Variant Forms of Saprophytic Mycelium Grown from Uredospores of *Puccinia graminis* f. sp. *tritici*

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*(Accepted for publication 7 September 1970)*

**SUMMARY**

Saprophytic cultures obtained from uredospores of *Puccinia graminis* f. sp. *tritici* race 126-ANZ-6,7 varied. Cultures were placed in two general categories: those which formed macroscopic colonies within 2 weeks of inoculation, and those which formed colonies 4 or more weeks after inoculation. The fast-appearing colonies contained binucleate cells and were the more common; growth ceased by staling or formation of spore-bearing stromata. Slow-appearing colonies were formed at an erratic and low incidence; they were uninucleate, showed much less tendency to stale or form stromata than did fast-appearing colonies, and could be propagated by serial subcultures. Variation of uninucleate colonies during maintenance in axenic culture is described.

**INTRODUCTION**

An understanding of the nutrition, metabolism, and physiology of plant rusts has been restricted by the types of mycelium previously available. Vegetative mycelium has been isolated from flax cotyledons infected with *Melampsora lini* (Turel & Ledingham, 1957) and from bean leaves infected with *Uromyces phaseoli* (Dekhuijzen, Singh & Staples, 1967; Dekhuijzen & Staples, 1968). Since such mycelium is dependent on host tissue for continued growth, this approach cannot provide the experimental scope possible with free-living forms of the fungus, particularly in the study of nutrition. Another approach is to use mycelium grown on an artificial medium. Hotson & Cutter (1951) and Cutter (1951, 1952, 1959, 1960a, b) were successful in obtaining axenic cultures of *Gymnosporangium juniperi-virginianae*, *U. ari-triphylli*, and *Puccinia malvacearum* from infected plant callus tissue. Other investigators have not been able to repeat this work or obtain axenic cultures of a rust from other two-membered callus systems (reviewed by Scott & Maclean, 1969). However, Cutter's isolates could be maintained by serial subculture, and results are available on studies of the nutrition (Cutter, 1951; and unpublished experiments cited by Scott & Maclean, 1969) and metabolism (Wolf, 1956) of one axenic isolate of *G. juniperi-virginianae*.

More recently, a number of rusts have been cultured from uredospores on artificial media: *Puccinia graminis tritici* (Williams, Scott & Kuhl, 1966; Williams, Scott, Kuhl & Maclean, 1967), *P. recondita* (Singleton & Young, 1968), *P. graminis avenae* (J. L. Kuhl, D. J. Maclean, K. J. Scott & P. G. Williams, unpublished observations, *Melampsora lini* (Turel, 1969).

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Work in this laboratory to devise methods for the routine production of vegetative mycelium of *Puccinia graminis tritici* race 126-ANZ-6,7 has been hampered by the inconsistent growth pattern of cultures. The formation of saprophytic mycelium from uredospores is unpredictable (J. L. Kuhl, D. J. Maclean, K. J. Scott & P. G. Williams, unpublished observations), and even when such mycelium is formed its subsequent development is variable. Variant forms of saprophytic mycelium have been described in previous work. Williams *et al.* (1967) found that mycelium formed a dense stroma which bore uredospores and teliospores in a manner similar to uredial infections of wheat plants. In repeating this work, Bushnell (1968) obtained a dense stroma with an outer sporophore-like layer, but very few spores formed. Coffey, Bose & Shaw (1969) obtained mycelium which was less dense than the stroma obtained by the other workers, and clusters of non-pigmented spores were found within it.

In the present communication we distinguish the isolation and morphology of two kinds of mycelium, the growth characteristics of which are described.

