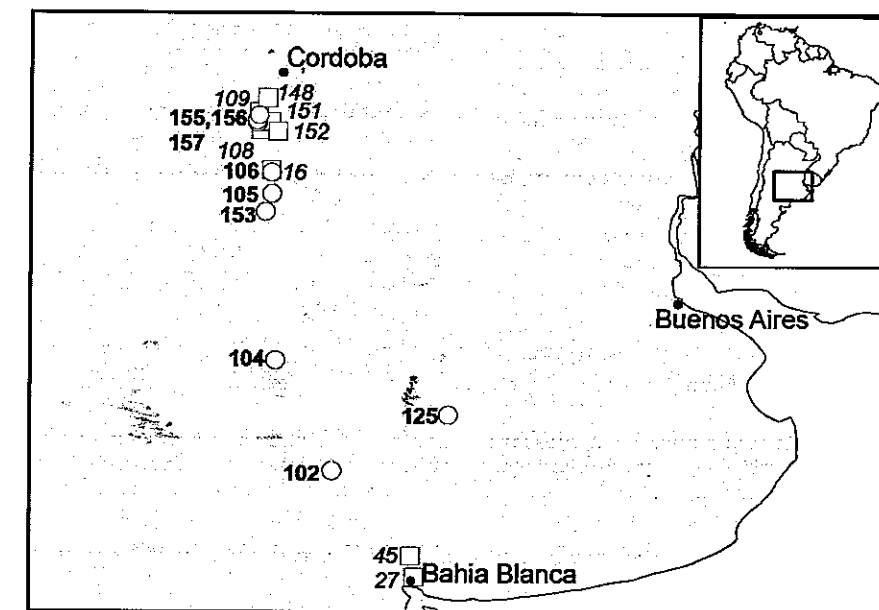


Fig. 2. Location of sites at which *Uromyces pencanus* was collected and spore types present. Sites were numbered in the order in which they were recorded, and these numbers, which are shown on the figure, were then used to identify rust isolates collected at these sites (e.g. UP27 was the *U. pencanus* isolate collected at site 27). ○ urediniospores present, □ urediniospores and teliospores present.

### Rust and plant propagation

As biotrophs, rusts can only be cultured in living plant tissues. Therefore, in order to produce inoculum of *U. pencanus* for experiments, it was necessary to establish a susceptible population of *N. neesiana* in the glasshouse. Previous experience with *P. nassellae* revealed differences in susceptibility among plant populations from various collection sites (Anderson et al. 2006). In order to deal with that possibility, seed of *N. neesiana* were collected at every site where the rust was sampled. The seed were stored in paper bags in the laboratory at room temperature for later use. In order to promote germination, glumes were removed with a scalpel and seeds were then placed in Petri dishes lined with wet cotton wool and filter paper and left on a glasshouse bench (temperature range 16–26°C) for ~7 days. At hypocotyl emergence, seedlings were planted individually into potting mix (10% organic matter, 70% ashes, 20% water, C/N: 18, pH 6.5) in plastic seedling trays. At the three leaves stage, plantlets were transferred to 10-cm-diameter plastic pots containing a 1:1 mixture of potting mix and local soil. Plants used in all experiments were grown in the same way.

Two rust isolates, UP27 and UP45 (see *Field surveys* section for naming of isolates) were cultured in the laboratory to provide inoculum for experiments. Cultures were started by transferring aggregations of urediniospores from a few clean pustules on naturally infected plants (from sites 27 and 45, respectively), onto the leaves of healthy plants grown from seed collected at these sites. Spore transfer was done with a sterile, fine-pointed needle under a stereomicroscope. Inoculated plants were misted manually with an atomiser and kept in dew chambers [100% relative humidity (RH) at 18–20°C under a 12-h light (fluorescent)/12-h dark regime] for the first 48 h. Dew chambers consisted of cube-shaped polyethylene boxes lined with water-soaked newspaper. After 48 h the plants were removed from the dew chambers and kept in controlled environment cabinets at ~70% RH, 18–20°C and a 12-h dark/12-h light (fluorescent, 1400 1×) regime. When pustules developed (15–20 days) spores from these pustules were used to inoculate other plants (applied as dry spores with a paint brush). All inoculated plants were kept in the environment cabinets while producing pustules. All inoculations performed in the experiments discussed here were undertaken as described above, unless otherwise stated.

Once rust cultures were established, plants from site 45 for isolate UP45 and from the Australian Capital Territory (ACT) for isolate UP27, were inoculated periodically to generate urediniospores for future experiments. Plants from the ACT were used for UP27 because, in preliminary inoculation experiments, this isolate was found to be more aggressive towards plants from the ACT site than to plants from site 27. For this same reason, ACT plants were used as positive controls for experiments involving UP27, unless otherwise stated. Spores were harvested under the stereomicroscope by means of a cyclone collector and stored, as described below, for later use.

