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NOTE

# Microscopic observations of a compatible host/pathogen interaction between a potential biocontrol agent (*Uromyces pencanus*) and its target weed (*Nassella neesiana*)

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**Abstract** *Uromyces pencanus* is a prospective biocontrol agent for *Nassella neesiana* in Australia and New Zealand. The infection process of *U. pencanus* urediniospores in leaves of its susceptible host *N. neesiana* was found to be similar to that of most other rust species. A pattern against which to compare nonhost reactions in host range experiments was achieved using a modification of Bruzzese and Hasan's whole leaf clearing and staining technique. The staining of microtome leaf sections provided supplemental information about this pathosystem.

**Keywords** Clearing and staining techniques · Infection process

## Introduction

*Nassella neesiana* (Trin. & Rupr.) Barkworth (Chilean needle grass, Poaceae) is a tussock-forming grass from South America that causes serious problems as a weed in Australia and New Zealand. It is a declared noxious weed of national significance in Australia (Thorp and Lynch 2000) and is targeted for containment or eradication in five regions of New Zealand (Auckland, Waikato, Hawke's Bay, Marlborough, and Canterbury) (MAF 2009). A biological control project was initiated in 1999 in Argentina to investigate pathogens with potential for the biological control of *Nassella trichotoma* (Nees) Hack. ex Arechav. (serrated tussock) and *N. neesiana* (Anderson et al. 2006) in the grasses' native range. The rust *Uromyces pencanus* Arth. & Holw. has since been identified as the most promising control agent for *N. neesiana*. It has been observed to have a negative impact on its host in the laboratory (Giordano et al. 2009) and in the field in Argentina, where it naturally infects *N. neesiana* and appears to be expanding its geographical distribution (Anderson et al. 2006, 2010). It is therefore expected to impose a negative effect on *N. neesiana* populations in Australia and New Zealand, should it eventually be introduced in these countries.

It is a regulatory requirement that selected biocontrol candidate agents are host-specific and pose a limited risk towards nontarget plant species (Morin et al. 2006). If host specificity can be demonstrated under optimal conditions for disease development in the laboratory/glasshouse, then that strongly indicates that nontarget species will not be affected in the field (Berner and Bruckart 2005). To this end, extensive host specificity testing of *U. pencanus* involving over 70 nontarget species within the Poaceae is now underway (Anderson et al. 2008). The desired outcome is that none of the nontarget species develop rust pustules or serious disease symptoms. However, the degree to which the infection has proceeded in such species, and the occurrence of resistance mechanisms at the cellular level, can also be relevant to decision makers. Indeed, these studies have at times provided decisive evidence leading to permission for biocontrol agents to be released (Evans 2000). In the present report, to study the infection process by urediniospores of *U. pencanus* microscopically, especially post-penetration events, two histological techniques

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were used to prepare infected leaves of the susceptible host *N. neesiana* for examination: staining of microtome leaf sections, and clearing and staining of whole leaf sections. This was done so as to establish a pattern against which to compare nonhost reactions during host range testing experiments.

### Staining of microtome leaf sections

Serial transverse microtome leaf sections, 10 μm thick, stained with safranine-fast green were prepared (Fig. 1a–k), as described in detail by Johansen (1940). The infected leaf samples had been fixed in formaldehyde-acetic acid-ethyl alcohol (FAE). The size and shape of haustorial sections were recorded. The diameter of circular haustorial sections, or the length of the major axis of elliptical or elongated haustorial sections, was measured. All measurements were made under a light microscope, using an ocular micrometer at a final magnification of 1000×. Mean (μm) ± standard error of the mean values, calculated over three to six replicates, are presented.

The observation of thin microtome leaf sections stained with safranine-fast green enabled the easy identification of most fungal structures, especially haustoria, as this technique rendered an excellent contrast between fungal and plant structures. Samples treated with this technique were taken at 14–18 days after inoculation, when pustules were first appearing through the epidermis, to make sure intercellular mycelia and haustoria were sufficiently well developed to be readily located on the slides. The type of plant cells in which haustoria were formed, and differences in the size and shape of haustorial sections could also be recorded with this technique (see below under "Infection process"). These differences in haustorial sections do not necessarily imply the existence of different types of haustoria, but instead probably correspond to different stages of haustoria formation (Harder and Chong 1984), or are the result of haustoria being in different positions within the cells.

The main disadvantage of microtome leaf sections is that the whole process, from sampling to the preparation of the slides for microscopic observation, is very time-consuming. In addition, a great number of leaf sections need to be examined before the exact place where the different fungal structures occur can be found. Thus, this method would be highly inconvenient in host range testing, when the relationship between the rust and the inoculated plant species needs to be investigated at the cellular level for dozens of plant species. However, it can be a complementary tool to study particular cases in which the interpretation of results using other techniques is doubtful. In the case of *U. pencanus* and *N. neesiana*, the observation of identifiable fungal structures in microtome sections made it easier to interpret events subsequently viewed from above in leaf samples stained following the technique of Bruzzese and Hasan (1983). Moreover, it would have been very difficult to identify the poorly stained haustoria in these samples if these structures had not been previously observed with the microtome technique.