**METHODS**

*Media.* The media used were modified from that of Williams *et al.* (1967). Medium I: 0.1 % yeast extract (Difco-Bacto, Detroit, U.S.A.), 0.2 % peptone (Evans Medical, Liverpool), 2 % (w/v) glucose, Czapek’s mineral solution (Ainsworth & Bisby, 1945) to 100 %. Medium II: medium I plus 0.3 % sodium citrate (Na₃C₆H₁₀O₇·5H₂O). Medium III: medium I with yeast extract and peptone each at 0.15 %. Medium IV: medium II but with glucose at 4 % (w/v) and peptone at 1.0 %. The pH was adjusted to 6.4. When solid media were required, 1.5 % agar (Difco-Bacto) was added before autoclaving. All media were sterilized by autoclaving for 10 min. at 121 °C.

Unless otherwise stated, plastic Petri dishes (8.5 cm. diameter) contained 30 ml. of solidified medium. Slants comprised 8 ml. of medium in 150 mm. × 17 mm. tubes plugged with cotton wool. Erlenmeyer flasks (150 ml.) containing 25 to 35 ml. of medium were used in liquid culture experiments.

*Preparation and inoculation of uredospores.*  Uredospores of *Puccinia graminis* (Pers.) f. sp. *tritici* (Eriks. and E. Henn.) race 126-ANZ-6,7 were obtained by methods of aseptic leaf culture previously described (Williams, *et al.* 1966). The leaves were dried under reduced pressure and uredospores removed by vibration. Each liquid culture was inoculated with approximately 10⁶ uredospores (from 2 to 3 leaf cultures) and the spores were dispersed by gentle agitation. Agar surfaces were inoculated with uredospores with the aid of a camel-hair brush.

*Subculture.* Pieces of mycelium were transferred to new medium in slants at 1 to 2 month intervals. Alternatively, uniform pieces of mycelium from the margins of colonies were transferred to Cellophane on agar in plastic Petri dishes. Pl. 1, fig. 2 shows such a culture 15 days after subculture. Thin commercially available Cellophane was boiled in distilled water for 10 min. and rinsed; this process was repeated three times. The Cellophane was immersed in distilled water and sterilized by autoclaving for 10 min. at 121 °C.

Dry weight was used as a parameter of growth, samples being dried for 15 h. at 90 °C. Results are expressed as mean dry wt/colony.

*Conditions of growth.* Cultures were incubated in the dark at high humidity. Uredospore-inoculated media were maintained at 17 °C. Initially subcultures were maintained at 17 °C, but later studies of growth were carried out at 23 °C.
RESULTS

Saprophytic mycelium derived from uredospores either staled, entered a reproductive phase, or formed a persistently vegetative mycelium. Staled cultures had a dull brown necrotic appearance and excreted brown pigments into the medium. This contrasted with reproductive cultures where the fungal thallus formed a dense brown–black stroma which usually bore uredospores or teliospores or both. Persistently vegetative cultures could be propagated by serial subculture, and had little tendency to stale or form stromata.

Development of staled or reproductive cultures on agar media

The formation of staled or reproductive cultures from a uredospore mass-inoculum seemed to follow a common pattern of early development. Sporelings formed macroscopic colonies within 2 weeks of inoculation, and growth centres expanded to form a combined mycelium within 3 weeks of inoculation. At this stage, cultures ranged from a spongy prosenchyma with profuse aerial hyphae to a dense interlocked mycelium; the former eventually staled (Pl. 1, fig. 5; Pl. 2, fig. 8), while the latter usually formed stromata (Pl. 1, fig. 4; Pl. 2, fig. 9 to 11). Uredospores or teliospores or both were formed in stromata in localized regions either on the outer surface (Pl. 2, fig. 11; cf. Williams, et al. 1967) or in clusters within the stromata (Pl. 2, fig. 9, 10). These spores showed the same pigmentation as found in uredial infections of wheat plants. The close arrangement of hyphae in the centre of a typical stroma is shown in Pl. 2, fig. 14; large thin-walled vesicles were often present. Sometimes spores formed in localized regions of a predominantly staled mycelium (e.g. Pl. 2, fig. 8).

Table 1. Morphology of cultures derived from uredospores on agar medium II

A sterile filter-paper template with holes was placed on the agar surface and uredospores spread over the exposed agar at a density of 500 to 1000 spores/mm². Each treatment was duplicated. The experiment was repeated with a different batch of uredospores. Results were consistent between replicates.

