Pathogens for the biological control of weedy stipoid grasses in Australia: completion of investigations in Argentina

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Summary

Nassella trichotoma (serrated tussock) and *Nassella neesiana* (Chilean needle-grass) are the two most widespread and damaging species of stipoid grasses that have been introduced into Australia. A project was set up in 1999 in Argentina to investigate the potential of pathogens as biological control agents for these species. A Corticiaceae fungus found at a few sites, growing in association with *N. trichotoma* plants severely affected by root and crown necrosis, could not be studied in detail because all attempts to isolate it in pure culture failed. Infection of inflorescences of *N. trichotoma*, *Nassella tenuis* and *Nassella tenuissima* was achieved in the glasshouse with the smut *Ustilago* sp. (within *U. hypodytes sensu lato*), seen causing drastic reduction in seed production on both target plant species in the field. However, technical difficulties regularly encountered during experimental work compromise the prospect of further studies on this pathogen. The bulk of the investigations concentrated on the rust *Puccinia nassellae* which infects both target plants and, on the basis of field data, showed the greatest potential for biological control. Rust isolates from *N. trichotoma* were previously found to infect a wide range of *N. trichotoma* accessions and a non-target native Australian species. Host-specificity tests conducted in this study showed that rust isolates from *N. neesiana* were able to develop mature uredinia on *N. neesiana* plants grown from seed collected in Australia, but none of the tested isolates infected the Australian native species *A. aristiglumis* and *A. scabra*. Further testing is still required to clarify the nature of this rust's life cycle and to investigate differences in specificity between isolates from both host species.

Keywords: *Austrostipa* species, biological control, *Nassella neesiana*, *Nassella trichotoma*, pathogens.

Introduction

Nassella trichotoma (Nees) Arech. (serrated tussock) and *Nassella neesiana* Trin. & Rupr. (Trin. & Rupr.) Barkworth (Chilean needle-grass) are the two most widespread and damaging species of stipoid grasses that have been introduced into Australia (McLaren *et al.* 1998). *Nassella trichotoma* has been estimated to infest over 1 million ha through New South Wales and Victoria (McLaren *et al.* 1998) and costs the Australian grazing industry in New South Wales alone, around $40 million per year (Jones & Vere 1998). *Nassella neesiana* is considered a very serious environmental weed that is spreading rapidly and threatens to infest extensive areas of native grassland in south-eastern Australia (McLaren *et al.* 1998). A project was set up in 1999 in Argentina to investigate the potential of pathogens as biological control agents for these species

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(Briese & Evans 1998, Briese *et al.* 2000). On the basis of preliminary field observations it was decided to prioritize for evaluation as potential biological control agents the smut *Ustilago* sp. (within *Ustilago hypodytes* (Schlecht.) Fr *sensu lato*) and the rust fungus *Puccinia nassellae* Arth. & Holw. A third species, a soil fungus believed to belong to the Corticiaceae, was included, with reservations, as a third prospective candidate.

The Corticiaceae fungus has been found at three of the 73 sites surveyed from 1999 to 2002 and was always associated with dying patches of *N. trichotoma* plants showing root and crown necrosis (Briese & Evans 1998, Anderson *et al*. 2002). All attempts to isolate the pathogen on artificial media have failed, as have artificial inoculations of plants. In glasshouse and field host-specificity tests reported in Anderson et al. (2002), which included target and non-target plant species, the control *N. trichotoma* plants were not infected by the Corticiaceae fungus under the given conditions, precluding any conclusions to be drawn on the specificity of this pathogen.

The smut *Ustilago* sp. was seen in the field preventing seed formation on severely attacked plants of *N. trichotoma* and *N. neesiana* (Anderson *et al*. 2002). However, the incidence of the disease in the field was usually low, with only a few exceptions. A severe outbreak of the disease on *N. trichotoma* was recorded at two of the surveyed sites, whilst such a high level of disease on *N. neesiana* was found at only one. Interestingly, at the latter site where *N. neesiana* plants were severely diseased, a large population of neighbouring *N. trichotoma* plants showed no signs of infection, suggesting that cross-infection between smut isolates from these plant species does not occur in the field (Anderson *et al*. 2002). In a preliminary host-specificity test, Anderson *et al*. (2002) demonstrated that the South American native *Nassella tenuissima* (Trin.) Barkworth was susceptible to a smut isolate from *N. trichotoma*. However, no infection could be recorded on the control *N. trichotoma* plants because they failed to flower, despite the application of gibberellic acid, which is known to trigger flowering. Therefore, this test could not be considered conclusive for the other plant species tested (*Austrostipa scabra* (Lindley) S.W.L. Jacobs & J. Everett, *Nassella tenuis* (Phil.) Barkworth and *N. neesiana*) that did not become infected.

