Growth of *Plasmodiophora brassicae* in Host Callus

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SUMMARY

Explants of young tumour tissue from several Brassica species infected with *Plasmodiophora brassicae* plasmodia gave rapid callus growth on medium containing 2,4-dichlorophenoxyacetic acid and coconut milk. Degeneration of the callus followed as resting spores were formed. The resting spores germinated *in situ* and primary plasmodia and zoosporangia developed in some cells, then secondary plasmodia reappeared and normal callus growth was resumed; thereafter all stages of the parasite life-cycle were present in active cells. This situation has been maintained for over 18 months by 8-weekly transfer of calluses to new medium. Callus clones containing only vegetative plasmodia were established on a coconut-milk + 2,4-dichlorophenoxyacetic acid medium. Sporogenesis did not occur readily in these clones unless callus was transferred to a kinetin + α -naphthylacetic acid medium or to other media which did not contain 2,4-dichlorophenoxyacetic acid. Stages of the *P. brassicae* life-cycle found in callus appeared similar to those in intact hosts, while zoosporangia were formed in the root hairs of *Sinapis alba* organ cultures infected with callus-produced resting spores. It was concluded that on suitable media *P. brassicae* may be maintained in complete balance with host callus.

INTRODUCTION

Tissue cultures offer convenient substrates for the growth of obligate parasites under controlled and sterile environmental conditions, making precise physiological investigations possible. The first report of the dual culture of an obligate parasite and its host was by Morel (1944), who grew *Plasmopara viticola* in callus cultures of vine. Since that time, with the exception of the work of Hotson & Cutter (1951) and Cutter (1959; 1960) involving the juniper + *Gymnosporangium juniperi-virginianae* and the *Arisaema triphyllum* + *Uromyces ari-triphylli* combinations, there have been few reports of the successful establishment and long-term maintenance of obligate parasites in host tissue cultures. Hotson & Cutter (1951) were able to establish their cultures by transferring explants of surface-sterilized juniper gall tissue, infested with *Gymnosporangium juniperi-virginianae*, to callus medium. A similar procedure was adopted by Strandberg, Williams & Yukawa (1966) in the first report of the culture of *Plasmodiophora brassicae* in callus of *Brassica oleracea* var. *capitata*, Badger Shipper. However, meristematic regions of infected calluses had to be transferred each week on a medium containing α -naphthylacetic acid + kinetin. No apparent difference was found between *P. brassicae* grown in intact hosts and in callus, and callus tissues, like intact host tissue, reacted to the parasite by cell and nuclear hypertrophy and increased

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starch content. These observations were further substantiated by electron microscope studies (Williams & Yukawa, 1967).

The present study, begun before publication of the work of Strandberg *et al.* (1966), confirms their findings for growth of infected *Brassica* callus on a kinetin + α -naphthylacetic acid medium, and also indicates that *Plasmodiophora brassicae* may be grown for long periods in complete balance with host callus on a coconut milk + 2,4,-dichlorophenoxyacetic acid medium.

METHODS

Organisms. Infected plants of *Brassica rapa*, Golden Ball turnip and Balmoral turnip, *B. napobrassica*, Wilhelmsburger swede, *B. napus* var. *annua*, Giant English rape, *B. oleracea* var. *capitata*, Early Drumhead cabbage, and *B. chinensis*, Wong Bok, were obtained by transplanting 10-day seedlings into John Innes potting compost containing approximately 1×10^8 *Plasmodiophora brassicae* resting spores/g. The strain of the parasite used has arbitrarily been denoted S and has been shown to be pathogenic to a wide range of Crucifers (I. J. McEvoy, personal communication). Plants harvested after 4 to 6 weeks of growth under greenhouse conditions had normally developed large clubs. Healthy plants were grown in sterile John Innes potting compost in the greenhouse.

