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Morphological Investigations and Infection Studies of the Rust-Causing Fungi of Roses

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ABSTRACT

Rose rust is caused by members of the genus *Phragmidium*, four of which are: *P. mucronatum* (Pers.) Schlect., *P. tuberculatum* Mull., *P. fusiforme* Schroet. and *P. rosae-pimpinellifoliae* (Rabh.) Diet. In the present study, preliminary identification of *Phragmidium* species was based on host range. Identification was then supported by measuring the dimensions of urediospores and teliospores. Axenic cultures of *P. tuberculatum* were initiated on culture media but these cultures were unable to reinfect rose leaves, therefore cultures of *P. tuberculatum* on leaf discs of 'Mme. Grégoire Staechelin' were initiated from urediospores. The infectivity of dry spores and spore suspensions of urediospores of *P. tuberculatum* directly from host tissue did not differ significantly; however, spore density of 30×10^4 per ml proved to be more infective than higher or lower densities. Surface-sterilized detached leaves showed significantly lower infection rates than leaves that were not surface sterilized. The urediospores on the leaf discs, survived when stored at 4°C for four weeks, but when stored for 12 weeks at the same temperature, they lost their viability and were not able to infect leaf discs.

Keywords: axenic culture, *Phragmidium* spp., *P. tuberculatum*, *Rosa* L., storage
Abbreviations: SEM, scanning electron microscopy

INTRODUCTION

Rust fungi belong to the class Basidiomycetes, order Uredinales, and are identified by the presence of rust-coloured spores in powdery pustules or gelatinous horns at some stage in their life cycle (Maclean 1982). The rust genus *Phragmidium* is predominantly found in the northern hemisphere and compromises about 60 species (Cummins and Hiratsuka 2003). Rose rust is caused by nine species of fungi of the genus *Phragmidium*; the four most important being *P. mucronatum* (Pers.) Schlect., *P. tuberculatum* Mull., *P. fusiforme* Schroet. and *P. rosae-pimpinellifoliae* (Rabh.) Diet. Attempts have been made to grow rust fungi in axenic culture from spores, including *P. tuberculatum* (Bhatti 1984), *P. mucronatum* (Bhatti and Shattock 1980), *P. violaceum* (Bhatti and Shattock 1980), *Puccinia recondite* (Raymundo and Young 1974) and *Puccinia graminis* (Foudin and Wynn 1972). Urediospores have been used almost exclusively because they can be used directly to reinfect susceptible plants. However, despite careful attempts to standardize techniques and optimise concentrations of nutrients, it has been difficult to draw a general conclusion about the value of a particular medium or growth condition.

According to Maclean (1982), no standard technique has been developed which can guarantee the successful axenic culture of different isolates of rust species. *In vivo*, physical stimuli caused by contact with the leaf surface are evidently involved in the induction of fungal growth. Scanning electron microscopy (SEM) indicates that the stomatal lip may be responsible for inducing the infection process (Wolf 1982).

The growth and development of pathogenic fungi on their host(s) are controlled by genes for resistance in the host and genes for virulence in the pathogen. The concept of a gene-for-gene relationship has been of great value in explaining the host specificity of pathogenic fungi using classical genetics (Kamoun 2006).

Many types of work in plant pathology require readily available microorganisms, and delays incurred in acquiring and re-isolating them can be limiting for reliable and reproducible research. It is also quite common that an organism has been isolated once but repeated attempts to re-isolate it have failed. Sufficient stocks must be laid down to provide identical experimental replicates, and if these are not prepared and stored correctly, long-term work may be jeopardized. Some species of rust fungi have been successfully preserved. *Puccinia coronata* and *Puccinia graminis* urediospores were preserved by lyophilization without change in pathogenicity (Sharp and Smith 1952). Urediospores of *Puccinia graminis* f. sp. *tritici* were stored in liquid nitrogen for 10 years with little deterioration in viability (Kilpatrick *et al*. 1971).

The aim of the present investigation was to 1) identify *Phragmidium* species based on their morphological characteristics, 2) describe a method for culturing and preserving the rust fungus and 3) characterize the resistance of a group of rose cultivars to *P. tuberculatum*.