### Spore storage

Tests were run to determine the best methods for short- and long-term storage of urediniospores. The effect of storage at

low temperatures on spore germination was tested by comparing germination rates of stored v. fresh spores. Spores were dried before storage by depositing them in open gelatine capsules and then placing the capsules in small plastic containers containing silica gel. The containers were stored at room temperature for 3 or 4 days, then six gelatine capsules, each containing ~0.01 g of spores, were closed and placed in another plastic container and left in a refrigerator (at ~4°C). Parallel to that, another batch of 12 closed capsules containing urediniospores were placed in a freezer (at -70°C). Subsequently, one gelatine capsule from each treatment was recovered from storage each month. Spores were dusted on to water agar and then incubated for 24 h at 18°C in the dark, to avoid a possible photo-inhibition of germination (Staples and Macko 1984; Mueller and Buck 2003). The germination of 100 randomly selected spores from each treatment was then assessed. To this end, the surface of the plate was scanned under a microscope (×10 objective) and the first 100 spores that were found were assessed for germination. A spore was considered to be germinated when its germ tube was equal to, or longer than, the spore diameter. The experiment was repeated once.

### Optimum temperature for spore germination

Tests were run to determine the optimal and limiting temperatures for urediniospore germination so that conditions during the experiments would be appropriate for germination and infection. Freshly collected urediniospores were dusted on to the surface of a plate containing water agar and incubated for 17 h in the dark at 5, 10, 15, 18, 25, 30 and 35°C. Samples (100 randomly selected spores each) were observed under the stereomicroscope, and the percentage of spores which germinated was evaluated as described above. The experiment was repeated once.

### Preliminary host range tests

Preliminary experiments were undertaken to ascertain the likelihood of UP27 being sufficiently host-specific to be used as a biocontrol agent. UP27 was applied to four plants of each of three grass species native to Argentina (*Nassella tenuissima*, *Stipa brachychaeta* and *Poa ligularis*) and two Australian species (*Austrostipa aristiglumis* and *A. scabra*). The methodology of inoculation was the same as described in the *Rust and plant propagation* section. The experiment was repeated once.

Two accessions of *N. trichotoma* from Argentina were also tested, as this species is also being targeted for biocontrol. That is, it would be highly desirable for the isolate of *U. pencanus* to be introduced against *N. neesiana* to also be effective against *N. trichotoma*. Two isolates, UP27 and UP45, were tested against *N. trichotoma* plants collected at sites 27 and 45, respectively. Plants tested (four from each site) had been well watered and subjected to 100% RH for 24 h before inoculation. *N. trichotoma* has very tightly rolled leaves and this procedure was known from previous experience to induce a very slight unrolling. This unrolling makes it possible to apply spores, with a fine needle, onto the adaxial (inner) side of the leaf blades, *U. pencanus* urediniospores are known to enter the leaf through the stomata (A. C. Flemmer, F. E. Anderson,

P. V. Hansen, D. A. McLaren, unpubl. data) and the stomata are on the adaxial surface in this species. The experiment was repeated once.

### Development of inoculation protocol for comprehensive host range testing

Given the high diversity of the Poaceae and the economic importance of numerous species belonging to this family, the list of plants to be tested for susceptibility to a potential biocontrol agent for *N. neesiana* will, by necessity, be extensive. Consequently, the development of an efficient and reliable method of inoculation, for assessing the host-specificity of the potential biocontrol agent, is a critical issue for this project. Such a method should ensure consistent disease development on susceptible plants, without being overly laborious, and without requiring excessive quantities of urediniospores. Experiments were conducted to test the efficacy of different inoculation methods and inoculum concentrations, and to determine ideal conditions for incubating plants after inoculation. In all these experiments, all of the mature pustules formed within the inoculated sections were counted 3–4 weeks after inoculation. When the boundary between two or more adjacent pustules could not be established, they were counted as one. This criterion was followed at all times when pustules were counted.

