EFFECTS OF TETRACYCLIC AND PENTACYCLIC TRITERPENOIDS ON GROWTH OF *PHYTOPHTHORA CACTORUM*[1]

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ABSTRACT.—The growth effects of adding 16 tetracyclic and pentacyclic triterpenoids (at 10 mg/l) to cultures of *Phytophthora cactorum*, a fungus unable to epoxidize squalene, have been examined. Tetrahymanol, β-amyrin and α-amyrin promoted growth; lupeol, lanosterol, cycloartenol, betulin, and oleanolic acid had no significant effect on growth relative to a control. Hop-22(29)-ene, lupeone, friedelin, and the cucurbitacins a, b, c, d, and e inhibited growth. The ability of certain pentacyclic triterpenoids to stimulate growth was unexpected since the tetracyclic sterol molecule was thought to be necessary for growth stimulation of pythiaceous fungi. That pentacyclic and various tetracyclic triterpenoids inhibit growth of *Phytophthora* inddicates these compounds may be naturally occurring fungistatic agents.

Sterol synthesis which proceeds through 2,3-oxidosqualene occurs in all groups of fungi except in certain oomycetes (1, 2). The absence of sterol production is a characteristic of the family Pythiaceae and, thus, represents an important chemotaxonomic marker for phylogenetic and functional reasons (3). While *Phytophthora* spp. and other members of the Pythiaceae do not require sterols for growth, they possess an obligatory nutritional requirement for certain sterols with strict requirements of stereochemistry in the ring and side chain (4, 5) as agents for the induction of sexual reproduction (6, 7).

The role of sterols as architectural components of membranes (8–10) and as hormones (9–12) in *Phytophthora* and *Pythium* appears secure. However, the function of triterpenoids remains speculative. The purpose of this paper is to shed new light on the role of triterpenoids by the examination of the response of *P. cactorum* to a variety of common naturally occurring tetracyclic and pentacyclic triterpenoids when added to vegetatively growing mycelia in liquid and solid cultures.

METHODS AND MATERIALS

The triterpenoids used in this study were obtained from the following sources: β-amyrin, α-amyrin, and tetrahymanol were gifts from W. R. Nes; hop-22(29)-ene was a gift from A. Marsilli; cycloartenol, betulin, and oleanolic acid were gifts from H. Kircher; and lupeol, lupeone, and the five cucurbitacins were gifts from M. J. Thompson. Friedelin was isolated from *Kalmia latifola* (13), and lanosterol, a commercial product, was purified by chromatography on LH-20 Sephadex. The purity of all triterpenoids, except the cucurbitacins, was checked by glc (14, 15). Only betulin was less than 95% pure. Sitosterol and cholesterol, used for sterol-treated controls, were gifts from W. R. Nes. The relative purity of the two sterols was greater than 99%, and their nmr spectra at 220 MHz have been reported (13).

Culture conditions for *Phytophothora* have been described elsewhere (4, 14). Sterols or triterpenoids were added at levels of 10 mg/liter to both solid and liquid media. These were added in ethanol solution (2 ml/liter) to *Phytophthora* growing on agar plates which we have operationally defined as solid culture; we have operationally defined growth in 50 ml liquid medium/250 ml flasks as liquid culture. Liquid cultures were grown at room temperature under normal laboratory light conditions, while solid cultures were maintained at 28° in an incubator in the dark.

Mycelia from the liquid cultures were harvested every 3 days up to 18 days after inoculation. They were collected on filter paper, washed 3 times with distilled water, and lyophilized for 24 hr. Mycelia harvested during days 6–15 were analyzed for metabolites of triterpenoids.

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During that time mycelia were in their log phase of growth. The freeze-dried mycelia were extracted for 24 hr with chloroform-methanol (2:1). The lipid extract was chromatographed as a streak on a thin layer of Silica Gel G and developed in benzene-ether (9:1). Cholesterol and lanosterol were routinely included in each chromatogram as representative standards of 4-desmethyl sterols and 4,4-dimethyl sterols, respectively. The sterol and triterpenoid bands were revealed by a 2',7'-dichlorofluorescein spray. Bands matching cholesterol and lanosterol were eluted from the tlc plate and further analyzed by glc or combined gc-ms as previously described (4, 14). The polar lipid band (origin) was scraped from the tlc plate in sitosterol- and β-amyrin-treatments and in the control. To the silica gel-containing polar lipid, 10 ml of 2% sulfuric acid was added in methanol, and the mixture was allowed to reflux for 2 hr at 70°. After the reaction was complete, 10 ml of water was added and the solution was extracted 3 times with 20 ml of hexane. The 3 hexane layers were pooled and dried under reduced pressure. The fatty acid methyl esters which were formed after the transesterification process were examined by glc as previously described (16).