MATERIALS AND METHODS

Leaf material for infection studies

Leaves of 'Queen Elizabeth', 'Paul Nyron', *R. spinosissima* L. and 'Alba Maxima' were obtained from the gardens of the Royal National Rose Society, Chiswell Green, UK. Fully expanded leaves of 'Agnes', 'Allgold', 'Frensham', 'KORbin' (='Iceberg'), 'MACel' (=Elizabeth of Glamis®), 'Mme. Grégoire Staechelin' (='Spanish Beauty'), 'Peace' and *R. canina* L. were obtained from private gardens in the UK.

Detached leaves were surface sterilised by washing under

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running tap water for 2 hrs, 70% ethanol for 30 sec and 15 min in bleach (20% (v/v) Domestos Ltd., UK, containing approximately 0.8% (w/v) sodium hypochlorite plus added surfactants). This was followed by washing 3 × 15 min in sterile distilled water. The lower surface of the leaves was scratched (2-3 shallow cuts) with a scalpel blade.

Measurements of spore dimensions

The spores were obtained from infected specimens of: 'Mme. Grégoire Staechelin', 'Paul Nyron', and 'Queen Elizabeth' (expected to be infected by *P. tuberculatum*); 'Alba Maxima' (expected to be infected by *P. mucronatum*); and *R. spinosissima* (expected to be infected by *P. rosae-pimpinellifoliae*) (Wilson and Henderson 1966). The spores were observed using a Nikon Optiphot light microscope and measurements were made by reference to a micrometer slide using an eye-piece graticule. The diameter of urediospores, and the length of the teliospores, their pedicels and apiculus and the number of cells in the teliospore were recorded (**Fig. 1**). Unless otherwise noted, means were presented with standard errors.

The morphology of the outer surface of the urediospores and teliospores were observed by SEM. In order to determine variability, measurements of different sections of teliospores and diameter of urediospores for three species of *Phragmidium* were carried out on 25 spores for each strain and standard errors were calculated.

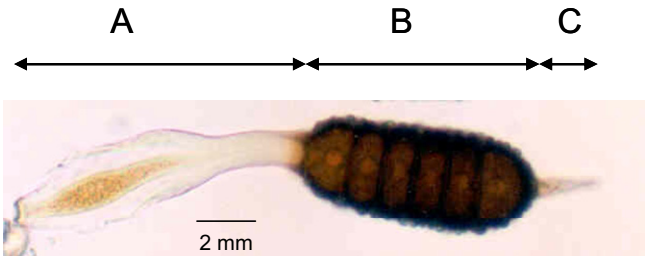

Fig. 1 Light microscope photograph of the pedicel (A), the teliospore (B) and apiculus (C) of *Phragmidium tuberculatum*.

Source of inocula of *P. tuberculatum*

Leaves of 'Mme. Grégoire Staechelin' infected with *P. tuberculatum* were surface sterilised and newly produced urediospores were used to inoculate culture media or the lower surface of leaf discs (14mm diameter) of 'Mme. Grégoire Staechelin' cut from surface sterilised leaves. Successfully infected leaf discs were used as a source of urediospores for subsequent experiments.

Axenic culture and culture of *P. tuberculatum* on rose leaves

All experiments were carried out using urediospores of an isolate of *P. tuberculatum* from the rose 'Mme. Grégoire Staechelin' and maintained on leaf discs of 'Mme. Grégoire Staechelin'.

Medium M1 consisted of 2 g/l of yeast extract (Oxoid Ltd, Basingstoke, UK), 2 g/l of peptone (Oxoid Ltd), 36 g/l Czapek Dox broth (Oxoid Ltd), 2 g/l casein hydrolysate (Oxoid Ltd) and 10 g/l Bactoagar (Difco Laboratories, East Molesey, UK). Medium M2 had a similar composition, except that the amount of yeast extract, peptone and Czapek Dox broth was reduced to 1, 1 and 3.5 g/l, respectively; casein hydrolysate was excluded and the amount of Bactoagar was increased to 20 g/l. Media M1 and M2 were used by Bhatti (1984) in axenic culture of *P. tuberculatum*. Dry urediospores were evenly dispersed on the media by a camel hair brush. The mycelia were sub-cultured on these media for several months and were tested for their ability to infect leaf discs of 'Mme. Grégoire Staechelin' at four-week intervals.

