The Effect of Some Pterocarpanoid Phytoalexins on Germ Tube Elongation of Stemphylium botryosum

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ABSTRACT

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Bioassays involving the continuous measurement of individual germ tubes of *Stemphylium botryosum* showed that maackiain (15 to 50 μg/ml) temporarily stopped elongation of the germ tubes and that the rate at which germ tubes resumed growth was dependent on the maackiain concentration. In up to 16% of treated germ tubes new growth began by branch formation just behind the apex. Medicarpin or a 1:1 mixture of maackiain and medicarpin had a similar effect on germ tube elongation, but phaseollin at a comparable concentration appeared to kill the germ tubes. Diffusates obtained from *Helminthosporium* carbonum-inoculated red clover leaflets and containing 20-30 μg/ml of maackiain plus medicarpin had the same effect on germ tube elongation as purified maackiain. The renewal of growth by maackiain-treated germ tubes of *S. botryosum* coincided with changes in the rates of glucose-^{14}C uptake. The rate of loss of ^{14}C-labeled compounds from germinated spores initially increased over threefold after maackiain treatment but by 8-12 hr was comparable to the rate of the control. That the resumption of growth was not the result of the previously described conversion of maackiain to noninhibitory compounds by *S. botryosum* was suggested by two methods. Firstly, germinated *S. botryosum* spores in which maackiain conversion was induced by small additions (2 μg/ml) of maackiain were as inhibited by later additions of maackiain (15 μg/ml) as were noninduced controls. Secondly, germ tube growth of *H. carbonum*, which cannot metabolize maackiain, was temporarily delayed by maackiain.

Additional key words: membrane permeability, dihydromaackiain, inermin, 3-hydroxy -8,9-methylenedioxypterocarpan, demethylhomopterocarpan, 3-hydroxy-9-methoxypterocarpan.

In previous work on phytoalexins of alfalfa and red clover, it was noted that the pterocarpanoid phytoalexins maackiain (inermin; 3-hydroxy-8,9-methylenedioxypterocarpan) and medicarpin (demethylhomopterocarpan; 3-hydroxy-9methoxypterocarpan) inhibit the germ tube growth of some fungi much more severely than they inhibit mycelial growth (3, 5). Although the reasons for this difference are unknown, it has been proposed (3) that the fungus has more time to convert the phytoalexin to nontoxic derivatives in the 2- to 3-day mycelial growth bioassays than in the few hours required for the germ tube growth bioassay.

This report involves a detailed study of the effect of the phytoalexin maackiain from red clover (*Trifolium pratense* L.) on germ tube growth of the alfalfa pathogen *Stemphylium botryosum* Wallr. This fungus is inhibited by maackiain in germ tube growth bioassays, but not by comparable levels of maackiain in radial mycelial growth bioassays (2, 3). In addition, it is known (6) that *S. botryosum* readily converts maackiain to its isoflavan derivative, dihydromaackiain, by an induced system. Although similar information on inhibition and conversion is available for the phytoalexin medicarpin, which is produced by red clover and alfalfa, maackiain is more readily isolated in large quantity from clover roots. The effect of medicarpin and phaseollin, a phytoalexin from *Phaseolus vulgaris*, was compared with the effect of maackiain. A preliminary report of this work has been published (7).

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MATERIALS AND METHODS

Phytoalexins.—The maackiain used was crystalline material (m.p. 179-182 C, uncorrected) isolated from red clover roots (6) and the phaseollin was crystalline material isolated previously (4) from bean. The medicarpin was prepared from jackbean seed inoculated with *Helminthosporium carbonum* Ullstrup by the method of Keen (8) except that final purification was by column chromatography on Sephadex LH-20 (Pharmacia Ltd., 2044 St. Regis Blvd., Dorval P.Q. H9P 1H6) (2) to remove maackiain which was present at a concentration ratio about 1:10 that of medicarpin.

Germ tube growth bioassays.—The *S. botryosum* (ATCC 26881) and *H. carbonum* isolates were those used in previous studies (2, 6) and cultures were maintained as described previously (2). A spore suspension of about 4×10^3 spores/ml was prepared by aseptically transferring dry spore masses from culture plates to sterile medium [half-strength Czapek Dox broth (Difco Laboratories, Detroit, MI 48232) prepared in 0.05 M citrate-phosphate buffer, pH 6.0, with ethanol added to 2% after autoclaving]. Drops containing 25 μliters of this suspension were put on sterile microscope slides inside

