The growth of *Peronospora farinosa* f. sp. *betae* and sugar beet callus tissues in dual culture

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Summary

Callus cultures established from various sugar beet varieties on complex and chemically defined media were infected with the sugar beet downy mildew fungus *Peronospora farinosa* under sterile conditions. The host callus and the parasite grew in complete balance in culture, and new infected cultures could be established by transferring explants of infected callus to fresh medium. *Peronospora farinosa* in callus culture produced normal intercellular hyphae with digitate haustoria and conidiophores with conidia, and remained pathogenic to sugar beet plants for nearly 2 years. The fungus grew for short distances away from infected calluses on the surface of an agar culture medium, but did not grow axenically when connections with these calluses were severed. Resistance to *P. farinosa* was not expressed normally in sugar beet calluses.

Introduction

The established techniques of plant cell and tissue culture are now being widely exploited for studies on crop improvement through plant breeding and clonal multiplication (Nickell & Torrey, 1969), for studies of crop-plant physiology, as in the work with sugar cane by Nickell and collaborators (e.g. Nickell, 1964, 1967; Heinz & Mee, 1969, 1971), and for studies of crop-plant diseases, particularly those caused by obligate fungal parasites. However, little work has hitherto been done on the pathology and physiology of sugar beet through the techniques of tissue culture. Indeed, Nickell & Torrey, in 1969, noted that little success had then been reported in culturing certain crop plants, especially the sugar beet. Although some obligate downy mildew and related fungi have been grown in host-tissue cultures (Morel, 1944; 1948; Singh, 1963; Izard, Lacharpagne & Schiltz, 1964; Nakamura, 1965; Griffin & Coley-Smith, 1968; Ingram, 1969b; Tiwari & Arya, 1969), dual culture of sugar beet and the sugar beet downy mildew *Peronospora farinosa* has not previously been described.

Methods

The downy mildew fungus, *Peronospora farinosa* (Fr.) Fr. f. sp. *betae*, obtained from Dr G. E. Russell, Plant Breeding Institute, Cambridge, was maintained on detached sugar beet cotyledon pairs (varieties Maris Vanguard or Bush Mono) by a method similar to that described by Ingram (1969b). The cotyledons, together with 1.0 cm. of hypocotyl, were removed from 7- to 9-day old seedlings and were infected with *P. farinosa* by dipping them in a suspension containing about $3 \times 10^6$ conidia/ml. water or by spraying with a similar suspension. The cotyledon pairs were then laid out in rows on moist crinkled grey filter paper in $17 \times 11 \times 6$ cm. clear plastic boxes and were incubated at 15°C in an incubator (Gallenkamp IH-280) lit for 12 h. each day by six 8 W daylight fluorescent tubes. Sporulation of *P. farinosa* occurred after 7 to 9 days of incubation, and conidia were used to
inoculate a fresh batch of cotyledons. Non-sterile cultures of the fungus could be maintained indefinitely by this method.

When aseptic conidia of _Peronospora farinosa_ were required, cotyledon pairs were inoculated as described above, but were incubated for only 72 h. after inoculation and were then surface-sterilized by immersion in 95% (v/v) ethanol for a few seconds followed by immersion in the filtrate from a 5% (w/v) calcium hypochlorite suspension, containing a drop of detergent, for 5 min. The cotyledons were then washed several times in sterile distilled water and placed on the surface of the solidified coconut milk medium (CM) described below, contained in 150 x 25 mm. Pyrex glass test-tubes, and were incubated in the lighted 15° incubator. Seven days after surface-sterilization the fungus normally sporulated on those cotyledons which remained free of contaminants (about 20 to 50%).

**Sugar beet tissue cultures.** Three commercial varieties of sugar beet (_Beta vulgaris_) were used in our experiments; these were Maris Vanguard, Sharpe's Klein E and Bush Mono. Both Maris Vanguard and Sharpe's Klein E exhibited nonspecific or partial resistance to _Peronospora farinosa_ (Russell, 1968, 1969a, b). Two noncommercial varieties of sugar beet, line F and line G, supplied by Dr G. E. Russell, were also used. Both were inbred lines of American origin: line G, a homozygous diploid, was very susceptible to attack by _P. farinosa_, while line F was highly resistant through the operation of a single dominant gene. The resistance of line F was apparently due to a hypersensitive reaction of the host cells to infection by the pathogen (Russell, 1968, 1969a, b).

