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# Characterization of *Phytophthora clandestina* races on *Trifolium subterraneum* in Western Australia

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**Abstract**

*Phytophthora clandestina* is a causal agent of root rot disease of subterranean clover in Western Australia (W.A). As a significant number of isolates of *P. clandestina* from W.A. could not previously be designated using existing differentials, a comprehensive set of subterranean clover (*Trifolium subterraneum*) cultivars was used as differentials to delineate a broader range of races of the pathogen. One hundred and one isolates of the pathogen collected from W.A. were screened on nine subterranean clover cultivars, of which seven were found to be useful as host differentials. A total of 10 races (in contrast to the five recognized previously) were defined and differentiated using octal nomenclature**,** presenting a clearer picture of the racial distribution of *P. clandestina* among W.A. isolates. Differences were found in the race populations between Australian states and are therefore important to the selection/breeding of cultivars for specific regions of Australia to counter the predominant race populations and for enforcing quarantine measures in relation to seed movements within and outside Australia. The octal nomenclature used provides a sound basis for follow-up studies and future race designations. Races 173 and 177 in this study were widely distributed and were the most common races in W.A., and together constitute 80% of the isolates characterized. While six of the seven host differentials were resistant to isolates belonging to race 001 and all were resistant to 000, it is of concern that only one differential was resistant to 157 and 173 and that none of the host differentials were resistant to 177. Our approach to *P. clandestina* race delineation is clearly conservative and is different from previous studies. The octal nomenclature we applied in this study is not only scientifically sound but also will facilitate rapid recognition and characterization of the races.

## Introduction

*Phytophthora clandestina* was first isolated in Victoria, Australia from rotted tap roots of subterranean clover, *Trifolium subterraneum* (Taylor et al., 1985a), and its life cycle has been described by Erwin and Ribeiro (1996). *Phytophthora clandestina* has been recorded only in Australia, and it is a major cause of decline in the productivity of subterranean clover pastures (Greenhalgh and Taylor, 1985). Greenhalgh (1992) estimated that this disease could reduce annual production of subterranean clover by more than 90%, particularly in long growing seasons and in irrigated

areas. While it is possible to manage this disease with fungicides (Barbetti et al., 1987; Greenhalgh et al., 1994), sowing root rot resistant cultivars is considered to be a more cost-effective long-term control measure. In Victoria, *T. subterraneum* var. *yanninicum* cvs Larisa, Trikkala and Meteora were reported to have high levels of resistance to this disease, as do some cultivars of *T. subterraneum* var. *subterraneum* such as Leura and Seaton Park (Taylor et al., 1985b; Greenhalgh and Flett, 1987; Taylor and Greenhalgh, 1987). Barbetti (1989) reported that the most susceptible cultivars to Western Australian (W.A.) isolates of *P. clandestina* were Woogenellup, Green Range, Mt Barker and Esperance, whereas Karridale, Dinniup, Larisa, Daliak and Trikkala were the most resistant to the W.A. *P. clandestina* isolates tested.

These early studies assumed the existence of only a single race of *P. clandestina*. However, a later study by Flett (1994) of 10 *P. clandestina* isolates on five subterranean clover cultivars, found that cvs Larisa and Trikkala, which previously had been classified as resistant, were susceptible to two isolates. Therefore, earlier isolates were designated race 0 while the two latter isolates were designated as race 1 (Flett, 1994). Subsequently, Purwantara et al., (1998, 2001) identified three additional races (*viz*. races 2, 3 and 4) using four to six cultivars and 25 (Purwantara et al., 1998) or 112 (Purwantara et al., 2001) isolates of *P. clandestina* collected from different geographic regions across southern Australia. However, race specificity of some isolates of *P. clandestina* previously collected from W.A. could not be identified using the limited cultivar differential sets used by Flett (1994) or Purwantara et al. (1995, 1998, 2001). This suggested a need for the isolates of this pathogen to be differentiated into races using a larger number of differentials. This would allow additional races to be characterized, as has been done for determination of races of *P. sojae* attacking soybean roots (Athow et al., 1974; Haas and Buzzell, 1976; Laviolette and Athow, 1977). For example, a further three races of *P. sojae* were discriminated from among a large group of isolates previously classified as race 6 by adding two additional host differentials (Laviolette and Athow, 1981). The number of defined races in *P. sojae* has grown from 25 in 1986 (Layton et al., 1986) to 55 in 2000 (Leitz et al., 2000), partly as a consequence of including additional host differentials.