RESULTS AND DISCUSSION

The growth effects of 16 pentacyclic and tetracyclic triterpenoids added to liquid cultures of *P. cactorum* have been examined. Tetrahymanol, β-amyrin, and α-amyrin promoted growth at the same rate; lupeol, lanosterol, cycloartenol, betulin, and oleanolic acid had no significant effect on growth relative to a control. Hop-22(29)-ene, lupeone, and friedelin slightly inhibited growth, and the cucurbitacins a, b, c, d, and e strongly inhibited growth. The effects of four representative triterpenoid treatments on the growth of *P. cactorum* in liquid culture are shown in fig. 1. The coefficient of variation between the growth rates for

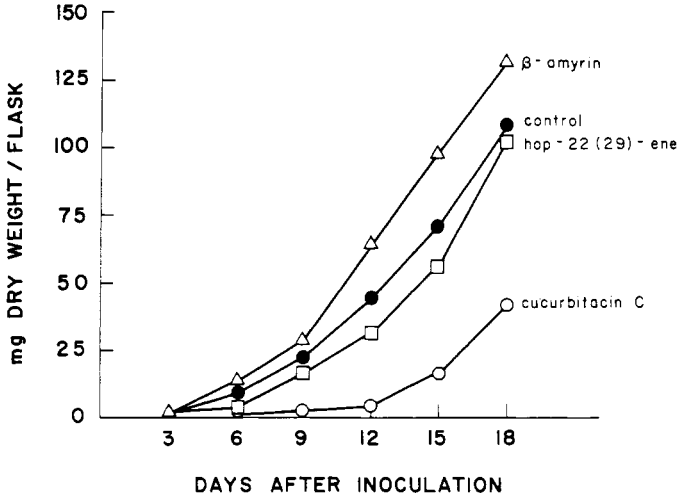

FIG. 1. Growth of *Phytophthora cactorum* mycelia in liquid culture as affected by various triterpenoid additions to the growth medium (at 10 mg/liter).

each particular triterpene treatment did not exceed ±5% in the late log phase but was as high as ±10% in the early log phase. Structures of some of the pentacyclic triterpenoids incubated in the present study are shown in fig. 2.

As previously reported, cholesterol, campesterol, and sitosterol stimulate growth at the same rate when added to liquid cultures (14). The dry weights of mycelium per flask were approximately 125 mg at 12 days and 200 mg at 18 days after the addition of sterol to the culture medium, while the corresponding

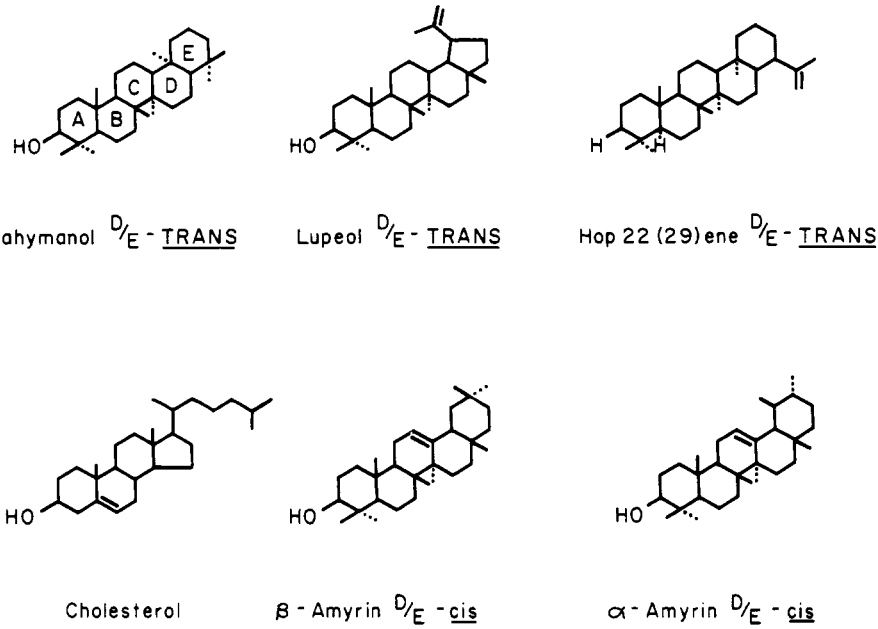

FIG. 2. Structures of some of the pentacyclic triterpenoids and cholesterol incubated with *Phytophthora cactorum*.

control flasks contained approximately 45 mg and 112 mg of mycelium, respectively. The mycelial dry weight of 200 mg flask approached the capacity of the flask, and after 18 days only certain sterol treatments possessed such a large mass of mycelium. As is shown in fig. 1, after 18 days β-amyrin (α-amyrin and tetrahymanol have similar dry weights at 18 days) possesses a dry weight of approximately 135 mg dry weight flask. Thus, our results show that although certain pentacyclic triterpenoids are capable of stimulating growth of *Phytophthora* in liquid culture, they do not promote growth at the same rate as the sterols.