Experiment 1: This experiment had two objectives: first, to determine whether inoculum should be placed adaxially or abaxially in order to produce adequate infection levels; and, second, to test how dry urediniospores mixed with talcum powder compared with urediniospores suspended in water in inoculations. For all treatments and controls, spores of *U. pencanus* isolate 27 were applied, either dry or suspended in distilled water, to *N. neesiana* plants from the ACT, with 10 strokes of a paintbrush on marked (with a marker pen) 20-cm-long sections of selected leaf blades. All inoculated plants were misted with water after inoculation. Three leaves per plant and three plants per treatment were inoculated as shown in Table 1. Inoculated plants were misted manually with an atomiser and kept in dew chambers (100% RH) at 18–20°C under a 12-h dark/12-h light (fluorescent, 1400 1×) regime. After 48 h the plants were removed from the dew chambers and kept in controlled environment cabinets under

Table 1. Treatments for experiment 1

Inoculation method	Inoculum
<b>Controls</b> <sup>A</sup> . Spores applied dry to adaxial side of broadest section of leaves	Dry spores alone
Spores applied in solution to adaxial side of a leaf, 20 cm measured from base of blade upwards	4 × 10 <sup>5</sup> spores/mL in water
Spores applied in solution to adaxial side, 20 cm measured on broadest section	4 × 10 <sup>5</sup> spores/mL in water
Spores applied in solution to abaxial side of broadest section	4 × 10 <sup>5</sup> spores/mL in water
Spores mixed with talcum powder, applied dry to adaxial side of broadest section	Dry spores in talcum powder. Ratio of spores: powder = 1:50 (by weight)

<sup>A</sup>Words in bold text are used to distinguish treatments in Fig. 5.

the same conditions as above but with RH lowered to around 70%. After 4 weeks they were inspected under the stereomicroscope for pustule development. Pustules were counted as explained above.

Experiment 2: The relationship between disease severity and the concentration of dry urediniospores applied was investigated. Here urediniospores of UP27 were diluted in talcum powder and applied to *N. neesiana* plants from the ACT as described above. Four concentrations of spores in powder (w/w) were tested: 2.5, 3.3, 5 and 10%. The spore concentrations chosen for testing were based on the speed with which spores can be produced given the resources available. That is, if a concentration greater than 10% were found to be optimum, it would not be possible to produce enough spores to use such a concentration for host range tests. After inoculation, plants were misted with water. Two positive control plants were included and inoculated as explained for experiment 1. After inoculations, plants were treated as described for that experiment and results assessed in the same way. The experiment was repeated once.

Experiment 3: The presence of moisture (dew) after inoculation promotes pustule development. This experiment was to determine what length of 'dew period' after inoculation would be optimal for this. Two leaves of each of 15 plants from the ACT were inoculated with UP27 urediniospores mixed in talcum powder (3.3% spores w/w). Inoculation was performed as described above. Inoculated leaves were sprayed with water. Inoculated plants were put in a dew chamber at 100% RH and three plants were removed after each of the following time intervals: 6, 8, 12, 24 and 48 h. Plants removed from the dew chamber were kept in a controlled environment cabinet at around 70% RH, 18–20°C, and under a 12-h dark/12-h light (fluorescent, 1400 1×) regime, for 4 weeks. After that the number of developed pustules was counted as explained above. The experiment was repeated once.

### Statistical analysis

Results of experiments 1, 2 and 3 were analysed through fixed-effect nested ANOVA with the plants as the nested factor and the leaves as replicates. Data were transformed following Box and Cox (1964) procedures: square-root transformations were used for experiments 1 and 2 and a logarithmic one for experiment 3.

## Results

### Field surveys

*U. pencanus* was found infecting *N. neesiana* plants at 17 sites from Bahía Blanca, Buenos Aires Province in the south, to Alta Gracia, Córdoba Province, in the north (Fig. 2). Samples were collected at each of the sites, many of which were revisited over the years. Spores belonging to seven different isolates, including UP27, were obtained. Heavy infections with *U. pencanus* alone (Fig. 1a, c) were observed killing infected foliage, especially under hot, dry conditions. Seed production was often observed to be greatly reduced in rust-infected plants as compared with healthy plants at the same site. This was noticed both at sites where only *U. pencanus* was observed (e.g. site 108), and at sites where mixed infections of

*U. pencanus* and *P. graminella* were found (e.g. site 155). The rust spore stages found at each site where *U. pencanus* was collected are indicated on Fig. 2. Both urediniospores and teliospores of *U. pencanus* were found to be common in the field, but aeciospores of that rust were never encountered on *N. neesiana*.

Infected *N. neesiana* plants growing at site 27 were monitored periodically over a whole year and later visited sporadically over a period of 5 years. During the systematic surveillance of the first year, only uredinia were present most of the time, with peaks of infection in the autumn and spring. Telia developed at the end of spring to the beginning of the summer. No aecia were ever recorded at this site – neither on *N. neesiana* plants nor on other plant species growing in association with them.

#### Rust propagation

The method adopted for rust propagation was found to be satisfactory. Urediniospores could be collected weekly from infected plants and stored for later use. Spores can usually be harvested many (up to six) times from each infected leaf.

