Axenic cultures of *Uromyces dianthi* (Pers.) Niessl were grown from urediospores, by using techniques and media similar to those of Williams, Scott & Kuhl (1966) and Williams, Scott, Kuhl & Maclean (1967). Extended periods of incubation were necessary for the development of colonies. Media containing yeast extract, peptone and casein hydrolysate, singly or in combination, initiated and supported cultures of *U. dianthi*. The hyphal segments were typically binucleate. Growth was optimal at 18–20 °C, on media of pH 5.8–6.2. Spore-like cells were found in the mycelium of older colonies. These spore-like cells were capable of germination and of initiating new saprophytic growths.

In recent years three different rust fungi have been grown from uncontaminated urediospores in axenic culture. Williams, Scott & Kuhl (1966) and Williams et al. (1967) found that an agar medium consisting of Czapek Dox salts, sucrose, Difco Yeast Extract, and Evans's Peptone not only initiated and supported the saprophytic growth of race ANZ-126-6 of *Puccinia graminis* f.sp. *tritici* from germinating urediospores, but also enabled the fungal colony to sporulate. Other workers have successfully cultured *Puccinia recondita* f.sp. *tritici* (Singleton & Young, 1968) and *Melampsori lini* (Turel, 1969; Coffey, Bose & Shaw, 1969) on similar media. Bushnell & Rajendren (1970) found media supplemented with casein hydrolysate useful when culturing *P. graminis* f.sp. *tritici*. The results suggested that the problem of culturing obligate fungal plant parasites was primarily one of nutrition. The carnation rust fungus, *Uromyces dianthi* (Pers.) Niessl., was selected for axenic culture experiments which are reported here.

**MATERIALS AND METHODS**

Carnation plants (*Dianthus caryophyllus* var. Bookham Bounty) infected with *U. dianthi* were obtained from a grower at Wolverhampton. Stocks of the rust were maintained on young plants of *D. caryophyllus* var. Grenadin Scarlet in a heated glasshouse.

By using techniques similar to those of Williams et al. (1966) large numbers of uncontaminated urediospores were obtained. The upper surface of young uninfected detached carnation leaves (var. Grenadin Scarlet) was sprayed with atomized water and dusted with urediospores. With their adaxial surfaces uppermost the leaves were then floated on water in Petri dishes sealed with adhesive tape. After incubation for 48 h at 20 °C the Petri dishes were moved to a growth cabinet (12 h day,
Three or four weeks after inoculation the characteristic early flecking symptoms became visible. The infected leaves were then surface-sterilized by 10 min immersion in a solution of 5% sodium hypochlorite plus a few drops of diluted Tween 80. This sterilization procedure was found to kill most of the phylloplane bacteria, the main source of contamination, and yet enable the sori to develop and produce urediospores. After washing for 10 min in three changes of sterile distilled water, the leaves were placed on the surface of nutrient agar in Petri dishes. The Petri dishes were then stacked in a desiccator and returned to the growth cabinet. The aseptic urediospores became exposed as the sori ruptured the epidermis, and were placed directly on the surface of the various agar media with sterile inoculating needles.

In later initiation experiments, urediospores were taken directly from growing plants. The spores were removed with a sterile scalpel blade from young opened sori and quickly transferred to the agar medium. In one experiment 20% of the total transfers were contaminated. However, the percentage germination of these urediospores was noticed to be higher, subsequent germ-tube growth more profuse, and saprophytic initiation more common, when compared with the behaviour of spores from leaves subjected to the surface-sterilization procedure.

The cultures were grown in plastic Petri dishes sealed with adhesive tape to reduce evaporation. All the agar media were autoclaved at 120°C for 15 min and their pH adjusted with 0.01 N-HCl. Saprophytic hyphae were stained with cotton blue in lactophenol, and drawn with the aid of a camera lucida.