This paper describes the use of a set of nine subterranean clover cultivars as differentials to characterize races of *P. clandestina* and to determine if certain races are specific to states within Australia. Knowing the distribution of races is an essential prerequisite, both to breeding cultivars with appropriate resistances for particular areas within W.A. and to the development and implementation of quarantine or sanitation precautions in relation to controlling the movement of contaminated seed material between states to prevent wider distribution of specific races.

## Materials and methods

For race characterization experiments, a complete randomized design was used, with three replicates for each host differential. Two experiments were carried out in a controlled environment room and with a 12-h photoperiod, a light intensity of 150 μE $m^{-2}$ $s^{-1}$, and at constant 20 °C for the growth of differentials. The second experiment, to confirm the key race characteristics defined in Experiment 1, was identical to the first, except that only sixteen isolates were used instead of the 101 isolates used in Experiment 1.

### *Subterranean clover cultivars and* Phytophthora clandestina *isolates*

Nine subterranean clover cultivars were used *viz*. Woogenellup, Leura, Trikkala, York, Larisa, Goulburn, Denmark, Seaton Park LF and Meteora. These were chosen to include the existing six differentials (*viz*. Woogenellup, Meteora, Larisa, Trikkala, Leura, and Seaton Park LF) used by Purwantara et al. (2001), plus an additional three cultivars (Goulburn, Denmark and York) known to exhibit various levels of resistance to other diseases such as those caused by *Kabatiella caulivora* (Barbetti, 1995) and *Uromyces trifolii-repentis* (Barbetti and Nichols, 1995). One hundred and one *P. clandestina* isolates collected from various locations in W.A. were used. Ninety-seven of these isolates from 32 sites were collected as part of a national survey of the incidence of *P. clandestina* across Australia (isolates from 14 sites were used by Purwantara et al., 2001), and four isolates (viz. WAC4954, WAC4955, WAC4956, WAC4957) were collected from W.A. during the 1980s.

*Inoculum*

Inoculum was prepared using a procedure modified from Flett (1994). Briefly, 500 ml of No. 3 grade vermiculite was amended with lima bean broth at a ratio of 5:1 (v/v) in a 1 l conical flask and autoclaved three times on three consecutive days at 120 °C for 30 min. The lima bean broth was prepared by soaking 1 kg of lima bean flour in 5 l of deionized water for 2 h at room temperature, and then straining through eight layers of Muslin cloth. Two-week-old *P. clandestina* colonies growing on lima bean agar plates were cut into 1 mm squares and used to inoculate each flask containing the prepared vermiculite and lima bean broth mix. The flasks were incubated in the dark at 22 °C for 2 weeks and shaken vigorously every two days to ensure uniform colonization.

*Glasshouse trial*

The vermiculite/lima bean-based inoculum was uniformly mixed with pasteurized soil mix (University of California Soil Mix; Baker 1957) at a rate of 1% w/w as used by Flett (1994). Twenty seeds (surface sterilized in 70% ethanol and rinsed three times in sterile deionized water) were evenly sown at 1 cm depth into the soil. Pots (10 cm diam) were flooded with deionized water immediately after sowing for 1 h and watered every day to excess with deionized water. One week after sowing, all pots were again flooded for 2 h with deionized water. Two weeks after sowing, whole seedlings were carefully collected and the roots washed free of soil under running tap water. The severity of root rot on each seedling was rated on a scale of 0–5 based on that used by Flett (1994) and later modified by Aldaoud et al. (1997) (*viz.*: 0, healthy roots; 1, lateral root discolouration; 2, 1/3 tap roots discoloured; 3, 2/3 tap roots discoloured; 3.5, tap roots rotted away but new lateral roots growing; 4, tap roots rotted away, no new lateral roots; 5, seedling dead). To confirm that disease symptoms observed were in fact caused by *P. clandestina*, 2 cm root segments from all differential cultivars were taken from each pot and floated in separate Petri dishes containing sterile deionized water for 2 days at 22 °C and then examined microscopically for typical sporangia of *P. clandestina* as illustrated by Erwin and Ribeiro (1996).