Callus-tissue cultures were established from the hypocotyl or cotyledons of sterile sugar beet seedlings; or from surface-sterilized segments of leaf or hypocotyl from pot-grown sugar beet plants at the first leaf stage of development. The aseptic seedlings were grown from sugar beet fruits which had been surface-sterilized by immersion in 95% (v/v) ethanol for a few seconds and then in the filtrate from a 5% (w/v) calcium hypochlorite solution for 10 min. followed by three washes in sterile distilled water. Segments from pot-grown plants to be used for callus initiation were surface-sterilized by the same method.

In our early studies callus cultures were initiated on a salts, vitamins, and sucrose medium supplemented (per litre) with 2,4-dichlorophenoxyacetic acid (2,4-D, 6·0 mg.), 1-naphthalene acetic acid (NAA, 0·1 mg.), coconut-milk (150 ml.) and agar (6·0 g.) (Ingram, 1969a – 'coconut milk medium' [CM]). In later experiments a basal medium containing the salts, micronutrients, vitamins and sucrose of Murashige & Skoog (1962) was used. This was supplemented (per litre) with Oxoid agar no. 3 (7·0 g.), kinetin (1·5 mg.) and one of the following auxin sources: 2,4-D (6·0 mg.), NAA (1·0 mg.), or 4-chloro-2-oxobenzothiazolin-3-yl acetic acid (Benazolin, 5·0 mg.). All media were adjusted to pH 5·5 and were autoclaved at 120° for 10 min. Infected and noninfected sugar beet calluses were usually grown in 150 x 25 mm. Pyrex glass test-tubes containing 15 ml. solidified medium, or in 100 ml. wide-neck Erlenmeyer flasks of Pyrex glass containing 50 ml. solidified medium. Noninfected sugar beet calluses were normally initiated and maintained by monthly transfer at 25° in a room lit by a single 100 W tungsten lamp; calluses infected with _Peronospora farinosa_ were maintained by monthly transfer in the lighted 15° incubator.

Calluses to be used for histological studies were fixed in formalin–acetic acid–alcohol and were embedded, sectioned and stained with safranin and fast green or with Delafield's haematoxylin according to the methods of Ingram (1969b), or with resorcin blue (Griffin & Coley-Smith, 1968), which is specific for callose and has been used to detect the presence of haustorial sheaths in downy mildew fungi (Fraymouth, 1956).
Dual culture of Peronospora and sugar beet

RESULTS AND DISCUSSION

The initiation and growth of sugar beet callus cultures

Callus cultures of the commercial sugar beet varieties Maris Vanguard (mature hypocotyl, and seedling hypocotyl and cotyledon) and Sharpe’s Klein E (mature hypocotyl and first leaf) and of the experimental lines F and G (hypocotyl, cotyledon and first leaf) became established readily on the coconut milk medium (CM medium). With the exception of line G all grew well as friable brown callus which usually became firm and white with successive transfers; some friable brown clones remained, however, even after two years in culture. Segments of line G tissue usually produced very slow growing, dark and often mucilaginous callus on the CM medium, although the quality of growth did improve with successive transfers. Callus cultures were also initiated and maintained on chemically defined media, but growth was not always as rapid on these media as on the CM medium. Successful firm, white callus cultures of line F leaf were initiated and maintained on the Murashige and Skoog basal medium (M and S medium) supplemented with kinetin and 2,4-D or NAA. Line G leaf tissue formed slow growing friable brown callus on these media, but formed rapidly growing white callus with adventitious roots on the M & S basal medium supplemented with kinetin and benazolin. This medium also initiated rapid growth of line F leaf callus. Although calluses of the varieties Maris Vanguard and Sharpe’s Klein E were not initiated on defined media, they did continue to grow as hard white callus when transferred to the M & S basal medium supplemented with kinetin and 2,4-D or NAA. However, the rate of callus growth on these defined media was somewhat slower than on the complex media on which they were initiated.

The production of dual cultures of Peronospora farinosa and sugar beet callus

Two different procedures were used to establish dual cultures of Peronospora farinosa and sugar beet callus. In the first procedure, established calluses of Maris Vanguard, Sharpe’s Klein E and line G were infected with P. farinosa under sterile conditions by placing contaminant-free sugar beet cotyledon pairs on which the fungus was sporulating freely (such as the pair illustrated in Fig. 1a) on to the surface of each callus, and then incubating at 15° in the lighted incubator. In many instances the fungus spread from the infected cotyledons and became established in the sugar beet callus, a weft of aerial mycelium being produced over the entire surface of each infected culture. Further dual cultures were established by placing small explants of the infected callus in contact with non-infected calluses. In such cases the fungus often grew on to, and became established in, the new callus tissue (Fig. 1b). Many calluses did not become infected when inoculation was attempted, but this apparent resistance did not appear to be related to the nonspecific resistance known to occur in the varieties Maris Vanguard and Sharpe’s Klein E (Russell, 1968, 1969a, b): calluses derived from line G, which does not exhibit marked nonspecific resistance as an intact plant, failed to become infected with P. farinosa at about the same frequency as calluses derived from the other two varieties. Unusual patterns of resistance are known to occur in callus culture when infections with downy mildew and other fungi are attempted (Ingram, 1969b).

