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ORIGIN OF STEROLS IN UREDOSPORES OF *UROMYCES PHASEOLI**

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Key Word Index—*Phaseolus vulgaris*; Leguminoseae; runner bean; *Uromyces phaseoli*; bean rust; fungi; sterols; sterol synthesis.

Abstract—The sterols from healthy bean leaves are β-sitosterol, stigmasterol, campesterol and 28-isofucosterol. An additional sterol observed in bean leaves infected with *Uromyces phaseoli* was identified as 7,(Z)-24(28)-stigmastadien-3β-ol, which is the major sterol of the uredospores of the fungus. The fungus appears to stimulate sterol synthesis, but most of the increased sterol content of infected leaves can be attributed to the sterol of the uredospores.

INTRODUCTION

IT HAS been suggested that some obligate parasitic fungi draw upon their host plants for at least part of their sterol requirements.[1,2] In previous studies we observed that germinating bean rust uredospores (*Uromyces phaseoli*) had the ability to synthesize sterols from simple precursors such as acetate, mevalonate and methionine[3] suggesting that the fungus might be capable of synthesizing sterols throughout its life cycle. Our approach to the question of whether the host plant or parasite was responsible for the synthesis of sterols found in uredospores produced by rust-infected plants was to identify the sterols in the host plant (Great Northern bean) and compare them with the sterols present in uredospores. This paper describes quantitative determinations of sterols during different stages of infection and also of sterol synthesis by uredospores from endogenous carbon sources.

RESULTS

Identification of sterols from bean leaves

The sterols were extracted from bean leaves and isolated by TLC. Acetylation followed by preparative TLC with $AgNO_3$ impregnated plates[3,4] gave three bands which were designated, in the order of their mobility as A, B and C (R_f, 0·65, 0·6 and 0·38, respectively). The sterol bands gave a blue color which developed slowly with the Liebermann-Burchard reagent and gave a negative Tortelli–Jaffé reaction indicating that they were Δ^5 sterols.[5,6]

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GLC on QF1 indicated the presence of two sterols in band A. After conversion to their TMS derivatives, the major sterol (85%) had the same R_t as β-sitosterol and the minor component (15%) had the same R_t as campesterol. The MS of recrystallized band A sterol acetates showed a weak peak for the M^+ of β-sitosterol acetate (M_s^+) at *m/e* 456. A peak corresponding with the M^+ of campesterol (M_c^+) at *m/e* 442 was also observed. The relative intensity of the peaks, *m/e* 396 ($M_s - 60$) and *m/e* 382 ($M_c - 60$), formed by the loss of acetic acid, were 100% and 21% respectively. Other peaks characteristic of these compounds,[7-11] were observed. The MS of band A sterols, after recrystallization also exhibited peaks characteristic of β-sitosterol and campesterol. The IR and NMR[12] spectra of the acetate derivatives were identical to authentic β-sitosterol.

GLC of the TMS derivative of band B sterol showed only one component on QF1 and OV17 and its R_t corresponded with that of stigmasterol TMS ether. The MS, IR[7,13] and NMR[12,14] spectra of band B sterol were also identical to those of stigmasterol.

Band C sterol also showed only one component by GLC on QF1 and OV17. The acetate derivative had a low R_f on $AgNO_3$-TLC suggesting that it could be fucosterol or 28-isofucosterol[15] (Δ^5-avenasterol) and GLC indicated that the sterol was probably 28-isofucosterol.[8] The MS of the acetate derivative did not exhibit a M^+ but an intense peak at *m/e* 296, characteristic of both fucosterol and 28-isofucosterol. However, the relative intensities of the ions at *m/e* 55, 296 and 394 indicated that the compound was 28-isofucosterol acetate.[8] As pointed out by van Aller *et al.*[16] fucosterol has never been isolated from plants that also contain a sterol with a 24-ethyl group such as β-sitosterol.

Identification of sterols of rust-infected bean leaves

Identical isolation and purification procedures for the sterols from rust-infected leaves (7-day infection) were used and after $AgNO_3$-TLC the acetylated sterols gave four bands. Three of the bands had R_f values identical to those obtained from the sterols of healthy leaves; the fourth band, designated band D, appeared between bands B and C and had a R_f value of 0·5.