To inoculate the lower surface of the leaves, either dry spores (transferred by a camel-hair brush) or spore suspension (in sterile distilled water) were used. Spore suspensions were prepared at densities of 1, 3, 10, 20, 30, 50 and 100 × 10^4 per ml. The leaves were either surface sterilised as described above or washed with tap water for two hours. In all of the experiments 30 leaf discs (14 mm diameter) were inoculated with 20 µl of spore suspension per leaf disc unless otherwise stated. The leaf discs were placed on Petri dishes with two layers of filter paper (90 mm, Whatman, GA, USA) moistened with 2 ml of sterile distilled water.

In all experiments, the plates were placed under high pressure metal halide lamps (HQI-T, Osram, Germany; PPFD 60 µmol m^{-2} s^{-1} at the plant surface) on a 16/8 hour light/dark cycle in a culture room maintained at 25 ± 2°C on a temperature-controlled bench maintained at 18 ± 2°C. Chi-square tests (calculated in Excel, Microsoft, 2003) were carried out to compare the different methods.

Tests of spore viability and observation of spore germination

Viability of spores was tested according to Ueda and Hirata (1989): urediospores were put onto a microscope slide, mixed with a 9.6 × 10^{-6} M solution of fluorescein diacetate (Sigma, Poole, England) in an aqueous solution of sucrose (0.4 M) and incubated for 10 min at room temperature. Spore viability was recorded as the percentage of fluorescent grains under UV light with an excitation filter (450-490 nm) and barrier filter (520 nm). The viability of urediospores on leaf discs (placed in small plastic vials) was investigated at -196°C (in liquid nitrogen and rapid thawing), -20°C (freezer and rapid thawing), 4°C (refrigerator) and 18°C (control). The results were recorded four and twelve weeks after storage and for each treatment at least 500 spores were observed.

To observe the germination of urediospores on the lower surface of leaf discs, the leaf discs were stained with a 0.1% solution of Calcofluor (Sigma Poole, England) in 100 mM Tris-HCl buffer, pH 8.5. After 1 to 2 min in the dye solution, samples were briefly rinsed in water and mounted on glass slides and were observed with a UV microscope.

Testing roses for resistance to *P. tuberculatum*

Urediospores were used to inoculate leaf discs of 'Agnes', 'Allgold', 'Frensham', 'KORbin', 'MACel', 'Peace', 'Queen Elizabeth' and *R. canina* . Spore suspensions (30 × 10^4 per ml) were used to inoculate 45 leaf discs (20 µl per leaf disc) for each cultivar and the infection rate was recorded as the percentage of infected leaf discs after 21 days.

Table 1 Measurements (mean ± SE) of different sections of teliospores and diameter of urediospores for three species of *Phragmidium* (expected from the rose cultivar). Ranges indicated by Wilson and Henderson (1966) are shown in parentheses.

Host	Species of rust indicated by host preference	No. of cells per teliospore	Length of structures of teliospores and associated structures (µm)			Diameter of urediospores (µm)
			Apiculus	Teliospore	Pedicel	
'Mme. Grégoire Staechelin'	*P. tuberculatum*	5.1 ± 0.25	19.9 ± 1.02	84.9 ± 5.8	96.3 ± 2.91	22.8 ± 0.55
'Queen Elizabeth'	*P. tuberculatum*	6.1 ± 0.18	20.4 ± 0.83	82.9 ± 3.30	95.7 ± 2.79	22.7 ± 0.55
'Paul Nyron'	*P. tuberculatum*	5.5 ± 0.13 (4-6)	17.3 ± 1.22 (22)	78.8 ± 2.77 (55-110)	80.4 ± 3.79 (55-110)	22.76 ± 0.38 (20-25)
'Alba Maxima'	*P. mucronatum*	6.6 ± 0.15 (5-9)	14.2 ± 1.33 (7-13)	86.2 ± 2.04 (64-90)	112.3 ± 4.16 (96-135)	22.62 ± 0.30 (20-28)
R. spinosissima	*P. rosae-pimpinellifoliae*	4.1 ± 0.16 (6-8)	5.0 ± 0.57 (14-16)	53.0 ± 2.06 (70-115)	65.2 ± 5.58 (105-173)	21.1 ± 0.50 (18-25)

