Inhibition of Growth and Sitosterol-Induced Sexual Reproduction in *Phytophthora cactorum* by Steroidal Alkaloids

W. David Nes, Patrick K. Hanners, George A. Bean, and Glenn W. Patterson

Research chemist, Plant Physiology and Chemistry Research Unit, Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Berkeley, CA 94710; and undergraduate research assistant and professors, respectively, Department of Botany, University of Maryland, College Park 20742.

Accepted for publication 3 July 1981.

ABSTRACT

Nes, W. D., Hanners, P. K., Bean, G. A., and Patterson, G. W. 1982. Inhibition of growth and sitosterol-induced sexual reproduction in *Phytophthora cactorum* by steroidal alkaloids. Phytopathology 72:447-450.

As part of a study designed to examine the influence of polycyclic isopentenoid structure in fungi normally auxotrophic or heterotrophic for sterols, we have investigated the effects of 14 synthetic and naturally occurring sterols, nine steroidal alkaloids, and one sapogenin on radial growth, hyphal morphology, and oospore production in *Phytophthora cactorum*, a pathogenic fungus unable to synthesize sterols. When the 24 polycyclic isopentenoids were tested individually, only the steroidal alkaloids and the sapogenin were fungistatic. The extent of growth inhibition was dependent on the concentration of steroidal alkaloid and sapogenin added to the medium and on whether the compound was added in the presence or absence of sitosterol. Certain sterols stimulated growth or had no effect on growth relative to that of the control. In cultures treated with sterols that promoted growth, 24-ethyl sterols gave maximal stimulation of hyphal extension. In some of the steroidal alkaloid treatments, growth inhibition was annulled by adding sitosterol to medium containing azasteroid. Although the addition of sitosterol (10 μg/ml) appeared to protect the mycelium and allow for continued vegetative growth in media containing up to 15 μg of azasteroid per milliliter, sitosterol-induced oospore production was nearly or completely inhibited by the azasteroid. The extent of inhibition was dependent on the concentration and combination of the sterol-steroidal alkaloid pairs in the medium. The results are interpreted to imply that the molecular feature of the steroidal alkaloid responsible for inhibiting sitosterol-induced sexual reproduction is the imino function present in the ring structure.

Additional key words: glycoalkaloids, oospore, steroid sapogenin.

The role of sterols and other polycyclic isopentenoids as natural regulators of growth and reproduction in the pythiaceous fungi is well established (6,12,20). The phylogenetic implications have also been discussed (26). During the last decade, much new information has helped to elucidate the mechanisms of regulation. For instance, stimulation of growth by sterols is believed to result from the incorporation of sterol into mycelial membranes (6,19), which induces changes in membrane permeability (3,28,33) and glucose utilization (31). On the other hand, the fungitoxic action, which is observed as growth inhibition, resulting from the presence of steroid and triterpenoid glycosides such as the *Solanum* (1,29) and *Veratrum* alkaloids (10), lucerne saponins (9), and steroid saponins found in many higher plant families (16,35), is believed to be the result of disruption of the sterol-containing mycelial membranes through the complexing of membranous sterol with the glycoside rather than with its genin (4,28,30). Similar arguments have been presented for growth inhibition resulting from sterol-polyene antibiotic complexes (8). Sterol-induced sexual reproduction is believed to result from the metabolism of the dietary sterol to steroid hormones in the fungus (5,18), which are analogous to the polar steroid hormones, antheridiol and oogoniol, produced in *Achyla* from fucosterol (18).

Recently, we examined the influence of polycyclic isopentenoid structure on the growth and reproduction of *Phytophthora cactorum* (20–22) and other fungi (27). *Phytophthora* spp., like other members of the Pythiaceae, are pathogens of tracheophytes. Moreover, because of their inability to synthesize sterols (6,12), they represent good eucaryotic model systems for studying structure-activity relationships. No competition from endogenously formed sterols exists and thus, the effects on growth and reproduction of various sterols and structurally related polycyclic isopentenoids added to the culture medium may be examined.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1982.

Previous studies on the relationship between polycyclic isopentenoid structure and growth response in pythiaceous fungi have focused primarily on a few sterols, glycoalkaloids, saponins, and their genins (1,3,4,9,15,16,28). Only the study by Hendrix and co-workers (13) reports on sterol-steroid interactions with other polyisopentenoids in vitro and their effects on oospore production. These researchers found that estradiol prevents cholesterol-induced sexual reproduction by *Pythium periplocum*. The purpose of the present investigation was to shed additional light on the effects that polycyclic isopentenoids, many of which have not previously been examined, have on growth and reproduction in *P. cactorum*.