petri dish moisture chambers. Wax pencil rings drawn on the slides generally were used to prevent spreading of the drops. After incubation at 25 C for 4 hr (about 2 hr for *H. carbonum*), a second 25-μliter aliquot of culture medium containing a known quantity of phytoalexin in 2% ethanol was added to the drop on each slide to give a 50-μliter drop. For the "noncontinuous" method of assay, the slides were then incubated at 25 C, growth was stopped at specified times by adding cotton-blue in lactophenol and the longest germ tube of each of 50 spores on three slides for each treatment was measured. For the "continuous" method of assay, after the addition of the phytoalexin, a sterile cover glass was placed over the drop with each corner supported by fragments of broken cover glass. As soon as possible thereafter, a microscopic field (at × 100) containing 10 separate germinated spores was selected for each slide, a sketch made of the spores to facilitate subsequent examinations, and the longest germ tube of each of the 10 spores was measured. For each different treatment, three slides (30 spores) were used and the same germ tubes were remeasured at 1- to 2-hr intervals for 6 to 8 hr after the addition to maackiain. Results are given as the average increase in length of all germ tubes, not just of those that had resumed growth.

Rate of glucose-^{14}C uptake.—Spores of *S. botryosum* were washed twice in sterile 0.005% Tween-20 solution by centrifugation and resuspension in a modified Czapek medium (salts at one-half concentration, sucrose replaced by 0.1% glucose, and 0.05 M phosphate buffer with 0.005% Tween-20 and 1% ethanol). The spore suspension was adjusted to about 1.3×10^5 spores/ml (about 0.8 mg dry weight/ml) and 5.0 ml of this suspension were added to each of nine sterile 50-ml flasks. One μCi of D-glucose-UL-^{14}C (200 mCi/mM, ICN Pharmaceuticals Inc., 1956 Bourdon St., Montreal P.Q. H4M 1V1) in 10 μliters of 20% ethanol was added to each flask, and the flasks were incubated at 25 C. Immediately after the addition of glucose-^{14}C, a 0.2-ml sample of medium was transferred from each flask to scintillation vials by pipetting from just below the surface to avoid removing the spores which had settled to the bottom of the flask. After 3 hr, another 0.2-ml sample was removed from each flask and then maackiain in 23 μliters of 95% ethanol was added to give 50 μg/ml, 25 μg/ml, or 0 μg/ml (ethanol control). Samples (0.2 ml) then were removed after 4, 5, 6, 8, 10, 12, and 24 hr of incubation. Ten ml of "Aquasol" (New England Nuclear, 575 Albany St., Boston, MA 02118) were added to each sample immediately after sampling. Samples were counted on a Packard Model 3375 Tri-carb Liquid Scintillation Counter for two cycles of 2 min each.

Loss of ^{14}C-labeled compounds.—Spore suspensions were prepared as for the glucose-^{14}C uptake experiment with 2 μCi of D-glucose-UL-^{14}C per flask added to the nongerminated spores. After 10 hr of incubation at 25 C, the contents of each of six flasks were filtered on a membrane filter (GA-1, 5-μm pore size, Gelman Instrument Co., Ann Arbor, MI 48106), then the germinated spores were washed with 20 ml of sterile distilled water and resuspended in 5 ml of fresh nonlabeled medium. After removing one 0.2-ml spore-free sample of medium from each flask, maackiain in 23 μliters of ethanol was added to each of three flasks to give 50 μg/ml, and 23 μliters of ethanol only were added to each of the three control flasks. Samples of 0.2 ml of medium were taken 1, 2, 3, 4, 6, 8, 10, and 12 hr after the addition of maackiain, then Triton X-100 was added to each flask to 1% and the flasks were incubated overnight before a final sample was taken to determine the total "leakable-^{14}C" remaining. Addition of Aquasol and counting procedures were the same as for the uptake experiment.

Cytological observations.—Observations of the immediate cytological effect of phytoalexins on the germ tube apex were attempted with the method of VanEtten and Bateman (13) in which the phytoalexin is added to the edge of the cover glass. However, most germ tubes of *S. botryosum* stopped growing when the unsupported cover glass was added. Most observations and photographs were made with interference contrast optics and with spore suspensions prepared as for the continuous bioassay method. In some experiments, the supported cover glass was added prior to the addition of the phytoalexin and the phytoalexin was added later to the edge of the cover glass while a growing germ tube was under observation.

RESULTS

Effect of maackiain and medicarpin on germ tube elongation.—With the continuous method of bioassay, maackiain at 25 μg/ml completely inhibited elongation of the germ tubes of *S. botryosum* for at least 2 hr; elongation recommenced between 2 and 4 hr after the addition of maackiain (Fig. 1-B). Between 6 and 8 hr, the rate of elongation was comparable to that of the controls between 4 and 6 hr. The percentage of germ tubes that resumed elongation increased with time. The lower the maackiain concentration, the earlier elongation was re-initiated (Fig. 1-A). Control spores usually had several germ tubes that briefly stopped growing; this may have been caused by the addition of the ethanol and was more pronounced in preliminary experiments in which the germination medium did not contain ethanol initially. About 10-15% of the germ tubes measured in controls never resumed growth. At 25 μg/ml maackiain, 16% of the germ tubes had visibly re-initiated growth by "branch" formation (Fig. 2) just behind the original hyphal apex. Presumably, other germ tubes had resumed growth in a similar manner but because of the orientation of the hyphae and the low magnification used for measurements, the old apex was not visible.