RESULTS

Identification of *Phragmidium* species

The number of cells per teliospore, the length of teliospore and pedicel, and the diameter of urediospores obtained for three varieties of roses ('Mme. Grégoire Staechelin', 'Queen Elizabeth' and 'Paul Nyron') were within the range specified by Wilson and Henderson (1966) for *P. tuberculatum*. The apiculus was shorter than the single value (22 µm) given by Wilson and Henderson (1966) (**Table 1**). As the full range of apiculus length was not provided by Wilson and Henderson (1966) and as the other parameters were in agreement, the provisional identification of *P. tuberculatum* on the basis of host range was accepted.

The number of cells per teliospore, the length of teliospore and pedicel and the diameter of urediospores obtained for spores taken from leaves of 'Alba Maxima' were within the range stated by Wilson and Henderson (1966) for *P. mucronatum* (**Table 1**). The length of apiculus (14.2 ± 1.33 µm) was longer than the range (7-13 µm) stated by Wilson and Henderson (1966). However, because of the large range, the identification of *P. mucronatum* on the leaves of 'Alba Maxima' was accepted.

The number of cells per teliospore and the length of teliospore, apiculus and pedicel were smaller in spores collected from *R. spinosissima* than those obtained by Wilson and Henderson (1966) for *P. rosae-pimpinellifoliae* (**Table 1**). In view of large discrepancies between the parameters of the results obtained from teliospores from *R. spinosissima* and those detected for *P. rosae-pimpinellifoliae* by Wilson and Henderson (1966), the identification based on host range was not accepted. It was noted that the dimensions were more similar to those recorded for *P. tuberculatum*.

SEM of the outer surface of the urediospores and teliospores showed that both urediospores and teliospores had verrucose surfaces. In the teliospores of *P. tuberculatum* the verrucae were only observed on the cells of the spores. The apiculus and pedicel had smooth surfaces (**Fig. 2**).

Axenic culture of *P. tuberculatum*

The urediospores obtained from 'Mme. Grégoire Staechelin' inoculated on M1 and M2 media produced germ tubes and cultures similar to those described by Bhatti and Shattock (1980) and Bhatti (1984) (**Fig. 3**). The colour of colonies was initially white, slowly changing through cream and yellow to brown. Colonies on M1 were larger, more dense and had more elaborate hyphae than on M2, where growth

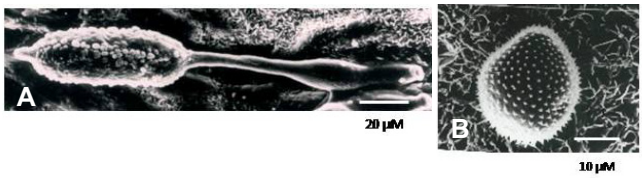

Fig. 2 the outer surface of (A) teliospores and (B) urediospores taken from leaves of 'Mme. Grégoire Staechelin' using scanning electron microscopy.

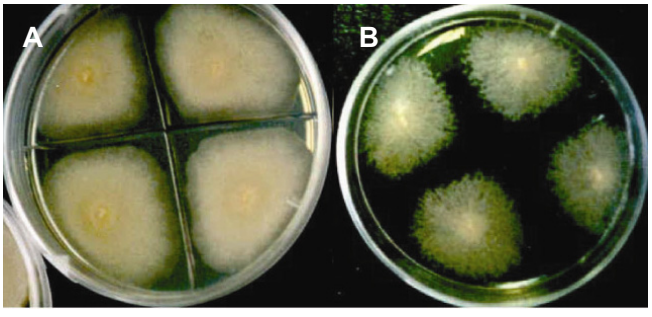

Fig. 3 The mycelial cultures of *Phragmidium tuberculatum* on (A) M1 and (B) M2 media in Petri dishes of 90 mm diameter.

Fig. 4 Germ tube from a urediospore of *Phragmidium tuberculatum* entering a stoma on the leaf of 'Mme. Grégoire Staechelin'.