In order to determine whether mycelia growing in solid culture respond to added sterol or triterpenoid in an analogous manner as when grown in liquid culture, seven triterpenoids were added to solid culture medium. The seven triterpenoids were chosen on the basis of their previous growth effects in liquid culture and reflected every aspect of growth-response: stimulation, no effect, and inhibition. The results of adding triterpenoids to solid culture are shown in table 1. Sitosterol was added to solid culture as a control for growth-stimulation. Unexpectedly, β-amyrin promoted radial growth to the same extent as sitosterol. Another unexpected finding was that cucurbitacin c did not inhibit radial growth as strongly as in liquid culture.

Different activities of the various triterpenoids could result from differences in the character of mycelial growth in solid and liquid substrates. Physiochemical differences in substrates could influence the rate at which added compounds are taken up by the mycelium. Another possibility is that, while growth in liquid cultures over an 18-day period provides an opportunity for both hyphal elongation and cell division which we measure as dry weight, radial growth on solid medium

over a 6-day period may principally involve hyphal elongation with statistically less cell division. The difference between growth in liquid and solid media may have some biological importance, since *Phytophthora* normally is not an aquatic parasite but, rather, infects solid surfaces such as tracheophyte fruits, leaves, and stems. Thus, the extent to which sterols or triterpenoids influence cell division, hyphal elongation, or both is an important matter and requires further study.

The combined mycelia from 5 triterpenoid treatments (β-amyrin, lanosterol, cycloartenol, tetrahymanol, and α-amyrin) were harvested on days 6–15 and extracted, and the added compounds were isolated, unchanged, as the free alcohols. Each isolated compound on tlc showed a single band which chromatographed in the lanosterol region and, after elution, exhibited a peak on glc corresponding to the respective compound added (15). The zone corresponding to sterols was also examined for each triterpenoid treatment by means of glc. No Δ^5-sterols could be detected (>.001% dry weight mycelium). We have incubated [2-^{3}H] cycloartenol and [2-^{3}H] lanosterol [10 mg of tritiated triterpenoid/ℓ of each (16)] in liquid cultures. There was no detectable conversion of these triterpenoids to 4-desmethyl sterols.

TABLE 1. The effect of added triterpenoid on the growth of *P. cactorum*.[a]

Treatment	Colony Diameter (mm)	
	Trial I	Trial II
control	31.5	30.0
lupeol	31.5	29.5
cycloartenol	31.0	31.0
lanosterol	31.0	30.5
hop-22(29)-ene	31.0	30.0
cucurbitacin c	30.0	28.0
betulin	32.0	30.5
oleanolic acid	31.0	29.0
β-amyrin	35.0	35.0
sitosterol	35.0	35.0

[a]Each trial represents the average of 5 petri dishes containing triterpenoid at 10 mg/liter and the coefficient of variation for the colony diameters obtained for each dish did not exceed ± 2.0%. Sitosterol treatments were added as an additional control. Cultures were incubated for 6 days at 25° in the dark on solid medium.

The compounds eluted from the triterpene region of thin-layer plates from cycloartenol- and β-amyrin-treated mycelia were analyzed by gc-ms to determine whether they contained the unchanged triterpenoids or whether the material identified by tlc and glc corresponded to some metabolites having the same mobilities as cycloartenol and β-amyrin. The mass spectrum of the material eluted from the thin-layer chromatogram of cycloartenol treatments showed the parent peak at $M^+(m/e)$ 426, thus confirming the molecular weight of cycloartenol. In addition, we observed the diagnostic peak of an intense fragment at m/e 286 which indicates the presence of the 9,19-cyclopropyl group of tetracyclic triter-

penoids (17). The latter fragmentation pattern confirms the molecule was of the cycloartane and not lanostane skeleton. The corresponding material from the β-amyrin treatments had a M^{-}(m/e) 426 and the diagnostic base peak of an intense fragment at m/e 218, characteristic of the amyrins (18).