#### Spore storage

Spores were successfully stored in the refrigerator (~4°C) for up to 4 months, but germination rates did drop swiftly: with a 50% decrease (from 40 to around 22%) in just 2 months (Fig. 3). For long-term storage, a freezer (-70°C) was found to be much more effective. There was a small decrease in spore germination after the first month of storage at -70°C (as compared with freshly collected spores), but germination rates remained more or less constant thereafter (Fig. 3).

#### Optimum temperature for spore germination

Germination of urediniospores occurred under a wide temperature range but was higher between 15 and 25°C (Fig. 4). Infection rates were the highest between 18 and 20°C in preliminary inoculation tests (data not shown). Therefore, that range was selected for maintenance of plants during all artificial inoculation experiments.

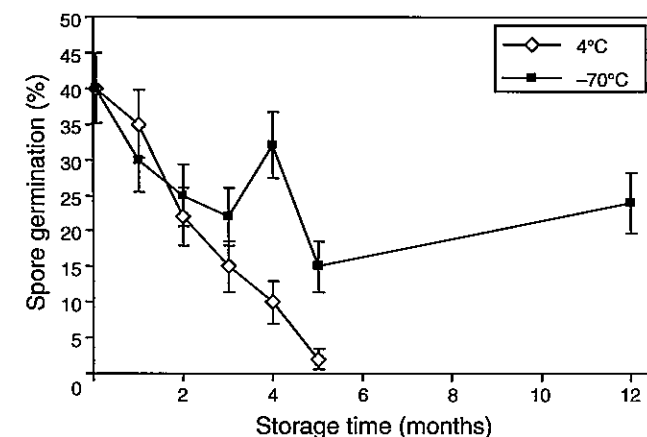


Fig. 3. Spore germination and storage time at two different temperatures (bars are  $\pm$ s.d. for binomial probabilities expressed as percentages).

#### Preliminary host range tests

Inoculation with *U. pencanus* isolate 27 did not result in pustule formation on any of the five non-target species tested. Positive control plants from the ACT did develop pustules. Isolates UP27 and UP45 failed to infect plants of *N. trichotoma* collected at sites 27 and 45, respectively, whereas they did infect all of the *N. neesiana* plants from the corresponding sites that were inoculated as positive controls.

#### Development of inoculation protocol for comprehensive host range testing

Experiment 1: Apart from the 'positive control', the treatment which resulted in the most pustules was the application of a urediniospore suspension to the adaxial side of the broadest part of the leaf ('broadest' treatment, Fig. 5). When dry spores were applied on the same section of leaves, few pustules were produced ('talcum' treatment, Fig. 5). No pustules were formed when inoculum was placed abaxially ('abaxial' treatment). While there was a large variation between treatments with respect to the mean number of rust pustules formed, these

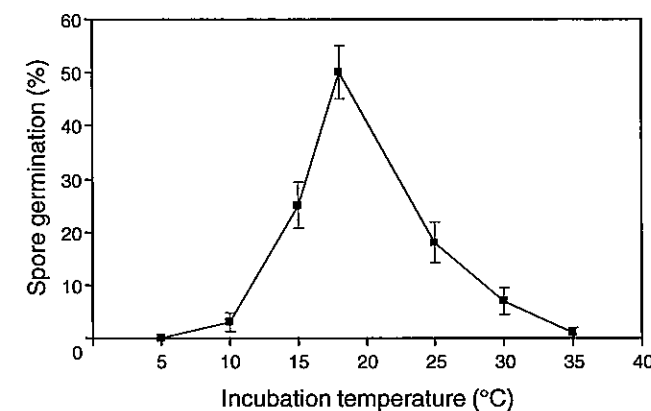


Fig. 4. Spore germination and incubation temperature (bars are  $\pm$ s.d. for binomial probabilities expressed as percentages).

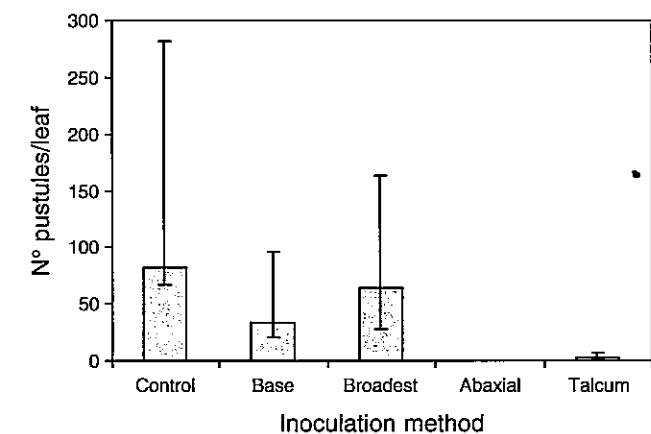


Fig. 5. Numbers of pustules/leaf (mean  $\pm$  s.e.) obtained with different inoculation methods (experiment 1). See Table 1 for details of inoculation methods.

differences were not statistically significant ( $P = 0.439$ ). This was mainly because of the highly significant variation between plants within each treatment ( $P < 0.0001$ , see error bars Fig. 5). None of the methods tested rendered better results than the 'control' treatment i.e. the application of dry spores alone.