RESULTS

Axenic cultures

The first successful U. dianthi culture was established on a medium consisting of 2 g Oxoid Mycological Peptone, 2 g Difco Yeast Extract, 36 g Difco Czapek Dox Broth and 15 g Difco Bacto-Agar per litre of distilled water. The pH of the medium after autoclaving was 6.1. The urediospores were incubated in the dark at 16°C and germinated normally. There followed a seemingly inactive period for over 8 weeks. During this time the cytoplasm in a number of germ-tubes remained visible, and some germ-tubes were seen to be slowly growing. Since an axenic culture seemed unlikely to form, observations ceased after 2 months. Twenty-three weeks after inoculation the urediospores were re-examined and found to be surrounded by a fungal colony. The colony was unlike most fungal growths, being a pale yellow, dome-shaped mycelial mass, 5 mm in diameter. The surface of the colony was irregular, with a number of comparatively large spine-like projections. At its margin branched, hyaline, septate hyphae were growing out over the agar surface (Text-fig. 1A; Pl. 4, fig. 1). Protruberances resembling clamp connexions were seen between some hyphal cells. The colonizing saprophytic hyphae closely resembled the intercellular parasitic hyphae found in freeze-microtome sections of infected carnation leaves. The mass of the colony consisted of densely interwoven and highly branched hyphae (Text-fig. 1B). Small
knob-like branches which resembled haustoria were seen. Fixing in acetic acid-ethanol and staining with Giemsa revealed that the hyphal cells possessed two nuclei. In later experiments the development of similar colonies in axenic culture was followed microscopically from urediospore germination to the formation of colonial saprophytic hyphae. These saprophytic hyphae were morphologically identical with those of the original growth, thus establishing it as a *U. dianthi* colony.
Fragments of the original colony 0.5–1.0 mm diam were successfully subcultured on two other peptone–yeast extract media, A (pH 6.10) and B (Table 1). These subcultures were incubated at 18 °C in the dark. Usually they required 1–2 weeks to establish themselves and to begin active growth. Subcultures from colonies growing on medium A were later established on media C, D and E (Table 1); C and D contained casein hydrolysates. Colonies on media C, D (Pl. 4, fig. 2) and E media appeared to grow as well as those on media A and B. Subcultures from colonies on medium E have also been established on media F, G and H (Table 1). These media contained yeast extract, peptone or casein hydrolysate as single supplements. Oxoid Czapek-Dox Agar (pH 5.9 and pH 6.4) alone did not support the growth of *U. dianthi*.

Soon after subculturing, the young colonies appeared white in colour. As the cultures aged and grew larger, the colour changed to yellow and then to pale orange. On medium A at 18 °C this process took about 5 weeks. Two to three months after subculturing the colonies stopped growing over the agar. The diameter of the orange dome-shaped cultures at this stage was usually 8.0–14.0 mm. Optimum growth of subcultures was obtained at 18–20 °C on agar media of pH 5.8–6.2. The pH limits for growth of subcultures were approximately 5.4 and 6.8.

*U. dianthi* was also successfully subcultured in 25 ml of a liquid medium (medium C minus agar) at pH 6.1. The fungus was incubated at 18 °C in 100 ml flasks plugged with cotton wool. Although submerged, the subcultures established themselves and formed small spherical colonies.

In further experiments, vegetative growths of *U. dianthi* were initiated from spores on medium A between pH 5.4 and 6.3. At 18 °C development of saprophytic hyphae began 6–8 weeks after germination. At 16 and 20 °C this process took 7–13 weeks. No axenic cultures developed at 22 °C.

In another series of experiments, colonies were initiated from spores at 18 °C on media I, J, K, L and M (Table 1).

Development of saprophytic hyphae took 5–6 weeks, except on medium L, where 11–13 weeks were needed.

### Table 1. Culture media (values in g/l of distilled water)

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<th>Medium</th>
<th>Basal medium</th>
<th>Peptone</th>
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<tr>
<td>Code</td>
<td>Difco Czapek</td>
<td>Difco Czapek</td>
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<td>letter</td>
<td>Dox broth</td>
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In these experiments approximately 65 % of all uncontaminated urediospore inoculations initiated axenic growths.