The tlc, glc and ms data confirm that cycloartenol is not metabolized to lanosterol, a process operating in certain other Phycomycetes (19). The inability of *P. cactorum* to metabolize tritiated cycloartenol to a 4-desmethyl sterol is in agreement with the lack of growth stimulation activity of cycloartenol as well as its inability to induce oospore production (4). Thus, it is reasonable to assume *P. cactorum* lacks several enzyme systems involved in the pathway from squalene to Δ^{7}- and $\Delta^{5,7}$-sterols. The latter sterols, however, can be metabolized to Δ^{5}-sterols by *P. cactorum* (20).

The chromatographic and mass spectral data also show that β-amyrin is not metabolized to a sterol or to a more highly oxidized triterpenoid, a process operating in photosynthetic higher plants (21). All 3 growth stimulating triterpenoids, β-amyrin, α-amyrin, and tetrahymanol, were isolated unchanged as the free alcohols, and their identities were confirmed by glc and, in the case of β-amyrin, by ms. In the triterpenoid-stimulating mycelial extracts there was no detectable sterol. Thus, the administered pentacyclic triterpenes were still present in the mycelia and had not undergone nuclear transformations such as desmethylation, hydrogenation, or dehydrogenation. Oleanolic acid is the oxidation product of β-amyrin in the known oxidation sequence of β-amyrin metabolism (21). Since oleanolic acid was also reisolated from the mycelium in unchanged form and did not stimulate growth in liquid or solid culture, we may assume that β-amyrin was not metabolized to a more highly oxidized form responsible for regulating growth.

The fatty acid composition of the polar lipids represents membranous fatty acids (22). In other biological systems, sterol depletion leads to a change in the fatty acid composition of phospholipids (23). Since *P. cactorum* does not synthesize membranous sterol or triterpenoids, we considered the reverse possibility, namely, that the addition of certain compounds may influence the membrane fatty acids, and the compensatory change in the fatty acid composition would be responsible for growth stimulation. There was no significant difference between the fatty acid composition of the polar lipid fraction of the β-amyrin- and sitosterol-treatments on the one hand and the control cultures on the other. The percentage of fatty acid composition of the polar fatty acyl lipids of the control cultures was as follows: 12:0 (10%), 14:0 (13%), 16:0 (33%), 18:0 (3%), 20:0 (traces), 16:1 (1%), 18:1 (15%), 18:2 (18%), 18:3 (3%), and unknown (4%). That sterols do not influence the cellular fatty acid composition has also been reported for the pythiaceous fungi (24, 25) and *Acholeplasma laidlawii* (26).

Our present and previous studies (4, 14) show that, while sitosterol stimulates growth and induces oospore production, β-amyrin stimulates only growth. Analogous to the structural function of certain sterols in vegetative mycelia, we have demonstrated that β-amyrin, α-amyrin, and tetrahymanol acted in a non-metabolic role, because they stimulated growth without undergoing chemical change. Our results also indicate that *P. cactorum* has a "broad specificity" for sterol-induced growth stimulation, since certain pentacyclic triterpenoids can also stimulate growth.

Structural features common to pentacyclic triterpenes which stimulate growth and the sterols are the free 3β-hydroxyl group and the planar A/B-*trans* ring juncture. A Δ^{5}-bond in ring B is not necessary for triterpenoids to stimulate growth as it is for sterols to stimulate growth (14). The D/E ring juncture is *cis*

in the amyrins but *trans* in tetrahymanol. This structural variation does not appear to be detrimental to biological activity in our systems. The inability of cycloartenol and lanosterol to stimulate growth cannot be due to the presence of the 4,4-dimethyl moiety because all three of the growth-stimulating pentacyclic triterpenes possess the *gem*-dimethyl grouping at C–4. The lack of stimulation by cycloartenol and lanosterol could possibly be due to the 14α-methyl group because this substituent destroys their α-face planarity. While tetrahymanol, β-amyrin, and α-amyrin also possess a 14α-methyl group, this substituent does not alter the 3-dimensional (conformational) planarity of the 3 growth stimulating triterpenoids (21).

The results of the present investigation represent the first time that free 3β-hydroxy pentacyclic triterpenoids have been shown to stimulate growth in a eukaryotic organism other than in protozoa. Recently, Bloch and co-workers studying the effect of tetracyclic and pentacyclic triterpenoids on the growth-response of the prokaryote sterol-requiring mycoplasmas demonstrated that these mycoplasmas utilized certain triterpenes, e.g., β-amyrin, in a structural mode similar to sterols (27). Moreover, the results of the present and previous studies (4, 14) show that polyhydroxy sterols and triterpenes act as fungistatic agents *in vitro*. Whether polyhydroxylated sterols and triterpenes play a phytoalexin role remains uncertain, but their presence in the host plant may affect the rate of infectivity since they have the potential of inhibiting mycelial growth *in vitro*.

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