Observation of germ tube apices by interference contrast optics at high magnification (× 800) showed that nontreated apices appeared relatively smooth, the only visible feature being elongated mitochondria which appeared as lengthwise striations. Changes in the apex after maackiain treatment were gradual and relatively subtle, but by about 30 min the apex was visibly rough due to clumping of vesicles, and movement of inclusions often was rapid and random (Fig. 2-A). As growth resumed, the apex resumed its normal appearance (Fig. 2-B, C, D).

Maackiain and medicarpin at 15 μg/ml and maackiain plus medicarpin (7.5 μg/ml of each) were compared for effect on germ tube elongation. The effect of medicarpin on germ tube elongation was similar to that of maackiain, and maackiain and medicarpin combined gave results

similar to each alone (Fig. 3). Because the experimental procedure prevented the measurement of enough germ tubes for good statistical comparisons in such a variable system, no attempt was made to determine whether the mixture gave significantly less inhibition than the phytoalexins alone.

Maackiain (50 μg/ml) also caused temporary cessation of growth of germ tubes of *H. carbonum* (Fig. 4) but the formation of appressoria by *H. carbonum* in the continuous bioassay system complicated interpretation of the results. In control treatments, about 50% of the germ tubes eventually formed appressoria which decreased the rate of elongation of these germ tubes. In contrast, in maackiain treatments, no appressoria formed (3); therefore, once growth had resumed it continued unimpeded. As a consequence, the degree of inhibition of *H. carbonum* actually was more severe than is apparent from Fig. 4.

Effect of phaseollin on germ tube elongation.—Phaseollin was tested at concentrations of 15 μg/ml or higher by the continuous bioassay method. In two of the experiments, no resumption of growth had occurred by the end of the experiment (5.5 hr in Expt. 1 and 8 hr in Expt. 2) and in a third experiment only 10% of the germ tubes had resumed growth by 8 hr after treatment. Germ tubes treated with phaseollin became granular in appearance almost immediately and the cytoplasm appeared to withdraw from the tip [Fig. 5-(A to C)]. Spores examined 24 hr after treatment with 15 μg/ml phaseollin had growing hyphae (Fig. 5-D) which were about twice the diameter of untreated hyphae but the original germ tubes were still granulated (Fig. 5-E) and had not elongated. In one noncontinuous bioassay, germ tubes existing at the time of the phaseollin (15 μg/ml) treatment had not begun growing by 8 hr, but 52% of the spores had produced new thicker germ tubes.

Effect of diffusates on germ tube elongation.—Because it was possible that the temporary inhibition of germ tube elongation was caused by a synergistic action of maackiain and the ethanol solvent, diffusate preparations from red clover leaves were tested directly to eliminate the need for an organic solvent. The diffusates were obtained by incubating spore suspensions of *H. carbonum* (about 5 × 10^4 spores/ml) on red clover leaflets for 23 hr as described previously (2). Controls consisted of filtrates of a portion of the spore suspension incubated as shallow drops in sterile petri plates for 23 hr, or diffusate treated to remove medicarpin and maackiain. In one experiment, this treatment involved filtering the diffusate through a membrane filter (5-μm pore size) on which 66% of the phytoalexins was adsorbed; in a second experiment, the

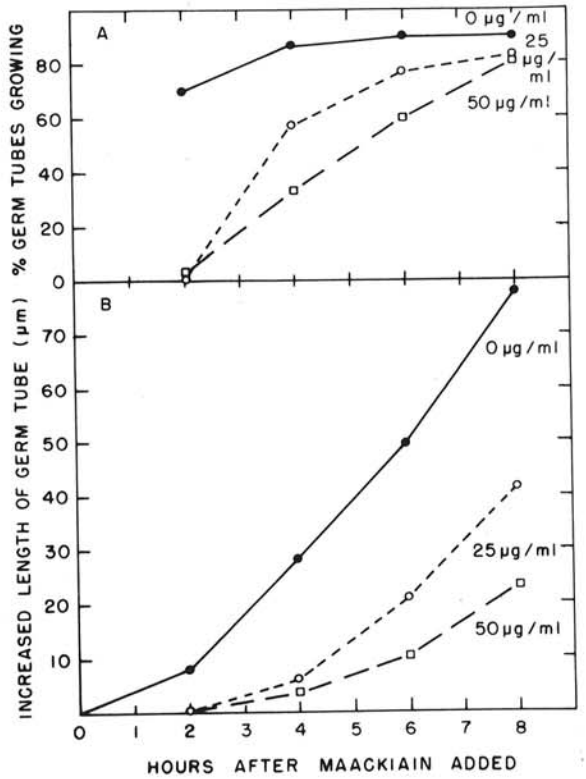

Fig. 1. Effect of 0 (control) (—•—), 25 (- -o- -) or 50 μg/ml (—□—) of maackiain on germ tube elongation of pregerminated *Stemphylium botryosum* spores. The longest germ tube of each of thirty spores/treatment was measured immediately following treatment (0 hr) and remeasured at the later time intervals.