<table>
<thead>
<tr>
<th>Volume of agar in Petri dish</th>
<th>Diameter of uredospore mass-inoculum (mm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>7 ml.</td>
<td>±</td>
</tr>
<tr>
<td>12 ml.</td>
<td>+</td>
</tr>
<tr>
<td>20 ml.</td>
<td>+</td>
</tr>
</tbody>
</table>

- = Loose, spongy, and mostly staled tissue; + = dense stroma with spore formation; ± = texture of mycelium intermediate between + and -.

As shown in Table 1 and in Pl. 1, fig. 1 stroma formation was favoured by a greater volume of agar and by confining the uredospores inoculum to a small area. This indicates that mycelial development was modified by a fungus–medium interaction in which nutrient depletion or accumulation of fungal excretory products or both would be important factors. However, small colonies staled on rare occasions, even when grown on a relatively deep layer of agar.

Both staling and reproductive colonies stopped growing within 4 to 6 weeks of inoculation. Subcultures ceased growth after one or two transfers.
Previous work has shown that rust mycelium which develops in a way similar to that described above contains binucleate cells at early (Williams et al. 1966) and later (Coffey et al. 1969) stages of growth.

Occasionally, colonies which appeared to be persistently vegetative formed on agar medium when the uredospore inoculum produced uneven and erratic growth. No subcultures were made from these colonies. This type of colony was more closely studied during a series of liquid culture experiments.

**Growth from uredospores on liquid medium**

All the types of growth mentioned above have also been observed in liquid cultures. In a typical experiment, uredospores were floated on liquid medium (Table 2), and after 17 days incubation 7 of the original 15 flasks remained uncontaminated. Only three of these flasks produced significant amounts of saprophytic growth (Table 2). An attempt to subculture the mycelium from one flask by fragmentation was unsuccessful. During incubation of the remaining flasks for a further six days, the colonies staled or formed stromata.

**Table 2. Growth of rust mycelium from uredospores on liquid medium**

<table>
<thead>
<tr>
<th>Condition of culture</th>
<th>Number of flasks exhibiting</th>
<th>After 17 days</th>
<th>After 23 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contaminated</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Germination only</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mycelium over part of liquid surface</td>
<td>1</td>
<td>1 (stroma)†</td>
<td></td>
</tr>
<tr>
<td>Mycelium over entire liquid surface</td>
<td>2*</td>
<td>1 (staled)†</td>
<td></td>
</tr>
</tbody>
</table>

* Mycelium in one flask was harvested.
† The combined fresh weight of mycelium from these two flasks was 1.2 g.

Extended incubation of liquid cultures occasionally led to the appearance of persistently vegetative colonies, particularly when uredospores had germinated but produced little or no saprophytic growth within the first 3 weeks. Such colonies were first isolated in 1967 as a result of two experiments designed to produce large quantities of mycelium. At the end of 3 weeks incubation growth was erratic in all flasks. Re-examined 2 to 3 months after inoculation, a total of 13 small white mycelial tufts 1 to 2 mm. in diameter were visible in three flasks on the liquid surface amidst the necrotic remains of sporelings and primary mycelium. These tufts were transferred to agar slants where they grew to a diameter of 3 to 8 mm. 68 days after transfer. None had staled or formed stromata. One was propagated by serial subculture and has now been maintained for 3 years; this isolate is termed v 1 (i.e. vegetative isolate 1).

The occurrence of persistently vegetative colonies was unexpected and we have experienced difficulty in re-observing this phenomenon. However, an experiment in June 1968 resulted again in the formation of persistently vegetative colonies (Table 3). As shown in this table, the formation of these colonies was favoured in flasks where least growth took place during the first 3 weeks of incubation. Pl. 1, fig. 6 shows flask...
Variation in axenic rust cultures no. 8 (Table 3) 60 days after inoculation; the discrete white tufts could be clearly distinguished from the necrotic remains of sporelings. Incubation for a further 20 days resulted in the appearance of more tufts, and some flasks then contained up to 30 colonies.

