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Pathogens On and Variation in *Nassella trichotoma* (Poales: Poaceae) in Australia

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Abstract

Serrated tussock (*Nassella trichotoma* (Nees) Hack. ex Arechav.)) is arguably the most important pasture weed in south-eastern Australia. For biological control, pathogens that are effective on all populations are needed.

Two fungi, *Dinemasporium* sp. Léveillé and *Ascochyta* sp. Lib., form leaf spots associated with death of serrated tussock north-west of Melbourne. Inoculation with spore suspensions from culture reduced germination. Inoculation of seedlings with *Dinemasporium* sp. resulted in infection and necrosis in pot experiments. Germination was also reduced by an unknown white fungus and *Fusarium* sp. Link ex Fries isolated from abundant external white mycelium on the base of the culms and associated with root rot. These fungi have potential for biological control of serrated tussock in Australia.

Preliminary tests on plants from Argentina and Victoria, Australia, by RAPD PCR with six 10-mer primers showed that populations had distinctive banding patterns, but did not group by locality. SEM (scanning electron microscopy showed that the ornamentation on the lemma and at the base of the awn did vary with locality. In Argentina, the lemma prickles were mostly single, large and domed. In NSW (New South Wales), Australia, the prickles were single, large and crater-like. In Victoria, the prickles formed rounded ridges with peaks at intervals. There were stout bristles at the base of the awn in Argentina and NSW, whereas these were absent in Victoria. This variation requires further investigation to ensure that pathogens used for biological control can attack all populations in Australia.

Keywords: *Nassella trichotoma*, biological control, RAPD PCR, fungus, serrated tussock

Serrated tussock (*Nassella trichotoma*) is an unpalatable and invasive grass that can cause up to 97% reduction in carrying capacity of pasture (Campbell 1998). It has now invaded 1 million ha of pasture in south-eastern Australia and climate modelling suggests that it has the potential to spread to 32 million ha (McLaren *et al.* 1998). The main method of control, particularly in non-arable land, has been the herbicide flupropanate (Campbell 1998), but this has recently become unavailable. While glyphosate can exert some control (Campbell 1998, Miller 1998), it too has environmental and economic costs and requires constant maintenance by the land manager, making other methods of control desirable.

For biological control to be effective, not only must the pathogens be found, but also all populations of the weed must be susceptible to them.

Nine pathogens with biological control potential for *N. trichotoma* were found in surveys of 18 sites in central, western and north-western Argentina (Briese and Evans 1998). Their evaluation and importation to Australia is likely to take several years, making it more attractive to find and use pathogens in Australia. Two fungi (*Zinzipegasa argentinensis* (Spegazzini) Nag Raj and *Fusarium* sp.) associated with dead and dying serrated tussock have previously been reported from one infested area in Australia, north-west of Melbourne (Hussaini *et al*. 1998). This paper reports on further fungi associated with diseased serrated tussock in Australia and their evaluation for biological control.

For biological control to be effective, all populations of serrated tussock must be susceptible. Reproduction is by both chasmogamous and cleistogamous flowers (Taylor 1987). Traditional classification uses morphological variation. Long- and short-awned seeds have recently been found in Australia, and it now seems likely that there has been more than one introduction into Australia from Argentina (Surrey Jacobs, Herbarium of New South Wales, pers. comm.). Scanning electron microscopy of the ornamentation of plant parts, including seeds and leaves, has been useful in other species and could assist here (GilAd 1998). Genetic polymorphism can be assessed using RAPD PCR (random amplification of polymorphic DNA in the polymerase chain reaction) (Williams *et al.* 1990), for example in *Sporobolus* species (Hetherington and Irwin 1999). This paper therefore also investigated morphological and genetic differences between serrated tussock in Australia and Argentina using RAPD-PCR.

Materials and Methods

***N. trichotoma* collection.** Populations of *N. trichotoma* were surveyed for pathogens associated with dead plants within a 50-km radius of Melbourne during 1996-7 (Table 1). Seeds were also collected in Victoria. In addition, landholders, Landcare groups, shires and councils from Victoria and New South Wales donated seeds and infected material (Table 1). Argentinian seeds were collected in 1996 (Table 1), donated by Dr Mark Gardener, and handled in AQIS-approved facilities at the Keith Turnbull Research Institute.

