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STEROLS IN RESTING SPORES OF *PLASMODIOPHORA BRASSICAE*

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Abstract—Sterols have been isolated from resting spores of *Plasmodiophora brassicae* and from healthy root material of various members of the Cruciferae. The results suggest that the parasite may draw upon the host plant for at least part of its sterol requirement.

INTRODUCTION

THE ROOT gall forming organism *Plasmodiophora brassicae* Woronin is obligate to the family Cruciferae and is responsible for the disease known as club-root. Studies of the organism[1,2] have shown that there is an increase in synthesis of protein, DNA, RNA, amino acids, sugars, starch and lipids in the host plant during the plasmodial stage of *P. brassicae*. During the succeeding sporulation stage, however, there is a tendency to a degradative phase of metabolism although protein DNA and lipid levels remain high. By means of studies of the enzyme activities involved during these stages, results have been obtained which seem to show that the parasite operates an over-riding control of the host regulatory systems.[2]

In a detailed study of lipid metabolism, Strandberg[3] has shown that radioactively labelled precursors were more rapidly incorporated into sterols and other lipid classes in infected hypocotyl tissue of cabbage than into healthy hypocotyl. It was not clear whether this extra sterol synthesis arose in the host or within the plasmodia of the invading organism. In an attempt to determine the origin of sterols in *P. brassicae*, sterols were isolated from healthy root material and from resting spores isolated from mature "clubs" and the results so obtained are described in the present paper.

RESULTS

Healthy plants of *Sinapis alba* L. (white mustard), *Malcomia maritima* [L.] R. Br. (Virginia stock) and *Lesquerella lyrata* (S. Wats.) Rollins were grown from seed and transplanted into soil or compost after 1 week. Infected plants were obtained similarly by transplanting into soil which had been previously infected with resting spores of *Plasmodiophora brassicae* or by watering seedlings once with an aqueous suspension of resting sporangia of *P. brassicae* after transplanting. A bulk of extract of spores was obtained from *Brassica rapa* L. (turnip) and was used partly for the above inoculum and also for a detailed study of lipids of *P. brassicae*.

[1] P. H. WILLIAMS, N. T. KEEN, J. O. STRANDBERG and S. S. MCNABOLA, *Phytopathology* **58**, 921 (1968).
[2] N. T. KEEN and P. H. WILLIAMS, *Phytopathology* **59**, 778 (1969).
[3] J. O. STRANDBERG, *Lipid Metabolism in Clubroot of Cabbage*, Ph.D. Thesis, University of Wisconsin, 1968. University Microfilms 68-5, 356.

Spores were obtained by macerating mature "clubs" in water followed by differential centrifugation. Sterols were released by boiling a suspension of the spores in 6 N HCl covered with a layer of toluene[4] and were isolated from the neutral fraction by digitonin precipitation.

Acid hydrolysis was used to penetrate the spore wall and to ensure liberation of sterols from lipid droplets within the spores. It was felt that the use of a modified form of the Vestergaard hydrolysis technique[4] would protect the sterols liberated from being decomposed by prolonged exposure to hot acid. In this process the sterols are continuously extracted into benzene or toluene whilst the hydrolysis is proceeding. The modifications to the Vestergaard hydrolysis used in the present work were the substitution of toluene for benzene and of HCl for H_2SO_4 and also the reaction was carried out for a longer period. The yield of material from a bulk extraction of spores from *B. rapa* was 0·16 per cent. Healthy root material was washed free of soil, air dried and sterols isolated as previously described.[5]

Analysis by GLC of the sterol fractions indicated β-sitosterol to be the main component in all cases examined (see Table 1) and showed that cholesterol (1–3 per cent) and campesterol

TABLE 1. STEROLS OF HEALTHY ROOT, AND OF RESTING SPORES ISOLATED FROM ROOT INFECTED WITH *P. brassicae* EXPRESSED AS A PERCENTAGE OF TOTAL STEROL

Source	Percentage of each sterol*					
	1	2	3	4	5	6
Brassica rapa L. (root)	5·5	1·6	15·8	6·4	70·7	—
(spores)	1·4	—	14·1	T	84·5	—
Sinapis alba L. (root)	1·3	—	18·4	4·4	75·9	—
(spores)	3·3	T	23·8	6·7	64·1	2·1
Malcomia maritima (L.) R. Br. (root)	1·3	4·3	22·8	9·0	62·6	—
(spores)	3·2	2·7	17·5	9·1	67·1	—
Lesquerella lyrata (S. Wats.) Rollins (root)	1·4	0·5	27·4	4·3	66·4	—
(spores)	2·2	2·4	18·4	13·2	63·8	—

* Key: 1 = cholesterol, 2 = brassicasterol, 3 = campesterol, 4 = stigmasterol, 5 = β-sitosterol, 6 = Δ^5-avenasterol and T = trace.

(14–24 per cent) were also present in all cases. Identity of compounds was presumed from comparison of GLC data with authentic compounds and from the known data relating to sterols in the Cruciferae.[5] The other sterols present in some or all cases were brassicasterol (24-methyl-$\Delta^{5,22}$-cholestadien-3β-ol), stigmasterol (24-ethyl-$\Delta^{5,22}$-cholestadien-3β-ol) and Δ^5-avenasterol (24-ethylidenecholesterol).