The bulk of the investigations concentrated on the rust fungus *P. nassellae*, which infects both target weed species and has been partially reported on previously (Anderson *et al.* 2002). Only uredinia of the rust have been found on *N. trichotoma*, whilst both uredinia and telia have been recorded on *N. neesiana.* Levels of infection in the field depend highly on environmental conditions, ranging from hardly detectable after prolonged dry periods to severe outbreaks that kill plants under favourable wet conditions. Cross-inoculations of *P. nassellae* isolates between the two target stipoid species have not resulted in any infection, indicating the presence of different strains of the rust adapted to specific hosts (Anderson *et al.* 2002). Anderson *et al.* (2002) found that rust isolates from *N. trichotoma* infected all tested accessions of this plant species, including representatives from Australian populations. Two of the tested isolates infected and developed mature uredinia on the Australian native species *Austrostipa aristiglumis* (F. Mueller) S.W.L. Jacobs & J. Everett, but none of the isolates infected either the three tested South American stipoid grasses from different genera or the Australian native *A. scabra*. In contrast, preliminary tests showed that rust isolates from *N. neesiana* were capable of infecting only the plant accessions from which they originated (Anderson *et al.* 2002).

In this paper, we report on the most recent findings obtained during the last phase of the project in Argentina. These include results from further host-specificity tests with *Ustilago* sp. and isolates of *P. nassellae* from *N. neesiana*. We also report on further attempts to elucidate the life cycle of *P. nassellae*, as well as results from screening additional rust isolates from *N. neesiana,* in order to identify one that is pathogenic on an Australian accession of *N. neesiana*. Future possible courses of action for this project are discussed in the light of these findings.

Materials and methods

Ustilago sp.

Cross-inoculation test: Pre-germinated seeds of *N. neesiana* and *N. trichotoma* collected at site 64 (Table 1) were dusted with large quantities of dry freshly harvested smut spores and then sown at a 2-cm depth in potting mix contained in plastic trays with 3-cm diameter cavities. Undusted pre-germinated seeds of these two species were planted in a different tray as a control. Spores collected from smut-infected inflorescences of *N. trichotoma* at site 07 were used to inoculate *N. neesiana*, whilst spores collected from smut-infected inflorescences of *N. neesiana* at site 64 were used to inoculate *N. trichotoma*. A total of 42 *N. neesiana* and 16 *N. trichotoma* plants emerged from inoculated seeds in the glasshouse (temperature range 16–26ºC) while 22 and 19 plants, respectively, emerged from the untreated control seeds. After three months, all plants were transferred to 10-cm pots containing potting mix and kept in the glasshouse until the onset of flowering. Plants were monitored weekly to detect the first appearance of smut symptoms on the inflorescences.

Host-specificity test: Pre-germinated seeds of different accessions of *N. neesiana* and *N. trichotoma* from Argentina and Australia, *A. scabra* from Australia, and *N. tenuis*, *N. tenuissima*, *Piptochaetium napostaense* (Speg.) Hack , *Stipa clarazii* Ball and *Stipa gynerioides* Phil. from Argentina were inoculated, using the same method as above, with dry smut

spores collected from smut-infected inflorescences of *N. trichotoma* at site 07. Undusted pre-germinated seeds of each of the species were planted as control. Inoculated and untreated seeds were sown as above in potting mix contained in different plastic trays in the glasshouse and plants that emerged were transferred to pots after 12 weeks. Plants were monitored weekly to detect the first appearance of smut symptoms on the inflorescences.

Table 1. Details of sites mentioned in the text.