Band D sterol gave reactions of a Δ^7-sterol with the Liebermann-Burchard[5] and Tortelli-Jaffe' tests.[6] GLC of the TMS ether derivative showed only one component that had the same R_t as the major sterol, 7-(Z)-24(28)-stigmastadien-3β-ol, isolated from bean rust uredospores.[3] The MS and IR spectra were also identical with that of this sterol.[3]

GLC, chemical tests, IR, MS and NMR showed that the sterols of band A, B and C were identical to those of healthy bean leaves.

The qualitative and quantitative determination of sterols of healthy and rust-infected leaves

Quantitative analyses of sterols at various stages of infection were performed together with those of healthy plants of an equivalent age. No significant differences in sterol con-

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tent (mg/g, dry wt) was observed between the healthy plants of different ages used for controls. The presence of 7,(Z)-24(28)-stigmastadien-3β-ol could not be detected until four days after inoculation of plants; then its concentration increased continuously over the next 6 days. Brown pustules began to appear 8 days after inoculation but at 7 days only white flecks were visible. After 10 days the plants were severely dehydrated and dying. The analyses shown in Table 1 are for 4 days after inoculation, the first day the fungal sterol was detectable and for 7 days after inoculation, the stage where the fungal sterol was high yet no uredospores were visible.

TABLE 1. QUANTITIES OF STEROLS IN HEALTHY AND RUST-INFECTED LEAVES

Source of extract	Days after inoculation	Total	(mg/g dry weight)* 1	2	3	4	5
Healthy leaves	4	1·97	0·89	0·79	0·18	0·11	ND†
Infected leaves	4	2·09	0·88	0·89	0·13	0·12	0·08
Healthy leaves	7	1·92	0·95	0·68	0·19	0·09	ND†
Infected leaves	7	3·88	1·23	0·87	0·24	0·11	1·42

* Total sterol content was determined gravimetrically and individual sterols were determined by GLC peak area.

† ND = not detected.

1 = β-Sitosterol; 2 = stigmasterol; 3 = campesterol; 4 = 28-isofucosterol; 5 = 7,(Z)-24(28)-stigmastadien-3β-ol.

The total sterol weight was calculated as 25·3% of the weight of the digitonide precipitate,[17] and the individual sterols were determined by GLC. The total weights of sterols extracted from bean leaves and rust-infected leaves were essentially the same on the 4th day. On the 7th day, the total weight of sterols extracted from infected leaves was about twice that of non-infected tissue and appeared to be related with the increase in fungal mass. Except for 7,(Z)-24(28)-stigmastadien-3β-ol there did not appear to be large differences in the levels of the other sterols between healthy leaves and rust-infected leaves either on the 4th or the 7th day.

In other experiments similar results were obtained although the level of 7,(Z)-24(28)-stigmastadien-3β-ol varied with the stage and the severity of infection.

Synthesis of sterols by uredospores

Previously we found that uredospores germinated on water with ^{14}C-labeled acetate and mevalonate would synthesize ^{14}C-sterols.[3] In the present work further experiments were performed to determine if sterol synthesis takes place in the absence of exogenous substrates. Uredospores, uniformly labeled with ^{14}C, were produced on bean plants, collected and then germinated on distilled water by procedures described by Trocha and Daly.[18] At 0, 3, 6, 9 and 12 hr after germination was initiated the levels of label in the sterol fraction were 1625, 2460, 3100, 2750 and 2450 dpm/mg spores, respectively. These

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data are the average of two separate experiments in which the maximum deviation of duplicates was 170 dpm/mg spores. Thus the quantity of sterols increased during the first 6 hr then decreased to a level that was still 1·5 times greater than the initial level during the next 6-hr period. During the 12-hr period ^{14}C in the total lipid extract decreased continuously from an initial level of about 70000 to 20000 dpm/mg spores. The loss of triglycerides and fatty acids during germination has been shown previously for ^{14}C-uredospores of *Puccinia graminis* f. sp. *tritici*.[19]

Since we have obtained cell-free preparations from uredospores that contain enzymes involved in sterol biosynthesis,[20] it appears that not only are uredospores capable of synthesizing sterols, they do so, actively, at the expense of endogenous carbon sources during the germination process.