Pathogen isolation. Stems and roots with visible fruiting bodies or mycelium were washed thoroughly, surface-sterilised with 2-5% sodium hypochlorite for 5 min, rinsed with sterile distilled water and placed on half-strength potato dextrose agar (½PDA) (Oxoid) agar with antibiotics (chloramphenicol and gentamycin), using standard procedures. Fungi were transferred as necessary on to ½PDA to obtain pure cultures and identified by reference to standard texts, e.g. Barnett and Hunter (1972), Nag Raj (1993).

Inoculum preparation and application. Conidial suspensions were made by flooding 14 d old ½PDA plates of sporulating fungi (*Dinemasporium* sp. and *Fusarium* sp.) with 10 mL plate^{-1} 0.1% Tween 20 in sterile distilled water and rubbing with a sterile glass rod. For *Ascochyta* sp., pycnidia were picked off 21-d-old plates, crushed and mixed in 0.1% Tween 20. Conidial numbers were estimated by counting four 7 µL samples on a haemocytometer. For non-sporulating isolates, mycelial mats collected from 14 d old plates were ground in 10 mL 0.1% Tween 20 in a mortar and pestle and used as inoculum;

Table 1.
Samples of *Nassella trichotoma* used in this study.

Location	Material	Description	Collection date	Isolation	Use and ID code SEM[b]	RAPD-PCR[c]
Victoria, Australia						
Werribee 37° 56' S, 144° 37' E	Senescent culms, seeds, leaves	Basal 1-5 cm of white mycelium in tussock centre	June 1996 Dec. 1996	*Fusarium* sp.	5	Werribee 3, 5-8
Tullamarine 37° 36' S, 144° 44' E	Senescent culms,	Black spots, reduction in flowering and seed set	Dec. 1997 Jan. 1998	*Zinzipegasa argentinensis* *Dinemasporium* sp.		
(1) Diggers Rest 144° 42' S, 144° 45' E	Senescent culms	Basal 1-3 cm of external white mycelium in tussock centre	Dec. 1996 April 1997	*Fusarium* sp.		
(2) Diggers Rest 144° 42' S, 144° 45' E	Senescent culms	Black spots, reduction in flowering and seed set	Feb. 1998	*Zinzipegasa argentinensis,* *Dinemasporium* sp.		
Near Geelong 37° 54' S, 144° 33' E	Senescent culms	Basal 1-3 cm of external white mycelium in tussock centre	Aug. 1997	Unidentified fungus 1		
VUT St Albans campus 37° 54' S, 144° 34' E	seed, seedling leaf	short-awned, long-awned	Dec. 1997		1-4	
Darraweit Guim 37° 25' S, 144° 55' E	mature leaf				6	

Location	Material	Description	Collection date	Isolation	Use and ID code SEM[b]	RAPD-PCR[c]
Rocklands, near Anakie 23 33' S 147 44' E	mature leaf					Rocklands
NSW[a], Australia						
Abercrombie 33° 56' S, 146° 19' E	seeds, leaf	Plants and seeds healthy			15-16	
Argentina						
(1) Pimar 38° 43' S, 62° 10' W	seed, pot plant leaf	Seeds healthy, one plant	Nov. 1996 June 1998		7, 8	 Arg1
(2) Estancia Pervilh 36° 18' S, 60° 16' W	seed, pot plant leaf	Many seeds immature or aborted.	Nov. 1996 June 1998		9, 10	 Arg2
(3) 70 km S of Olavarria 36° 18' S, 60° 16' W	seed, pot plant leaf	Seeds healthy, immature.	Nov. 1996	 June 1998	11, 12	
(4) Near Castelli 25° 56' S, 60° 16' W	seed, pot plant leaf	Seeds healthy, mature	Nov. 1996 June 1998		13, 14	

[a]NSW New South Wales, [b]SEM scanning electron microscopy;

[c]RAPD-PCR random amplification of polymorphic DNA by the polymerase chain reaction.

colony-forming units were counted on dilution plates of half-strength potato dextrose agar.

Seed preparation and inoculation. Seed collected from St Albans and Werribee was surface sterilised with 5% sodium hypochlorite for 5 min. Twenty seeds per treatment were placed on 1.5% tap water agar in plastic screw-capped tubs (7.5 cm diameter), with five replicates of each treatment and control. Each tub was inoculated with 3 mL of conidial or mycelial suspension (10^6 CFU (colony-forming units) mL^{-1}). Control tubs were inoculated with 3 mL of 0.1% Tween 20. Tubs were incubated at 25°C at 27 μE $m^{-2}.s^{-1}$ provided by 36W Osram 'Warm White' lights in a 12 h photoperiod. Germination was scored at 3 weeks after inoculation, but tubs were kept for 3 months to check for any further germination.