The callus culture medium consisted of tobacco medium salts and micronutrients (Hildebrandt, 1962). Ferric tartrate was omitted and replaced with $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.0398 g./l.) and Na_2EDTA (0.0532 g./l.). This medium was supplemented with calcium pantothenate (2.5 mg./l.), thiamine hydrochloride (0.1 mg./l.), glycine (3.0 mg./l.), α -naphthylacetic acid (NAA, 0.1 mg./l.), 2,4-dichlorophenoxyacetic acid (2,4-D, 6.0 mg./l.) and coconut milk (150 ml./l.), and was solidified with 6.0 g. Davis agar/l. (coconut milk medium). In a chemically defined medium (the kinetin medium) 2,4-D and coconut milk were omitted, the NAA content was raised to 0.5 mg./l. and kinetin was added at 1.0 mg./l. (P. H. Williams, personal communication). In some experiments the kinetin medium was further supplemented with extra vitamins and micronutrients (pyridoxine HCl 0.1 mg./l.; nicotinic acid 0.5 mg./l.; mes-oinositol 0.2 mg./l.; biotin 4.0 $\mu\text{g.}$ /l.; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.02 mg./l.; H_2MoO_4 0.017 mg./l.) or with an amino acid mixture based on the analysis of the amino acids of coconut milk of Tulecke, Weinstein, Rutner & Laurencot (1961). All tissue culture media were adjusted to pH 5.5 with NaOH or HCl and were then autoclaved at 115° for 10 min.

Callus cultures were always incubated at 26° in a culture room lit continuously with a single 100 W. tungsten lamp.

Infected cultures were initiated from *Plasmodiophora brassicae* clubs from 4- to 6-week-old plants: pieces of club approximately 1.0 cm. diameter and 2.0 cm. long were washed in water, immersed in 95 % (v/v) ethanol in water for 1 min., then in 1 % (w/v) HgCl_2 in water for 4 min. After four washes in sterile distilled water the clubs were trimmed to remove dead tissues contaminated with mercuric chloride, and were cut into 1.5 mm^3 to 2.0 mm^3 explants which were placed on 15 ml. medium contained in $25 \times 150 \text{ mm}$. Pyrex glass tubes and incubated at 26° . A similar procedure was used for the initiation of callus cultures from organs of healthy plants grown for 4 to 6 weeks in sterile soil.

When callus cultures had become established, those which were to be transferred

to fresh medium every 7 to 14 days were always grown singly on 15 ml. medium contained in 25 × 150 mm. Pyrex glass tubes, while those which were to be transferred every 8 weeks were grown on 30 ml. medium contained in 100 ml. Pyrex glass Erlenmeyer flasks (3 calluses/flask). The growth of established, infected callus tissue of *Brassica rapa*, Golden Ball, on different media was compared by using 15 ml. batches of medium contained in 150 × 25 mm. glass tubes. These were inoculated singly with 1.5 mm³ explants of actively-growing callus tissue, previously maintained for 12 months on coconut-milk medium, and maintained for 1 week before beginning the test on medium without growth factors. The growth of the calluses was assessed visually and by fresh and dry weights after being transferred each week for 5 weeks on the test media.

Root organ cultures were established from sterile seedlings of *Sinapis alba* in a liquid medium composed of salts, micronutrients, vitamins and sucrose (Street & Henshaw, 1966) supplemented with *meso-inositol* (50.0 mg./l.), kinetin (0.00125 mg./l.) and α -naphthylacetic acid (0.0001 mg./l.). This medium was adjusted to pH 4.8 with NaOH or HCl and autoclaved at 115° for 10 min. Sterile seedlings were grown as follows: seeds of *S. alba* were washed in 95 % (v/v) ethanol in water for 1 min., immersed in 5 % (w/v) filtered calcium hypochlorite solution for 7 min. and washed three times in sterile distilled water; they were then germinated in the dark at 26° on potato + glucose agar. After 3 days of growth, 0.5 cm. root-tip segments were removed from the seedlings and placed in 20 ml. medium contained in 100 ml. Pyrex glass Erlenmeyer flasks. Further incubation of the cultures was at 26° in the dark.

