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Autor(en): **Stössel, Peter / Hohl, Hans R.**

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Sterols in *Phytophthora infestans* and their role in the parasitic interactions with *Solanum tuberosum*

by *Peter Stössel* and *Hans R. Hohl*

Cytological Laboratory, Institute of Plant Biology, University of Zürich

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Working with *Phytophthora infestans* on *Solanum tuberosum Hohl* and *Suter* (1976) found abundant sporangia on infected leaves of the susceptible cultivar Bintje but few or none on the resistant cultivar Eba. Since fungi are primarily distributed by their spores, lack of sporulation must be considered a very important factor of resistance.

It is well documented that sterols induce reproduction in pythiaceous fungi including *Phytophthora* (*Hendrix* 1964, 1965, 1970, *Elliott* 1977). These fungi are not capable of synthesizing sterols but must obtain them from their environment (e.g. *McCorkindale* et al. 1969, *Gottlieb* et al. 1978, *Schlösser* and *Gottlieb* 1966, *Weete* 1973). For these reasons we investigated sterol content and composition of a resistant and a susceptible potato cultivar, since variations could severely affect sporulation and might, therefore, represent factors of resistance against *Phytophthora infestans*. Studies were restricted to sterols with a free 3 β-hydroxy group, a double bond in ring B (Δ 5) and an aliphatic side chain with 8 to 10 carbon atoms, for these molecules have been found to be more active in inducing sporangia and oospores in pythiaceous fungi than others such as ergosterol, lanosterol, 7-dehydrocholesterol or cholestan (*Elliott* 1972, *Hendrix* 1974, *Langcake* 1974).

We also reinvestigated the proposition by *Elliott* and *Knights* (1969) about a positive correlation between cycloartenol: β-sitosterol ratio and field resistance. This relationship could not be confirmed by *Langcake* (1974). Furthermore, a series of experiments was performed to gain information about the influence of fungal nutrition, temperature and light on uptake and esterification of sterols and their effect on growth and reproduction in *P. infestans*.

Material and Methods

Phytophthora infestans Mont. (de Bary) strain S (race 4) was grown on either kidney bean (30 g homogenized beans per liter of distilled water) or P-1 medium (*Hohl* 1975) containing 10 g of sucrose per liter instead of the original 30 g per liter. P-1L (*Hohl* 1975) denotes the P-1 medium to which 200 mg per liter of lecithin (Sigma) has been added. The two modifications of P-1, namely P-1 mod A and P-1 mod B have various amounts of cholesterol (Sigma), 7-dehydrocholesterol (Sigma, pfs), stigmasterol (Merck) and β-sitosterol (Sigma, practical grade) added as indicated in Table 5. The 40 mg of sterols/1 medium were gaschromatographically analysed for their exact composition. The kidney bean medium contains about 18 mg/l of sterols as determined by gas chromatography.

To measure hyphal growth, the fungus was grown in 50 ml medium / 250 ml Erlenmeyer flask at either 16 or 24° C in total darkness or continuous light (‚Gallenkamp' incubator, mod. Compenstat). Sporangia were produced in cultures grown on the same media with 1.5% agar added and incubated at 16° C and 100% rel. humidity (*Croisier* 1933).

Tubers of *Solanum tuberosum* L. cultivar Bintje (r) and Eba (R3) were allowed to germinate at room temperature, then planted in soil and kept in 14 h light/day. The upper leaves from 3 week-old plants were used for experiments. According to a scale based on infection rates under natural field conditions ranging from 1 to 9 (1 denoting highest resistance, 9 highest susceptibility). leaves of Bintje and Eba were graded 5 and 2, respectively [1]).

Sporangia from 10 days-old fungal cultures on kidney bean agar were collected in sterile water and allowed to germinate for 3 h at 4° C. Droplets of the zoospore suspension (10^5/ml) were spread on the lower side of the leaves and the control treated likewise with sterile water. After 70 h of incubation at 21° and 100% rel. humidity, the leaves were homogenized and extracted in boiling chloroform-methanol (2:1) for 1 h (*Dupéron* et al. 1971), adding 5 α-cholestan or cholesteryl-butyrate (Supelco) as an internal standard. To hydrolyse esterified sterols, the extracts were concentrated and then boiled for 1 h under nitrogen in 90% methanol containing 10% KOH (*Dupéron* et al. 1971). Distilled water was added and the neutral lipids extracted with petroleum ether (*Langcake* 1974), concentrated under reduced pressure and chromatographed on silica gel thin layer plates (Merck) developed with diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2 v/v; *Freeman* and *West* 1966). According to the method of *Skipski* and *Barclay* (1969), cochromatographed references were detected with $SbCl_3$ in chloroform, the sterol bands scraped off, eluted in chloroform-methanol (1:1), concentrated and then allowed to react with BSTFA (Bis(trimethylsilyl)-trifluoroacetamide, Merck) at 60° C. Prior to extraction, the fungal mycelium and sporangia were rinsed with distilled water and dried overnight at 60° C. The amount of sterols adsorbed by the hyphae was determined by briefly dipping sterol-free mycelium into sterol-containing medium and was subtracted from the total.