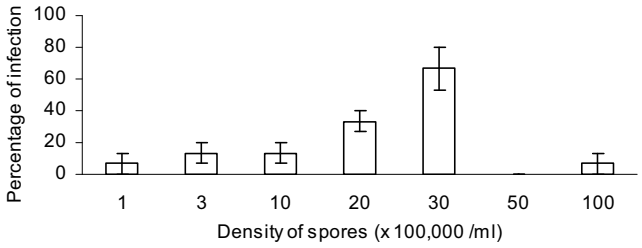

Fig. 5 Effect of inoculum spore density on the percentage of leaf discs infected. Bars show standard error of the mean.

was slower. The cultures were successfully sub-cultured on the respective media for several months, but did not infect either scratched or unscratched leaf discs of 'Mme. Grégoire Staechelin'.

Culture of *P. tuberculatum* on leaves

Infected leaf discs of 'Mme. Grégoire Staechelin' produced fresh batches of urediospores in sufficient quantities to be collected and used for experiments 14 days after they had been washed and surface sterilised. The leaf discs of 'Mme. Grégoire Staechelin', inoculated with the urediospores taken from leaves of 'Mme. Grégoire Staechelin', produced fresh urediospores, usually 21 days later. Urediospores germinated on the lower surface of leaf discs and the germ tubes were visible using a light microscope equipped with UV light when the leaf discs were stained with Calcofluor. The bright blue fluorescence of fungal spores and germ tubes, which entered the leaf through stomata, was observed (**Fig. 4**).

To optimise the rate of infection of leaf discs of 'Mme. Grégoire Staechelin', spore suspension densities of 1, 3, 10, 20, 30, 50 and 100 × 10^4 per ml were tested. The highest percentage of infection rate was obtained at a spore density of 30 × 10^4 per ml (66.6 ± 13.3%) (**Fig. 5**).

In order to test the possibility that surface sterilisation of the leaves prior to cutting leaf discs might affect the viability of the urediospores, infection rate was compared in leaf discs from surface sterilised and unsterilised leaves. The unsterilised leaves were washed in tap water in the same manner as with the sterilised leaves, but were not exposed to 70% ethanol or bleach. The rate of infection in the surface sterilised leaves (43.3 ± 8.81%) was approximately half that of the leaves that were not surface sterilised (76.6 ± 8.81%), and statistical analysis (chi-square test) indicated that there was a significant difference between the two methods (χ^2 = 31.4, df = 1, P <0.01).

The infectivity rates of dry spores was compared with that of spore suspensions. The spore suspension density was adjusted to 30 × 10^4 per ml, but it was not possible to quantify the inocula of dry spores. The infection rate for the leaves inoculated with the spore suspension (56.6 ± 6.75%) was not significantly different from the leaves that were inoculated with dry spores (58.8 ± 5.10%) (t = 0.26, df = 10, P = 0.79).

Storage of spores

The viability of urediospores stored on leaf discs at -196°C (in liquid nitrogen), -20°C (freezer), 4°C (refrigerator) and

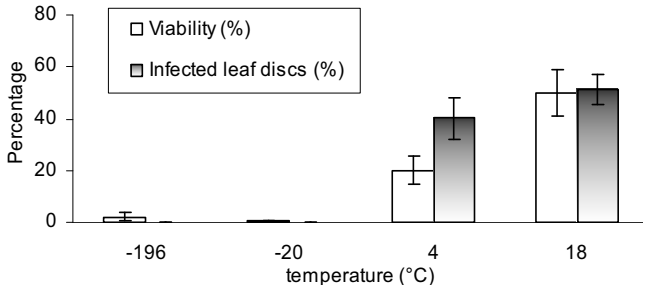

Fig. 6 Percentage of viability and infectivity of spores stored at -196, -20, 4 and 18°C (controls). Bars show standard error of the mean.