*Statistical analysis*

Levels of seedling root rot caused by *P. clandestina* in the nine differentials were analysed using the analysis of variance functions of GENSTAT V. The seedling root rot severity of each differential cultivar caused by each *P. clandestina* isolate was used to construct histograms for determining the spectrum separation points for resistance or susceptibility of each differential cultivar and whether the differences in resistance and susceptibility were assumed to be due to single major gene effects and as such could be used as host differentials (Figure 1).

P. clandestina *race nomenclature*

Octal nomenclature (Goodwin et al., 1990) was used to code *P. clandestina* isolates according to their pathogenicity (expressed as disease severity) on the differentials in which race codes were based on arranging the differentials from right to left. However, to adhere to this nomenclature we had to discard two differentials, viz. cvs Meteora and Denmark, from the nine differentials tested (see Figure 1). The seven remaining cultivars used as differentials were grouped in three sets according to their genetic inheritance and continuity with previous studies. For example, cvs Seaton Park LF, Leura and Woogenellup were grouped as one set or triplet (digit 0) because they all belonged to *Trifolium subterraneum* var. *subterraneum* while York, Larisa and Trikkala were grouped as another triplet (digit 1) because two of them belonged to *T. subterraneum* var. *yanninicum* (Larisa and Trikkala) and were used previously, while *T. subterraneum* var. *subterraneum* cv. York was added to complete this triplet. Cultivar Goulburn was grouped as an incomplete set (digit 2) because it was not included in the previous studies of Flett (1994), or Purwantara et al. (1998, 2001). As there is only one cultivar in this set it could be completed with the addition of two more differentials in any follow-up studies. Octal numbers were assigned based on the pathogenicity of the isolates on and interaction with each differential within a set in which 0 indicates a non-pathogenic reaction (or resistance of the differential) and 1 indicates a pathogenic reaction (or, susceptibility of the differential). Octal digits were assigned as follows: 000 = 0; 001 = 1; 010 = 2; 011 = 3; 100 = 4; 101 = 5; 110 = 6; 111 = 7 (Table 1). The digit with an