Seedling preparation and inoculation. Ten 3-month-old (2-3 leaf) seedlings in each of 5 90 mm x 130 mm punnets from seeds collected at St Albans and Werribee were sprayed to run-off with *Dinemasporium* conidial suspension (10^6 mL^{-1}) in 0.1% Tween 20. Controls were inoculated with 0.1% Tween 20 alone. Seedlings were incubated for 48 h in 100% humidity in dew chambers made from aquarium tanks, with cool steam provided by an ultrasonic room humidifier (Humidaire, KT-100A). Seedlings were assessed for infection and health at 3 months after inoculation. Conidiomata were harvested from seedlings, surface-sterilised as before and crushed in sterile distilled water. Loopfuls were streaked over ½PDA. The remainder was inoculated on to 25 10-week-old Werribee seedlings in a tub as for seeds and infection recorded at 2 months relative to the control (uninoculated).

Scanning electron microscopy. Seeds from several Victorian, Argentinian and one NSW locality were studied (Table 1). Leaves were excised for St Albans short- and long-awned populations from 3-month-old seedlings grown in a glasshouse in natural light and 15-25°C. For Argentinian populations, leaves were excised from 12-month-old plants grown in quarantine in a glasshouse at Keith Turnbull Research Institute. Seed (still enclosed in the lemma), and short (5-10 mm long) pieces from the centres of fresh leaves were fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, overnight at 4°C, dehydrated in ethanol, critical-point dried, fixed to stubs, sputter-coated with gold and examined with a Jeol JSM-35CF scanning electron microscope.

DNA extraction. DNA was isolated by a method modified from that of Dellaporta *et al.* (1983). For each sample, 800 g of fresh leaf was chopped into small pieces, covered with liquid nitrogen and ground to a fine powder. Fifteen mL extraction buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 50 mM EDTA, 10 mM mercaptoethanol) and 1 mL 20% sodium dodecyl sulphate were added, shaken vigorously and incubated at 65ºC for 10 min. Eight mL of 5 M potassium acetate was added, and the solution was shaken vigorously for 1 min, incubated on ice for 2 h and centrifuged at 20,000 g for 20 min at 4ºC. The supernatant was filtered through one layer of Miracloth, 10 mL of isopropanol was added and the mixture was shaken and incubated at -20ºC for 12 h. The solution was centrifuged at 15,000 g for 15 min at 4°C, the supernatant discarded, and the pellet was air-dried and resuspended in 0.7 mL Tris/EDTA (100 mM Tris-HCl, pH 7.2, 0.02 mM ethylene diamine tetra-acetic acid). The solution was centrifuged at 20,000 g for 10 min and the supernatant transferred to a fresh Eppendorf tube. Seventy-five μL 3 M sodium acetate and 500 μL of isopropanol were added, the solution was mixed and DNA was precipitat-

ed at -20°C for 12 h before centrifuging at 20,000 g for 1 min. The pellet was washed in 70% ethanol, air-dried and resuspended in 500 µL TE (10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA). DNA concentration was estimated by gel electrophoresis against uncut λ DNA (Boehringer Mannheim, Castle Hill, Australia).

RAPD PCR. DNA was amplified by the method of Theodore *et al.* (1995), but with the cycles of Richardson *et al.* (1995). Each 25 µL reaction mixture contained 1 U *Tth Plus* DNA polymerase (Fisher-Biotech), 2.0 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, 100 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of each primer and 1 µL of DNA extract containing 5-20 ng DNA. The parameters used for the PCR in a Perkin-Elmer Cetus thermocycler were: 5 cycles of: 30 s denaturation at 94°C, 2 min annealing at 40°C, 1.5 min extension at 72°C; 35 cycles of: 5 s denaturation at 94°C, 25 s annealing at 45°C, 1.5 min extension at 72°C; and finally 10 s denaturation at 90°C, 20 s annealing at 45°C, 5 min extension at 72°C. Products were stored at 4°C before amplified products were separated by gel electrophoresis on a 1% agarose gel containing 1 µg mL^{-1} ethidium bromide at 90 V for 30 min. Spp1/*Eco*R1 and pUC/*Hpa*II (Progen) were used as molecular weight markers. Negative controls contained all components except DNA extracts.