18°C (control) was recorded four weeks after storage. A spore suspension (30×10^4 per ml) of these spores was used to inoculate leaf discs and percentage of infected leaves was recorded after 21 days. Microscopic observations indicated that the stored urediospores had shrunken cytoplasm and were lighter in colour than the control spores. The viability based on staining of urediospores stored at -196°C and -20°C was reduced to 2.07% and 0.50%, respectively (**Fig. 6**) and they were not able to infect leaf discs. The viability of spores at 4°C (20.0 ± 5.51%) was significantly lower than the control spores (50.0 ± 9.03%, t = 2.82, df = 6, P = 0.03). The percentage of infected leaf discs inoculated with spores stored at 4°C (42.2 ± 8.02%), was not significantly different from the percentage of leaf discs inoculated with control spores (51.1 ± 5.87%, t = 0.89, df = 4, P = 0.4). However, after storage at 4°C for a period of 12 weeks, the urediospores lost their pathogenicity and were not able to infect leaf discs, and their viability was reduced to 3.06 ± 0.80% (**Fig. 6**).

Resistance of roses to *P. tuberculatum*

According to the survey carried out by Howden and Jacobs (1973), 'Allgold', 'Frensham', and 'KORbin' were resistant to *P. tuberculatum*. In our experiment, 'Allgold' and 'KORbin' showed resistance, but 'Frensham' showed a high infection rate (85.9 ± 0.70%), which was close to that observed for the susceptible control, 'Mme. Grégoire Staechelin' (86.6 ± 7.70%) (**Table 2**). Howden and Jacobs (1973) recorded 'MACel', 'Peace' and 'Queen Elizabeth' as susceptible to *P. tuberculatum*, but in the present investigation, 'Queen Elizabeth' showed susceptibility (24.4 ± 2.20%) and both 'Peace' and 'MACel' had low infection rates (4.40 ± 2.20% for both) (**Table 2**).

Howden and Jacobs (1973) indicated that plants of *R. canina* and 'Agnes' are not infected by *P. tuberculatum*. The low infection rate of these plants in our tests (4.40 and 0%, respectively) by an isolate of *P. tuberculatum* corresponds with their findings (**Table 2**).

DISCUSSION

Phragmidium tuberculatum is the most common species of *Phragmidium*, infecting the main classes of modern cultivated roses (Howden and Jacobs 1973). Comparison of the measurements of teliospores with those recorded by Wilson and Henderson (1966) confirmed the identification of spores taken from leaves of 'Mme. Grégoire Staechelin', 'Queen Elizabeth' and 'Paul Nyron' as three isolates of *P. tuberculatum*. Identification of one isolate of *P. mucronatum* on leaves of 'Alba Maxima' was also in accordance with Wilson and Henderson (1966). However, *R. spinosissima* belongs to the section *Pimpinellifoliae* (DC.) Ser., and according to Howden and Jacobs (1973) is expected to be infected by *P. rosae-pimpinellifoliae*. The measurements of teliospores taken from leaves of *R. spinosissima*, however, differed from those observed by Wilson and Henderson (1966) for *P. rosae-pimpinellifoliae*. This could have been the result of infection of this plant by another species of *Phragmidium*, e.g. *P. tuberculatum*. Howden and Jacobs (1973), suggested that species of *P. mucronatum* and *P. tuberculatum* are not cross-infective (the isolates of one species do not infect the susceptible hosts of the other), but they found *P. tuberculatum* on the leaves of *R. rugosa* Thunb. stocks, in particular *R. rugosa* 'Hollandica', which was expected to be infected by *P. rosae-pimpinellifoliae*. Gäumann (1959), on the other hand, stated that host ranges of rose rusts are not species-specific. Therefore, the infection of *R. rugosa* by *P. tuberculatum* in the investigation by Howden and Jacobs (1973) is explainable. The uncertain identity of the isolate of rust obtained from *R. spinosissima* and discrepancies in the length of the apiculus in the isolate tentatively identified as *P. mucronatum* draw attention to the need for further investigations of species of *Phragmidium*. In particular, host range and variability of spore dimensions need further characterisation as they are presently used as criteria for species identification.