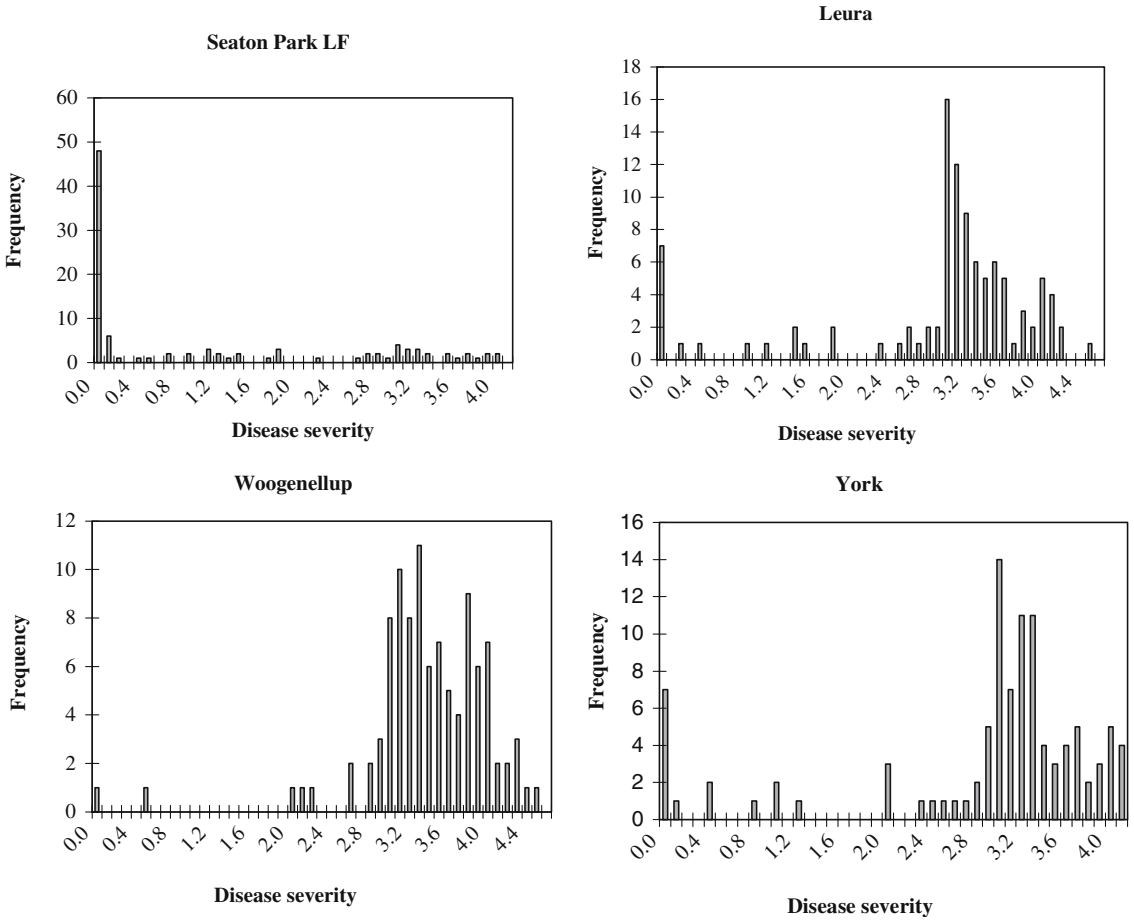


*Figure 1.* Histograms reflecting pathogenicity levels of 101 isolates of *Phytophthora clandestina* on nine cultivars of *Trifolium subterraneeum*. Disease severity rating $<2$ for Woogenellup, York and Denmark; $<1.4$ for Larisa and Goulburn; $<2.3$ for Leura; $<2.2$ for Seaton Park and $<1.8$ for Trikkala; are considered as resistant; disease severity rating $\geq$ above mentioned points are considered as susceptible. Cultivars Meteora and Denmark were excluded from the differential sets because they did not show bimodal responses to the pathogen.

underscored character indicates an incomplete set in which one or more differentials are missing.

## Results

There was a significant ($P < 0.01$) interaction between *P. clandestina* isolates and root rot severity on the tested subterranean clover cultivars. Seven of the nine cultivars chosen as differentials showed clear bimodal distribution (Figure 1). Analyses of histograms based on pathogenicity of 101 isolates of *Phytophthora clandestina* on seven differential cultivars showed that the resistance or susceptibility separation point of chosen differentials is 2.0 for Woogenellup and York, 1.4 for Larisa and Goulburn, 2.3 for Leura, 1.8 for Trikkala and 2.2 for Seaton Park LF (Figure 1). Where the disease severity rating was less than the separation point, it was considered as a resistance response**,** while a rating greater than and including the separation point was considered as susceptibility. On the basis of pathogenicity of *P. clandestina* isolates across the seven differential cultivars, 10 races were differentiated from among the 101 isolates collected from W.A. (Table 1).

Regression analyses showed that the results of the second experiment were positively correlated with those of the first experiment ($P < 0.001$) and, in addition, variance analyses indicated that there was no significant difference between the results from the two experiments ($P = 0.93$).