Organ cultures were infected with *Plasmodiophora brassicae* by placing 2 cm. root tip segments in 25 ml. Erlenmeyer flasks, containing 5 ml. of medium, and inoculating with callus-produced resting spores suspended in organ culture medium (see above). (The final concentration of spores in the flasks was about 1×10^6 /ml.). Resting spores were extracted from calluses under completely sterile conditions: pieces of callus tissue were placed in a small quantity of organ culture medium in a test-tube, frozen at -20° for 30 min. and then, after thawing at room temperatures, were homogenized with a glass rod. The resulting suspension was filtered through muslin to remove debris and added to the culture flasks after spores had been counted with a haemocytometer.

Histological techniques. Calluses were removed from the culture medium and fixed in 3 % glutaraldehyde in 0.1 M-phosphate buffer (0.994 g. Na₂HPO₄ + 0.4083 g. KH₂PO₄, all in 100 ml. water at pH 7.2) for 3.5 hr. They were then washed for two periods of 1 hr in the buffer, fixed in 1 % osmic acid in buffer for 1 hr, and finally given two further 1 hr washes in buffer. All procedures were done under vacuum at 4°. Calluses to be examined were embedded in wax and 8 μ sections stained with Delafield's haematoxylin (Ingram, 1967).

Stages of the *Plasmodiophora brassicae* life-cycle were identified by reference to: (a) sections of club tissue from intact plants; (b) whole roots of intact *Brassica* seedlings infected with the root hair stages of the parasite according to the method of Channon, Flint & Hinton (1964); (c) illustrations prepared by Karling (1942).

RESULTS

Initiation of Plasmodiophora brassicae infected callus cultures

When explants of 4-week club tissue from the Brassica species listed above were placed on the coconut-milk medium and incubated at 26°, a phase of rapid callus growth was normally initiated after 4 to 6 days which continued until at 4 weeks the fresh weight of calluses was between 60 and 140 mg. However, after 4 to 6 weeks of incubation cultures began to show signs of decline: the tissues became brown and unhealthy and growth virtually ceased. Continued incubation of such degenerate calluses led, after a further 3 to 4 weeks, to renewed and healthy tissue growth which arose from isolated points on the callus mass. Growth of the new callus continued without further interruption, explants being transferred to fresh medium every 8 weeks (Table 1). In contrast, explants of healthy tissue from roots and hypocotyls of the Brassica species being investigated after 4 to 6 days initiated callus growth which proliferated without check on the coconut-milk medium.

Table 1. *Development of callus, containing Plasmodiophora brassicae from explants of Brassica rapa, Balmoral club, on the coconut-milk medium*

Approx time on the medium (days)	Quality of callus growth	Stage of <i>P. brassicae</i> life-cycle present in the callus
7-27	Vigorous growth	Advanced vegetative plasmodia
28-42	Callus became unhealthy and growth ceased	Advanced and cleaved plasmodia and resting spores
43-56	Callus not growing	Resting spores and primary plasmodia and zoosporangia
57-63	Renewal of vigorous healthy growth	Resting spores, primary plasmodia and zoosporangia and young, vegetative plasmodia
> 64	Vigorous healthy growth continuing without check	All stages of the parasite life-cycle

Histological investigations of sample infected calluses of *Brassica rapa*, Balmoral and Golden Ball, and *Brassica oleracea* var. *capitata*, Drumhead, were made at the time of callus initiation, at intervals up to 9 weeks afterwards and, in the case of Golden Ball, at 10 and 14 months afterwards. Because the variation in the rate of growth of individual calluses was slight it was possible to trace the complete pattern of host and parasite development during the early stages of infected tissue-culture growth. The interrupted nature of these early stages of growth was found to correspond with clearly defined steps in the development of the parasite within the tissues.

At the time of tissue culture initiation clubs, which were to be used as a source of explants, contained numerous cells infected with *Plasmodiophora brassicae*. The infected cells were usually hypertrophied, with enlarged nuclei and nucleoli, and contained an abundance of starch. Several types of parasite plasmodia were present, the most numerous being at an advanced vegetative stage with vacuoles, many nuclei and an abundance of lipid droplets. Resting spores had not normally formed in the cells at this stage.