Extracts were analysed in a gaschromatograph ‚Fractovap 2101' (Carlo Erba, Milano) equipped with flame ionization detector, a glass capillary column (length 20 m, diameter 0.34 mm) coated with 1% OV-101 (manufactured by H. Jaeggi, Trogen, Switzerland), and hydrogen as a carrier with a flow rate of 5 ml/min. The samples were directly injected (*Grob* and *Grob* 1972) at 250° C injector and 25° C oven temperature and analysed under the following conditions: 180° C isotherm for 3 min, plus 8° C/min up to 240° C isotherm. Cholesterol, 7-dehydrocholesterol, stigmasterol and β-sitosterol were identified using chromatographic standards (Sigma, Applied Science Lab., Supelco), cycloartenol by comparison with *Langcake's* (1974) results [2]).

[1]) The authors thank Dr. F. Winiger, Eidg. Forschungsanstalt für landwirtschaftlichen Pflanzenbau, Zürich-Reckenholz, for providing potato tubers as well as information concerning field resistance of the various cultivars.

[2]) According to *Knights* (1973), the silicons OV-1 and OV-101 give comparable results.

Results

Sterols in leaves of two cultivars of Solanum tuberosum

The gaschromatographic analyses showed β-sitosterol, stigmasterol, cholesterol, campesterol and cycloartenol as the major components of the sterol fraction from uninfected, fresh leaves of Bintje (r) as well as Eba (R3). In both cultivars β-sitosterol showed the highest concentration of the four (Table 1). The total of all identified free and esterified sterols in leaves was 0.21 mg/g dry wt. in the cultivar Bintje and 0.23 mg/g in Eba.

Infected leaves still contained the same major sterols analysed in fresh leaves but they contained more β-sitosterol, less stigmasterol and little less cholesterol and campesterol respectively (Table 1). Uninfected, but similarly treated controls showed very similar alterations (Table 1), indicating that the changes are due to ageing of the tissue and not to the presence of the parasite.

Relative amounts of cycloartenol and β-sitosterol in resistant and susceptible tissues were also investigated. The results showed that leaves of the susceptible cultivar Bintje contained half as much cycloartenol as β-sitosterol (ratio: 0.53) whereas leaves of Eba with a higher field resistance contained significantly less (ratio: 0.38).