DISCUSSION

From the examination of sterols in spores and infected plant tissue Knights[1] concluded that the club root fungus, *Plasmodiophora brassicae*, draws upon its host for the sterols found in its resting spores. Jackson and Frear[2] have also suggested that the sterols in the uredospores of *Milampsora lini* (flax rust) are obtained from the host plant. However, Nowak *et al.*[21] found that the major sterol of uredospores of *Puccinia graminis tritici* (wheat stem rust) was detected in only 1 of 11 samples of non-infected wheat leaves. Further they observed that the level of stigmast-7-enol appeared to be correlated with the amount of fungal tissue in the host when susceptible and resistant plants were compared. Our results also showed a marked difference between the sterols present in the healthy plant and in uredospores but sterol metabolism in these organisms must be somewhat different since different sterols are involved. Nevertheless in the cases where the sterols of the fungal spore are different from those of the host plant the possibility that the fungus draws its sterol requirements from the host can only be explained by a few hypotheses. A sterol intermediate could be produced by the host and utilized by the fungus in the synthesis of its sterols. Perhaps 4,4'-dimethyl-sterols could serve as an intermediate but we could not detect any 4,4'-dimethyl-sterols in our work. However, they are undoubtedly present in concentrations below our methods of detection. If a common intermediate sterol was involved, its transport from plant to fungal cells might be expected to result in higher levels than that observed for an intermediate involved in a single cell. It is also conceivable that the parasite could exert an overriding control on the types of sterols produced by the host.

For *U. phaseoli* the sterols of the uredospores and the host plant are different. The levels of sterols characteristic of the host are not altered greatly by fungal growth and the increase in sterol content of infected leaves before uredospore formation is primarily due to the synthesis of the major sterol of uredospores. Since uredospores contain the enzymes for synthesis of sterols from acetate, mevalonate and methionine[3] this genetic information must be present, although not necessarily expressed, during the particular stage of the fungus' life cycle on the bean plant. Thus we have concluded that fungal enzymes are probably responsible for the synthesis of sterols required by bean rust uredospores and that only simple substrates such as carbohydrates are drawn from the host to synthesize the more complex molecules needed for uredospore formation.

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EXPERIMENTAL

Plants. Seeds of Great Northern beans (*Phaseolus vulgaris* L.) were germinated in soil at about 27° for 7 days with illumination, then moved to a growth chamber at 20°. After 1 week in the growth chamber, half of the plants were inoculated with uredospores, *Uromyces phaseoli* (Pers.) Wint. Var. typica Arth. The rust-infected leaves were removed on the 4th and 7th day after inoculation and healthy leaves were also removed on the same day.

Isolation the sterols. The sterols of the bean leaves and of the rust-infected leaves were isolated and purified in the same way. Primary leaves (40 g) were cut into small strips and homogenized in 200 ml of MeOH–$CHCl_3$ (1:2). After removal of the debris by filtration the residue was washed 3 × with 200 ml of the same solvent. The combined extracts were washed with 50 ml H_2O and dried under a stream of N_2. Phospholipids were precipitated by Et_2O–$(Me)_2CO$ (1:5) and removed by filtration. The filtrate was evaporated and the residue obtained saponified by refluxing with 5% ethanolic KOH for 1 hr. The non-saponifiable fraction was extracted 3 × with Et_2O after the addition of 100 ml H_2O. The Et_2O fraction was washed with H_2O dried and evaporated to dryness under N_2. The digitonide ppt was prepared, washed and cleaved as described by Keller.[17] Chromatography of the recovered sterols was performed on preparative TLC[4] plates (Silica Gel G) using Et_2O–heptane–HOAc (70:30:1). The sterol bands were removed and extracted from the absorbent with Et_2O. The sterol content was converted to a dry weight basis by drying 5 g of fresh tissue to constant weight at 40°. Separate ratios were determined for samples of each type and age.

Analysis of ^{14}C-uredospores. For each sample, 20 mg of the labeled spores were placed on 20 ml H_2O and allowed to germinate for a prescribed period of time. The spores were removed, homogenized in $CHCl_3$–MeOH (2:1) and extracted twice with the same solvent. The total extract was reduced in vol and subjected to TLC using Et_2O–heptane–HOAc (70:30:1) and the sterol fraction isolated. The bands corresponding to the various other lipid fractions were also removed from the plates and eluted from the absorbent with Et_2O. An aliquot was taken for counting by liquid scintillation.