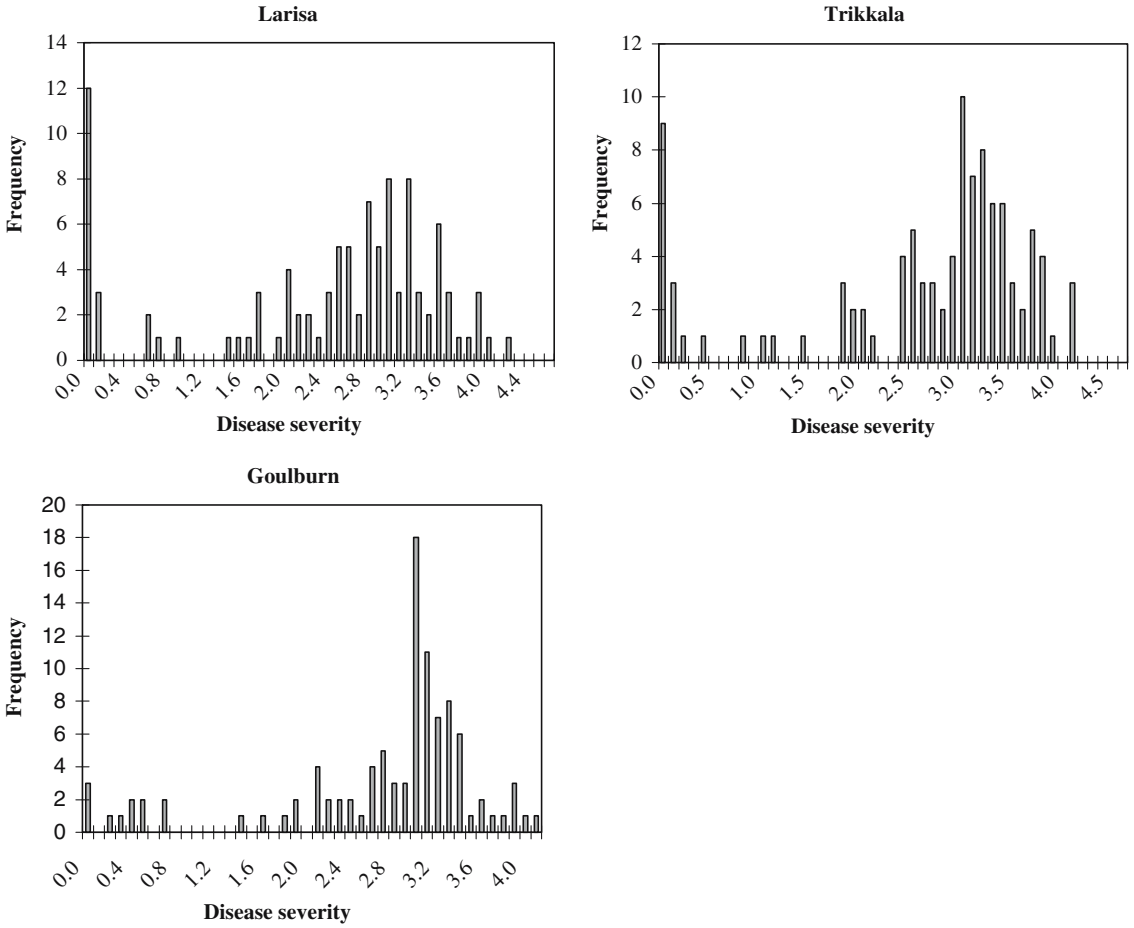


*Figure 1.* Continued.

## Discussion

In our study, inclusion of cvs Goulburn and York and the exclusion of cv. Meteora enabled an improved delineation of a total of 10 *P. clandestina* races, while allowing scope for a theoretical potential to delineate up to 128 races in the future. Purwantara et al. (1995) concluded that isolates of *P. clandestina* differ in virulence against different cultivars and have varying abilities to attack cultivars with different levels of resistance. In contrast, their initial study used only four cultivars (Woogenellup, Meteora, Larisa and Trikkala) and six *P. clandestina* isolates to examine race specificity. Three of these isolates were specifically pathogenic on cv. Woogenellup, two isolates were pathogenic on cvs Larisa and Trikkala in addition to cv. Woogenellup, and one isolate was pathogenic on both cvs Meteora and Woogenellup. Consequently, they designated these as races 0, 1 and 2, respectively. Subsequently, Purwantara et al. (1998) identified two additional races (3 and 4) using four cultivars (Woogenellup, Leura, Meteora and Seaton Park LF) and 25 isolates collected from different geographic regions in southern Australia. More recently, Purwantara et al. (2001) confirmed the existence of these same five races based on their virulence across a set of six differential cultivars (Woogenellup, Leura, Meteora, Larisa, Trikkala, and Seaton Park LF) and 112 isolates collected from southern Australia (including W.A.). Their inability to delineate many W.A. isolates of the pathogen prompted our study.