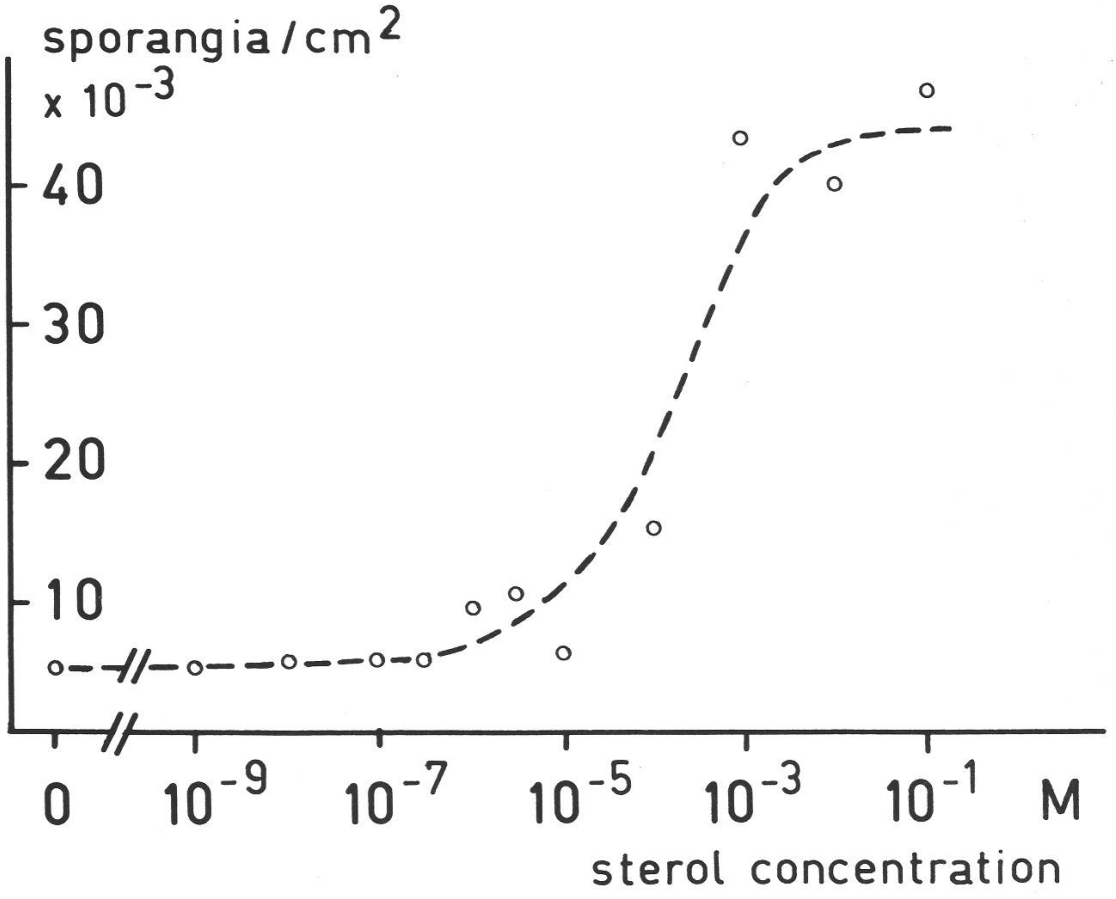

Fig. 1:
Number of sporangia formed by *P. infestans* grown for 15 d on P-1L medium containing varying amounts of stigmasterol and β-sitosterol (1:2 w/w).

Table 1:

The four major 4-desmethyl sterols in fresh, in detached (control) and in infected leaves of the two cultivars Bintje (susceptible) and Eba (resistant) after alkaline hydrolysis, expressed as percentage of total sterols. Infected and control leaves were incubated for 70 h at 21°C and 100% relative humidity.

Leaf Sample	Cultivar	Cholesterol	Campesterol	Stigmasterol	β-Sitosterol
Fresh	Bintje	17	5	31	47
	Eba	9	7	28	56
Infected	Bintje	9	6	23	62
	Eba	8	4	18	70
Control	Bintje	12	5	23	60
	Eba	8	5	17	70

Table 2:

Accumulation of cholesterol in the mycelium of *P. infestans* following various periods of incubation at 16°C and continuous illumination in 50 ml of P-1 medium (10 g of sucrose/liter) containing 2 mg of cholesterol. Values obtained after alkaline hydrolysis.

Incubation time in weeks	Mycelial dry weight in mg	Total cholesterol absorbed in µg	µg cholesterol per mg mycelial dry weight
1	2	20	10.0
2	30	180	6.0
3	52	250	4.8
4	80	260	3.3
5	88	380	4.3
6	94	300	3.2

Table 3:

Concentration of cholesterol and cholesterylbutyrate in the mycelium of *P. infestans* following growth at 16°C and continuous illumination in 50 ml of P-1 medium (10 g of sucrose/liter) containing 2 mg of purified (thin-layer chromatography) cholesterylbutyrate without gaschromatographically detectable cholesterol.

Incubation time in weeks	Cholesteryl-butyrate in µg/mg dry wt.	Concentration of cholesterol	
		in µg/mg dry wt.	in % of total
2	8.9	0.2	2.7
5	1.0	0.05	5.9

Accumulation of extracellular sterols in the mycelium

Uptake of cholesterol by the mycelium was measured during a six week growth period in the P-1 medium containing 40 mg/liter of cholesterol. As shown in Table 2, the highest amount of cholesterol accumulated relative to mycelial dry weight was obtained after 1 week, from where the values steadily decreased.

P. infestans also absorbed a sterol ester (Table 3). After feeding cholesteryl butyrate most of the absorbed sterol remained in the ester form. With prolonged incubation the proportion of free sterol slightly increased.