The persistently vegetative colonies described in Table 3 were similar to those described in the previous experiment as they could be propagated by serial subculture and showed no tendency to stale or form stromata. Three isolates from this experiment are still being maintained and are designated v2, v3 and v4.

Table 3. Incidence of persistently vegetative rust isolates from uredospores on liquid medium

Each flask (containing 25 ml. liquid medium II) was inoculated with approximately $10^6$ uredospores and incubated for 59 days. The degree of staling was estimated visually by the intensity of brown pigments in the medium. This was roughly correlated with the amount of saprophytic mycelium formed within 2 to 3 weeks of inoculation. Flasks which had produced no visible saprophytic mycelium in the first three weeks gave a colour rate $+\rightarrow +$ on the staling scale.

<table>
<thead>
<tr>
<th>Flask no.</th>
<th>Degree of staling</th>
<th>No. of macroscopically visible mycelial tufts after 59 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$++++++$</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>$+++++$</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$+++++$</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>$+++$</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>$+++$</td>
<td>23</td>
</tr>
<tr>
<td>6</td>
<td>$+++$</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>$++$</td>
<td>14</td>
</tr>
<tr>
<td>8</td>
<td>$++$</td>
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<td>15</td>
<td>$+$</td>
<td>20</td>
</tr>
<tr>
<td>16</td>
<td>$+$</td>
<td>10</td>
</tr>
<tr>
<td>17</td>
<td>$+$</td>
<td>13</td>
</tr>
</tbody>
</table>

Growth characteristics of persistently vegetative colonies

Subcultures form compact white colonies when grown in the dark (Pl. 1, fig. 2). Colonies become orange–yellow on exposure to the light, the pigment being localized in lipid globules in the hyphae (Pl. 2, fig. 13). All isolates are now maintained by placing pieces of mycelium on a Cellophane layer over an agar medium. Hydrolysis of Cellophane by the fungus has never been observed.

Attempts were made to grow subcultures of v1, 15 months after its isolation, in submerged liquid culture (medium I, medium II). These cultures were inoculated with surface-grown colonies, either as large pieces or as a suspension of fragments. This resulted in a few viable cultures, growth being diffuse and extremely slow. After optimal conditions for surface growth had been determined ($25^\circ$, 4% w/v glucose, 1% peptone—cf. medium IV; D. J. Maclean & K. J. Scott, unpublished observations),
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and provided that autoclaving time was restricted to a maximum of 10 min. at 121° (N. K. Howes & K. J. Scott, unpublished observations), submerged growth was greatly improved.

Nuclear content

An examination of VI 2 years after its isolation, and V2, V3 and V4 1½ years after their isolation, showed that constituent cells were uninucleate (Pl. 1, fig. 3). The nuclei appeared to be in the expanded condition described by Savile (1939). The development of persistently vegetative colonies when first isolated differed markedly from that of mycelium which staled or formed stromata. This suggested that the persistently vegetative colonies were uninucleate even when first isolated, but no study was made of the nuclear condition of these isolates at that time.

Sectoring

Sectoring was not often observed. However, when VI was serially subcultured for a few generations on a medium of high peptone concentration (medium IV) for the first time about 2 years after its isolation, some colonies grew faster than others. Slow-growing (VI A) and fast-growing (VI B) colonies were selectively subcultured for 6 months. Then, after 43 days growth on medium IV, colonies of VI A had a mean dry weight one-third that of VI B (11 mg. as compared with 32 mg.). On medium II, which had a lower peptone concentration, the difference in growth rates was less but still significant.

For more than a year after isolation subcultures of V2 formed small heaped colonies, the mycelium being relatively dense and fine in texture (V2 A). Later subcultures of this isolate occasionally formed faster-growing colonies (V2 B) which were more diffuse and showed no tendency to revert to the slow-growing type. Sectors of V2 B were observed to arise from the edge of colonies of V2 A in contrast to the slow separation of sectors in VI.