Notably, for the first time, differentiation of races among previously uncharacterized isolates of *P. clandestina* could be made. The use we made

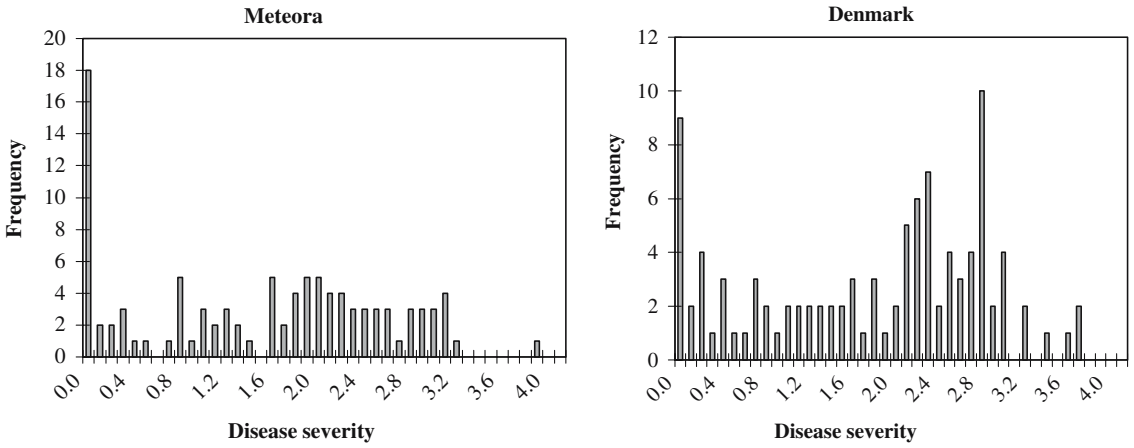


*Figure 1.* Continued.

of octal nomenclature for naming race codes will enable future studies to simply, where required, add more differentials by adding their codes to the left of those currently used. This will facilitate rapid recognition and characterization of the races, including their pathogenicity in relation to the differentials. This improved differentiation of *P. clandestina* races not only enables a much more accurate description of each race but also provides the basis for effective monitoring of race distribution and/or spread across southern Australia.

Newly designated races 173 and 177 were the most commonly isolated races in W.A. We found only one isolate each for races 121, 141, 151 and 157, suggesting that these races are not common in the regions we sampled within W.A. However, this situation may change with alterations in the adoption of cultivars of pasture legume species used within or between the regions sampled. The cultivars we used as differentials together represent a significant portion of the area sown to subterranean clover in W.A. (Nichols et al., 1996). None of the differential cultivars we used had resistance to race 177, while only cv. Seaton Park LF had resistance to race 173 and cv. Larisa was the only cultivar with resistance to race 157. Cultivars York and Trikkala have been widely sown in W.A. and have resistance to four races (000, 001, 101 and 121) for cv. York, and to six races (000, 001, 101, 121, 141 and 143) for cv. Trikkala. However, these two cultivars are likely to suffer significant losses from the other races we identified. Cultivars Goulburn, Larisa and Leura, have also been widely sown in W.A. These cultivars, however, are likely to suffer significantly from eight races (101, 121, 141, 143, 151, 157, 173, 177) for cv. Goulburn; from three races (121, 173 and 177) for cv. Larisa and from four races (143, 157, 173 and 177) for cv. Leura. Race 177 has the potential to cause the most widespread yield losses as none of the seven differentials tested, which are also commercially recommended cultivars, showed resistance to this particular race.

Reliable race differentiation, a pre-requisite for appropriate measures to address quarantine concerns, can be taken for *Phytophthora* species such as *P. clandestina* that could potentially be seed-borne or carried in plant or soil residues associated with seeds (Erwin and Ribeiro, 1996). Only once the full race spectrum and its distribution is defined, can relevant precautions be taken in relation to seed sanitation and appropriate decisions made to restrict seed movement between Australian states. Where possible, unnecessary yield losses and associated costs resulting from disease outbreaks caused by the introduction of new races to a region should be avoided. The absence of previously designated race 2 (Purwantara et al., 2001) in our study and the increased number of races of *P. clandestina* determined for W.A., together mean that caution is required when moving seed within or outside Australia.