To determine the degree of selectivity of sterol uptake, the fungus was cultivated on P-1 medium containing cholesterol, 7-dehydrocholesterol, campesterol, stigmasterol and β-sitosterol (a total of 40 mg sterol/liter, in two combinations, see Table 5; concentrations of individual sterols determined by gas chromatography) and in the kidney bean medium containing its own sterols (determined gaschromatographically, see Table 5). The concentration of the sterols in the fungus material was determined after 6 weeks and 10 d, respectively, from cultures kept at 16 or 24°C in the dark or under continuous light. The results are given in Tables 4 and 5.

The following points emerge:

(1) Uptake of sterols from synthetic but not from natural medium is strongly affected by light and temperature. Continuous light reduced and higher temperatures usually increased the uptake (Table 4).

Table 4:

Free and esterified sterols in the mycelium of *P. infestans* grown under various temperature and light conditions in 50 ml of three different media.

L = cultures illuminated, D = cultures kept in darkness.

Medium	Incubation		µg sterols per mg myc. dry weight	Percentage of free sterols	Percentage of esterified sterols
P-1 mod A*	6 wks at 16°C	L	0.8	82	18
		D	1.4	30	70
	24°C	L	1.2	65	35
		D	1.9	73	27
P-1 mod B*	6 wks at 16°C	L	2.0	73	27
		D	3.2	30	70
	24°C	L	1.0	65	35
		D	3.4	76	24
Kidney beans*	10 d at 16°C	L	0.62	92	8
		D	0.67	79	21
	24°C	L	0.63	97	3
		D	0.67	92	8

* For concentration of sterols see corresponding lines in Tab. 5.

(2) Neither light nor temperature had any selective influence on uptake of individual sterols under the conditions tested (Table 5). With the exception of 7-dehydrocholesterol the rates of the various sterols taken up by the fungus generally reflected their extracellular supply. 7-dehydrocholesterol was absorbed in small amounts only.

(3) The metabolism of the sterols was affected by light and temperature conditions (Table 4). At 16°C the amount of esterified sterols was higher in darkness than in continuous light, attaining values up to 70% of the total intracellular sterols. At 24°C this relationship was reversed.

(4) Though kidney beans merely contain traces of cholesterol, this compound accounted for 6–12% of all sterols absorbed by the mycelium (Table 5).

Table 5:

Cholesterol, 7-dehydrocholesterol, campesterol, stigmasterol and β-sitosterol concentrations in % of total sterol content (after alkaline hydrolysis) of mycelia of *P. infestans* following growth for 6 weeks in three different media. The cultures were kept at 16 and 24°C, respectively, and incubated in the light (L) or in the dark (D).

Medium	Incubation Temp. (°C)	Light condition	Cholesterol	7-dehydro-cholesterol	Campesterol	Stigmasterol	β-sitosterol
P-1 mod A			(40)	(8)	(7)	(34)	(11)
	16	L	40	4	9	34	13
	16	D	43	4	8	33	12
	24	L	43	3	8	33	13
	24	D	41	4	8	35	12
P-1 mod B			(22)	(18)	(16)	(20)	(24)
	16	L	26	10	18	21	25
	16	D	26	11	17	20	26
	24	L	25	6	18	22	29
	24	D	29	8	17	21	25
Kidney beans			(1)	nd[1)]	(6)	(32)	(61)
	16	L	6	nd	5	30	59
	16	D	8	nd	5	29	58
	24	L	12	nd	6	28	54
	24	D	12	nd	5	28	55

Values in brackets refer to sterol compositions of fresh media, also gaschromatographically analysed.

1) not detectable

Table 6:
Growth of *P. infestans* under various conditions in P-1 medium (10 g of sucrose/liter) with no (control) or added sterols (P-1 mod A and P-1 mod B, see Table 5 for sterol composition). Incubation time was 6 weeks, temperatures 16 or 24°C. The cultures were incubated in the light (L) or in darkness (D). Values are expressed as mg mycelial dry weight/50 ml of medium.

Medium	Mycelial dry weight (mg) after growth at 16°C		24°C	
	Light	Darkness	Light	Darkness
Control	77	128	71	95
P-1 mod A*	63	104	88	86
P-1 mod B*	59	98	90	89

* For sterol composition see corresponding lines in Tab. 5.

Effect of sterols on hyphal growth

Without any sterol supplement, the fungal growth on the defined medium is markedly affected by light and temperature conditions. Total darkness and the lower temperature of 16°C increased the growth rate (Table 6). Sterols only increased hyphal growth under suboptimal conditions such as continuous light combined with a temperature of 24°C (Table 6). Sterols extracted from 3.5 g fr. wt. of leaves of either potato cultivar and added to 10 ml of medium P-1 increased the linear growth rate of *P. infestans* at 24°C by 8% only.