The pattern of development of callus from club tissues was essentially the same in all three species investigated. During the first 4 weeks of callus growth cell division took place throughout the explants and a large body of new tissue was produced. However, division of hypertrophied, infected cells was not evident, and newly-formed callus cells did not contain the parasite. Stages of sporogenesis of *Plasmodiophora brassicae* plasmodia (i.e. cleavage and formation of resting spores) became evident in infected cells of the inoculum after about 4 weeks of incubation, when a rapid decline in callus vigour was recorded. At the same time the walls of infected cells became brown and did not take up stain. At approximately 6 weeks all plasmodia had undergone sporogenesis, and resting spores were the only part of the parasite life-cycle represented in the tissue cultures.

After about 8 weeks of incubation when callus proliferation was renewed, it was noted that many enlarged, brown-walled cells, which had obviously once contained resting spores, were partially or completely empty, the place of the spores being occupied by amorphous debris. Within such cells, or close to them, stages of the *Plasmodiophora brassicae* life-cycle normally found in the root hairs of intact plants (Karling, 1942) were noted. These infected cells did not contain starch, and frequently appeared to be dead. The parasite consisted either of small primary plasmodia with 1 or 2 nuclei and no lipid, or clusters of primary zoosporangia. The latter (Pl. I, fig. 1, 2) often contained from 3 to 16 incipient zoospores. Primary plasmodia and zoosporangia in callus closely resembled similar structures observed in the root hairs of intact infected *Brassica* seedlings.

Following the appearance of zoosporangia, secondary plasmodia soon developed in adjacent newly-formed cells. The appearance of secondary plasmodia (Pl. I, fig. 3, 4) coincided with renewed callus growth, which then continued unchecked. Many of the secondary plasmodia developed to form resting spores, and subsequent sections of calluses revealed all stages of the *Plasmodiophora brassicae* life-cycle in the tissues. Even after 14 months, Golden Ball calluses, which had been transferred on 30 ml. coconut-milk medium contained in 100 ml. Erlenmeyer flasks every 8 weeks and in which no part of the original clubroot inoculum remained, contained representatives of each stage of the parasite life-cycle. Vegetative plasmodia were usually in the most recently-formed cells of small meristematic centres scattered through the calluses, while other stages, including resting spores and primary zoosporangia, were in the cells of the older parts of the callus. Infected callus cells resembled infected cells of intact hosts in frequently being hypertrophied, in usually possessing enlarged nuclei, and in containing considerably more starch than uninfected cells. Indeed, healthy calluses, unlike infected calluses, contained very little starch. The parasite was anatomically indistinguishable from the parasite observed in intact hosts.

Control of the parasite life-cycle in tissue culture

By selection of callus tissues and frequent transfer to fresh coconut-milk medium it was possible to establish clones of *Brassica rapa*, Golden Ball, callus containing only vegetative plasmodia. During the establishment of these clones from club tissue the parasite went through the stages of sporogenesis and resting spore germination already described. As soon as new callus growth containing vegetative plasmodia formed on the inoculum it was removed to fresh medium. Thereafter, only meristematic tissues were transferred to 15 ml. fresh medium contained in 25 × 150 mm. Pyrex glass tubes

every 7 to 14 days. The parasite present in calluses maintained in this way (Pl. I, fig. 3, 4) did not appear to develop beyond the vegetative plasmodial stage and resting spores were never formed. Despite this, new tissues always contained plasmodia in the cells.

Table 2. *Mean fresh weights of two replicate calluses, and stages of callus and parasite development when Brassica rapa, Golden Ball, tissues, infected with Plasmodiophora brassicae, were maintained without transfer on 15 ml. and 60 ml. batches of the coconut-milk medium*

Time on the medium (weeks)	Fresh weight (mg.) of calluses on:		Callus and <i>P. brassicae</i> development on:	
	15 ml. medium	60 ml. medium	15 ml. medium	60 ml. medium
2	83.70	78.50	A*	A*
4	165.50	156.75	A	A
6	311.80	308.00	B	A
8	529.75	667.90	C	A
10	453.50	922.75	D	B
12	473.15	1140.26	D	C
16	400.50	1049.50	D	D

* A = healthy callus growth with vegetative plasmodia; B = callus senescing at centre, plasmodia vegetative; C = callus senescent and containing a few spore-filled cells (medium becoming dehydrated); D = callus completely dead and medium showing extreme signs of dehydration.