MATERIALS AND METHODS

The nine steroidal alkaloids were obtained from the following sources: 15-aza-24 methylene-D-homocholestadienol was a gift from L. W. Parks; jervine and muldamine were gifts from R. F. Keeler; solanidine, tomatine, tomatidine, solanine, solasonine, and solasodine were gifts from D. F. Johnson; and tigogenin was purchased from Steraloids. Sources of reference sterols have previously been reported (20–22). Most of the compounds were of high purity (>95%), and their configurational purity has been established by ^{1}H nuclear magnetic resonance (25). *P. cactorum*, strain IMI 21168, was a gift from C. Elliott.

Culture conditions are described in detail elsewhere (20–22). For mycelia grown on solid media, the sterols were added as 10 mg/L in ethanol solution at 2 ml/L. The pH of the medium was 4.6 before inoculation. Cultures were inoculated with 5-mm plugs containing mycelia previously cultured on a completely synthetic, sterol-free medium for over 1 yr. After inoculation, each treatment group, comprising five petri dishes (50 mm), was incubated at 25 C in the dark for 6 days; then radial diameters were measured in the light, and the dishes were returned to the dark incubator for another 15 days. In the treatment with polar steroids, the volume of ethanol used to solubilize the compound remained constant (2 ml of ethanol per liter of medium), regardless of the steroid concentration. A concentration range of 1–15 mg of steroidal

alkaloids and sapogenins per liter was chosen. Use of higher levels was precluded by sample shortage.

Methods for the isolation and identification of sterols (23) from mycelia cultured on liquid medium have previously been described (22), as have the methods for counting oospores (5,21). In view of known influences of light (7,17) and temperature (11) on growth and reproduction, all quantitative comparisons were carried out at the same time. For instance, all the sterol treatments were inoculated at the same time, whereas the steroidal alkaloid and sapogenin treatments were examined separately. Mass spectra were obtained through the courtesy of Sam Dutky at the U.S. Department of Agriculture, Beltsville, MD.

RESULTS AND DISCUSSION

Growth response to added sterols. To determine the influence of sterol structure on vegetative growth measured as hyphal extension, 14 sterols commonly found throughout the evolutionary hierarchy of algae to tracheophytes were incubated on agar medium. As shown in Table 1, 24-ethylsterols promoted greater hyphal extension than 24-methylsterols or 24-desalkylsterols when the cultures were incubated at 25 C in the dark. Those sterols that failed to stimulate hyphal extension (Table 1) did not inhibit growth. We have previously shown, however, that when cholesterol (a 24-desalkylsterol) and sitosterol (a 24-alkylsterol) were added to mycelia growing vegetatively in liquid cultures under normal laboratory environmental conditions, both compounds stimulated growth, measured as dry weight, in a similar manner (26). Langcake (15), studying the effects of sterols on vegetative growth of *Phytophthora infestans* cultures grown on solid and liquid substrates, similarly found that certain sterols produced different degrees of growth stimulation when added to solid and to liquid cultures. The growth response of *Phytophthora* on solid substrates appears to be a better representation of mycelial growth in nature, because *Phytophthora* is not normally an aquatic parasite but rather infects and reproduces on the solid surfaces provided by the leaves, fruits, roots, and stems of the host plant.

TABLE 1. Effects of 24-desalkylsterols and 24-aklylsterols on the radial growth of *Phytophthora cactorum*

Trivial name	Structure based on changes of 5_α-cholestan-3_β-ol	Percent stimulation of growth relative to control[a]
Ergosterol	$\Delta^{5,7 trans-22}$-24_β-methyl	0
7-dehydrodihydrobrassicasterol	$\Delta^{5,7}$-24_β-methyl	0
22,23-dihydrobrassicasterol	Δ^{5}-24_β-methyl	0
Brassicasterol	$\Delta^{5, trans-22}$-24_β-methyl	0
Ergostanol	24_β-methyl	0
Cholestanol	...	0
Cholesterol	Δ^{5}	0
26-Homocholesterol	Δ^{5}-26(27)methyl	0
Campesterol	Δ^{5}-24_α-methyl	9.8
Campestanol	24_α-methyl	9.8
Spinasterol	$\Delta^{7, trans-22}$-24_αethyl	18.1
Chondrillasterol	$\Delta^{7, trans-22}$-24_β-ethyl	18.1
Stigmasterol	$\Delta^{5, trans-22}$$24_\alpha$-ethyl	18.1
Sitosterol	Δ^{5}-24_α-ethyl	18.1

[a] Growth was measured 6 days after inoculation; sterols were added at a level of 10 μg/ml. Radial growth of the control was 30.5 ± 1.78 mm based on least significant difference at $P = 0.05$. Sterol-supplemented cultures varied to the same extent as the control.