Site ID	Site location (nearest town)	Coordinates	
		ºE	ºS
07	Villa La Gruta	38.15033	62.08607
16	Alcira	32.73529	64.34049
27	Bahía Blanca	38.66602	62.23448
30	Villa Ventana	38.03206	61.98911
45	Tornquist	38.36551	62.28152
52	Coronel Suarez	38.02574	61.38724
64	El Crucero	31.90762	64.52332
94	Napaleofú	37.40969	58.97501
99	Tandil	37.41076	59.15270

Puccinia nassellae

Life cycle: *Nassella neesiana* plants were grown from seed collected at site 16 in potting mix contained in 3-cm diameter pots. Two different methods were used to inoculate plants (*ca.* 2-months-old) with telia (*ex*. 16) that had previously been treated to break dormancy (Anderson *et al*. 2002). In one method, treated but ungerminated teliospores were transferred with a needle onto the upper surface of leaves under a stereomicroscope. The second method involved an adaptation of the "leaf-disc method" used by Morin *et al.* (1992), which consists of inverting a Petri dish containing telia with germinating teliospores stuck to the surface of water agar over plants, thus allowing basidiospores to fall freely onto leaves. Basidiospores recovered from the surface of the water agar were inspected under the microscope to check germination. Inoculated plants were transferred to a controlled environment cabinet at 18ºC, approximately 100 % relative humidity and a 12 h photoperiod (fluorescent 18W). Plants were visually assessed for any type of symptoms or development of aecia after 2–3 weeks. Ten inoculations involving six to eight plants each were performed over time using each of the methods.

Host-specificity test: A series of trials was performed to test the susceptibility of different accessions of *N. neesiana* from Argentina and Australia, *A. aristiglumis* and *A. scabra* from Australia, and *S. clarazii* from Argentina to isolates of *P. nassellae* recovered from *N. neesiana* at sites 27, 52, 94 and 99. Leaves of healthy plants (*ca*. 2 months old), grown in potting mix contained in 3-cm diameter pots, were inoculated by dusting dry urediniospores using a small paint brush under the stereomicroscope (27, 94 and 99 isolates) or by spraying to run-off a suspension of urediniospores in distilled water (52 isolate) onto plants. Urediniospores that had been dried and kept in the fridge at 4°C for approximately 3 months were used for the 94 and 99 isolates, whilst freshly harvested urediniospores were used for the 27 and 52 isolates. Inoculated plants were misted with water and placed in a controlled environment cabinet (conditions as above) for 2–3 weeks. Plants were then visually assessed for presence of fully developed uredinia.

Results

Ustilago sp.

Cross-inoculation test: Slightly more than 80% of the plants of each species grown from inoculated seeds produced inflorescences. For plants grown from the control untreated seeds, 100 and 63% of the *N. neesiana* and *N. trichotoma* plants, respectively, flowered. None of the inflorescences of the control or inoculated plants developed symptoms of the smut fungus.

Host-specificity test: For seven of the accessions of the various species, more plants grown from untreated seeds flowered than those grown from smut-inoculated seeds (Table 2). However, *N. tenuissima* plants grown from inoculated seeds flowered as well as plants grown from untreated seeds, whilst more of the inoculated *A. scabra, P. napostaense* and *S. gynerioides* plants produced inflorescences. Only a small percentage of flowering plants of *N. tenuis* (12%), *N. tenuissima* (3%) and the Australian accession of *N. trichotoma* (5%) were found to be susceptible to the *Ustilago* sp. isolate from *N. trichotoma* collected at site 07 (Table 2).

Puccinia nassellae

Life cycle: Telia incubated under the described conditions germinated profusely producing hundreds of basidiospores which were also observed to germinate readily on water agar. However, none of the *N. neesiana* plants inoculated with either method developed any sign of infection.

Host-specificity test: Rust isolates collected from *N. neesiana* at various sites infected accessions of *N. neesiana* from which they originated, but also infected at least one other *N. neesiana* accession (Table 3). Two of the four isolates tested on an Australian accession of *N. neesiana* developed mature uredinia on some of the inoculated plants. Neither of the rust isolates *ex.* 94 and 99 tested against the *Austrostipa* species infected plants.