Experiment 2: The number of pustules formed in response to inoculation with different concentrations of spores in talcum powder tended to increase with spore concentration, albeit not proportionally (Fig. 6). The number of pustules developed at the highest tested concentration (10% spores) was higher than in the positive controls (100% spores). No statistically significant differences ( $P = 0.453$  and  $P = 0.293$  for repetitions 1 and 2, respectively), were detected between treatments, the effects of which were masked by the very significant differences between plants within treatments ( $P < 0.0001$  for both repetitions). The highest percentage of infected plants and leaves was obtained with the highest concentration tested (Fig. 6).

Experiment 3: Plants that were subjected to a 24-h period in the humidity chamber at 100% RH developed more than twice as many pustules as those subjected to 6, 8 or 12 h of saturated humidity (Fig. 7). The 48-h dew period gave a similar number of pustules to the 24-h period. Results also indicated that 6 hours, the shortest period tested, was enough for pustule development, albeit in low numbers. There was no clear tendency in the percentages of infected plants and leaves in relation to the duration of the dew period. No significant differences were detected in the number of pustules that developed in response to the different amounts of time spent in the humidity chamber ( $P = 0.367$  and  $P = 0.738$  for the initial experiment and the repetition, respectively). Once again, this was probably due to the significant variation ( $P < 0.001$  and  $P < 0.05$  for each repetition, respectively), between plants within each treatment overcoming the variations due to the treatment.

#### Discussion

*U. pencanus* was found to be widespread in the northern-central temperate parts of Argentina. Climatic conditions vary

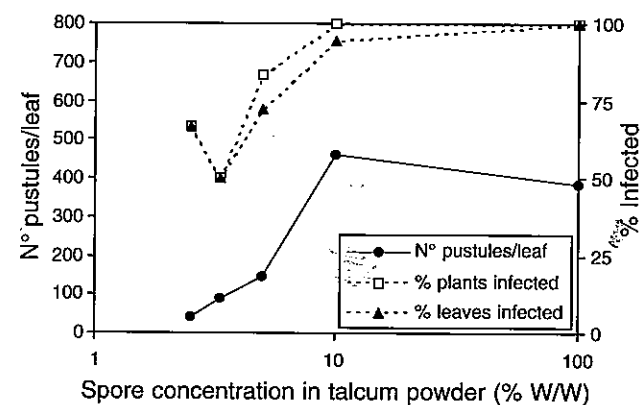


Fig. 6. Mean number of pustules per leaf (back-transformed) obtained with four different concentrations of spores in talcum powder. Data pooled for both repetitions of experiment 3. Percentage of infected plants and leaves for each concentration is also shown. Differences between treatments were not statistically significant (see main text for details). Note that the scale of the x-axis is logarithmic.

significantly within this large distribution area, with more rainfall and milder winter temperatures at sites in Córdoba, compared with those in the Buenos Aires province. The most promising isolate, UP27, was collected from site 27, which is in Buenos Aires province.

*U. pencanus* causes significant damage to *N. neesiana*, both in the field and under controlled conditions, and is therefore expected to have a significant impact on weed populations should it be introduced into Australia and/or New Zealand as a biological control agent. Heavily infected plants developed intense chlorosis, which hastened their senescence. *U. pencanus* has the typical effects of a biotrophic pathogen, imposing a chronic energy drain on its host reserves (Charudattan 2005). The accumulated effect of rust infection throughout host development may substantially inhibit seed production (Paul and Ayres 1986). This effect was observed in the field, with less seed being produced by infected plants than by sympatric healthy ones. At sites where *P. graminella* was also present and causing mixed infections with *U. pencanus*, an even more marked reduction in seed production was observed. This suggests that the two pathogens may have a complementary or synergic adverse effect on the host. Plants stressed by rust infection have been shown to decrease their competitive ability with other plant species (Groves and Williams 1975; Paul and Ayres 1987; Müller-Schärer and Rieger 1998). It is anticipated that infection with *U. pencanus* will exert a similar negative effect on the competitive ability of *N. neesiana* in Australia and New Zealand, leading to its gradual replacement by beneficial species. This is consistent with the philosophy underpinning the classical approach in biological control of weeds (Evans et al. 2001).