Results

Symptoms and pathogen isolation. Of the five fungi noted on serrated tussock, four were isolated into pure culture (Table 1), two from the leaf spots and two from the mycelial growth around the crowns. A *Dinemasporium*-like fungus was isolated from dark leaf spots (acervuloid conidiomata), surrounded by dark, multicellular, rigid pointed setae containing 1-celled fusiform hyaline conidia with an appendage at each end (Nag Raj 1993); in culture on half-strength potato dextrose agar, it formed white fluffy growth with acervuli surrounded by dark setae. *Ascochyta* sp. was isolated from dark leaf spots containing embedded pycnidia with 2-celled ovoid hyaline conidia (Barnett and Hunter 1972). Unidentified fungus 1 and *Fusarium* sp. were isolated from mycelial growth around the crown and lower 1-3 cm of dead and dying tillers that were easily pulled up from the centre of tussocks. *Fusarium* sp. had previously been recorded on serrated tussock (Hussaini *et al*. 1998) and was isolated into culture; it had a flat white obverse, a patchy wine-red reverse and sporulation characteristic of the genus, whereas the unidentified fungus 1 grew as velvety white mycelium without any sporulation.

Inoculation trials. In tubs of seeds, *Dinemasporium* sp. was the most successful inoculum, as it stopped seed germination completely. The other three fungi also reduced seed germination significantly, by 70±1% (*Aschochyta* sp. and unidentified fungus 1) to 80±1% (*Fusarium* sp.). In punnets of seedlings, *Dinemasporium* produced superficial acervuloid conidiomata on the leaves of 1-3 Werribee seedlings per punnet; seedlings did not die, although the infected leaves showed complete necrosis above the acervuli. No control or St Albans seedlings showed any disease symptom. Only *Dinemasporium* was re-isolated, thus satisfying Koch's postulates, and all inoculated tub Werribee seedlings died within 2 months.

Scanning electron microscopy. Ornamentation varied with origin, with Victorian, NSW and Argentinian populations all different. In Victorian seed, adjacent prickles amal-

Figs. 1-4. Lemma surface on seeds from Victoria and New South Wales, Australia, and Argentina, showing prickles amalgamated into ridges in Victorian specimens.

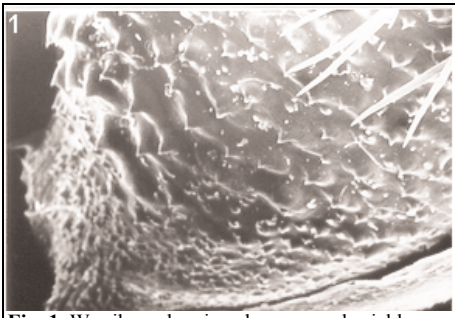

Fig. 1. Werribee, showing sharp curved prickles on ridges.

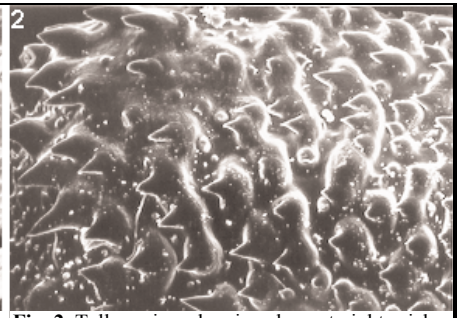

Fig. 2. Tullamarine, showing sharp straight prickles on ridges.

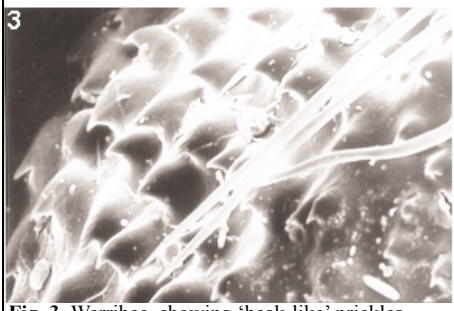

Fig. 3. Werribee, showing 'beak-like' prickles.

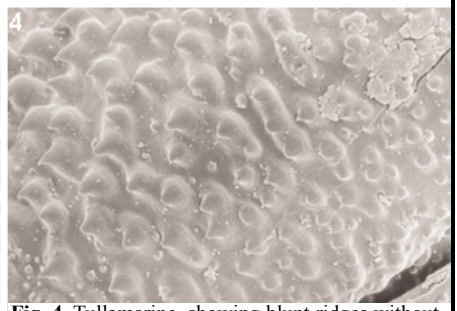

Fig. 4. Tullamarine, showing blunt ridges without prickles.