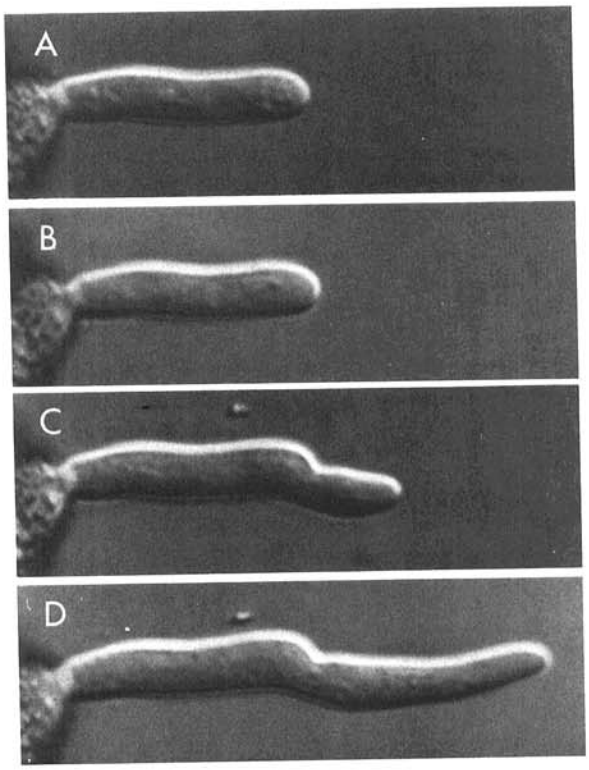

Fig. 2-(A to D). Effect of maackiain (15 μg/ml on the microscopic appearance of the germ tube apex of *Stemphylium botryosum* after exposure to maackiain for: **A)** 1.5 hr, **B)** 2 hr, **C)** 2.5 hr, and **D)** 3 hr (× 1,452). Maackiain was applied to germinated spores as for the continuous bioassay method.

diffusate was partitioned three times with an equal volume of carbon tetrachloride (CCl_4) to remove the medicarpin and maackiain. In all bioassays, 75 μliters of the solution tested was added to 25 μliters of *S. botryosum* spore suspension. Diffusates with a maackiain plus medicarpin concentration of about 18 or 20 μg/ml in the bioassay had an effect on germ tube elongation (Fig. 6, Table 1) similar to that of pure maackiain (Fig. 3) in 2% ethanol. Partial (Fig. 6) or complete (Table 1) elimination of the inhibitory effect of the diffusates was accomplished by treatments that removed part or all of the maackiain and medicarpin from the diffusate. Because of the experimental procedure, diffusates not treated to remove the phytoalexins were not sterile.

Effect of maackiain on glucose-^{14}C uptake of germinated spores.—Previous data (3) showed that maackiain and medicarpin affected the rate of oxygen uptake of germinated *S. botryosum* spores. To evaluate further the effect of maackiain on the metabolism of *S. botryosum*, the rate of change in glucose-^{14}C uptake by germinated spores treated with 25 or 50 μg/ml of maackiain was determined (Fig. 7). There was no marked change in the rate of glucose uptake for the first hour after treatment; for the next 2-hr period minimal uptake occurred in treated spores whereas the uptake by control spores continued at a fairly constant rate. By 8 hr, the rate of glucose uptake had increased in treated spores. If one assumes a constant rate of uptake from 12 to 24 hr, the rates were 4,692, 4,439, and 3,564 cpm/hr for the control, 25 μg/ml, and 50 μg/ml maackiain, respectively (data for 24 hr are not shown in Fig. 5). Thus, by 24 hr, the 25 μg/ml treatment had achieved a rate comparable to that of the control. Microscopic observation of germ tubes at 8 hr showed subapical branch formation in maackiain-treated germ tubes, indicating that growth had resumed by that time.

Effect of maackiain on loss of ^{14}C-labeled compounds from germinated spores.—Phaseollin has been reported (11, 13) to cause plasma membrane damage resulting in increased leakage of cell components into the external medium. To determine if such damage might be responsible for the temporary inhibition of germ tube elongation, the effect of maackiain on membrane permeability was measured by the rate of loss of ^{14}C-labeled cell constituents after treatment of germinated spores with 50 μg/ml of maackiain. An incubation period of about 10 hr was required for the germinating spores to accumulate sufficient glucose-^{14}C, therefore the germ tubes were considerably longer than in other experiments.