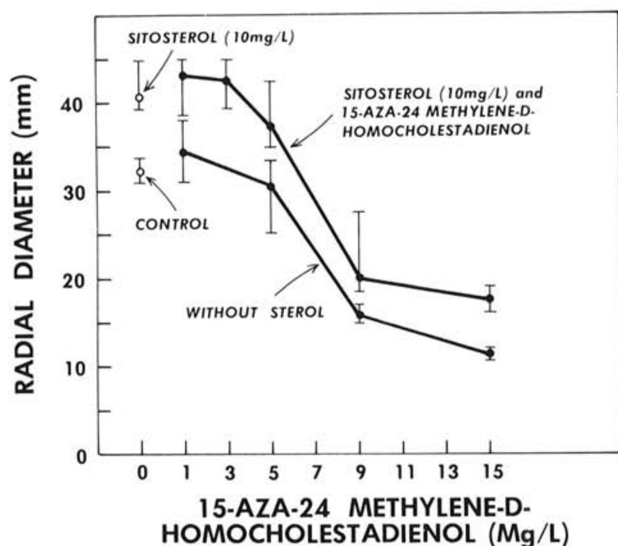

Fig. 1. Growth curves of *Phytophthora cactorum* mycelia, as affected by increasing amounts of 15-aza-24 methylene-D-homocholestadienol in the presence and absence of sitosterol (at 10 μg/ml). Points represent mean values of the radial diameter from five petri dishes per treatment and vertical bars indicate the range of replicate determinations. Sixty plates representing 10 treatments (one treatment was contaminated) were inoculated at the same time with 5-mm mycelial plugs and grown in the dark at 25 C.

Growth stimulation or the lack thereof could conceivably be due to a sterol metabolite or to a failure of the sterol to enter the mycelium. To assess this possibility, we selected five representative sterols exhibiting no stimulation or some stimulation of growth on solid media and added these sterols (10 μg/ml) to mycelia growing in liquid cultures. The sterols—cholesterol, campesterol, 22,23-dihydrobrassicasterol, 26-homocholesterol, and campestanol—were reisolated as the free alcohol and were not metabolized. Confirmation of the structure of the sterols was by gas-liquid chromatography and mass spectroscopy (23,25). More recently, we have added numerous radioactive compounds, eg, (^{14}C)-labeled cholesterol, campesterol, sitosterol, cycloartenol, and β-amyrin, singly and in equal amounts (10 μg/ml of each), to cultures grown on solid medium (25; and Nes, Saunders, and Heftmann, *unpublished*). The sterols were assimilated to a greater extent than were the triterpenoids by cultures grown on solid medium. When the sterols and triterpenoids were isolated from the fungus, none of the compounds were metabolized in the nucleus, or in the case of sterols, the side chain. However, the sterols were converted to esters and glycosides, whereas the triterpenoids were converted only to esters. Differences in the uptake and conversion to esters and glycosides of various polycyclic isopentenoids may have significant influence on structure-activity comparisons. This phenomenon is currently under investigation.

Growth response to polar steroids. When *P. cactorum* was incubated with increasing amounts of either an azasteroid or a sapogenin, the radial diameter decreased. In some of the treatments, the addition of sitosterol to the culture medium appeared to reverse the fungitoxicity. Two representative growth curves of *P. cactorum* mycelia affected by increasing amounts of