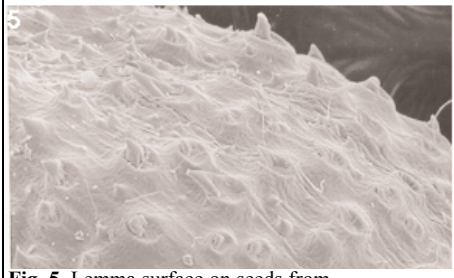

Fig. 5. Lemma surface on seeds from Abercrombie, New South Wales, Australia, showing straight prickles arising from crater-like bases.

Fig. 6. Lemma surfaces on seeds from Castelli, showing predominantly single slightly curved thick prickles. Bar, 100 µm.

gamated to form ridges (Figs. 1-2); individual prickles were short and curved, resembling a parrot's beak (Fig. 3), and varied in sharpness. Prickles in Victorian populations from Werribee (Fig. 3) were much sharper than from St Albans (Fig. 4), but long- and short-awned seeds did not differ at St Albans. In NSW seed, prickles were single, had a crater-like base and had much stouter prickles (Fig. 5). In Argentinian seed, prickles were mostly single (Figs. 6-10), sometimes sparse and less curved than in Victorian populations (Figs. 6-7), resembling more those from NSW, but without the crater-like base (Fig. 8),

Figs. 7-10
Lemma surfaces on seeds from Argentina.

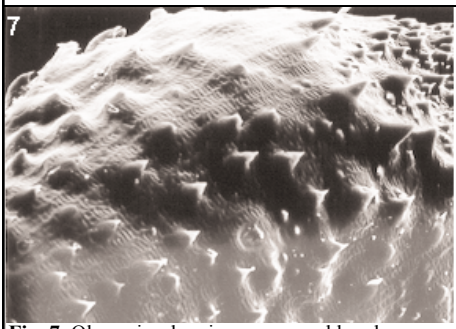

Fig. 7. Olavarria, showing sparse and barely curved prickles.

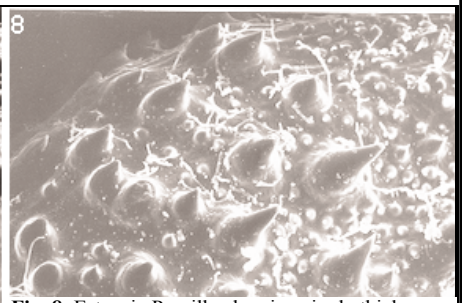

Fig. 8. Estancia Pervilh, showing single thick prickles and small protuberances on lemma surface.

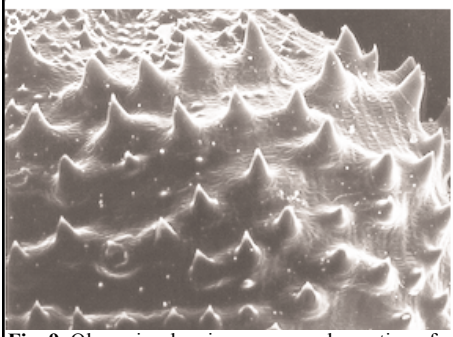

Fig. 9. Olavarria, showing some amalgamation of prickles into ridges near the awn.

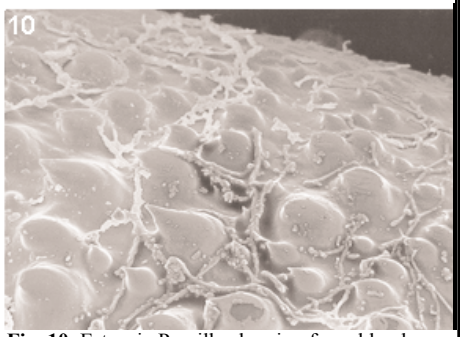

Fig. 10. Estancia Pervilh, showing fungal hyphae winding between prickles, Bar, 100 µm.

although some from Olavarria amalgamated into ridges near the awns (Fig. 9). In Argentinian samples only, fungal hyphae were common, winding over the surface (Fig. 10); these were most common from Estancia Pervilh. The closest similarity between Australian and Argentinian provenances was Victorian and Olavarria samples, as both had simple prickles amalgamating into ridges.