*Initiation of saprophytic growth*

The first urediospores germinated 1–2 h after inoculation on the agar medium. Germination was usually complete within 24 h, but this depended on the number of spores present; at high concentrations there was an inhibition of germination. After 48 h normal germ-tube growth had ceased. Some germ-tubes burst at their apex, discharging their contents on the agar. Fusions between germ-tubes occurred in areas of high spore density. Aggregations of germ-tube protoplasm were also formed not far above the agar surface (Pl. 4, fig. 3). These aggregations closely resembled the urediospore germ-tube 'fusion bodies' described by Rodenheiser & Hurd-Karrer (1947). They seemed to bind crossing hyphae together, and to withdraw cytoplasm from the hyphae and pull them taut. In some instances appressoria were formed, from which structures resembling substomatal vesicles and infection hyphae developed (Pl. 4, fig. 4). The cytoplasm in the majority of germ-tubes and infection hyphae became disorganized and they ceased to grow. A number of germ-tubes, however, continued to grow slowly over the agar surface. After a few days, aerial hyphae usually began to develop from these tubes. These hyphae were about 3 µm in diameter and initially possessed very few septa. Urediospores seeded on medium L at 18 °C gave rise to unusually profuse growth of aerial hyphae, some up to 1·50 mm in length (Pl. 5, fig. 5). Aggregations of closely associated branched, anastomosing aerial hyphae developed in places. A number of aerial hyphae grew downwards to the agar surface.

After a seemingly inert period or ‘lag phase’ of 4–13 weeks (depending on temperature, medium and pH), hyphae which colonized the agar surface and formed the mass of the colony were produced in certain areas of high spore concentrations. These colonial hyphae were 4–7 µm in diam, highly branched, regularly septate and often bore knob-like projections; they resembled those found in the original colony and in subsequent subcultures. Microscopic examinations during the ‘lag phase’ revealed aerial and agar-surface hyphae of slightly differing morphology. This suggested that the initial hyphae slowly differentiated to the colonial hyphae through various transitional types. Colonial hyphal cells were in fact observed developing from morphologically intermediate hyphae, which were rather more highly septate and branched than the initial hyphae.

Once initiated, successful saprophytic colonies spread relatively rapidly. A subculture developing on medium A (pH 6·3) at 20 °C attained a diameter of 3 mm after 3½ weeks incubation. Growth of colonies usually stopped after 2–3 months.

Williams et al. (1967) reported the formation of urediospores and teliospores on *Puccinia graminis* f.sp. *tritici* cultures growing on media containing 0·1 % Evans’ Peptone. *U. dianthi* cultures did not form urediospores or teliospores on any of the media used. Angularly globose hyphal cells, usually 16–25 µm in diam with colourless walls 0·5–2 µm thick, were,
however, fairly common in the mycelium of older colonies (Text-fig. 1 C). They were also formed at the margins of colonies transferred to unfavourable media. These cells resembled aeciospores. When separated from old colonies and placed on new suitable media, they were observed to germinate and initiate new growths of colonial saprophytic hyphae (Pl. 5, figs. 6, 7). Under the phase-contrast microscope most of these spore-like cells were seen to possess two nuclei. Small pieces of hyphae (minimum one cell) also seemed capable of initiating colonies (Pl. 5, fig. 8).

In some cultures the spore-like cells and hyphal fragments were allowed to initiate and develop small growths in the presence of large colonies. Observations showed that the small colonies stopped growing out over the agar at the same time as the large older colonies. When transferred to fresh media the small colonies began growing again. This suggests that growth in the small colonies was inhibited by certain toxic metabolites diffusing through the agar from the large colonies.

Attempts to infect growing and detached susceptible aseptic carnation leaves with *U. dianthi* cultures have so far proved unsuccessful.