In the second procedure contaminant-free cotyledon pieces or single cotyledons of Maris Vanguard, on which Peronospora farinosa was sporulating freely, were left on the CM medium and were allowed to form callus at their cut surfaces. In many cases this callus contained systemic mycelium of P. farinosa and could be removed to fresh medium where dual growth continued.
(a) Sporulation of *Peronospora farinosa* on a pair of contaminant-free sugar beet cotyledons.

(b) A non-sporing dual culture of *Peronospora farinosa* and sugar beet callus grown on the coconut-milk medium.

(c) Conidia and a conidiophore of *Peronospora farinosa* grown on sugar beet callus.

(d) Sporulation of *Peronospora farinosa* on sugar beet cotyledons infected with a small piece of a sugar beet callus – *P. farinosa* dual culture.

(e) Growth of *Peronospora farinosa* away from the parent callus on the surface of the M & S medium supplemented with kinetin (1.5 mg./l.) and Benazolin (5.0 mg./l.) (viewed from the underside). The site of removal of a strip of agar severing all connexions with the callus is indicated by an arrow.
Dual culture of Peronospora and sugar beet

Balanced growth of host and parasite in culture

One of the problems involved in growing obligate parasites in host tissue cultures is that of maintaining a proper balance between the growth rate of the fungus and the growth rate of the host callus tissue (Brian, 1967). Such balance, although obtained with Plasmoidiophora brassicae and Brassica callus (Strandberg, Williams & Yukawa, 1966; Ingram, 1969a), has not been obtained with many host–parasite combinations. For example, when downy mildew fungi have previously been grown on host callus it has been found that the tissues were killed by the parasite, and that dual cultures could be perpetuated only by inoculating fresh calluses with small pieces taken from infected cultures (Morel, 1944, 1948; Griffin & Coley-Smith, 1968; Ingram, 1969b). This was not the case with the Peronospora farinosa and sugar beet combination; infected calluses, although usually covered with a dense weft of aerial mycelium (Fig. 1b), were not killed, and new dual cultures could be initiated simply by transferring small pieces of infected callus tissue to fresh medium where they grew and enlarged in a similar way to noninfected calluses.

Table 1. The dry weight of tissue produced by inocula of Maris Vanguard callus heavily infected with Peronospora farinosa, and of healthy Maris Vanguard callus, on two media

The calluses were weighed after 28 days of growth in the 15°C lighted incubator, and the figures are the means of 10 replicates.

<table>
<thead>
<tr>
<th>Medium*</th>
<th>Weight of callus (mg.)</th>
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<tr>
<td></td>
<td>Noninfected</td>
<td>Infected</td>
<td></td>
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<tr>
<td>CM medium</td>
<td>42.5 ± 7.2</td>
<td>42.5 ± 7.0</td>
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<tr>
<td>M &amp; S basal medium + 1.0 mg. kinetin/l. and 0.5 mg. NAA/l.</td>
<td>21.6 ± 2.4</td>
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* For details see text.

Further evidence for the stable and balanced nature of the Peronospora farinosa and sugar beet dual cultures was obtained when the growth rates of infected and noninfected cultures were compared. Examples of the results obtained are presented in Table 1, which gives the final weight of callus produced by explants of noninfected and preinfected Maris Vanguard callus on two media. It is clear that the presence of P. farinosa in Maris Vanguard callus had no detrimental effect on its growth. Similar results were obtained with noninfected and infected Sharpe’s Klein E callus.

As with many plant diseases caused by obligate parasites, a degree of balance exists between Peronospora farinosa and infected sugar beet leaves grown under normal conditions. However, this balance does not persist indefinitely, and infected leaves are eventually killed. In tissue culture, where most nutrients, including sugar, were supplied in the culture medium, P. farinosa and sugar beet appeared to be capable of living in complete balance indefinitely. This suggests that under natural conditions host death could be brought about in part by nutritional factors, such as inhibition by the parasite of host carbohydrate synthesis.

Histological investigation of infected sugar beet callus cultures

Microscopic examination of stained sections of infected Maris Vanguard and Sharpe’s Klein E callus revealed that the bulk of the vegetative growth of Peronospora farinosa in tissue culture was in the form of aerial mycelium extending over the surface of the callus. This aerial mycelium usually consisted of a mixture of sterile hyphae and of branched
FIG. 2

(a) A haustorium of *Peronospora farinosa* in a sugar beet callus cell. (Stained with Delafield’s haematoxylin.)

