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A REASSESSMENT OF THE ROLE OF STEROIDAL ALKALOIDS IN THE PHYSIOLOGY OF *PHYTOPHTHORA*

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Abstract—*Phytophthora cactorum* was incubated *in vitro* with solasodine, [^{3}H] solanidine, and [^{3}H] solanine in the presence and absence of an equal weight of [^{14}C] cholesterol. The extent to which the two genins and the glycoalkaloid inhibited sterol-induced sexual reproduction was dependent primarily on the extent to which the fungus assimilated the steroidal alkaloids Cholesterol increased the uptake of solasodine and [^{3}H] solanidine by the mycelium, but the genins affected neither cholesterol-induced growth responses, measured as radial diameter or dry wt, nor the accumulation of [^{14}C] cholesterol, nor its conversion to esters and glycosides The genins were not converted to esters or glycosides, but [^{3}H] solanine was hydrolysed to solanidine Solanine was not significantly assimilated by the fungus

INTRODUCTION

Studies on the effect of steroidal alkaloids on fungal growth *in vitro* [1–7] and *in vivo* [8–11] have led to the hypothesis [8, 12] that fungitoxicity, usually measured as a decrease in radial diameter of mycelia cultured on agar media, is due to complex formation between the glycoalkaloid and mycelial membrane sterol Levels required for fungitoxicity are reported to be $< 60\,\mu$g/ml of medium or tissue [11] However, no evidence was produced to show that fungi are capable of incorporating the unaltered glycoalkaloid into the mycelium (much less into the membrane systems)

We have recently examined the effects of the principal glycoalkaloids and genins of *Veratrum* and *Solanum* on the growth, measured as radial diameter (i e hyphal extension), and sitosterol-induced sexual reproduction, as determined by counting the number of oospores, of *Phytophthora cactorum* [1] While the alkaloids completely, or almost completely, abolished sterol-induced oospore production at levels of $> 15\,\mu$g/ml, fungal growth was generally not affected In those treatments where some fungistatic action occurred, no correlation between alkaloid structure and fungitoxicity was apparent The aim of the present study was to determine the ability of pythiaceous fungi to discriminate between sterols and steroidal alkaloids in terms of their uptake and effects on growth and reproduction

RESULTS

The radial diameter and dry wt of *P cactorum* are increased by cholesterol (Table 1) In the absence of cholesterol, solasodine inhibits growth, as measured by radial diameter, but not as measured by dry wt In the presence of cholesterol, solasodine has no significant effect on growth but decreases oospore production The neutral lipid fraction of the mycelia cultured on cholesterol, solasodine and equal amounts of cholesterol and solasodine was analysed by GC without hydrolysis, because only minor quantities of sterol could be obtained by hydrolysis in preliminary experiments Moreover, the free sterol is the principal molecular form incorporated into the mycelial membranes of pythiaceous fungi [13–15] While the total amount of free cholesterol accumulated by the mycelia was unaltered by solasodine addition to the medium, cholesterol addition increased the solasodine content The ratio of free cholesterol–solasodine in the mycelium was *ca* 3 1 when the two compounds were added to the same culture at 1 μg/ml of each, but *ca* 10 1 when each compound was added to a separate culture Incubation of *P cactorum* with [^{14}C] squalene ($1\,5 \times 10^5$ cpm/250 μg) for 3 weeks showed, as expected [16], no synthesis of Δ^5-sterols or steroidal alkaloids from this precursor

Solasodine and solanidine are unable to affect cholesterol uptake, as shown by incubation experiments with [^{14}C] cholesterol (Table 2) Moreover, they had only a minor effect on the conversion of cholesterol to esters and no significant effect on its conversion to glycosides After incubation of *P cactorum* with [^{3}H] solanidine in the presence and absence of cholesterol, the neutral lipid fraction was analysed by TLC with solvent system 1 followed by solvent system 2, and then scanned Only one significant radioactive zone was detected, corresponding to solanidine The zone corresponding to solanidine (R_f 0 3) and the chromatographic origin (which should contain the glycoalkaloids) were eluted from the plate and analysed in a liquid scintillation counter The solanidine band contained most of the radioactivity The eluted solanidine was further chromatographed in solvent system 3 Again the radioactivity was found to correspond to solanidine (R_f 0 63) The zone, eluted from the second chromatogram, analysed by GC, was indistinguishable from authentic solanidine (RR_t, 1 1) [^{3}H] Solanine was not significantly incorporated into the mycelium either in the presence or absence of cholesterol When the mycelium was examined for hydrolytic products of solanine by TLC and scanning, only labeled solanidine was detected In [^{3}H] solanine treatment, the ratio