To establish host ranges of different pathotypes, axenic cultures of *P. tuberculatum* are needed in order to obtain single spore isolates. Bhatti and Shattock (1980) recorded the formation of germ tubes from urediospore-like structures in an axenic culture of *P. mucronatum*, which induced a few small brown raised pustules on *R. laxa* Retzius, the susceptible host. However, in the present investigation the mycelium grown on the M1 and M2 media from urediospores taken from leaves of 'Mme. Grégoire Staechelin' were not able to infect scratched or unscratched leaves of 'Mme. Grégoire Staechelin'. The purpose of scratching the leaves was to injure the surface tissue to allow the mycelium an alternative mode of entry to the interior of the host leaf. This strategy was suggested by the observation that saprotrophically grown mycelium from *P. graminis* were able to infect wheat leaves when the mesophyll was exposed by prior removal of a portion of the abaxial epidermis (Maclean 1982), but this method was unsuccessful in the present investigation. Maclean (1982) stated that races of *P. graminis* f. sp. *tritici* (wheat rust), *P. graminis* f. sp. *avenae* (oat stem rust) and *P. graminis* f. sp. *secalis* (rye stem rust) differ in the ease with which they can be cultured. It is possible that mycelium of the isolate of *P. tuberculatum* used in the present investigation was physiologically, as well as genetically, different from the isolate used by Bhatti and Shattock (1980).

Table 2 Percentage of infected leaf discs of cultivars of roses (mean ± SE) when inoculated with *Phragmidium tuberculatum* from leaves of 'Mme. Grégoire Staechelin' and susceptibilities previously recorded by Howden and Jacobs (1973).

Rose tested	Rate of infection (%)	Howden and Jacobs (1973)[a]:		
		P. tuberculatum	*P. mucronatum*	*P. rosae-pimpinellifoliae*
'Mme. Grégoire Staechelin' (control)	86.6 ± 7.70	N/A	N/A	N/A
'Frensham'	85.9 ± 0.70	R	R	R
'Queen Elizabeth'	24.4 ± 2.20	S	R	R
'Peace'	4.40 ± 2.20	S	R	R
'MACel'	4.40 ± 2.20	S	R	R
Rosa canina	4.40 ± 2.20	R	S	R
'KORbin'	0	R	R	R
'Allgold'	0	R	R	R
'Agnes'	0	R	R	S

[a] S: recorded as susceptible; R: no infection observed, presumed resistant; N/A: cultivar not observed by Howden and Jacobs (1973).

The inability of axenic cultures of urediospores taken from 'Mme. Grégoire Staechelin' to re-infect leaves of susceptible hosts, led to an investigation on optimising the infection rates on leaves of a susceptible host. It has been known that fungal spores in dense populations, either in pustules or in suspensions, do not germinate at all or do so only at a reduced rate as a result of self-inhibition by substances present in, or produced by, the spores themselves (Wolf 1982). In the present investigation, lower infection rates at spore densities greater than 30×10^4 per ml suggested a density related self-inhibition of spores of *P. tuberculatum*. Lower infection rates at densities below 30×10^4 per ml could have been due to the limited number of spores that successfully find a stoma to enter through.

Comparing two methods of preparing the leaves prior to inoculation showed that the infection rate was significantly lower in surface sterilised leaves than the leaves that were not surface sterilised. This could have been the result of the effect of ethanol and bleach residuals on the leaf surface, thus, further tests are needed to investigate other methods of surface sterilisation.

There was no significant difference between spore suspensions and dry spores as sources of inocula. Spore suspensions have the advantage that spore densities can be quantified as a basis for a reproducible procedure. On the other hand, the dry spore method is easier to perform and could be used as a simple method for routine multiplication of spores in cultures. Furthermore, the dry spore method is more efficient in terms of recovering high spore numbers.

It is not always convenient or practical to maintain inocula on living host plants for long periods. Furthermore, there are hazards such as the possibility of genetic changes in culture, contamination with other pathogens, or even accidental loss of the culture (Clifford 1973). However, if storage is necessary, stored inocula must remain viable and maintain their pathogenicity. Urediospores stored at 4°C for four weeks were viable and were able to infect the leaves of a susceptible host, but lost their viability and pathogenicity considerably after 12 weeks. Clifford (1973) also reported a decline in germination rates of urediospores of *Puccinia hordei* after long-term storage at 2°C.