Resting spores were not formed readily by *Plasmodiophora brassicae* maintained in a vegetative state in Golden Ball callus for 12 months. Table 2 illustrates the results of a test where 2 mm³ explants of Golden Ball callus, containing *P. brassicae* maintained in a vegetative state for 12 months, were transferred singly to 15 ml. batches of coconut-milk medium contained in 25 × 150 mm. test-tubes, or to 60 ml. batches of coconut-milk medium contained in 40 × 200 mm. test-tubes and incubated without further transfer. Two sample calluses were removed from each treatment at intervals, weighed and examined by freehand section for the presence of resting spores. Initially calluses grew well on both volumes of medium, with senescence at the centre of the tissue masses becoming evident after 4 to 6 weeks. By 8 weeks the 15 ml. batches of medium had started to dry out and calluses senesced rapidly. The 60 ml. batches of medium did not show extreme signs of dehydration until 10 or 12 weeks, but when they did, calluses senesced rapidly. Resting spores had developed after about 8 weeks in a few cells of calluses maintained on 15 ml. batches of medium, although young vegetative plasmodia were still abundant in the tissues. When senescence took place the parasite died with the tissues and no more resting spores were formed. The situation was essentially the same in calluses maintained on the 60 ml. batches of medium, although senescence and the appearance of a few resting spore cells did not take place for approximately 12 weeks. None of the calluses ever contained a large number of cells filled with resting spores.

However, resting spores did develop after 4 to 5 weeks in most of the infected cells of Golden Ball calluses previously transferred weekly on the coconut-milk + 2,4-D medium, when these were maintained without transfer on medium containing no growth factors or on medium supplemented with coconut milk alone. Resting spores

did not develop readily in Golden Ball calluses maintained without transfer on medium containing 2,4-D alone.

*Growth of Plasmodiophora brassicae infected callus on a
kinetin + α -naphthylacetic acid medium*

Calluses infected with *Plasmodiophora brassicae* were established on the kinetin medium from 4- to 6-week-old clubs of *Brassica rapa*, Golden Ball, and *B. napobrassica* Wilhelmsburger. Early stages of callus growth and parasite development were similar to those described for establishment of infected callus on the coconut-milk medium. However, infected tissues did not normally grow as well on the kinetin medium as on the coconut-milk medium: callus growth was slower and normally looked brown and unhealthy. Furthermore, it was not possible to maintain callus containing all stages of the *P. brassicae* life-cycle for long periods on the kinetin medium. Unless meristematic tissues containing only vegetative plasmodia were rigorously selected and transferred to fresh medium every 7 to 14 days the parasite rapidly underwent sporogenesis, cell walls became lignified and the callus mass died after about 4 weeks. Sporogenesis took place rapidly even in *B. napobrassica* calluses, where *P. brassicae* had been kept in a vegetative state for over a year by weekly transfer, when the calluses were left untransferred for 4 weeks on the kinetin medium.

Table 3. *Mean fresh and dry weights of five replicate calluses after 5 weeks of growth from inocula of infected Brassica rapa, Golden Ball, callus tissue on the coconut-milk medium and on the kinetin medium, unsupplemented and supplemented with extra vitamins and trace elements and with an amino acid mixture*

(1) Extra vitamins: pyridoxine HCl (0.1 mg./l.), nicotinic acid (0.5 mg./l.), mesoinositol (0.2 mg./l.), biotin (4.0 μ g./l.). (2) Extra trace elements: $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.02 mg./l.), H_2MoO_4 (0.017 mg./l.). (3) Amino acid mixture: based on an analysis of the amino acids of coconut milk (Tulecke *et al.* 1961).