### Whole leaf clearing and staining technique

The whole leaf clearing-staining technique proposed by Bruzzese and Hasan (1983) has been included in the host range testing of many rust fungi selected as prospective candidates in biological control programs, especially in projects in which a high number of species must be tested against the pathogen (Bruzzese and Hasan 1986; Evans and Tomley 1994; Parker et al. 1994). The technique is easy to perform and has the advantage of allowing a relatively rapid examination of the rust infection process on leaf samples of weeds and cultivated plants (Bruzzese and Hasan 1983). It has been shown to work very well with whole leaf portions of the flat leaves of dicotyledonous species (Bruzzese and Hasan 1986; Evans and Tomley 1994; Parker et al. 1994) or of some grass species such as *Dactylis glomerata* (Bruzzese and Hasan 1983), maize, oat, or wheat (personal observations).

When applied on the leaves of *N. neesiana* (Fig. 1l–r) this technique did not render totally satisfactory results, because both fungal structures and plant cells stained in similar shades of blue, and because of the anatomy of the leaves, characterized by prominent veins and deep intercostal areas. As stomata are located at the bottom of the latter, it was very difficult to focus them under the microscope. It was therefore necessary to modify the technique by changing immersion times in the clearing-staining solutions, as explained below, and to remove prominent veins with a scalpel (Wood 2006). This was done after clearing the sample, just before mounting leaf portions on the microscope slides.

Samples were taken at 7 and 30 days after inoculation. These sampling dates were the same as those chosen for the host range testing experiments (our unpublished data). Whole leaf portions about 1 cm long were stained by immersion for 6 h in the clearing-staining solution (Bruzzese and Hasan 1983), rinsed in distilled water, and finally cleared in a concentrated chloral hydrate solution (Bruzzese and Hasan 1983) for 24 h. The vials containing the leaf portions in the different solutions were placed on a rotary shaker at low speed and at room temperature (20–25°C) for the duration of immersion. Samples were finally rinsed with distilled water and mounted on microscope slides in a permanent polyvinyl alcohol mounting medium (Omar et al. 1978).