Accumulation of sterols in sporangia

Grown on the kidney bean medium, the sporangia accumulated almost as much sterol as 10 day-old mycelium, i.e. 0.7 µg/mg dry wt. By comparison, cultures on the defined medium accumulated only about 0.2 µg/mg dry wt. in their sporangia. Whereas all the 4-desmethylsterol concentrations measured in the mycelium correlated well with their extracellular supply, the sporangia contained a proportionately greater amount of cholesterol (Table 7). Again 7-dehydrocholesterol was found in small quantities only.

Effect of sterols on the production of sporangia

In this experiment the two major sterols in potato leaves, stigmasterol and β-sitosterol (both slightly contaminated with cholesterol and campesterol), were used. As shown in Figure 1, little sporulation occurred in the absence of sterols but a concentration as small as 10^{-6} to 10^{-5} M was sufficient to measurably improve formation of sporangia. Sterols from potato leaves of Bintje or Eba added to the P-1 medium (extract from 1 g fresh weight of leaves per ml of medium) induced 1.2×10^4 and 1×10^4 sporangia/cm^2, respectively.

Table 7:

Sterol composition (after alkaline hydrolysis) of sporangia of *P. infestans* produced during 20 days of growth at 16°C in continuous light on three different media. The results are given as % of total sterol concentration with corresponding values of sterols present in the fresh medium provided in brackets ().

Medium	Cholesterol	7-dehydro-cholesterol	Campesterol	Stigmasterol	β-sitosterol	sporangia per cm²	(%) indirect germination
P-1 mod A	68 (40)	2 (8)	6 (7)	13 (34)	11 (11)	8500	20
P-1 mod B	50 (22)	5 (18)	18 (16)	10 (20)	17 (24)	14500	20
Kidney bean	7 (1)	nd[1] (nd)	5 (6)	30 (32)	58 (61)	7000	70

1) not detectable

Discussion

In leaves of the resistant and the susceptible cultivar the total amount of the morphogenetically active 4-desmethylsterols reached about 0.02% of the dry weight which is about twice that found in tuber tissue by *Dupéron* et al. (1971). Despite of minor differences in the concentration of individual sterols, lack of sporulation and reduced growth of *P. infestans* on the resistant host tissue can hardly be explained solely by differences in supply of host sterols since it has been shown (e.g. *Hendrix* 1970, *Langcake* 1974) that above a certain minimum concentration of total sterols sporulation is not decisively affected by concentration changes of individual sterols in a mixture. According to *Hendrix* (1970) optimal reproduction in vitro is obtained with sterol concentrations from 10^{-5} to 10^{-4} M. The concentrations of sterols found in our potato cultivars (appr. 5×10^{-5} M) fall well within this range. Furthermore, sterols extracted from Eba and Bintje promote growth and sporulation of *P. infestans* to a comparable degree. In essence, then, our results confirm and extend the conclusions of *Langcake* (1974) that sterols are not likely to be primarily responsible for the differential sensitivity of potato cultivars towards *Phytophthora.*

The suggested correlation between ratio of cycloartenol: β-sitosterol and field resistance (*Elliot* and *Knights* 1969) could not be substantiated by *Langcake* (1974). Using different cultivars, our results also show a reverse relationship. Thus, the possible importance of this ratio in the disease remains unclear.

Saponins like the steroidglycoalkaloids solanine and chaconine interfere with sterols in fungal mycelia (*Schlösser* 1972). While this interaction might be important in some host-parasite systems (*Wolters* 1968, *Défago* et al. 1975, *Lüning* et al. 1978), in potatoes no correlation between resistance against *P. infestans* and glycoalkaloid content could be established (*Deahl* et al. 1973, *Frank* et al. 1975). It is also unlikely that there are pronounced differences in accessibility of sterols in various cultivars

since sterols generally occur in cytoplasmic membranes. The simplest explanation for the parasite's inability to sporulate on the resistant host would be that there is too little mycelium formed to permit sporulation. Observations (*Hohl* and *Hächler*, unpublished) that some hyphal growth does occur in the resistant tuber tissue and that this amount can be increased considerably by raising inoculum density without affecting sporulation, however, points to a specific inhibition of sporulation in the resistant cultivar.