Methods have been successfully developed for maintaining a pure culture of the rust in the glasshouse and for storing urediniospores. Storage in the freezer at -70°C proved to be the best method to keep spores viable in the long term as it has been shown they keep well for at least up to 12 months at this temperature. A mycoparasite covered the pustules on some of

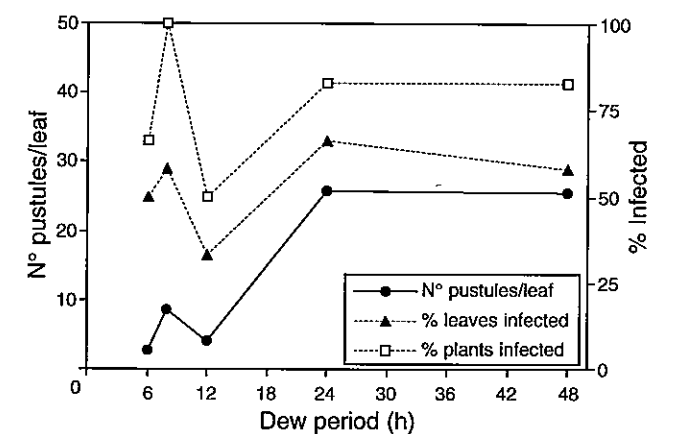


Fig. 7. Mean number of pustules per leaf (back-transformed) obtained after subsection to different periods at 100% humidity. Data pooled for both repetitions of experiment 4. Percentage of infected plants and leaves with each concentration is also shown. Differences between treatments were not statistically significant (see main text for details).

the inoculated plants during the early stages of the mass rearing process. It was identified as *Simplicillium* sp., formerly within *Verticillium* section *Prostrata* (Zare and Gams 2001), a group known to comprise, among others, species which can attack other fungi (Gams and Zare 2001). While this hyperparasite was not obvious in the field, it thrived under our experimental conditions, thus interfering with the multiplication of spores. Storage of spores in the freezer was found to eliminate the mycoparasite from our system, as its conidia do not survive such low temperatures. There is still insufficient information to allow the assessment of the impact of hyperparasitism on both fungal and plant populations in nature, but at least in some cases, it has been shown that naturally occurring hyperparasites can significantly reduce the infection pressure of pathogens on their host plants (Kiss 2001). Field infection of *U. pencanus* by *Simplicillium* sp. has not been observed in Argentina, but nevertheless care should be taken to ensure that *U. pencanus* isolates to be introduced into Australasia are free from this mycoparasite.

Before a biological control organism can be considered for importation into Australia or New Zealand, its life cycle should be fully documented. *U. pencanus* was reported to be autoecious, i.e. to complete its entire life cycle on one host (Arthur 1925; Greene and Cummins 1958; Cummins 1971; Lindquist 1982). However, no aecial stage that could potentially belong to *U. pencanus* was observed during the field surveys. Teliospores did not germinate under any of the various methods applied to induce the production of basidiospores (these methods will be described in a separate publication). In the absence of basidiospores, it was not possible to elucidate the life cycle experimentally. It is possible that the rust is heteroecious, but despite considerable effort it was not possible to link *U. pencanus*-infected *N. neesiana* and aecia-bearing dicotyledons in the field (F. E. Anderson, M. L. Díaz, J. Barton, A. C. Flemmer, P. V. Hansen and D. A. McLaren, unpubl. data).

It is hypothesised that teliospores may have become redundant in the *U. pencanus* life cycle and that the rust would cycle in the form of urediniospores on its grass host. It can be very difficult or even impossible to prove a negative, such as the non-existence or irrevocable loss of spore stages (Evans 1993; Ellison et al. 2006). Authorities in Australia and New Zealand are known to consider prospective agents on a case-by-case basis. In Australia, other agents have previously been approved for release despite an incomplete knowledge of their life cycle, as it was concluded the benefits of such a release outweighed the potential risks it posed (Ellison et al. 2006). As *U. pencanus* seems a promising biocontrol agent in all other aspects investigated to date, approval for its introduction may be granted on the same grounds.

The success of biological control programs depends, among other factors, on finding a precise genetic match between the target weed genotypes and the pathogen isolate/s selected (Charudattan 2005). Several isolates of *U. pencanus* were applied to different Australasian accessions of the target weed and were found to differ with respect to the weed populations they could damage (F. E. Anderson, unpubl. data). Isolate UP27 caused disease in most of the Australian populations of *N. neesiana* that were tested and was, therefore, subsequently

chosen for further host range testing. However, this isolate showed differences in its aggressiveness towards the different accessions, being consistently aggressive towards plants from Canberra in the ACT and Goulburn in New South Wales but showing great variation towards plants from Laverton and Truganina in Victoria (F. E. Anderson, unpubl. data).