Medium	Weight of callus (mg.)	
	Fresh	Dry
Coconut-milk medium	291.94 $\pm$ 31.37	24.88 $\pm$ 2.77
Kinetin medium	175.94 $\pm$ 22.11*	19.84 $\pm$ 2.94
Kinetin medium $\pm$ extra vitamins and trace elements	162.66 $\pm$ 12.80*	18.00 $\pm$ 1.37
Kinetin medium $\pm$ extra vitamins + trace elements + amino acids	182.08 $\pm$ 37.85*	20.98 $\pm$ 4.44

* These calluses contained many cells filled with resting spores at the end of the experiment.

The growth of infected calluses could not be improved markedly, nor could sporogenesis of the parasite be delayed, by supplementing the kinetin medium with an extra vitamin and micronutrient mixture (pyridoxine HCl; nicotinic acid; meso-inositol; biotin; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$; H_2MoO_4) or with an amino acid mixture based on an analysis of the amino acids of coconut milk (Tulecke *et al.* 1961). Plate 2, fig. 6 and Table 3 refer to a growth test where explants of infected *Brassica rapa*, Golden Ball, callus were incubated for 5 weeks on the coconut-milk medium and on the kinetin medium, unsupplemented and supplemented with the extra vitamins and micronutrients mixture and with the amino acid mixture.

Infection of root organ cultures

When 2.0 cm. tip segments from root organ cultures of *Sinapis alba* were infected with 1×10^6 resting spores of *Plasmodiophora brassicae* (obtained from infected calluses grown on the kinetin medium) primary plasmodia and zoosporangia developed in the root hairs after 4 to 9 days. Only a small proportion (less than 1 %) of the root hairs were infected, but the plasmodia and zoosporangia, when examined microscopically in unstained preparations using bright-field or phase-contrast illumination, appeared identical to similar structures formed in the root hairs of *S. alba* seedlings infected with resting spores from intact plants according to the method of Channon *et al.* (1964). Clubs did not develop on organ cultures, even after 10 weeks of incubation.

DISCUSSION

Maintenance of both host and parasite in an active state is an important problem in growing obligate parasites in tissue culture. Hotson & Cutter (1951) and Cutter (1959) overcame this problem and were able to grow clones of Juniper callus infected with *Gymnosporangium juniperi-virginianae* for several years on artificial medium. Other workers (Constabel, 1957; Rossetti & Morel, 1958; Maheshwari, Hildebrandt & Allen, 1968), however, have been less successful with rust fungi, and the author (unpublished data) found that *Uromyces fabae* did not colonize callus formed on infected segments of *Vicia faba* stem, although sporulation did take place on those segments. *Plasmodiophora brassicae* has been grown in callus of *Brassica oleracea* var. *capitata* over an extended period (Strandberg *et al.* 1966), but active infected tissues had to be transferred on a kinetin + α -naphthylacetic acid medium every week. The present study indicates that *P. brassicae*, either in a vegetative plasmodial form or going through its whole life-cycle, may be maintained in complete balance with host callus for long periods on suitable media.

It is interesting that *Plasmodiophora brassicae* went through all stages of its life-cycle during callus culture initiation, and that the various stages of the life-cycle were reflected in the quality of growth of the callus tissues. Sporulation of *P. brassicae* plasmodia soon after transfer of tissue explants to the callus medium was probably inevitable, since the plasmodia in the clubs used as a source of inoculum were at such an advanced stage of development. Since hypertrophied infected cells of the inoculum did not divide, callus tissues produced initially were not infected with vegetative parasite plasmodia, and became infected only after resting spores had germinated *in situ* and primary plasmodia and zoosporangia had been formed. In intact hosts, *in situ* germination of resting spores is unlikely, and in callus some constituent of the tissue culture medium, such as auxin or EDTA, may have been responsible for inducing the process. Both the sequence of events during callus initiation and the behaviour of established infected calluses when left untransferred on the kinetin medium suggested that sporulation of *P. brassicae* was inhibitory to callus proliferation, even though many cells in the tissue mass did not contain the parasite. However, *P. brassicae* continued its life-cycle in established *Brassica rapa*, Golden Ball, callus for nearly 2 years, during which time no inhibition of tissue growth was noted, possibly because the ratio of plasmodia undergoing sporogenesis to the total number of infected cells in such calluses was very small.