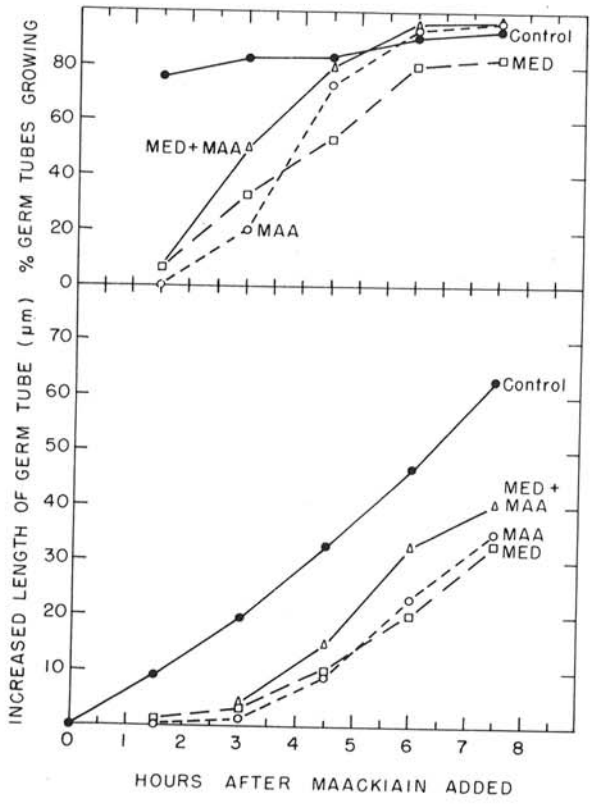

Fig. 3. Effect of 15 μg/ml of maackiain (- -o- -), 15 μg/ml medicarpin (—□—), 7.5 μg/ml maackiain plus 7.5 μg/ml maackiain (—Δ—) or ethanol only (—•—) on germ tube elongation of pregerminated *Stemphylium botryosum* spores. The longest germ tube of each of 30 spores/treatment was measured immediately following treatment (0 hr) and remeasured at the later time intervals.

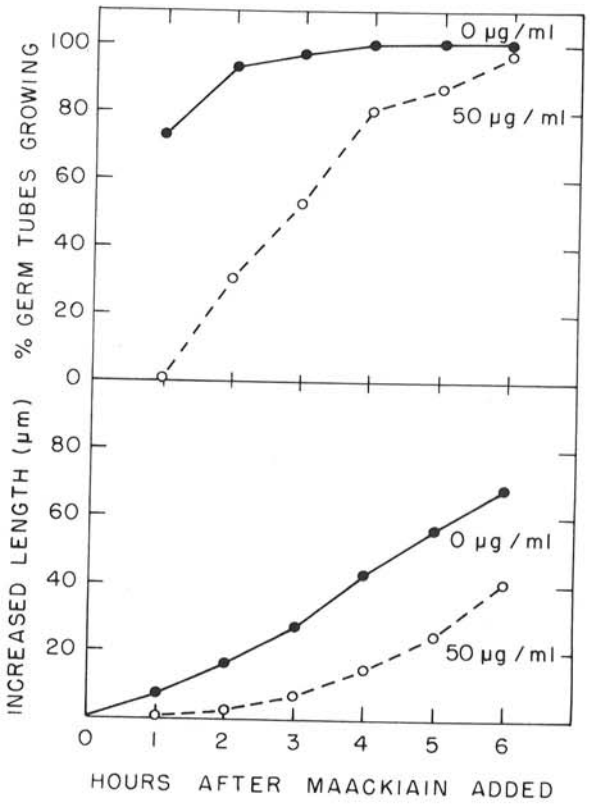

Fig. 4. Effect of 0 (control)(—•—) or 50 μg/ml maackiain (- -o- -) on germ tube elongation of pregerminated *Helminthosporium carbonum* spores. The longest germ tube of each of 30 spores/treatment was measured immediately following treatment (0 hr) and remeasured at the later time intervals.

Control spores lost 350 cpm/hr/0.2 ml of medium for the first 4 hr of incubation and 225 cpm/hr/0.2 ml of medium thereafter (Fig. 8). Maackiain-treated spores had an initial rate of loss of 1,225 cpm/hr in the first hour, 850 cpm/hr from 1 to 4 hr, 625 cpm/hr from 4 to 8 hr, and 250 cpm/hr from 8 to 12 hr. After the addition of Triton X-100 to all flasks at 12 hr, 0.2 ml of medium contained 17,995 ± 1,021 cpm and 18,155 ± 559 cpm for the control and maackiain treatments, respectively.