Awns also varied with origin, with Victorian populations different from those in NSW and Argentina. Argentinian and NSW seed (Figs. 11-12) had long, dense, stout trichomes (hairs) at the base of the awn, whereas the base of the awn lacked hairs and hairs on the awn were smaller on Victorian seed (Fig. 13).

Leaves did not vary noticeably in surface structure, regardless of origin, age and method of growth; all had sparse, single, slightly curved hairs pointing towards the top of the leaf (Fig. 14). Some Argentinian populations had hairs with a bulbous base (Fig. 16), but this character was also found in Victorian populations from Darraweit Guim (Fig. 15).

RAPD-PCR. Most OPM primers (2, 4, 5, 6 and 12) differentiated amongst populations by the presence or absence of products (differences in intensity were ignored), whereas OPM-01 did not (Figs. 17-22). PCR reactions without DNA gave no product.

Figs. 11-13. Awn bases at attachment to lemma, showing differences in hairs.
Figs. 14-16. Leaf surfaces, showing similarities.

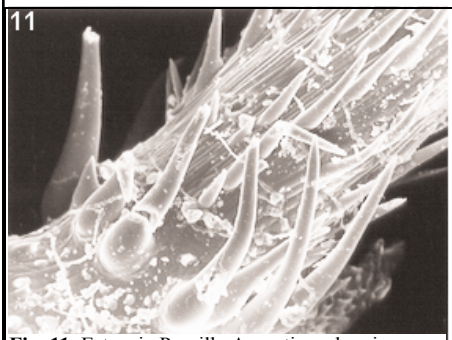

Fig. 11. Estancia Pervilh, Argentina, showing long, dense, stout hairs at the base of the awn.

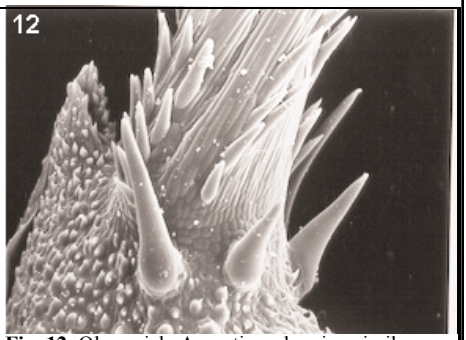

Fig. 12. Olavarriak, Argentina, showing similar hairs.

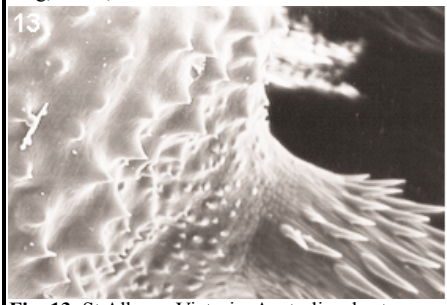

Fig. 13. St Albans, Victoria, Australia, short-awned seed showing lack of stout hairs at the base of the awn and short, straight hairs at the awn.

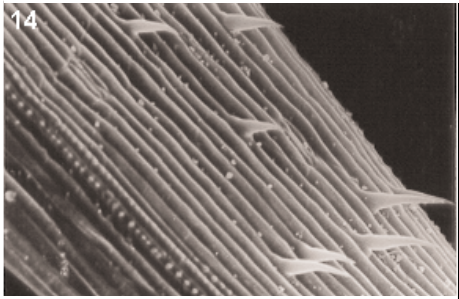

Fig. 14. St Albans, Victoria, Australia, seedling leaf surface from short-awned seed, showing short curved prickles.

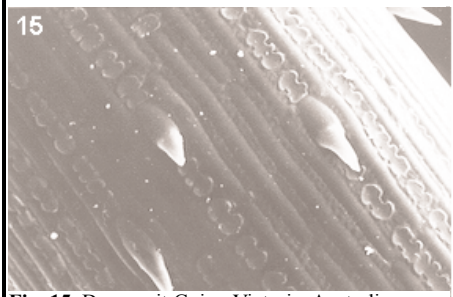

Fig. 15. Darraweit Guim, Victoria, Australia, mature leaf surface showing bulbous base on prickles.