Clones of *Brassica rapa*, Golden Ball, callus containing only vegetative plasmodia were established by selection and frequent transfer on the 2,4-D + coconut-milk medium. Sporogenesis did not occur readily in such clones once they had become well established, even when the tissues were allowed to senesce. Sporogenesis was induced, however, when the tissues were transferred to other media which did not contain 2,4-D, suggesting that the auxin status of culture media may be important in the initiation of sporulation of *Plasmodiophora brassicae* in callus.

Histological investigations of calluses infected with *Plasmodiophora brassicae* indicated that both the parasite and host-cell reactions to infection in tissue culture are the same as in intact hosts, thus confirming the observations of Strandberg *et al.* (1966).

Brassica seedlings have been infected with callus-produced resting spores (P. H. Williams, personal communication), and further evidence for the viability of resting spores formed in callus is provided by the demonstration of their ability to infect root hairs of *Sinapis alba* root organ cultures. Smith (1956) showed that *Plasmodiophora brassicae* resting spores germinated better in non-sterile soil than in sterile soil, an effect which could be reproduced by treating spores with macerating enzymes in sterile conditions. A similar phenomenon was demonstrated by Mosse (1962), where certain bacteria or macerating enzymes were necessary for infection of sterile host plants by *Endogone* species. It is possible that the reasons why only a small proportion of the root hairs of *S. alba* organ cultures became infected after inoculation with callus-produced resting spores may be explained in similar terms. However, no effort was made to wash resting spores used for root organ culture inoculation and some inhibitor of germination produced in the calluses might have been carried over in the inoculum. Despite these possibilities, resting spores did germinate within calluses, as discussed above. The fact that club formation did not occur in organ cultures may indicate a fault in technique, for Smith (1956) has reported growth of *P. brassica* in excised root cultures.

Hotson & Cutter (1951) reported that *Gymnosporangium juniperi-virginianae* grew out of host calluses onto the culture media; this result could not be achieved by other workers (Constabel, 1957; Rossetti & Morel, 1958). Although a large number of calluses infected with *Plasmodiophora brassicae* were maintained during the present study, no evidence was found that the parasite ever grew out of the tissues. Even when infected callus tissues were grown in liquid suspension culture (unpublished data), *P. brassicae* was never detected free in the culture medium. However, it is significant that primary plasmodia and zoosporangia formed in calluses were frequently seen to be in cells which were dead, indicating a lesser degree of dependence on the host cells than secondary plasmodia, which were always associated with living cytoplasm. This possibility is supported by observations that many Crucifers and non-Crucifers, though not susceptible to the secondary stages of the *P. brassicae* life-cycle, are susceptible to the primary (root hair) stages (Köle & Philipsen, 1956).

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EXPLANATION OF PLATES

PLATE 1

Stages of the *Plasmodiophora brassicae* life cycle from tissue cultures grown on the Coconut milk medium.

Fig. 1 and 2. Primary zoosporangia, with incipient zoospores, in cells which had previously contained resting spores, formed during callus culture initiation from *Brassica rapa*, Balmoral, club tissues. (Stained with Delafield's haematoxylin.)

Fig. 3 and 4. Vegetative (secondary) plasmodia released from thick hand sections of 12-month-old *Brassica rapa*, Golden Ball, callus. (Unstained preparation.)

PLATE 2

Fig. 5. Resting spores of *Plasmodiophora brassicae* formed in *Brassica rapa*, Balmoral, callus grown on medium without growth factors. (Unstained preparation.)

Fig. 6. Five replicate examples each of *Brassica rapa*, Golden Ball, callus after 5 weeks of growth on (A) coconut-milk medium, (B) kinetin medium, (C) kinetin medium+extra vitamins and micro-nutrients, (D) kinetin medium+extra vitamins+micronutrients+an amino acid mixture.

D. S. INGRAM

(Facing p. 18)

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