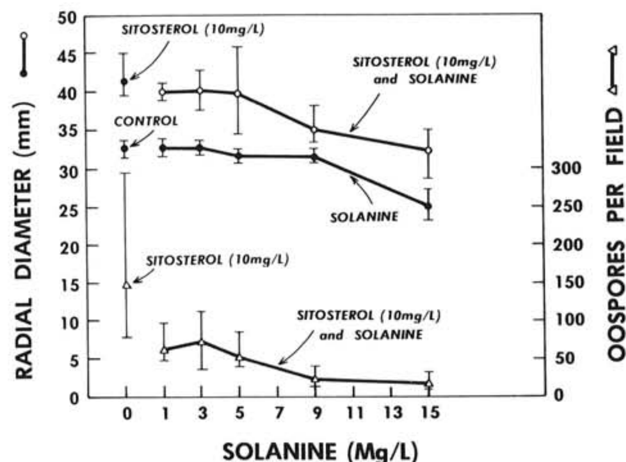

Fig. 2. Effect of solanine on growth and reproduction of *Phytophthora cactorum* in the presence and absence of sitosterol. Points represent mean values of data from five petri dishes and vertical bars indicate the range of replicate determinations.

steroidal alkaloids in the presence and absence of sitosterol are shown in Figs. 1 and 2. Growth-response data for nine steroidal alkaloids and one sapogenin added to the culture medium at 15 μg/ml in the presence and absence of sitosterol (added at 10 μg/ml) are shown in Table 2. In five out of nine cases, sitosterol in the medium protected *Phytophthora* from the deleterious influence of this level (15 μg/ml) of azasteroids. Maximal inhibition of growth did not necessarily occur with azasteroids possessing a derivatized C-3 hydroxyl group, ie, glycoalkaloids. Comparison of the extent of growth inhibition induced by solanine, tomatine, solasonine with that induced by their aglycones (solanidine, tomatidine, and solasodine, respectively), shows that the aglycone does not in each case inhibit more growth than its corresponding glycoside (Table 2). The aglycone has been reported to be the active fungistatic agent of the glycoalkaloid molecule (32,34). Thus, hydrolysis of the steroid glycoalkaloid, which is known to operate in *P. infestans* (14), probably does not have a protective function, as is speculated for *Septoria lycopersici* on tomato (2). Although sitosterol reversed the inhibitory effect of certain steroidal alkaloid-treated cultures, eg, jervine, it enhanced the inhibitory effect of others, eg, tigogenin-treated cultures. The fact that structurally different azasteroids, containing an imino group in rings D or F, as well as a sapogenin are capable of inhibiting growth indicates that the fungistatic effect depends neither on the exact position nor on the presence of an imino group.

Influence of steroidal alkaloids and sapogenin on oospore production. None of the steroids in the concentration range tested stimulated oospore production. In the presence of sitosterol, all the steroidal alkaloids inhibited oospore production. Sitosterol was the sterol chosen to induce oospore production, because none of the sterols tested (Table 1) promoted greater growth or induced greater numbers of oospores than sitosterol-supplemented cultures (22) did. Inhibition of sitosterol-induced oospore production increased with increasing concentrations of the steroidal alkaloid (Table 3, Figs. 2 and 3). Tigogenin, added in concentrations from 1 to 15 μg/ml in the presence of sitosterol did not significantly alter oospore production relative to the sitosterol control treatment. The fact that solasodine, which differs structurally from tigogenin principally in the replacement of oxygen by an imino group in the F-ring, significantly inhibits sitosterol-induced oospore production implicates the imino group as the deleterious structural feature of the azasteroid. As is shown in Fig. 2, solanine significantly inhibits oospore production at levels at which mycelial growth is relatively unaffected. Similar observations were made with the other steroidal alkaloids (Fig. 3, Tables 2 and 3).

Microscopic studies of mycelia incubated with and without sterol. Cultures of four representative sterol treatments (ergosterol, cholesterol, sitosterol, and campesterol) and a control culture were examined by phase-contrast microscopy. The cultures were harvested 6 days after inoculation. No obvious morphological difference was found among cultures treated with different sterols. However, comparison of sterol-free with sterol-supplemented cultures showed striking morphological differences in the mycelia (Fig. 4). The latter have thinner hyphae, more branching, less mycelial disruption at the hyphal tips, and fewer aborted hyphae (appearing as protuberances on the surface of the individual hyphae) than the former. These morphological differences between sterol-free and sterol-supplemented cultures are consistent with the assumption that sterols induce beneficial effects on mycelia of *Phytophthora* (6).