Effect of maackiain on elongation of "induced" germ tubes.—*Stemphylium botryosum* (2, 6) converts maackiain to the isoflavan dihydromaackiain by a system that is induced by maackiain, or by structurally-related phytoalexins. Dihydromaackiain is further converted to noninhibitory compounds. If the re-initiation of growth of *S. botryosum* germ tubes is related to the conversion of maackiain, "induced" spores would be expected to recover from a maackiain treatment more quickly than "noninduced" ones. *Stemphylium botryosum* spores germinated with or without 2 μg/ml maackiain, were compared for their reaction to 15 μg/ml maackiain. There was no detectable difference between the two treatments (Table 2). Qualitative thin-layer chromatography assays (6) confirmed that the maackiain conversion system had been induced by the initial maackiain treatment.

DISCUSSION

The results of the germ tube growth bioassays showed that germ tube elongation of *S. botryosum* is completely stopped for one to several hours after treatment with either maackiain or medicarpin and then elongation resumes. Bailey et al. (1) noted a similar lag period in mycelial growth bioassays with *Colletotrichum lindemuthianum* and phaseollin but did not determine if the re-initiation of growth was due to the resumption of growth of the original hyphae or the production of new hyphae.

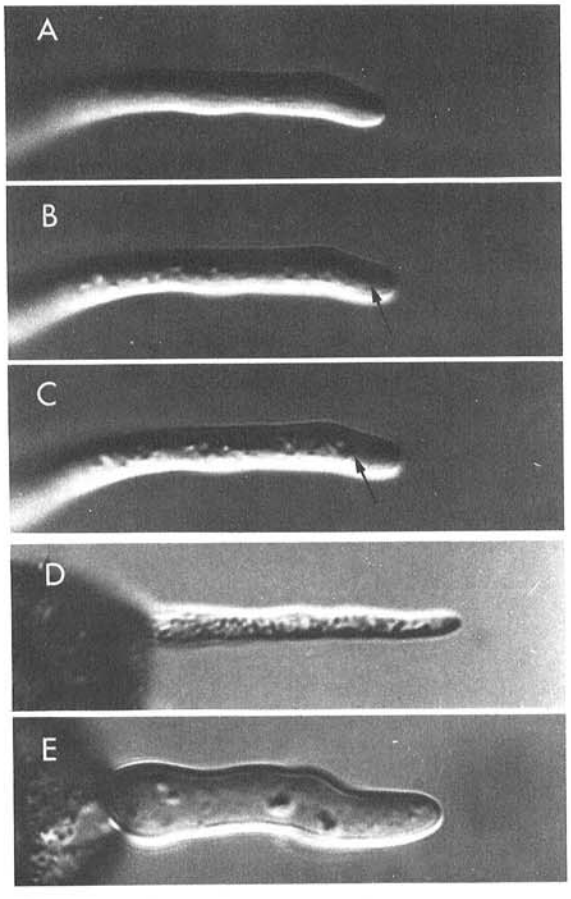

Fig. 5-(A to E). Effect of phaseollin (10 μg/ml) on the microscopic appearance of the germ tube apex of *Stemphylium botryosum*. Germ tubes **A)** before, **B)** 1 min, and **C)** 3 min after the addition of phaseollin to the edge of the cover glass. **D)** Germ tube 8 hr after exposure to phaseollin. **E)** Newly emerged germ tube 8 hr after treatment of the germinated spores with phaseollin (× 1,320). Arrow denotes point of retraction of the protoplasm from the apex.