The decrease in the concentration of sterols in ageing mycelium (see also *Knights* and *Elliott* 1976) is more likely due to an increased proportion of wall material relative to cytoplasm than to reduced uptake of sterols. *Langcake* (1975) found that *P. infestans* takes up more cholesterol than stigmasterol. This result was confirmed using a natural substrate but not with the defined medium P-1, where we found a pronounced correlation between uptake and supply for both compounds. The fact that *P. infestans* takes up sterol esters and splits the ester bond parallels similar observations made by *Knights* and *Elliott* (1976) with *P. cactorum*. The demonstrated varying amounts of sterols esters formed in the mycelium of several *Pythium* and *Phytophthora* species (*Hendrix* 1975), might be influenced also by temperature and light conditions.

The effect of sterols on hyphal growth of various pythiaceous fungi has already been intensively investigated (*Hendrix* 1964, 1970, *Hohl* 1975, *Langcake* 1975). Our results indicate that the effect of sterols is noticed best under suboptimal growth conditions such as continuous light and elevated temperature. Furthermore, the fact that growth promoting activity of sterols is temperature and light dependent might help to explain some inconsistencies in reported effects of sterols on these fungi (*Hendrix* 1970, *Langcake* 1974, *Hohl* 1975).

Sporangia from synthetic media contain about 0.2 μg sterols per mg dry weight or about 1/3 that of sporangia grown on the natural medium where the concentration of sterols in mycelium and sporangia is roughly equal. There is evidence that a reduced content of sterols in sporangia raised on artificial media is partially responsible for their greatly decreased capacity for zoosporogenesis (see Table 7; *Stössel* 1978).

Summary

The plant sterols investigated, campesterol, stigmasterol, β-sitosterol and cholesterol occurred in comparable concentrations in both the susceptible (Bintje) and the resistant (Eba)potato (*Solanum tuberosum*) cultivar. Minor differences are not likely to account for the lack of sporulation of *Phytophthora infestans* on the resistant and abundant sporogenesis on the susceptible host. No positive correlation between field resistance and the ratio of cycloartenol: β-sitosterol was found.

Uptake of sterols into the mycelium of the parasite was not selective but influenced by nutrient conditions, light and temperature. In continuous darkness at 16°C, where growth was optimal, sterol uptake and degree of esterification was highest. The sterol concentration of the mycelium decreased with age. In sporangia accumulation of cholesterol was selective. The sterol concentration of sporangia produced on the

synthetic medium amounted to 0.2 μg/mg dry weight which is about 1/3 of that produced from sporangia grown on a natural medium and only about 1/20 of that of the corresponding mycelium. Contrary to this the sterol concentrations from sporangia an mycelium grown on the natural kidney bean medium are roughly equal.

Zusammenfassung

Sterole und die parasitische Wechselbeziehung zwischen *Phytophthora infestans* und *Solanum tuberosum.*

Die Pflanzensterole Campesterin, Stigmasterin, β-Sitosterin und Cholesterin treten im anfälligen (Bintje) und im resistenten (Eba) Wirt *(Solanum tuberosum)* in vergleichbaren Mengen auf. Geringfügige Unterschiede in der Sterinzusammensetzung können kaum für das Fehlen der Sporulation auf dem resistenten und dem massenhaften Auftreten von Sporangien auf dem anfälligen Wirt verantwortlich gemacht werden. Auch ergibt sich keine positive Korrelation zwischen Feldresistenz und dem Verhältnis von Cycloartinol: β-Sitosterin.

Die Sterinaufnahme durch das Myzelium zeigt keine deutliche Selektivität und wird durch Nährstoffangebot, Licht- und Temperaturverhältnisse modifiziert. In Dunkelheit bei 16°C, wo der Pilz die beste Wachstumsrate erzielt, sind sowohl Sterinaufnahme ins Myzel wie Grad der Veresterung der aufgenommenen Sterine am höchsten. Die Sterinkonzentration nimmt mit dem Alter des Myzels ab. In den Sporangien dagegen wird Cholesterin selektiv akkumuliert. Die Sterinkonzentration der auf synthetischem Medium gebildeten Sporangien erreicht einen Wert von 0.2 μg pro mg Trockengewicht. Dies entspricht nur 1/20 der im entsprechenden Myzel enthaltenen Konzentration und etwa 1/3 des in Sporangien von einem natürlichen Substrat, hergestellt aus ‚kidney beans', vorgefundenen Wertes. Auf dem natürlichen Substrat gebildete Myzelien und Sporangien weisen vergleichbare Sterinkonzentrationen auf.

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Dr. P. Stössel und Prof. H.R. Hohl
Institut für Pflanzenbiologie
Cytologie
Zollikerstrasse 107
CH-8008 Zürich