(b) A haustorium of *Peronospora farinosa* in a sugar beet callus cell containing three intact nuclei. (Stained with Delafield’s haematoxylin.)

(c) Intracellular growth of *Peronospora farinosa* in sugar beet callus. (Stained with Delafield’s haematoxylin.)
conidiophores bearing conidia (Fig. 1c). Both the conidiophores and the conidia were identical with conidia of *P. farinosa* produced on intact sugar beet leaves.

Mycelial growth within the infected callus tissues was predominantly intercellular and was concentrated in the surface layers of cells, although intercellular hyphae were frequently observed at the centre of large infected calluses. These intercellular vegetative hyphae were coarse and apparently normal, and produced the characteristic digitate haustoria of *Peronospora farinosa*, which penetrated certain host cells (Fig. 2a). Although some of the cells penetrated by haustoria were dead, many more were obviously alive, and host cell nuclei were frequently seen to be closely associated with haustorial branches. For example, Fig. 2 (b) shows a callus cell penetrated by a digitate haustorium; three intact nuclei with distinct nucleoli are visible within the cell. The cell illustrated was abnormal in having three nuclei: such abnormalities were frequently observed in callus tissues and it is not thought that the condition was due to the presence of the parasite in the cell. A sheath staining blue with resorcin blue was sometimes visible around the base of haustoria. Structures associated with sexual reproduction were never observed in callus tissue.

Although the growth of *Peronospora farinosa* in callus culture was characteristically sparse and intercellular, there were regions of tissue which were dead and where the fungus had proliferated intracellularly so that the cells were packed with vegetative hyphae (Fig. 2c). Sometimes these regions were near the edges of fissures in the calluses, but it is possible that cell death may sometimes have been due to the presence of the fungus.

The growth of *Peronospora farinosa* in sugar beet callus was clearly very similar to the growth of the fungus in sugar beet leaves, apparently normal spores, hyphae and haustoria being produced. The fact that the fungus caused little mechanical damage to the infected host tissues is a reflexion of the balance existing between host and parasite in culture. Ingram (1969 b) found that the growth of *Peronospora parasitica* in Brassica callus was more abundant than in infected leaves, and that in culture the fungus took on many of the characteristics of an aggressive parasite so that infected calluses were killed very quickly. In contrast *Plasmopara viticola* growing in vine callus (Morel, 1944, 1948) and *Pseudoperonospora humuli* growing in hop callus (Griffin & Coley-Smith, 1968) killed their host callus tissue only very slowly and mycelial growth of these fungi in culture resembled normal mycelial growth in infected host leaves.

**Sporulation and pathogenicity of Peronospora farinosa grown in callus culture**

Conidia were usually produced by *Peronospora farinosa* grown on sugar beet callus in the lighted incubator at 15°, although the number of spores produced by each dual culture was frequently quite low. Sporulation was sometimes improved by adding a small amount of sterile distilled water to the culture tubes to increase humidity. Many of the *P. farinosa* – sugar beet dual cultures lost their ability to produce conidia after an extended period (about 1 year). Such a nonsporulating culture is illustrated in Fig. 1 (b). The loss of ability to sporulate was not permanent, however, for when detached sugar beet cotyledons were infected with nonsporing *P. farinosa* mycelium by placing small pieces of infected callus on to them, together with a drop of distilled water, and incubating at 15° in the lighted incubator in plastic boxes lined with moist filter paper, infections were established and sporulation took place on the cotyledons 7 days later (Fig. 1 d). This result suggests that the loss of ability to sporulate in culture may have been due to a change in the host callus tissue rather than to a change in the *P. farinosa*.

The conidia produced by *Peronospora farinosa* grown in sugar beet callus were viable and germinated in distilled water on glass slides to produce germ tubes similar to those
produced by conidia from sugar beet leaves. The percentage germination of callus-grown conidia was often low (< 5%), although this figure was increased when conidia which had been washed from infected calluses in sterile distilled water were centrifuged at low speed and then resuspended in fresh sterile distilled water. In one experiment the percentage germination of conidia treated in this way was compared with the germination of untreated conidia. Three drops (0.05 ml./drop) of an untreated or of a centrifuged and resuspended conidial suspension containing \(4 \times 10^4\) conidia/ml. were placed on each of three glass microscope slides and incubated at 25° in Petri plates lined with moist filter paper. The percentage germination was assessed after 18 h. by counting the total number of germinated and ungerminated conidia in one low-power microscope field per drop (about 50 conidia/field). Only 2-4% of the untreated conidia produced germ tubes after 18 h. incubation compared with 26.7% germination of the centrifuged and resuspended conidia. When centrifuged conidia were resuspended in supernatant which had been concentrated by evaporation to one-fifth its original volume and incubated on glass slides germination was prevented.