In summary, the most effective inhibitors of sitosterol-induced oospore production have an imino group in the nucleus or side chain. Both steroidal alkaloids and sapogenins may inhibit growth, but the degree of fungitoxicity depends on their concentration in the culture medium and on whether a sterol is present. Maximal growth stimulation occurred with 24-ethyl sterols. The configuration of the ethyl alkyl group at C-24 is not critical for growth stimulation by sterols, but it is for sexual reproduction (22).

TABLE 2. Effects of polar steroids on the growth of *Phytophthora cactorum*[a] [b]

Steroid	Percent inhibition of growth[c]	
	With sitosterol	Without sitosterol
15-Aza-24 methylene D-homocholestadienol	47.3	64.6
Jervine	25.8	61.3
Muldamine	0	31.1
Tomatidine	46.6	27.9
Solanidine	15.2	17.9
Tomatine	32.1	31.1
Solanine	0	22.4
Solasonine	0	7.0
Solasodine	34.0	31.5
Tigogenin	40.0	10.7

[a] Radial growth was measured at 25 C, and the mean radial diameter value for each of the treatments rarely exceeded a variation of 1.78 mm based on least significant difference at $P = 0.05$.

[b] Steroids were added to the culture medium at a level of 15 μg/ml and sitosterol at 10 μg/ml.

[c] Inhibition, measured 6 days after inoculation, is relative to a control culture without sterol.

TABLE 3. Influence of increasing steroidal alkaloid concentrations on sitosterol-induced oospore production by *Phytophthora cactorum*

Steroid	Oospore production[a] (percent of control) at steroidal alkaloid concentrations (μg/ml) of				
	1	3	5	9	15
Muldamine	90.7	87.3	82.5	36.9	58.7
Jervine	62.2	5.7	3.8	0.0	0.0
Tomatidine	97.8	68.6	68.4	39.8	6.9
Tomatine	83.8	82.0	64.0	cont.[b]	10.4
Solanine	44.3	50.7	35.9	16.2	14.2
Solasodine	100	92.1	60.4	31.3	14.9
15-Aza-24 methylene-D-homocholestadienol	35.5	33.0	43.0	0.0	0.0

[a] Mean oospore count after 21 days divided by the mean count obtained with sitosterol (10 μg/ml) controls. Values are the mean of five petri plates per treatment. Deviation from the average rarely exceeded 20%.

[b] Contaminated cultures that were not examined further.

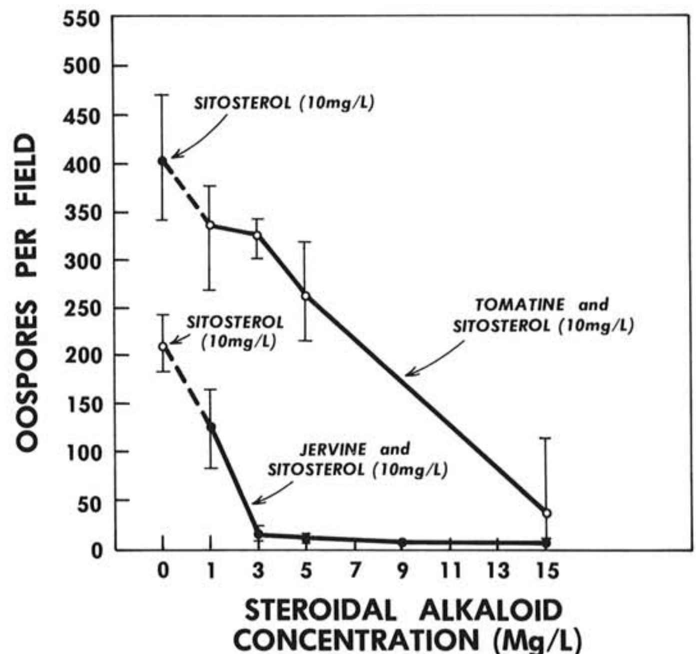

Fig. 3. Effect of increasing concentrations of two representative steroidal alkaloids on reproduction of *Phytophthora cactorum* in the presence and absence of sitosterol. Points represent mean values of data from five petri dishes and vertical bars indicate the range of replicate determinations. Sitosterol-treated cultures were incubated at the same time as the control treatment (broken lines).