Isolates V3 and V4 were distinguished from the other isolates. Colonies of V3 grown on medium II became water-logged more easily. Isolate V4 showed some resemblance to VI A and VI B in general appearance, but larger pieces of mycelium were required for its successful subculture. We have not yet observed sectors in cultures of V3 and V4.

The age and size of the mycelium piece used in subculturing was important. Subcultures from aged colonies often failed to grow, whereas pieces of young colonies (2 to 4 weeks after subculture) usually did. A dry weight of 0·15 mg. or more was required for the successful subculture of V2, V3 and V4. However, growth has been obtained with VI B from an inoculum of as little as 0·02 to 0·06 mg. dry weight.

Sporulation of uninucleate isolates

On rare occasions colonies of VI growing on Cellophane on an agar medium bore uredospores and teliospores dispersed throughout the thallus (Pl. 2, fig. 12). Neither this nor any other uninucleate isolate formed a dense stroma. Although the uredospores were similar to those produced under natural conditions, some teliospores were abnormal in shape (Pl. 2, fig. 12). The nuclear condition of these spores was not studied. Uninucleate mycelium can interact with wheat leaves to form uredospores and teliospores which are uninucleate (D. J. Maclean & K. J. Scott, unpublished observations).
DISCUSSION

The variant forms of saprophytic mycelium that we have observed fall into two general categories based on differences in morphogenesis and nuclear content. Both categories were obtained from wild-type uredospores of *Puccinia graminis*. These spores are characteristically binucleate; however, uninucleate uredospores have been observed in predominantly binucleate uredosori of rust fungi (Little & Manners, 1969). Mycelia in the first category formed macroscopic colonies within 2 weeks of inoculation which contained binucleate cells and terminated growth either by staling or formation of stromata. We were not able to obtain viable subcultures. Mycelia in the second category (persistently vegetative) did not form macroscopic colonies until 4 or more weeks after inoculation. These colonies did not tend to stale or form reproductive tissues, and serial subcultures were made. Although their nuclear condition was not determined immediately that they were isolated, subsequent subcultures were found to be uninucleate.

Cutter (1959, 1960a; and unpublished observations cited by Scott & Maclean, 1969) examined the nuclear condition of axenic isolates of *Gymnosporangium juniperi-virginianae* and *Uromyces ari-triphylli*. Telial galls, the material from which the cultures of *G. juniperi-virginianae* were obtained, characteristically contained a binucleate mycelium. Four of seven axenic strains were predominantly binucleate within 30 days of isolation, the others being completely uninucleate. After serial subculture, two of the previously binucleate strains became uninucleate. However, all five strains of *U. ari-triphylli* contained binucleate cells and were remarkably stable in culture. Our results show that *Puccinia graminis* resembles *G. juniperi-virginianae* in tending to lose the binucleate condition.

Bushnell (1968) and Coffey *et al.* (1969) obtained axenic cultures of the same race of rust used in the present study. The 60 day cultures of Coffey *et al.* were seen to contain binucleate cells and resembled the staled mycelium of our first category. The cultures described by Bushnell seem to be intermediate between our two categories both in morphology and in time taken for the mycelium to develop from sporelings. Subcultures of Bushnell’s mycelium were viable, and on ageing they formed stromata. We have not observed stroma formation during ageing of our uninucleate cultures. Bushnell’s results are difficult to interpret in view of our inability to subculture successfully mycelium of our first category.

It is informative to examine the conditions under which uninucleate mycelium was formed. This mycelium was derived from ‘persistently vegetative’ colonies. These colonies were formed when sporelings produced little or no primary mycelium within 3 weeks of inoculation. Under these conditions variant cells would have been relatively free of competition. A maximum of 30 small discrete colonies were isolated from any individual inoculum of about $10^6$ uredospores.