DISCUSSION

It is apparent from Table 1 that there is a close similarity between the sterol content of spores obtained from *Malcomia maritima* and *Lesquerella lyrata* and also that each extract is not qualitatively different from that of the sterol fraction of the corresponding healthy root material. A small qualitative difference was noted between spores from *Sinapis alba* and those from the other two species in that there was a small but measurable amount of Δ^5-avenasterol in the spores from *S. alba.*

[4] P. VESTERGAARD and B. CLAUSSEN, *Acta Endocrinol.* **39**, *Suppl.* 64, 35 (1962).
[5] D. S. INGRAM, B. A. KNIGHTS, I. J. MCEVOY and P. MCKAY, *Phytochem.* **7**, 1241 (1968).

It has been shown that the sterols in members of the Cruciferae vary in relative proportion during growth and physiological development.[5] Thus Δ^5-avenasterol is found in appreciable quantities in seed of *S. alba* (*ca.* 13 per cent of the sterol fraction) but its abundance was found to diminish rapidly following germination and to have virtually disappeared in 2 weeks. The finding of Δ^5-avenasterol in spores from *S. alba* but not in healthy root material therefore suggests that the parasite has taken at least some of its sterol from the host plant during the early stages of development when Δ^5-avenasterol might have been expected to be present in the seedling. Further, since it is known that the relative percentage of β-sitosterol rises and the level of campesterol falls during growth of the plant prior to the onset of flowering and that this trend is reversed after flowering, it is felt that the quantitative differences between spores and root material in all three cases are consistent with the hypothesis that the parasite draws upon the host for sterol during its early stages of development. This is based on the presumption that, since the plants from *S. alba* were not in flower, the percentage of campesterol in root of healthy plants would be expected to be lower than in spores which had utilized sterol from the plant and had also obtained them at an earlier stage of development; and also that, since *L. lyrata* and *M. maritima* had flowered prior to harvesting, the trend of sterol biosynthesis would have reversed and might have led (as was noted) to higher percentages of campesterol in the healthy plants than was noted in the spores.

The sterols of spores from *Brassica rapa* were both quantitatively and qualitatively appreciably different from the sterols in spores from other sources and from the root material of healthy plants. This infected material originated from commercially grown turnips at Cochno Farm, Hardgate, Dunbartonshire, the plants were much more mature before harvesting (*ca.* 6 months) and were probably older prior to infection compared with the age of healthy material grown in pots and harvested after 6 weeks for reference. In the light of the same general trend in sterol synthesis with growth in members of the Cruciferae[5] outlined above, this result also suggested that the parasite is drawing upon the host for sterols.

However, whilst the qualitative differences observed suggest that the parasite draws upon the host for its sterols, it must be pointed out that the original inoculum was probably a mixture rather than a single race of *Plasmodiophora brassicae* and the observed differences may arise from selection of different races by different hosts. Possibly also, the organism may synthesize sterols and respond rather differently to the external environment of the host cell. Since *P. brassicae* is an obligate parasite and cannot as yet be cultured other than in host cells of roots of Cruciferae, it is not possible to isolate and culture the organism in the presence of radioactive precursors. Further studies to determine more clearly the origin of sterols in *P. brassicae* therefore require a susceptible host plant in which the sterols are markedly different from those found in the species used in this work; such a species is as yet unknown.

EXPERIMENTAL

Seed of *Sinapis alba*, *Malcomia maritima* and *Lesquerella lyrata* were sown in trays of soil and were transplanted 1 week after emergence of the cotyledons. *S. alba* and *M. maritima* were transplanted into both clean and infected soil, the latter being obtained by manually mixing in washed spores at the rate of *ca.* 1×10^8/g soil and *L. lyrata* into a sand and peat mixture (1:1). Watering was performed with the modified Hoagland's solution previously described.[5] To obtain infected material from *L. lyrata*, pots and trays were watered once with a suspension of *Plasmodiophora brassicae* spores in water (*ca.* 1×10^8 spores/ml at 5 ml/plant). Harvesting was achieved by washing plants free of soil and root material separated. Healthy roots were air dried and *P. brassicae* infected roots were frozen and stored until required. Plants were harvested 8 weeks after transplanting for *L. lyrata* and *M. maritima* at which time both species were in flower. For *S. alba* plants were maintained for 5–6 weeks in a growth cabinet[5] under conditions of 20° with alternate periods of 8 hr illumination and 16 hr darkness, under which conditions growth is purely vegetative.

Spores of *P. brassicae* were obtained by macerating the frozen "clubs" in water and filtering the brei through three layers of muslin. The water suspension of spores was centrifuged at 2000 *g* and the supernatant discarded. The pellet was resuspended in water and recentrifuged several times to remove bacteria and other small particles contaminating the sample. Finally, spores were obtained free of starch grains by mechanically washing free the upper brown layer of spores from the pellet using a fine jet of water. They were then collected by centrifugation and transferred to a flask for hydrolysis. From *Brassica rapa*, spores were freeze dried and weighed prior to hydrolysis.

Hydrolysis. Spores were suspended in 6 N HCl (100 ml/g approx.), the aqueous layer covered with toluene (25% v/v) and the mixture boiled under reflux for 3 hr. After cooling, the toluene layer was separated, the acid layer extracted with ether (2 × ½ vols) and the organic layers combined, washed with water, $NaHCO_3$ solution (3 × ½ volumes of ½ saturated) and water. After drying (Na_2SO_4) the combined ether/toluene mixture was evaporated and sterols isolated by digitonin precipitation. Analysis by GLC was as previously described[5] using a 3% OV-17 column at 255°.

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