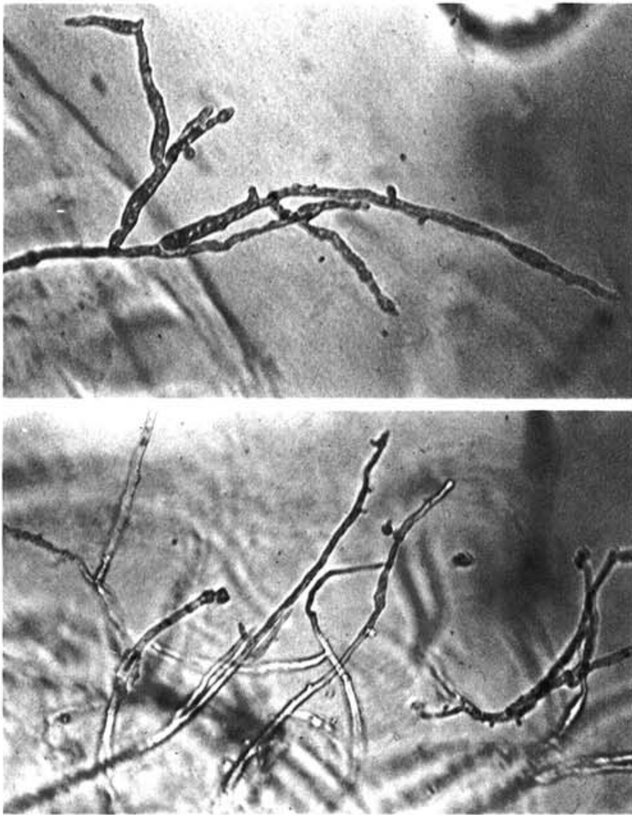

Fig. 4. Photomicrographs of hyphal tips of *Phytophthora cactorum*. Cultures were grown for 6 days on a completely synthetic solid medium in the dark at 25 C. Top, sitosterol-supplemented culture; bottom, sitosterol-free culture.

Phase-contrast micrographs of sterol-free and sterol-supplemented cultures showed that sterols induced beneficial effects in the hyphae.

Much speculation exists about the potential role of steroidal alkaloids in causing resistance to fungi in the Solanaceae (1,34). Our data appear to support this role. For instance, in vitro, the colony diameter and number of oospores (induced by sitosterol) diminished as the amount of azasteroid in the medium increased from 0 to 15 μg/ml.