Table 1 Steroid-induced physiological properties and incorporation of free solasodine and cholesterol by *Phytophthora cactorum*

Expt No	Treatment	% dry wt*	Dry wt† (mg)	Radial diameter‡	No of oospores produced§
1	Cholesterol	0 038	140	41 8	384
2		0 028	134	40 6	356
1	Cholesterol and solasodine	0 038‖ 0 026‖	122	41 5	200
2		0 011¶ 0 016¶	115	40 0	142
1	Solasodine	0 002¶	100	29 0	0
2		0 003¶	98	25 0	0
1	Control 1	N D**	115	38 0	0
			110	33 5	0
1	Control 2	N D **	74	36 0	0
2			64	35 0	0

*Cultures were incubated with 10 μg/ml cholesterol and/or 10 μg/ml solasodine on a synthetic medium for 3 weeks at 20° in the dark

†Combined mycelia of five Petri plates representing one steroid treatment Cultures were harvested and dried after the oospores were counted, i e after 21 days

‡Mean radial diameter of five Petri plates Deviation from the mean rarely exceeded 2 %

§Mean number of oospores per transect per Petri plate Cultures were incubated for 21 days Deviation from the mean did not exceed 10 %

‖Cholesterol content

¶Steroidal alkaloid content

**N D represents not detected, e g sterols or solasodine, at a level of < 0 001 % Each flask contained 10 μg/ml ethanol (control 1) or no ethanol (control 2)

Table 2 Incorporation and conversion to esters and glycosides of cholesterol and steroidal alkaloids by *Phytophthora cactorum*

Treatment	% incorporation of [^{14}C]- or [^{3}H]-labeled material obtained from the acetone extract of the mycelium	Proportions of radio activity on TLC plate		
		SG§	FS	SE
[^{14}C]Cholesterol*	61 2	2	72	26
[^{14}C]Cholesterol and solasodine*	54 2	2	89	9
[^{14}C]Cholesterol and solanidine*	60 0	2	79	19
[3H]Solanidine and cholesterol†	65 0	0	100	0
[^{3}H]Solanine and cholesterol‡	2 5	67	33	0
[^{3}H]Solanidine†	38 0	0	100	0
[^{3}H]Solanine‡	1 4	50	50	0

*Each Petri plate was incubated with *ca* 10 μg [^{14}C]cholesterol (290 000 cpm), 40 μg carrier cholesterol and, in two treatments, 50 μg of steroidal alkaloid

†Each Petri plate was incubated with *ca* 10 μg [^{3}H]solanidine (124 000 cpm), 40 μg carrier solanidine, and/or 50 μg cholesterol

‡Each Petri plate was incubated *ca* 10 μg [^{3}H]solanine (123 000 cpm), 40 μg carrier solanine, and/or 50 μg cholesterol

§SG represents steryl glycosides or steroidal glycoalkaloids FS and SE represent the free and esterified products, respectively Extracts of cultures incubated with [^{14}C]cholesterol were chromatographed with solvent system 1, the [^{3}H]steroidal alkaloids were chromatographed with solvent system 1 followed by solvent system 3

solanine–solanidine in the mycelium was *ca* 1 1 (cf Table 2) and in the medium *ca* 5 1

We have previously observed that solanine, solanidine and solasodine [1] inhibit sterol-induced reproduction However, in the present study solanine had no effect on cholesterol-induced oospore production (data not shown) This is probably the result of using a lower incubation temperature In the earlier study, cultures were incubated at 25° while in the present study the temperature was 20° We observed no change in the pH (4 6) of the medium during incubation Thus any activity of glycosidases was probably lower in the present experiments