Breeding for resistant cultivars has been acknowledged as the most economic long-term disease control measure**,** not only for annual pasture legumes in Australia (Barbetti et al., 1986), but also for many other crops. The information from our study highlights the presence of a larger number of races and will now enable clover breeders to make better and more informed decisions on the

*Table 1*. Races of *Phytophthora clandestina* determined by root rot severity on seven differential cultivars of *Trifolium subterraneum* where 0 indicates non-pathogenic/resistance reaction and 1 indicates a pathogenic/susceptible reaction on the differentials following inoculation

| Differential cultivar of subterranean clover | | | | | | | Race octal code | Previous race designation | Race frequency (%) |
|---|---|---|---|---|---|---|---|---|---|
| Goulburn | York | Larisa | T rikkala | Seaton Park LF | Leura | Woogenellup | | | |
| 0 | 0 | 0 | 0 | 0 | 0 | 0 | 000 | u | 2 |
| 0 | 0 | 0 | 0 | 0 | 0 | 1 | 001 | 0; u | 9 |
| 1 | 0 | 0 | 0 | 0 | 0 | 1 | 101 | 0; u | 2 |
| 1 | 0 | 1 | 0 | 0 | 0 | 1 | 121 | 0 | 1 |
| 1 | 1 | 0 | 0 | 0 | 0 | 1 | 141 | 0 | 1 |
| 1 | 1 | 0 | 0 | 0 | 1 | 1 | 143 | 0; u | 4 |
| 1 | 1 | 0 | 1 | 0 | 0 | 1 | 151 | u | 1 |
| 1 | 1 | 0 | 1 | 1 | 1 | 1 | 157 | u | 1 |
| 1 | 1 | 1 | 1 | 0 | 1 | 1 | 173 | 1;3;u | 52 |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 177 | 4; u | 27 |

Octal digits were assigned as follows: 000 = 0; 001 = 1; 010 = 2; 011 = 3; 100 = 4; 101 = 5; 110 = 6; 111 = 7. The octal digits are sorted according to the number of virulences per triplet (Goodwin et al., 1990). The digit with an underscored character of new race code indicates an incomplete set in which two differential hosts are missing. 'u' is an unknown race in previous designations (Purwantara et al., 1998).

development and utilization of appropriate cultivars for specific regions. In Australia, breeding of subterranean clover cultivars with resistance to *P. clandestina* root rot has relied upon incorporation of what now appears to be major gene-based resistance, presumably on the assumption that only a small number of races were present in the country. However, continued utilization of different major gene-based resistances may result in the further proliferation of new races of *P. clandestina* as has been recorded with *P. sojae* (Ryley et al., 1991) where the introduction of cultivars with dominant gene-based resistance has resulted in the rapid appearance of new races (Walker and Schmitthenner, 1984). Therefore, it is expected that further new races of *P. clandestina* will appear over time, especially in areas where major gene-based resistance has been or is being widely utilized. Polygenic or horizontal (quantitatively inherited/or partial) resistance offers a more stable long-term basis for managing this disease than does any total reliance on major gene-based resistance or immunity to disease (Schmitthenner, 1985; Athow, 1986; Tooley and Grau, 1982) because it does not encourage the rapid build-up of new races (Schmitthenner, 1985). Subterranean clover cultivars with durable resistance to *P. clandestina* could perhaps best be developed by identifying and utilizing strong polygenic resistance rather than relying solely upon major gene-based resistance for disease control.

Our approach to the race delineations is clearly conservative and based on separation of peaks in the histograms that represent susceptible and resistant responses of host differentials to the races of this pathogen. This would also suggest that all previous delineations of races (including race 2) may require re-evaluation. We believe that the scheme of Goodwin et al. (1990) we applied in our study is scientifically sound and is of great value in relation to the agronomic evaluation of subterranean clover as a pasture legume.

Further studies to analyse host resistance could be the way to provide ultimate clarification of genetic basis of race delineation in *P. clandestina*.

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