The mechanisms whereby uredospores gave rise to the various forms of saprophytic mycelium, particularly the uninucleate cultures, are obscure. Uninucleate hyphae could have arisen in ways such as: (1) formation of a diploid; (2) septation of a dikaryon followed by growth of one of the cells; (3) presence of uninucleate uredospores in the inoculum; (4) recombination followed by haploidization and septation. Preliminary results of an investigation by the authors with I. Tommerup indicate that nuclei in our uninucleate strains are haploid.
Variation has also been observed during the maintenance of uninucleate isolates by subculture. Two isolates (v1, v2) each produced faster-growing strains. If v1 and v2 contained a mixture of ‘fast’ and ‘slow’ cells when first isolated, one would expect the fast-growing cells soon to predominate. Since the new strains were observed more than a year after isolation, we conclude that new cell types arose during maintenance. Both the old ‘slow’ and the new ‘fast’ strains were uninucleate. Possible causes of this variation include mutation, segregation of cytoplasmic factors and parasexual mechanisms.

Mechanisms of somatic variation which occur in rust fungi maintained by uredial infection have not yet been satisfactorily elucidated (e.g. Watson & Luig, 1962; Bartos, Fleischmann, Samborski & Shipton, 1969; Little & Manners, 1969). Haploid isolates in axenic culture may be of value in clarifying them.

We thank Professor I. A. Watson, The University of Sydney, for generous gifts of wheat seed and the rust pathogen; Mr C. C. Ryan for criticisms of the manuscript; and Dr I. Tommerup for advice on cytological techniques. This work was supported in part by grants from the Rural Credits Development Fund of Australia, The Wheat Industry Research Council, and The Australian Research Grants Committee.

REFERENCES


Variation in axenic rust cultures


EXPLANATION OF PLATES

PLATE 1

Fig. 1. Petri dish (20 ml. medium, cf. Table 1) 44 days after inoculation with uredospores. Diameter (mm.) of inoculum is shown in parentheses. Some colonies have excreted brown substance(s) into the medium. ×0.85.

Fig. 2. Subcultures of isolate v1 growing on Cellophane disc on 30 ml. agar medium II. ×0.85.

Fig. 3. Uninucleate cell of isolate v1. Stained by Giemsa technique after 8 min. hydrolysis at 60° in N-HCl. ×750.

Fig. 4. Stroma formation (5 mm., fig. 1) showing fungus curling up from the agar. ×3.3.

Fig. 5. Staling mycelium (13 mm., fig. 1). ×3.3.

Fig. 6. Flask containing persistently vegetative colonies 60 days after inoculation with uredospores. The discrete white colonies can be distinguished from masses of necrotic sporelings. ×0.75.

Fig. 7. Uredospore and teliospore formation in a subculture of v1. ×400.

PLATE 2

Fig. 8. Staled culture: section through 13 mm. colony, fig. 1. Note aerial hyphae (*a*), loosely arranged hyphae in the agar (*b*). A cluster of small uredospores was formed (*c*), but such regions are not general in staled cultures. ×50.

Fig. 9. Stroma: section through 5-5 mm. colony, fig. 1. Uredospores and some teliospores were formed in clusters (*d*). This colony has curled up, enclosing aerial hyphae (*e*). ×50.

Fig. 10. One of the spore clusters in fig. 9. ×200.

Fig. 11. Uredospore formation (*f*) on outer surface of sporophore layer (*g*). Original inoculum at (*h*). ×200.

Fig. 12. Spores (*i*) formed in a subculture of isolate v1. Frozen section, unstained. ×33.

Fig. 13. Monopodial branching of v1 showing lipid globules in hyphae. Frozen section, unstained. ×730.

Fig. 14. Tightly interwoven hyphae in centre of a stroma. Hyphae are often swollen, sometimes forming large vesicles (*f*). ×200.

Figures 8, 9, 10, 11 and 14 are from 10 μm. wax sections, hydrated, and stained with aqueous crystal violet.