Russell & Evans (1968) found that the percentage germination of conidia of *Peronospora farinosa* harvested from sugar beet leaves was improved by washing in deionized water, which probably removed a water-soluble inhibitor of germination. They also noted that the presence of sugars in conidial suspensions of *P. farinosa* reduced germination on glass slides. The washing of conidia in our experiment may have served the dual purpose of removing a water-soluble inhibitor together with any sucrose which may have been taken into solution from the callus culture medium when the conidia were harvested.

Callus-produced conidia were pathogenic, and sporulating infections were established when they were used to inoculate healthy sugar beet calluses or detached sugar beet cotyledons.

The expression of major gene resistance to *Peronospora farinosa* in sugar beet callus cultures

Ingram & Robertson (1965), Ingram (1967) and Robertson et al. (1968) found that major genes for resistance to *Phytophthora infestans* appeared to be expressed in potato tissue cultures. Similarly, Warren & Routley (1970) showed that single genes for resistance to *P. infestans* were at least partially expressed in tomato callus. However, Ingram (1969b) showed that in Brassica callus, resistance to *Peronospora parasitica* was not expressed normally. A preliminary investigation was carried out to determine whether the major gene for resistance to *Peronospora farinosa*, known to exist in sugar beet line F leaves, was expressed in line F calluses. Six-month-old healthy line F and line G first leaf calluses, originally initiated on the CM medium, were each inoculated with 0.5 ml. of a suspension containing \(2 \times 10^4\) callus-grown conidia/ml. of distilled water. In another experiment small pieces of infected Maris Vanguard callus were placed in contact with six-month-old healthy line F calluses. In the experiment where conidia were used as inoculum, 8 out of 11 line G calluses, and 4 out of 8 line F calluses became infected. In the experiment where infected callus was used as inoculum, each of 12 line G calluses and 3 out of 12 line F calluses became infected. In both experiments the fungus subsequently produced aerial mycelium and conidia over the entire surface of the infected test calluses. The results of these preliminary tests suggest that resistance may not be expressed normally in sugar beet callus.

Growth of *Peronospora farinosa* on to the agar culture medium

A feature of dual cultures of *Peronospora farinosa* and sugar beet callus grown on tissue culture media was that mycelium frequently grew away from infected cultures over the surface of the agar. In some instances a dense weft of mycelium which extended for 1.5 to
2·0 cm. from the parent callus and only connected to it by a few hyphal strands (Fig. 1e) was produced. This phenomenon was particularly noticeable when dual cultures were grown in Petri dishes on the defined media rather than on media containing coconut milk. The hyphae of *P. farinosa* growing on the surface of the agar medium were coarse with distorted and bulbous tips which had frequently burst. Neither haustoria nor haustorium-like branches were detected, although perfectly formed conidiophores with conidia were often found at up to 1·0 cm. from the parent callus culture. Growth of the *P. farinosa* mycelium on agar did not continue when connexions with the parent callus were severed by removing a narrow strip of agar, as indicated in Fig. 1 (e). Mycelium beyond such barriers soon died, although regrowth occurred from the severed edges of the colony still connected to the parent callus, and hyphae grew over the bare Petri plate where the strip of agar had been removed. Griffin & Coley-Smith (1968) described a similar phenomenon with *Pseudoperonospora humuli* grown in hop callus. This fungus grew into the agar medium but, as with *P. farinosa*, axenic growth could not be achieved when connexions with the parent callus were severed. Little success has so far been achieved in culturing downy mildew and related fungi axenically. Guttenburg & Schmoller (1958) did obtain limited vegetative growth and conidiophores of *Peronospora brassicae* on beer-wort-phosphate agar medium, while Tiwari & Arya (1969) obtained saprophytic growth and sporulation of *Sclerospora graminicola* on an agar medium, but this did not persist after two transfers in the absence of host callus tissues. It is interesting that *P. farinosa* grew for some distance from the parent callus over the surface of the agar culture medium, suggesting a somewhat diminished dependence on the host tissues, and it would be worth investigating whether it can be grown on the surface of a membrane physically separating it from the host callus, as has been achieved with *Cronartium ribicola* recently (Harvey & Grasham, 1970) or in true axenic culture as with certain of the rusts (Scott & Maclean, 1969).

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