DISCUSSION

The results of the present and earlier [1] experiments show that (1) solanine is not significantly incorporated into the fungal mycelium, (2) solanidine, which is assimilated by the fungus, is not converted to the glycoside, (3) measurements of radial growth may not be an appropriate estimate of fungitoxicity, (4) compounds which do not alter the dry wt of the fungus may still significantly affect sexual reproduction of the fungus, (5) the uptake of cholesterol and its conversion to esters and glycosides is not correlated with the physiological effects resulting from incubation with steroidal alkaloids, (6) the fungicidal action of solasodine is not correlated with pH, and (7) steroidal glycoalkaloids were not synthesized *de novo* by *Phytophthora* as suggested by others [17] Although steroidal alkaloids inhibit sterol-induced oospore production, they do not necessarily inhibit growth, as measured by dry wt or hyphal extension (cf ref [1] and Table 1) The lack of fungitoxicity in *Phytophthora* by glycoalkaloids is not due to the absence of mycelial membrane sterol [2], but rather to the inability of this pythiaceous fungus to assimilate significant amounts of the glycoalkaloid Our results with *P cactorum* agree with the recent finding that *P infestans* [18] incorporates significant amounts of a nutritional supplement of solanidine but not solanine In studies on the fungitoxicity and membrane damaging action of various saponins and glycoalkaloids on mycelia of sterol-synthesizing fungi [19], cell membrane disruption was correlated with the extent of enzymatic conversion of the glycosides to the corresponding aglycone

Temperature and pH are known to affect the activity of glycosidases [19–21], as well as the binding capacity of sterol with the glycoalkaloid [4, 6, 8] Thus differences (radial diameter but not necessarily dry wt) in the fungicidal action of glycoalkaloids [8, 11], which are apparent during host–parasite interactions, may depend as much on the environmental conditions which could affect saponin hydrolysis as on the complex formation between the assimilated sterol and glycoalkaloid This implies that resistance to *Phytophthora* cannot be correlated simply with the content of glycoalkaloid synthesized by tracheophytes While it is not unreasonable to believe that excess amounts of glycoalkaloid in the culture medium combine with sterols added to the medium, this would simply decrease the amount of sterol in the medium available for assimilation The reduction in available sterol would affect growth rates of pythiaceous fungi Alternatively, the reduction in growth and lowering of sterol content in sterol synthesizing fungi observed by others [8, 11, 22], may also be attributed to the amount of genin formed after hydrolysis and its subsequent uptake by the fungus Thus the mode of action of steroidal alkaloids, which is dependent on their uptake and deposition into mycelial membranes, may be related to their effects on sterol synthesis or alter membrane function through interaction with proteins In support of this view, various synthetic and naturally occurring fungal azasterols have been shown to inhibit sterol synthesis and metabolism in yeast and insects and uncouple oxidative phosphorylation [23–25]

EXPERIMENTAL

Cholesterol was obtained from Applied Science Lab Solasodine, solanidine and solanine were obtained from the steroid reference collection (D F Johnson, NIH) [^{14}C] Cholesterol was purchased from Amersham [^{13}H] Solanine and [^{3}H] solanidine were gifts from M R Gumbmann The labeled and non-labeled sterols and steroidal alkaloids were purified by TLC on Si gel [solvent system 1, C_6H_6–Et_2O (9 1), solvent system 2, $CHCl_3$–MeOH (9 1), solvent system 3, EtOH–HOAc (19 1)] This was followed by reverse-phase HPLC on a column of Zorbax BP-ODS with 96% aq MeOH and 0 01% NH_4OH GC was performed at 260° on 3% SE-30 Cholesterol and solasodine were quantitated from a calibration curve of cholesterol concn vs peak height RR_t (cholesterol) solasodine, 1 86, solanidine, 1 10

The pH of the agar medium was measured for control, cholesterol and solasodine treatments with a Beckman Model 3550 digital pH meter equipped with a flat-surface electrode (pH 2000) The pH of the liquid medium before addition of agar was 4 6 After incubation for 3 weeks, the electrode was placed on the surface of the agar with slight constant pressure The pH reading slowly decreased over a period of 2 hr after which it remained constant at 4 6 in every case

The methods for culturing *P cactorum* on agar medium, the method for counting oospores, and for isolating and identifying radioactive and nonradioactive steroids were as described earlier [1, 26], with two principal differences The amount of EtOH used to solubilize the steroids was 10 μl/ml rather than 2 μl/ml Cultures incubated with labeled substrates were extracted in a Soxhlet apparatus with Me_2CO overnight, while mycelia with nonlabeled steroids were harvested, dried under vacuum in an abderhalden apparatus overnight and then weighed The dried material was ground to a powder and extracted with Me_2CO in the usual manner The medium for the [^{3}H]solanine treatment was extracted with BuOH and hexane according to ref [26] The neutral lipid was chromatographed as before and developed in solvent system 3

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