Discussion

Anderson *et al*. (2002) found inflorescences of both *N. trichotoma* and *N. neesiana* infected by *Ustilago* sp. at a number of field sites, but observed that cross-infection between the two stipoid species does not seem to occur. Although results from the cross-inoculation experiment presented here seem to be in agreement

Table 2. Susceptibility of various accessions of *Nassella neesiana*, *Nassella trichotoma* and other non-target plant species to an isolate of *Ustilago* sp. collected from infected inflorescences of *N. trichotoma* at site 07.

Plant species	Origin of plant accessions (Argentina site ID or Australian location)	Control seeds (untreated)			Smut-inoculated seeds		
		Total no. of plants emerged	Flowering plants (% of total emerged plants)	Infected plants (% of total flowering plants)	Total no. of plants emerged	Flowering plants (% of total emerged plants)	Infected plants (% of total flowering plants)
Austrostipa scabra	La Trobe, Vic., Australia	15	27	0	36	36	0
	Mont Park, ACT, Australia	19	47	0	23	22	0
Nassella neesiana	27	14	93	0	35	77	0
	ACT, Australia	23	91	0	48	85	0
N. tenuis	30	22	50	0	22	36	12
N. tenuissima	27	23	97	0	31	97	3
N. trichotoma	30	20	30	0	30	13	0
	Dalgety, NSW, Australia	23	70	0	37	54	5
Piptochaetium napostaense	27	20	40	0	45	42	0
Stipa clarazii	27	24	67	0	43	42	0
S. gynerioides	Caldenal, Argentina	28	93	0	17	100	0

Table 3. Susceptibility of various accessions of *Nassella neesiana* and other non-target plant species to isolates of *Puccinia nassella* collected from *N. neesiana* at different sites.

Plant species	Origin of plant accessions (site ID or country)	Disease incidence (% of infected plants)[a]			
		Origin of rust isolates (site ID)			
		99	94	52	27
N. neesiana	16	–[b]	–	70	–
	52	100[c]	80	100[d]	–
	94	–	10	–	–
	99	80	–	–	–
	ACT, Australia	0	0	10	62[e]
Austrostipa aristiglumis	Australia	0	0	–	–
A. scabra	Australia	0	0	–	–
Stipa clarazii	Argentina	–	0[f]	–	–

[a] Based on a total of 10 plants unless otherwise indicated.
[b] Not tested.
[c] Based on a total of four plants.
[d] Based on a total of 12 plants.
[e] Based on a total of 13 plants.
[f] Based on a total of five plants.

with these field observations they are by no means conclusive. The same comment is applicable for the results obtained in the host-specificity test. The very low rates of infection obtained in the tests reported herein and all previous inoculation trials involving the *Ustilago* sp. (Anderson *et al.* 2002) undermine the validity of the negative results obtained. Ideal conditions for infection to occur may not have been provided in these experiments. Nevertheless, it would appear that such conditions are not easily met in nature either since low disease incidence is the most common situation is the field. There may only be a very narrow window of opportunity during seed germination for infection to take place.

Results from the host-specificity test performed in this study concurred with previous findings (Anderson *et al.* 2002) demonstrating that *Ustilago* sp. collected from *N. trichotoma* can also infect other congeneric species such as *N. tenuis* and *N. tenuissima*. However, it is possible that additional species are also susceptible to this smut, but failed to develop symptoms in these host-specificity tests because of the very low rate of infection obtained. Apart from the fact that the low levels of infection obtained during experiments with *Ustilago* sp. hindered glasshouse studies on this pathogen, the low rates of disease spread within host populations observed in the field suggest that the potential of this pathogen as a classical biological control agent is most likely limited.