LITERATURE CITED

1. Allen, E. H., and Kuc, J. 1968. α-Solanine and α-chaconine as fungitoxic compounds in extracts of Irish potato tubers. Phytopathology 58:776-781.
2. Arneson, P. A., and Durbin, R. D. 1967. Hydrolysis of tomatine by *Septoria lycopersici*: A detoxification mechanism. Phytopathology 57:1358-1360.
3. Child, J. J., Defago, G., and Haskins, R. H. 1969. The effect of cholesterol and polyene antibiotics on the permeability of the protoplasmic membrane of *Pythium* PRL 2142. Can. J. Microbiol. 15:599-603.
4. Defago, G. 1978. Interactions entre les sterols fongiques et les glucosides de quelques plantes-hotes. Ann. Phytopathol. 10:157-170.
5. Elliott, C. G. 1979. Influence of the structure of the sterol molecule on sterol-induced reproduction in *Phytophthora cactorum*. J. Gen. Microbiol. 115:117-126.
6. Elliott, C. G. 1977. Sterols in fungi: Their functions in growth and reproduction. Adv. Microbiol. Physiol. 15:121-173.
7. Englander, L., and Turbitt, W. 1979. Increased chlamydospore production by *Phytophthora cinnamoni* using sterols and near ultraviolet light. Phytopathology 69:813-817.
8. Fowlks, E. R., Leben, C., and Snell, J. F. 1967. Sterols in relation to the influence of nystatin on *Pythium aphanidermatum* and *Colletotrichum lagenarium*. Phytopathology 57:246-249.
9. Gestetner, B., Assa, Y., Henis, Y., Birk, Y., and Bondi, A. 1971. Lucerne saponins: Relationship between their chemical constitution, and haemolytic and antifungal activities. J. Sci. Food Agric. 22:168-172.
10. Han, Y. B., and Woo, W. S. 1973. Antimicrobial activity of *Veratrum* alkaloids. J. Pharm. Soc. Korea (Eng.) 17:137-140.
11. Haskins, R. H. 1965. Sterols and temperature tolerance in the fungus *Pythium*. Science 17:1615-1616.
12. Hendrix, J. W. 1970. Sterols in growth and reproduction of fungi. Annu. Rev. Phytopathol. 8:111-130.
13. Hendrix, J. W., and Guttman, S. 1968. Annulment of sterol-induced sexual reproduction by estradiol in *Pythium periplocum*. Science 161:1252.
14. Holland, H. L., and Taylor, G. J. 1979. Transformations of steroids and the steroidal alkaloid, solanine, by *Phytophthora infestans*. Phytochemistry 18:437-440.
15. Langcake, P. 1975. Sterols in potato leaves and their effects on growth and sporulation of *Phytophthora infestans*. Trans. Br. Mycol. Soc. 63:573-586.
16. Lazur'evskii, G. V., Zhuchenko, A. A., Kintya, P. K., Balashova, N. N., Mashenko, N. E., Bobeiko, V. A., Para, S. P., Andryushchenko, V. K., and Goryanu, G. M. 1978. Effects of steroid glycosides on *Phytophthora infestans*. Dokl. Akad. Nauk SSSR 243:1076-1077.
17. Lilly, V. G. 1966. The effect of sterol and light on the production and germination of *Phytophthora* spores. 18th Symposium of Colston Research Society. University of Bristol Proceedings. Butterworths, London. pp. 259-272.
18. McMorris, T. C. 1978. Sex hormones of the aquatic fungus *Achlya*. Lipids 13:716-722.
19. Nes, W. D., and Heftmann, E. 1981. A comparison of triterpenoids with steroids as membrane components. J. Nat. Prod. 44:377-400.
20. Nes, W. D., and Patterson, G. W. 1981. Effects of tetracyclic and pentacyclic triterpenoids on growth of *Phytophthora cactorum*. J. Nat. Prod. 44:215-220.
21. Nes, W. D., Patterson, G. W., and Bean, G. A. 1979. The effect of steroids and their solubilizing agents on mycelial growth of *Phytophthora cactorum*. Lipids 14:458-462.
22. Nes, W. D., Patterson, G. W., and Bean, G. A. 1980. The effect of steric and nuclear changes in steroids and triterpenoids on sexual reproduction in *Phytophthora cactorum*. Plant Physiol. 66:1008-1011.
23. Nes, W. D., Patterson, G. W., Southall, M. A., and Stanley, J. L. 1979. Sterols and fatty acids of *Epifagus virginiana*, a nonphotosynthetic angiosperm. Lipids 14:274-276.
24. Nes, W. D., Saunders, G. A., and Heftmann, E. 1981. Metabolic discrimination between cholesterol and β-amyrin by *Phytophthora cactorum*. Lipids 16:744-748.
25. Nes, W. R., Krevitz, K., Joseph, J., Nes, W. D., Harris, B., Gibbons, G., and Patterson, G. W. 1977. The phylogenetic distribution of sterols in tracheophytes. Lipids 12:511-527.
26. Nes, W. R., and Nes, W. D. Lipids in Evolution. Plenum Press, New York. 244 pp.
27. Nes, W. R., Sekula, B. C., Nes, W. D., and Adler, J. H. 1978. The functional importance of structural features of ergosterol in yeast. J. Biol. Chem. 253:6218-6225.
28. Olsen, R. A. 1973. Triterpene glycosides as inhibitors of fungal growth and metabolism. Physiol. Plant. 29:145-149.
29. Roddick, J. G. 1974. The steroidal glycoalkaloid α-tomatine. Phytochemistry 13:9-25.
30. Roddick, J. G. 1978. Effect of α-tomatine on the integrity and biochemical activities of isolated plant cell organelles. J. Exp. Bot. 29:1371-1381.
31. Schlosser, E., and Gottlieb, D. 1968. The effect of sterols on the metabolism of *Pythium* species. Arch. Microbiol. 61:246-253.
32. Segal, R., and Schlosser, E. 1975. Role of glycosidases in the membranolytic, antifungal action of saponins. Arch. Microbiol. 104:147-150.
33. Sietsma, J. H., and Haskins, R. H. 1968. The incorporation of cholesterol by *Pythium* sp. PRL 2142, and some of its effects on cell metabolism. Can. J. Biochem. 46:813-818.
34. Sinden, S. L., Goth, R. W., and O'Brien, M. J. 1973. Effect of potato alkaloids on the growth of *Alternaria solani* and their possible role as resistance factors in potatoes. Phytopathology 63:303-307.
35. Wolters, B. 1968. Saponin als pflanzliche Pilzabwehrstoffe. Planta (Berl.) 79:77-83.