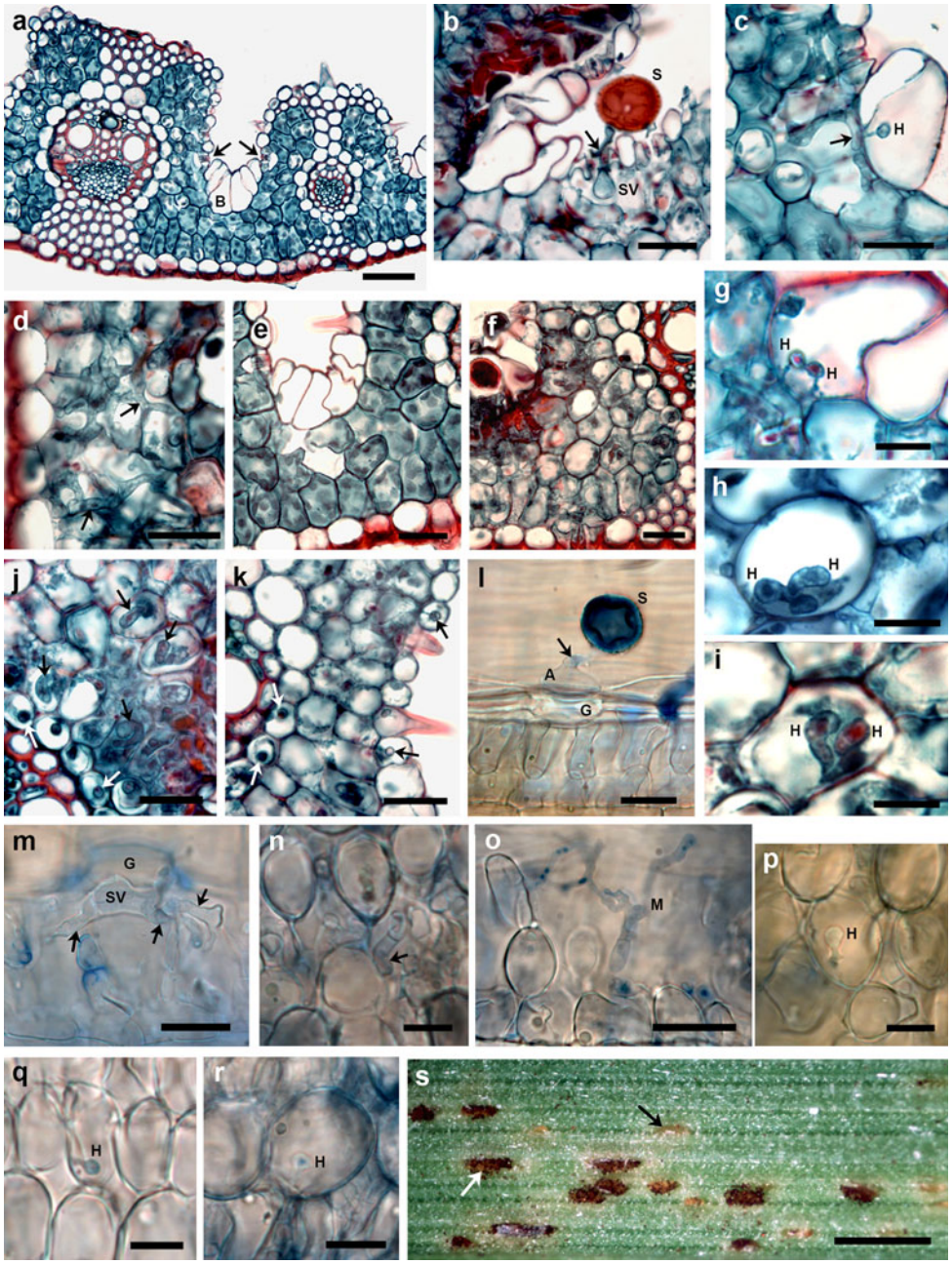


**Fig. 1** Infection process of urediniospores of *Uromyces pencanus* on *Nassella neesiana* leaves. **a**–**k** Transverse microtome sections of leaves stained with safranine-fast green. **a** Two prominent veins, and stomata (*arrowed*) and bulliform cells (*B*) located between them at the base of deep valleys (*intercostal areas*). **b** Spore (*S*) with an infection peg (*arrow*) growing between the guard cells to form a substomatal vesicle (*SV*). **c** Haustorial mother cell (*arrow*) which forms a haustorium (*H*) in the adjacent bulliform cell. **d** Intercellular mycelium (*arrows*) in the mesophyll. **e** Mesophyll cells at healthy sites. **f** Mesophyll cells with disorganized contents at infection sites. **g** Bulliform cell with two haustoria. **h**, **i** Mesophyll cells, each with two haustoria. **j** Elongated haustorial sections (*black arrows*) in mesophyll cells and circular haustorial sections (*white arrows*) in parenchymatous bundle sheath cells. **k** Circular haustorial sections in parenchymatous bundle sheath cells (*white arrows*) and in long epidermal cells (*black arrows*). **l**–**r** Surface view of whole leaf samples stained with the modified Bruzzese–Hasan technique after the elimination of prominent veins. **l** Germinated spore and appressoria (*A*) formed over the guard cells (*G*) and were separated from the germ tube by a septum (*arrow*). **m** Infection hyphae (*arrows*) developing from substomatal vesicle (*SV*). **n** Haustorial mother cell (*arrow*) in contact with a mesophyll cell. **o** Intercellular mycelium (*M*) in the mesophyll. **p**–**r** Haustoria (*H*) in mesophyll cells. **s** Surface view of an inoculated leaf showing different stages of pustule formation: a uredinium primordium (*black arrow*) and sporulating pustules with brown urediniospores (*white arrow*). *Bars* **a** 40 μm; **b**–**f**, **j**–**m**, **o** 20 μm; **g**–**i**, **n**, **p**–**r** 10 μm; **s** 0.35 mm

In sections treated this way it was possible to observe spore germination and appressoria formation, and to distinguish between fungal and plant tissues. For unknown reasons, most spore germination tubes and many appressoria stained intensely, but some failed to stain at all. These differences were sometimes observed on structures occurring on the same leaf portion. The observation of fungal structures inside the leaf was only possible when the prominent veins of the leaf had been removed. Using this technique it was possible to visualize haustoria, but only in mesophyll cells. Intercellular hyphae stained pale blue on most samples, in only slight contrast with plant cells.

## Infection process

Urediniospores of *Uromyces pencanus* isolate 27 (collected in Bahía Blanca, 38°40′S, 62°14′W, Argentina), one of the eight isolates collected and later selected for host range testing (Anderson et al. 2006), were obtained from a pure culture established under controlled conditions on *N. neesiana* plants. These were grown in a glasshouse from seeds collected in the Australian Capital Territory. Previous experiments had shown that the selected rust isolate was especially aggressive towards plants belonging to this accession (our unpublished data). Dry urediniospores were brushed onto the adaxial side of leaves of adult plants. Inoculated plants were sprayed with water and subjected to 100% relative humidity for the first 48 h. This was achieved by placing them in "dew chambers" (cube-shaped polyethylene boxes lined with water-soaked newspaper). For the rest of the incubation period (30 days) they were kept in a controlled environment cabinet, at 18–20°C, around 70% relative humidity, and a 12 h photoperiod.