**DISCUSSION**

The saprophytic growth of *U. dianthi* upon media similar to those used by other workers confirms the suitability of yeast extract, peptone or casein hydrolysate, together with sucrose and Czapek Dox minerals, for culturing rusts. The essential nutrients or growth factors provided by the complex organic supplements must be present in yeast extract, in peptone and also in casein hydrolysate.

The extended periods of incubation necessary for the initiation of the colonial hyphae indicate a slow metabolic adaptation of the rust to its new environment. This process, as suggested by Scott & Maclean (1969), may involve a change in the fungal genome from a parasitic to a saprophytic expression. Such a change could possibly result from nuclear interchanges following the fusions of germ-tubes and hyphae. Cytoplasm and possibly nuclei have been observed by Little & Manners (1969) to pass between fused urediospore germ-tubes of *Puccinia striiformis*.

The metabolic adaptation to the artificial environment seems to be associated with a gradual morphological change in the hyphae. This suggests a link between metabolism and morphological form; it is only when metabolizing successfully (fully adapted to their environment) that the hyphae have a stable morphology.

Bushnell (1968) working with *P. graminis* f.sp. *tritici* obtained saprophytic growth only in areas of high inoculum density (100–200 spores/mm²). Similarly, the initiation centres of *U. dianthi* colonies were always observed to lie in areas of very high spore concentrations (100–400 spores/mm²). It has been suggested (Scott & Maclean, 1969) that metabolites leaking from germinating urediospores are essential for saprophytic initiation and that usually only in regions of high spore densities are they found in effective concentrations. Microscopic examinations have shown that more protoplasm is present in germ-tubes closely associated with spore clusters than in germ-tubes from isolated and scattered urediospores.
Scott & Maclean (1969) classified the various types of saprophytic growth of *P. graminis* f.sp. *tritici* into three groups. The growth of *U. dianthi* resembles their second group, in that the rust needed extended periods of incubation before it formed colonies and that vegetative colonies could be maintained indefinitely by subculture. It differs in that at no time were urediospores or teliospores produced. However, spore-like cells resembling aeciospores were formed extensively in the mycelium of older colonies.

Wong & Willetts (1970), working with *P. graminis* f.sp. *tritici*, noticed that cessation of colony growth was closely associated with the formation and build-up of a brown colouring pigment, believed to be a toxic oxidized polyphenol. The cessation of growth of *U. dianthi* could not be associated with such a definite colour change. However, the orange colour of *U. dianthi* colonies slowly deepened after growth had stopped. It is believed that in *U. dianthi* cultures there is a slow build-up of toxic metabolites which inhibit growth when a critical level is reached. This may or may not be associated with the orange pigmentation.

The results of these and previous experiments suggest that most rust fungi will eventually be grown in axenic culture, and that the problem of culturing obligate fungal parasites is essentially nutritional.

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REFERENCES

EXPLANATION OF PLATES 4 AND 5

**PLATE 4. Uromyces dianthi**

Fig. 1. Colonizing saprophytic hyphae growing over the surface of medium A (pH 6.30). × 180.

Fig. 2. Subculture 43 days old growing on medium D at 18 °C.

Fig. 3. Fusion body (f) formed on medium A (pH 5.80) at 16 °C. Photographed 11 days after urediospore inoculation. × 260.

Fig. 4. Structures resembling appressorium (a), sub-stomatal vesicle (b) and developing infection hyphae (c) formed on medium A (pH 5.80) at 16 °C. Photographed 8 days after urediospore inoculation. × 400.

**PLATE 5. Uromyces dianthi**

Fig. 5. Aerial hyphae on medium L at 18 °C. Photographed 45 days after urediospore inoculation.

Figs. 6, 7. Colony initiation from germinating spore-like cells. Photographed after 7 days at 18 °C on medium A (pH 6.05). × 280.

Fig. 8. Colony initiation from a small fragment of saprophytic hyphae. Photographed after 7 days at 18 °C on medium A (pH 6.05). × 280.

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