On the basis of field observations, the rust *P. nassellae* showed the greatest potential for biological control of *N. trichotoma* and *N. neesiana* (Anderson *et al.* 2002). However, the nature of the rust's life cycle on either host species has still not been fully elucidated. The rust's life cycle could only be studied experimentally using isolates from *N. neesiana* because teliospores have never been found on *N. trichotoma* in the field. Although *N. neesiana* plants were subjected to a strong inoculum pressure of germinating basidiospores (*ex. N. neesiana*) in this study, no infection was obtained on plants originating from the same location as the rust isolate used. This finding strongly suggests that *P. nassellae* is not autoecious. It is noteworthy though that Holway, who made the collection in Bolivia of the type specimen of *P. nassellae* on *Nassella caespitosa* Griseb., reported that aecia were repeatedly found on surrounding *Desmodium* sp. plants associated with the rusted grass (Greene & Cummins 1958). During field surveys conducted in Argentina over the years for this project, aecia-bearing plants belonging to this genus or other genera in the Fabaceae have never been found associated with rust-infected *N. neesiana* plants (unpublished data). However, several other aecia-bearing species have been observed growing close to rust-infected *N. neesiana* plants, but no single species was consistently found to be seriously considered as an alternative host for *P. nassellae*. Nevertheless, further investigations of these aecia-bearing species are required before completely disregarding these as possible alternative hosts.

In contrast to results from previous experiments reported by Anderson *et al.* (2002), it was demonstrated in this study that isolates of *P. nassellae* collected from *N. neesiana* at different sites successfully infected *N. neesiana* plants that did not share the same origin as the isolates. Moreover, two of the isolates tested (*ex.* 27 and 52) were able to develop mature uredinia on *N. neesiana* plants grown from seed collected in Australia, suggesting that isolates from *N. neesiana* are not as specific as previously believed (Anderson *et al.* 2002). Notwithstanding, neither of the two additional tested isolates (*ex.* 94 and 99) infected the Australian native species *A. aristiglumis* and *A. scabra*. This suggests that rust isolates from *N. neesiana* may behave differently from those from *N. trichotoma*, which were found to develop mature uredinia on *A. aristiglumis* in previous work (Anderson *et al.* 2002).

In conclusion, technical difficulties regularly encountered during the investigations of two of the candidates, *U. hypodites sensu lato* and a member of the Corticiaceae, have not allowed a complete body of information to be built on them, thus not permitting a thorough evaluation of their potential to be made at this stage. Studies on the third prospective candidate, *P. nassellae*, have been more successful and proceeded further, but have provided conflicting evidence which needs to be resolved. Anderson *et al.* (2002) reported that isolates from *N. trichotoma* and *N. neesiana* did not infect congeneric *Nassella* species, but showed that *N. trichotoma* isolates were able to develop sporulating uredinia on *A. aristiglumis*. In contrast, the study presented here found that isolates from *N. neesiana* did not infect any of the three *Austrostipa* and *Stipa* species tested. These preliminary findings suggest that rust isolates from *N. neesiana* may pose a lesser risk to non-target plants than those from *N. trichotoma*, but may be adequate for the control of *N. neesiana* only, because of their higher specificity. Additional host-specificity testing and cross-inoculation trials between both target weeds using a wider range of isolates are required to fully clarify these issues.

No other severely damaging pathogens were encountered on *N. trichotoma* during the extensive field surveys conducted in Argentina, with the exception of a *Septoria* leaf spot, and this under exceptionally wet weather conditions (Briese & Evans 1998, unpublished data). The limited number of damaging pathogenic fungi on *N. trichotoma* in Argentina does not therefore offer other alternatives for the biological control of this weed in Australia. In contrast, the prospects for possible biological control of *N. neesiana* with *P. nassellae* or another rust species recently found are more encouraging. Trap plants of *N. neesiana* grown from seed from ACT (Australia) and planted in a field plot at Bahía Blanca recently became heavily infected with another rust fungus tentatively identified as

Uromyces pencanus (Diet. & Neger) Arth. & Holw. (unpublished data). This rust species had been reported previously on *N. neesiana* in Argentina by Lindquist (1982) and has been found only once on an Argentinean accession of this same host during this project (unpublished data). The fact that it had not been recorded during previous surveys may indicate that infection is dependent on uncommon environmental conditions, but since this rust fungus is one of the two autoecious species known to infect grasses of the genera *Stipa* and *Nassella* (Greene & Cummins 1958) and its host range appears to be confined to the genus *Nassella* (Greene & Cummins 1958, Lindquist 1982), it may prove profitable to explore its potential as a biological control agent for *N. neesiana.*

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