When intraspecific variation in resistance is detected, as is the case here, the ramifications for the biological control program can be significant, as pointed out by Morin et al. (2006). Additional isolates of *U. pencanus* will clearly be needed in order to supplement the effect of UP27.

The preliminary results of host range testing were promising. Positive control plants consistently developed pustules, whereas all five non-target species tested remained free of disease. Even the closely related weed *N. trichotoma* was resistant to the rust, probably indicating that the rust is highly host-specific. More comprehensive host range tests, involving more than 60 non-target species (all in the Poaceae), are presently underway. If the results of these tests are satisfactory, then *U. pencanus* isolate 27 will have excellent prospects as a biocontrol agent, at least for six populations of *N. neesiana* in Australia.

The results of experiments conducted to develop an inoculation protocol for comprehensive host range testing were affected by the fact that some of the plants did not become infected at all, although others, from the same accession and under the same treatment, became heavily infected. This masked the effect of treatments. That being said, these experiments did provide some guidelines for the development of a protocol. Spores should be placed on the adaxial, and not the abaxial, side of blades, and the broadest part of the leaf blade is suitable for inoculation. Although fewer pustules were obtained in the first experiment when spores were applied mixed in talcum powder, as compared with suspended in water, the former method was much easier than the latter. This is because the talcum powder is visible to the naked eye and that makes it possible to see the difference between inoculated and non-inoculated tissue without using a microscope. Higher concentrations of spores in powder were tested in the second experiment and it was observed that the number of pustules that developed tended to increase with spore concentration, albeit not proportionally. Pustule number did not level off at the highest concentration tested, so it is likely that the optimum spore concentration was higher than those tested. However, as discussed in Material and methods, it would not be practical to use higher spore concentrations. The main limitation for spore production is the availability of space in controlled environment cabinets. These cabinets must house both plants used for culturing the rust, and plants inoculated for host range testing, so there is a limit to the number of spores that can be produced for/during host range tests. The number of pustules obtained using the highest concentration of spores in talcum powder tested here (10% spores w/w) was higher than that obtained using spores alone, indicating that many spores would be wasted if applied undiluted. This could be because of a 'crowding effect' a phenomenon in which spores that contain self-inhibitors, and are crowded together, tend to germinate poorly or not at all (Staples and Macko 1984), or because the capacity of inoculated leaves to produce pustules can be

saturated (Gilles and Kennedy 2003). The concentration of 10% spores in talcum powder also gave the best results in relation to the percentage of infected plants and leaves, as compared with the other dilutions tested, so, this concentration of spores in talcum powder appears to be the best choice for host range experiments. Finally, results of the third experiment indicated that a 24-h dew period would be the most appropriate. Plants under this treatment developed more than twice as many pustules as plants subjected to any of the shorter dew periods tested and the longer period tested (48 h) did not lead to any further increase in pustule formation.

A wide variation in reactions of *N. neesiana* individuals to inoculation with the fungus was observed during our experiments. Although some of the problems might be related to some individuals tested being resistant, others involved individual plants known, from previous experiments, to be susceptible. The number of pustules observed after inoculation varied from zero to many, even for different leaves of the same plant. Fungal infection processes are affected by the developmental history of the leaves (Hatcher et al. 1995). For example, it has been suggested, for the *Betula pendula*-*Melampsorium betulinum* system, that differences in susceptibility among inoculated leaves could be the result of differences in physiological activities such as photosynthesis and carbohydrate content, which would in turn change with leaf age (Poteri et al. 2001). Inoculated leaves were deliberately chosen so that their aspect and developmental stage seemed alike, but it cannot be ruled out that some kind of difference existed between them. There are some indications that mite infestations can induce resistance to fungal infection (Karban et al. 1987). It was observed that heavy mite infestations of *N. neesiana* can inhibit infection by *U. pencanus* (F. E. Anderson, unpubl. data). For this reason, only plants that were free from mite infection were included in the studies. No plausible explanation can be provided at present for the differences in rust infection found between plants and leaves, but there must be some metabolic process by which some leaves can become resistant at a given stage and this fact should be born in mind if *U. pencanus* should eventually be used as a biocontrol agent.

*U. pencanus* is a highly damaging pathogen of *N. neesiana* and is relatively easy to mass rear and store. It has been shown that it can infect six out of seven tested Australian accessions of *N. neesiana* but not the two *N. neesiana* populations from New Zealand tested to date (Anderson et al. 2008). Preliminary host range experiments with closely related *Nassella* species and two Australian *Austrostipa* species suggest the rust can only attack the target weed. It is an excellent candidate for biological control and future work will involve searches for novel isolates of *U. pencanus* complementary to UP27, as well as completing the comprehensive host-specificity testing of this rust species.