GLC was performed on a FID instrument using two columns (stainless steel 2 m × 8 mm); column I packed with 3% QF-1 on Anakrom ABS 130-140 mesh, operated at 233° with a flow rate of 60 ml per min, column II packed with 5% OV-17 on Anakrom AB; 70/80 mesh, operated at 270° with a flow rate of 60 ml per min. Relative proportions of sterols were determined by peak areas which were measured with a disc integrator.

NMR, MS and IR spectra were determined as previously described.[3] MS Band A sterols; *m/e* (rel. intensity) 414(M_s^+, 100%), 400(M_c^+, 23%), 399(M_s^+-Me, 33%), 385(M_c^+-Me, 14%), 396(M_s^+-H_2O, 65%), 382(M_c^+-H_2O, 21%), 381(M_s^+-H_2O–Me, 38%), 367(M_c^+-H_2O–Me, 8%), 329(M_s^+-H_2O–C_5H_7, 58%), 315(M_c^+-H_2O–C_5H_7, 13%), 303(M_s^+-H_2O–C_7N_9, 77%), 289(M_c^+-H_2O–C_7H_9, 23%), 275(M_s^+-H_2O–C_9H_{13}, 30%), 261(M_c^+-H_2O–C_9H_{13}, 9%), 273(M_s^+ or M_c^+-side chain, 54%), 255(M_s^+ or M_c^+-side chain–H_2O, 66%), 213(M_s^+ or M_c^+-side chain–H_2O–42, 90%). MS Band and A steryl acetates; *m/e* 396(M_s^+-HOAc, 100%), 382(M_c^+-HOAc, 21%), 381(M_s^+-Me–HOAc, 10%), 367(M_c^+-Me–HOAc, 3%), 255(M_s^+ or M_c^+-side chain–HOAc, 14%), 213(M_s^+ or M_c^+-side chain–42–HOAc, 9%). Band B sterol; *m/e* 412(M^+, 100%), 397(M^+-Me, 9%), 394(M^+-H_2O, 23%), 379(M^+-Me–H_2O, 9%), 369(M^+-43, 20%), 351(M^+-43–H_2O, 29%), 299(M^+-Me–C_{23} to C_{27}–H, 14%), 273(M^+-side chain, 22%), 271(M^+-side chain–2H, 61%), 255(M^+-side chain–H_2O, 88%), 253(M^+-side chain–H_2O–2H, 15%), 231(M^+-side chain–42, 16%), 229(M^+-side chain–27–OH, 18%), 213(M^+-side chain–42–H_2O, 36%). MS Band B steryl acetate; *m/e* 394(M^+-HOAc, 100%), 351(M^+-43–HOAc, 21%), 255(M^+-side chain–HOAc, 74%), 253(M^+-side chain–HOAc–2H, 22%), 213(M^+-side chain–HOAc–42, 20%). MS Band C steryl acetate; *m/e* 394(M^+-HOAc, 28%), 379(M^+-Me–HOAc, 3%), 296(M-C_7H_{14}–HOAc, 100%), 281(296–Me, 22%), 253(M^+-side chain–HOAc–2H, 16%), 228(M^+-side chain–HOAc–27, 13%), 213(M^+-side chain–HOAc–42, 18%), and 55(C_4H_7, 40%). NMR Band A steryl acetates, δ 0·68 (*s*, C-18 proton), 0·85 (*d*, *J* 6·8 Hz, C-26 and C-27 protons, 0·86 (*t*, *J* 6·3 Hz, C-29 protons, 0·93 (*d*, *J* 6·1 Hz, C-21 proton), 1·01 (*s*, C-19 proton), 2·09 (*s*, acetyl). NMR Band B steryl acetate, δ 0·69 (*s*, C-18 proton), 0·83 (*t*, *J* 5·7 Hz, C-29 proton), 0·86 (*d*, *J* 6·4 Hz, C-26 and C-27 protons), 1·01 (*s*, C-19 proton), 1·03 (*d*, *J* 5·5 Hz, C-21 proton), 2·08 (*s*, acetyl).

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