The varieties of roses screened for *P. tuberculatum* showed different responses to the findings recorded by Howden and Jacobs (1973), who stated that genotypes of *R. canina* are not infected by *P. tuberculatum*. Whereas, Ritz *et al*. (2005) showed that two *Phragmidium* species, *P. mucronatum* and *P. tuberculatum* were able to infect the three dog rose (section *Caninae*) species examined (*R. canina*, *R. corymbifera* Borkh. and *R. rubiginosa* L.), indicating overlapping host range. They concluded that the frequent hybridization of dog roses in nature might have prevented host-induced speciation in the parasites and is therefore the cause of the wide host range of the two observed rose rust taxa. Rust fungi may be host-specific, and each species and isolate infects only certain types of hosts, therefore to screen a selection of roses for rust resistance, a collection of different isolates for each particular *Phragmidium* species is needed.

Although in this investigation the work was carried out on multi-spore cultures and the leaves of the susceptible host were infected successfully, obtaining single spore isolates of *Phragmidium* would be very desirable. Single spore isolates could be obtained by culturing the spores in axenic cultures and producing colonies from single spores, or by inoculating the leaves with spores taken from a single uredosorus. Different species of *Phragmidium* are distinguished according to their hosts, characteristics of their teliospores and the size of urediospores. However, characterization of different isolates of species requires further investigation. In particular, further attention to the fine morphological structures of the spores might lead to the identification of additional taxonomic discriminants and more systematic sampling could lead to more precise definition of the ranges of morphological variation.

REFERENCES

Bhatti RMH (1984) Axenic culture of rose rust fungus, *Phragmidium tuberculatum*. *Canadian Journal of Botany* **62**, 1098-1099

Bhatti RMH, Shattock RC (1980) Axenic culture of *Phragmidium mucronatum*. *Transactions of the British Mycological Society* **74**, 595-600

Clifford BC (1973) Preservation of *Puccinia hordei* urediospores by lyophilization or refrigeration and their subsequent germination and infectivity. *Cereal Rusts Bulletin* **1**, 30-34

Cummins GB, Hiratsuka Y (2003) *Illustrated Genera of Rust Fungi* (3rd Edn), American Phytopathological Society Press, St Paul, MN, 240 pp

Foudin AS, Wynn WK (1972) Growth of *Puccinia graminis* f. sp. *tritici* on a defined medium. *Phytopathology* **62**, 1032-1040

Gäumann E (1959) *Die Rostpilze Mitteleuropas mit besonderer Berücksichtigung der Schweiz*. *Kommissionsverlag Buchdruckerei Büchler & Co.*, Bern, Switzerland, 1407 pp

Howden JCW, Jacobs L (1973) Report on the rust work at Bath. *The Rose Annual*, 113-119

Kamoun S (2006) A catalogue of the effector secretome of plant pathogenic oomycetes. *Annual Review of Phytopathology* **44**, 41-60

Kilpatrick RA, Harmon DL, Loegering WQ, Clark WA (1971) Viability of urediospores of *Puccinia graminis* f. sp. *tritici* in liquid nitrogen. *Plant Disease Reports* **55**, 871-873

Maclean RD (1982) Axenic culture and metabolism. In: Scott KJ, Chakravorty AK (Eds) *The Rust Fungi*, Academic Press, London, New York, pp 37-12

Raymundo SA, Young HC (1974) Improved methods for the axenic culture of *Puccinia recondita* f. sp. tritici. *Phytopathology* **64**, 262-263

Ritz CM, Maier WFA, Oberwinkler F, Wissemann V (2005) Different evolutionary histories of two *Phragmidium* species infecting the same dog rose hosts. *Mycological Research* **109 (5)**, 603-609

Sharp EL, Smith FG (1952) Preservation of *Puccinia* urediospores by lyphilization. *Phytopathology* **42**, 263-264

Ueda Y, Hirata M (1989) Pollen fertility in roses. *Japanese Journal of Palynology* **35**, 1-7

Wilson M, Henderson DM (1966) *British Rust Fungi*, The University Press, Cambridge, 384 pp

Wolf G (1982) Physiology and biochemistry of spore gemination. In: Scott KJ, Chakravorty AK (Eds) *The Rust Fungi*, Academic Press, London, New York, pp 151-178