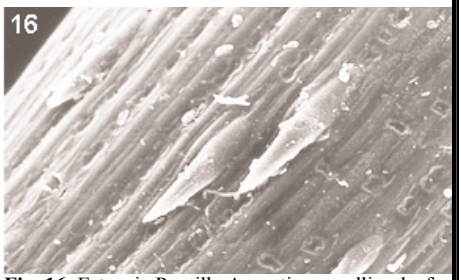

Fig. 16. Estancia Pervilh, Argentina, seedling leaf surface from seed collected on site and grown in Australia in a quarantine glasshouse, showing bulbous base on prickles. Bar, 10 µm.

Primers gave three (OPM-04 – Fig. 19) to seven (OPM-12 – Fig. 22) PCR pattern types for the nine serrated tussock types tested. There were no obvious groupings of locality by similar patterns. Apart from OPM-01, all primers distinguished between the Argentinian samples. Victorian populations (three types) were differentiated from Argentinian populations (two types) by OPM-05 (Fig. 20), but the remaining primers produced PCR types that included more than one locality or gave unique patterns for each sample.

Figs. 17-22. Products from RAPD-PCR amplification of DNA from leaves of *Nassella trichotoma* with OPM primers. Lanes are numbered left to right. Lane 1: Spp/*Eco*RI.

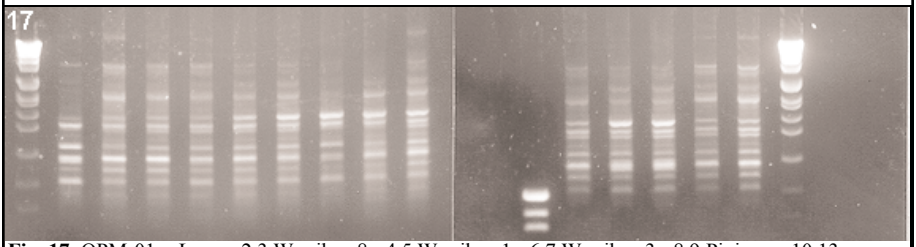

Fig. 17. OPM-01. Lanes: 2,3 Werribee 8; 4,5 Werribee 1; 6,7 Werribee 3; 8,9 Pinimar; 10,13 Rocklands; 11. control; 12 Puc/*Hpa*II; 14,15 Estancia Pervilh; 16,17 Werribee 7, 18 Spp/*Eco*RI.

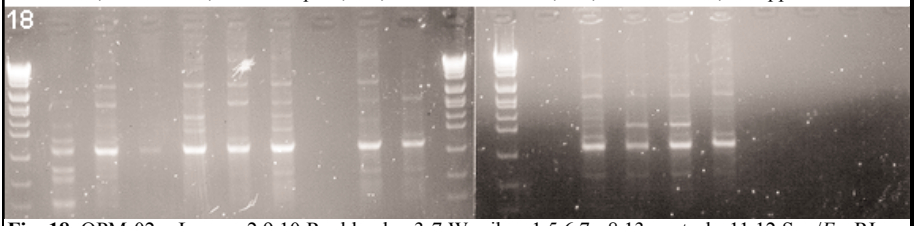

Fig. 18. OPM-02. Lanes: 2,9,10 Rocklands; 3-7 Werribee 1,5,6,7; 8,13 control; 11,12 Spp/*Eco*RI; 14,17 Pinimar; 15,16 Estancia Pervilh.

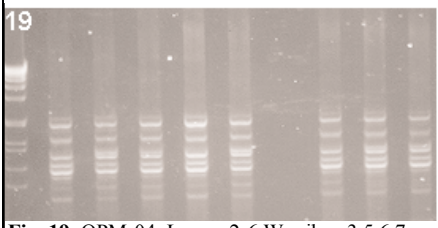

Fig. 19. OPM-04. Lanes: 2-6 Werribee 3,5,6,7; control; 7 Rocklands; 8,9 Pinimar, Estancia Pervilh.

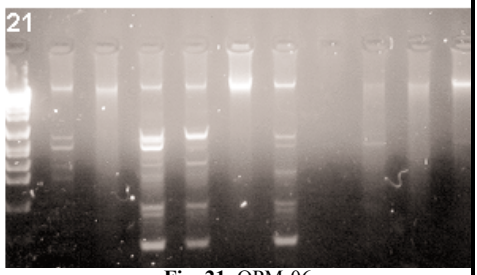

Fig. 21. OPM-06

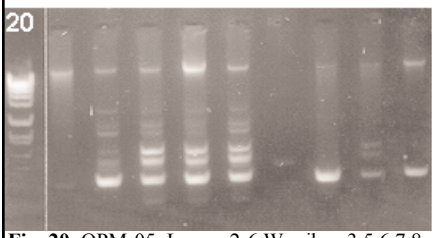

Fig. 20. OPM-05. Lanes: 2-6 Werribee 3,5,6,7,8; 7 control; 8,9 Pinimar, Estancia Pervilh.