Inoculated plants were examined weekly for external symptoms (macrosymptoms) of infection under a stereomicroscope. These observations were continued until the appearance of fully developed pustules. *N. neesiana* leaves showed numerous chlorotic specks at 5–11 days after inoculation with urediniospores of *U. pencanus*. Most of these specks were later confirmed to be infection sites when uredinia were formed within them. Under the tested conditions, pustule formation was evident under the stereomicroscope at 9–11 days after inoculation, when shiny yellowish slight elevations in intercostal zones of leaf blades appeared (Fig. 1s, black arrow). Sporulation was first evident under the stereomicroscope at 10–12 days after inoculation (Fig. 1s, white arrow), and with the naked eye from 15 days onwards.

Pre and post-penetration events were examined under a light microscope (Leica DMLS, Leica Microsystems Wetzlar GmbH, Germany). Once urediniospores of *U. pencanus* germinate, the germ tubes locate stomata and form appressoria that are separated from the germ tube by a septum (Fig. 1l). Subsequently, an infection peg grows between the guard cells to form a substomatal vesicle (Fig. 1b). Usually, two to four infection hyphae develop from the vesicle, one or two at each end (Fig. 1m), and grow between the mesophyll cells. Initially, the infection hyphae grow parallel to the long axis of the leaf, as observed for *Puccinia graminis* f. sp. *tritici* on wheat (Rowell 1984). Growth of the infection hyphae continues until the tip of each one, upon contact with a host cell, is delimited by a septum to form a haustorial mother cell (Fig. 1c, n) which in turn, produces a haustorium in the host cell (Fig. 1c). Then, intercellular mycelia develop between mesophyll cells (Fig. 1d, o) and form new haustoria. The mycelium that develops from one penetration site is restricted to the corresponding intercostal zone by the leaf veins, as observed for other rusts (McLean 1979). Hyphae were not observed passing to an adjacent intercostal zone. Probably, the tight arrangement of vascular bundle sheath cells surrounding the veins, and their extensions to both the abaxial and adaxial epidermis (Fig. 1a), prevented the growth of the intercellular hyphae.

Haustoria are formed in epidermal cells (except in guard and subsidiary cells), mesophyll cells, and parenchymatous bundle sheath cells (Fig. 1g–k, p–r). They are formed most commonly in mesophyll cells and there are often two or three haustoria per infected cell (Fig. 1h, i). The leaf sections examined were too thick for the shape and orientation within cells of whole haustoria to be determined (Kneale and Farrar 1985), so that only haustorial sections are described. These varied in shape from circular to elongated, regardless of the cell type in which they occurred, although elongated sections were not observed in long epidermal cells. Small circular haustorial sections were found to be more frequent in epidermal and parenchymatous bundle sheath cells (Fig. 1k), measuring around 2.94 (±0.16) and 3.16 (±0.12) μm in diameter, respectively. Elongated (7.63 ± 0.40 μm) and big circular (3.42 ± 0.20 μm) haustorial sections were more common in mesophyll cells.

The microscopic techniques applied in this study revealed normal mesophyll cells with intact chloroplasts at sites without infection (Fig. 1e), and an evident disorganization of mesophyll cell contents at infection sites where chloroplasts were not well defined and cells looked empty (Fig. 1f). The degradation of chloroplasts in cells in the infection zone, as observed by Sziráki et al. (1984), would probably account for these differences, because chloroplasts were easily visualized in mesophyll cells at healthy sites. However, necrosis or collapse of mesophyll cells was not observed at infected sites, as it has been in other studies of rust–susceptible host relationships (Bonde et al. 1976; McLean 1979).

In general, the sequence of events and fungal structures observed during the infection of susceptible *N. neesiana*

plants by *U. pencanus* urediniospores were similar to those observed for other rust species (Heath 1974; McLean 1979; Rowell 1984; Staples and Macko 1984; Hu and Rijkenberg 1998; Laurans and Pilate 1999). In agreement with the findings of Kneale and Farrar (1985), haustoria were most commonly formed in mesophyll cells, possibly because it is in this type of cell where the plant assimilates are produced.

In summary, the whole leaf clearing and staining technique did not provide fully satisfactory results for this pathosystem. It was possible to improve results by modifying this method, and to acquire complementary information on the infection process by inspecting stained fine microtome sections. Thus, the modified Bruzzese and Hasan technique can now be used to compare the series of events that occurs when nontarget species are inoculated with *U. pencanus* isolate 27, with those that occur on the susceptible target weed.

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