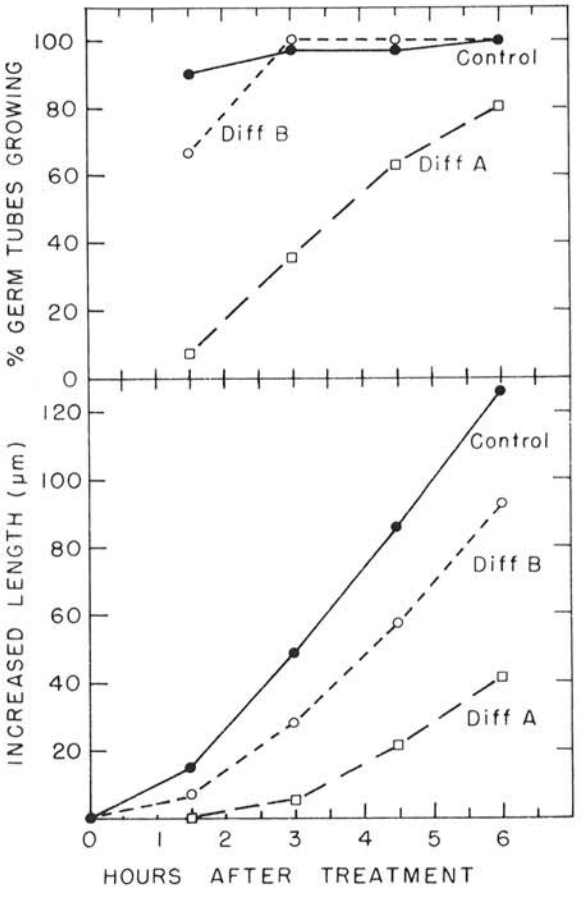

Fig. 6. Effect of diffusates from red clover leaves inoculated with *Helminthosporium carbonum* on germ tube elongation of pregerminated *Stemphylium botryosum* spores. The control (—•—) was supernatant from an *H. carbonum* spore suspension incubated as drops in a sterile petri plate. The diffusate was either untreated (DIFF A,—□—) or was passed through a 5-μm (pore size) membrane filter which adsorbed most of the phytoalexins (DIFF B,- -o- -). Untreated diffusate and filtered diffusate in the bioassay gave final maackiain plus medicarpin (approximately 1:1 ratio) concentrations of 18 and 6 μg/ml, respectively.

TABLE 1. Effect of diffusates from red clover leaves inoculated with *Helminthosporium carbonum* on germ tube elongation of *Stemphylium botryosum*

Treatment	Length of longest germ tube/spore (μm)[a] at:		
	0 hr[b]	2 hr	6 hr
Germination fluid[c]	25.6 ± 9.7	68.4 ± 19.4	208.6 ± 48.6
Complete diffusate[d]	25.6 ± 9.7	24.7 ± 9.0	45.6 ± 17.3
Diffusate minus CCl_4-soluble compounds[e]	25.6 ± 9.7	76.0 ± 17.7	208.8 ± 44.5

[a]Spores were killed and stained at each time and the longest germ tube/spore for 50 spores on each of three slides per treatment was measured except at 0 hr when only one set of three slides was used to determine the average germ tube length at the time of treatment. Values are ± standard deviation of the mean.

[b]Time at which the treatments were added to the spores which were pregerminated for 4 hr.

[c]Filtrate from spore suspension of *H. carbonum* in 0.05% Tween-20 solution incubated as a thin layer in sterile petri plates for 23 hr.

[d]Filtrate from spore suspension of *H. carbonum* in 0.05% Tween-20 solution incubated on the surface of red clover leaves for 23 hr before drops were recovered and pooled. Final maackiain plus medicarpin (approximately 1:1 ratio) concentration in the bioassay was 20 μg/ml.

[e]As for "d" above but maackiain and medicarpin were removed from the diffusate by partitioning three times with an equal volume of CCl_4.

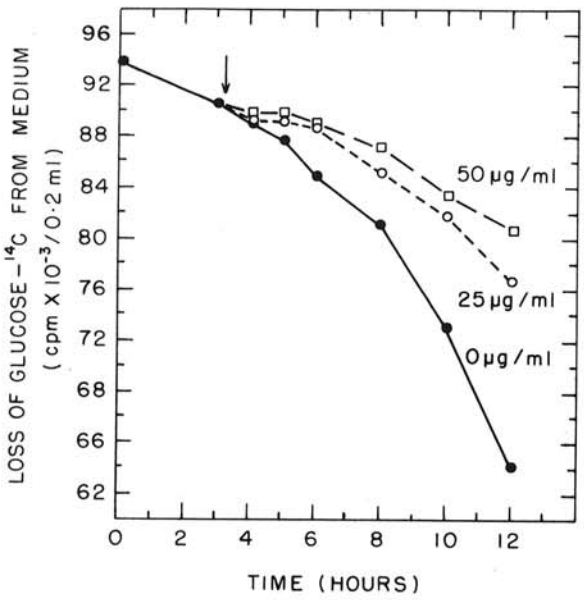

Fig. 7. Effect of 0 (control) (—•—), 25 (—□—) and 50 μg/ml (---o---) maackiain on the uptake of glucose-UL-^{14}C by pregerminated *Stemphylium botryosum* spores. Glucose-^{14}C was added at 0 hr and maackiain was added at 3 hr (arrow). At each time interval, 0.2-ml samples of culture medium were removed to determine the loss of ^{14}C.

In this study, use of the continuous bioassay method confirmed that it was the germ tubes present at the time of the phytoalexin treatment that resumed elongation and that the duration of the delay in elongation was dependent on the phytoalexin concentration. Unfortunately, it was not possible to determine if maackiain concentrations >50 μg/ml could inhibit elongation permanently because even at 50 μg/ml crystallization of maackiain frequently occurred and addition of higher maackiain concentrations was