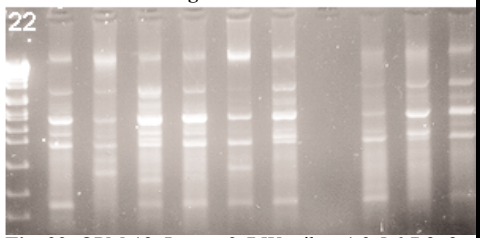

Fig. 22. OPM-12. Lanes: 2-7 Werribee 1,3,5,6,7,8; 8 control, 9,10 Pinimar, Estancia Pervilh; 11 Rocklands.

Discussion

Isolation of pathogens and pathogenicity trials. This isolation of a *Dinemasporium* sp. from leaf spots of serrated tussock is the first record in Australia. The fungus does not correspond in all respects to *D. strigosum* (Persoon: Fries) Saccardo, the only species recorded from an Australian specimen, of *Stipa* sp. from Meningie (Nag Raj 1993), as its

conidiomata are more open and dimensions of the setae and conidia are greater. Nor does it correspond to any *Dinemasporium* sp. so far described (Nag Raj 1993). Its conidiomata resemble those of *Z. argentinensis*, previously recorded on serrated tussock, but the setae are much more rigid and mostly pointed (Nag Raj 1993). The conidia also resemble one-celled conidia of *Z. argentinensis* (which has 1-4-celled conidia), but never develop more than one cell. Its exact status remains to be determined.

The significant reduction in germination by inoculation with four fungi isolated from dead or dying serrated tussock near Melbourne suggests that these fungi, in particular *Dinemasporium* sp., have potential for biological control of this weed. In addition, the infection of seedlings by *Dinemasporium* sp. suggests that is a primary pathogen, as it satisfied Koch's postulates. It was more virulent in seeds and seedlings in tubs than in punnets, as expected, since pathogenicity is influenced by the inoculum concentration and by the substrate upon which it is growing (Charudattan 1989). Further assessment under field conditions on more mature plants is needed before it can be evaluated for use as a mycoherbicide (Charudattan 1989). Although complete kill is unlikely in more mature plants, disease may reduce seed set and reduce the weed's competitiveness, thus aiding control as part of an integrated pest management system.

Variation in serrated tussock. The large variation in serrated tussock shown by both SEM and RAPD-PCR suggests that the weed varies widely genetically, and could be expected to vary in susceptibility to biological control agents (Hetherington and Irwin 1999.). This may explain the infection of Werribee seedlings by *Dinemasporium* sp., yet the lack of infection of St Albans seedlings inoculated simultaneously. Further direct testing of pathogens with a large range of provenances and genotypes is therefore necessary before a biological control agent is chosen.

The lack of correspondence between SEM and RAPD groupings suggests that the variation in SEM appearance of seeds is probably determined by environmental conditions rather than by genetics. Plants in New South Wales mature their seeds under hotter and drier conditions than those in Victoria and the more elaborate ornamentation may be a response to those conditions. The lack of similarity between Australian and Argentinian seed ornamentation may simply reflect lack of similarity between the environmental conditions. The large variation among Argentinian provenances may reflect the climate of their different habitats (ranging from coastal Pinimar to high-altitude Estancia Pervilh and Olavarria). However, the ornamentation in samples from Estancia Pervilh and Olavarria was different and may reflect differences in an environmental factor other than altitude.

The genetic variation shown by RAPD-PCR was mostly as great among plants, e.g. Werribee, as among provenances, suggesting that populations at a site are very heterogenous. Testing of any potential biological control agent on a large number of provenances will be required, to ensure that a full range of genetic types is included. The large range of variation within one paddock at Werribee suggests that the plants are mostly outcrossing rather than cleistogamous (Taylor, 1987), although breeding experiments would be needed to show the degree to which markers are heritable from parent to seed. OPM-05 was the only primer to group plants by provenance, but even it split Werribee plants into two genetic types. This suggests that RAPD-PCR may be less useful than less sensitive techniques such as microsatellites or ribosomal DNA internal transcribed spacer sequences (White *et al.* 1990) to study genetic variation.

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