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## Detection of three major RNA viruses infecting sugarcane by multiplex reverse transcription–polymerase chain reaction (multiplex-RT-PCR)

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**Abstract.** A multiplex reverse transcription–polymerase chain reaction (multiplex-RT-PCR) was developed for the detection of *Sugarcane mosaic virus* (SCMV), *Sugarcane streak mosaic virus* (SCSMV) and *Sugarcane yellow leaf virus* (SCYLV), three of the major RNA viruses widely prevailing in the sugarcane-growing regions around the world. Primers designed from the coat protein genes of the respective viruses amplified fragments of ~860 bp (SCMV), ~690 bp (SCSMV) and ~615 bp (SCYLV) in multiplex-RT-PCR. In this study, we could specifically detect the target viruses in disease-suspected sugarcane cultivars. Of nine samples tested, four were found infected with all three targeted viruses, two had infections of SCMV and SCSMV and the remaining three samples had the viruses found separately. Furthermore, multiplex-RT-PCR was found to be equally efficient to uniplex-RT-PCR to amplify the target viruses from sugarcane. This is the first report on detecting three viruses together in a single PCR reaction in sugarcane and this will have immense application in sugarcane quarantine programs.

### Introduction

Sugarcane is one of the major commercial crops growing widely in tropical and subtropical regions in the world. The cane production in these regions is limited by various etiological agents, viz. fungi, bacteria, phytoplasma and viruses. Mosaic and yellow leaf disease pose a potential threat to sugarcane cultivation worldwide. The potyviruses *Sugarcane mosaic virus* (SCMV) and *Sorghum mosaic virus* (SrMV) cause mosaic in sugarcane under natural conditions (Grisham 2000). However, in India, there is no molecular evidence for the occurrence of SrMV in sugarcane (Viswanathan et al. 2008a). Recently, the association of *Sugarcane streak mosaic virus* (SCSMV), an unassigned member of *Potyviridae* either alone or in combination with SCMV was established to cause sugarcane mosaic in India (Viswanathan et al. 2007) and we proposed a new genus name, 'Susmovirus' for the newly identified virus (Viswanathan et al. 2008a). These two viruses had previously been reported to cause mosaic in most Asian countries, including India (Chatenet et al. 2005). Yellow leaf disease (YLD) of sugarcane is caused by *Sugarcane yellow leaf virus* (SCYLV), a *Polerovirus* belonging to the *Luteoviridae*, and was described in 1988 in Hawaii (Schenck 1990) and in India in 1999 (Viswanathan 2002). SCYLV causes significant reduction in plant growth and yield (Comstock et al. 1994; Schenck et al. 1997).

Although there are several reports on the simultaneous occurrence of different mosaic causing viruses/strains on Poaceae family members, viz. SCMV and SrMV on sugarcane in the United States (Yang and Mirkov 1997) and Argentina

(Perera et al. 2007), SCMV-Eg and MDMV-2SA on maize in France (Marie-Jeanne et al. 2000), SrMV strains SCM, H, M and I in Louisiana from commercial sugarcane varieties (Grisham 1994), SCM+SCI and SCM+SCH (Perera et al. 2007), SCMV-A and SCMV-F from Pakistan, SCMV-A and SCMV-D from South Africa and SCMV-B and SCMV-J from Venezuela (Marie-Jeanne et al. 2000) and MDMV and SCMV on Poaceae in Yugoslavia (Krstic and Tomic 1995), no attempt was made to develop multiplex-RT-PCR for the detection of different RNA viruses occurring in sugarcane, except by Smith and Van de Velde (1994), who developed duplex-RT-PCR protocols for the simultaneous detection of SCMV and *Fiji disease virus*.

Since all three viruses (SCMV, SCSMV and SCYLV) are omnipresent in almost all the sugarcane-growing countries of the Asian continent (Hema et al. 1999; Gaur et al. 2003, 2006; Chatenet et al. 2005; Viswanathan et al. 2007, 2008b, 2008c), development of multiplex reverse transcription–polymerase chain reaction (multiplex-RT-PCR) has the potential advantage to detect all three viruses in a single reaction. To this end, coat protein gene-specific primers were designed to amplify all three viruses from symptomatic sugarcane and multiplex-RT-PCR conditions were optimised to detect all three viruses from sugarcane samples.

### Materials and methods

#### *Sugarcane samples used*

The first unfurled leaf in the whorl from nine symptomatic sugarcane varieties (Table 1) were collected during the 2006–07 crop season and the total RNA was isolated using