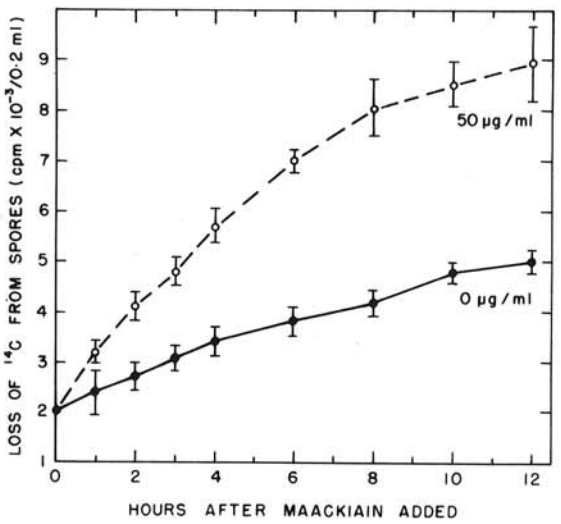

Fig. 8. Effect of 0 (control) (—•—) or 50 μg/ml (- -o- -) maackiain on the loss of ^{14}C-labeled constituents from *Stemphylium botryosum* spores germinated in the presence of glucose-UL-^{14}C for 10 hr. After the spores were washed, maackiain was added (0 hr) and 0.2-ml samples of the external medium were used to determine the increase in ^{14}C.

TABLE 2. Effect of maackiain (Maa) on germ tube elongation of *Stemphylium botryosum* spores "induced" to convert maackiain

Treatment	Length of longest germ tube/spore (μm)[a] at:		
	0 hr	2 hr	6 hr
Noninduced control[b]	38.9 ± 9.7	105.9 ± 21.5	279.6 ± 55.3
Induced control[c]	31.8 ± 7.5	81.2 ± 14.9	237.5 ± 53.3
Noninduced + Maa[b]	38.9 ± 9.7	38.5 ± 9.7	95.7 ± 23.9
Induced + Maa[c]	31.8 ± 7.5	36.3 ± 9.2	107.3 ± 28.2

[a]Spores were killed and stained at each time and the longest germ tube/spore for 50 spores was measured on three slides/treatment. Values are ± the standard deviation of the mean. Zero hour measurements were of spores killed at the time of the second maackiain treatment.

[b]The medium contained no Maa during germination but after 3 hr, maackiain was added to one-half the slides to give 15 μg/ml.

[c]The medium contained 2 μg/ml Maa during the germination period and after 3 hr, maackiain was added to one-half the slides to give 15 μg/ml.

impossible without increasing the amount of ethanol. Diffusates from *H. carbonum*-inoculated red clover leaves caused a similar delay of growth of germ tubes which confirmed that the delay was not the result of a synergistic effect between the ethanol solvent and maackiain.

Microscopically, maackiain affected the germ tube apex so that growth frequently was resumed by formation of a new apex just behind the original one. This response also occurs when the apex is affected by osmotic shock or heat (9) and occurred occasionally with *S. botryosum* when spores were germinated in the absence of ethanol and later were placed in 2% ethanol. Subtle cytological changes occurred in the treated apices, and after reinitiation of growth, apices were normal in appearance.

Germ tubes treated with phaseollin rarely resumed growth, instead new growth originated by the emergence of germ tubes which were abnormally large in diameter. This is somewhat similar to results with *C. lindemuthianum* (10) in which new growth in the presence of phaseollin emerged from a few cells that had survived the initial exposure. The previously described (1) delay in growth of *C. lindemuthianum* probably was due to the time required for this new growth to develop. Under the microscope, the effect of phaseollin on *S. botryosum* appeared much more severe than that of maackiain and the treated hyphae were similar in appearance to phaseollin-treated hyphae of *Rhizoctonia solani* (13) and *C. lindemuthianum* (10). From the extreme granulation of such treated hyphae, it was not unexpected that they did not resume growth.

As expected from the previous experiments on respiration (3), the pattern of glucose-^{14}C uptake by spores after maackiain treatment could be interpreted as reflecting the changes in germ tube elongation. Precise interpretation of the uptake data is complicated because both uptake and leakage of ^{14}C-labeled compounds may have occurred simultaneously. However, the contribution of ^{14}C-leakage products after only 3 hr of uptake was probably minimal. The leakage experiment suggests that there is an immediate effect of maackiain on the permeability of the plasmalemma as was proposed for the effect of phaseollin on *R. solani* (13). The decreasing rate of leakage may indicate a recovery that allows the resumption of growth by the germ tube.

Initially, it was assumed that the resumption of growth of germ tubes of *S. botryosum* was the result of conversion of maackiain to noninhibitory products, a process previously documented (6), but the results of two experiments suggest that such conversion was not involved. First, germinated spores in which the maackiain conversion system was induced by small additions (2 μg/ml) of maackiain were equally as inhibited by later additions of maackiain (15 μg/ml) as were noninduced controls. Second, germ tube growth of *H. carbonum*, a fungus which cannot metabolize maackiain (2), was similarly affected by maackiain. Smith (12) noted that a bean phytoalexin, kievitone, caused granulation and vacuolation of the protoplasm of hyphae of fungi whether or not they were sensitive to that phytoalexin in mycelial growth bioassays. He proposed that all of the fungi tested had a receptor site for kievitone but that the insensitive ones detoxified the kievitone and resumed growth. In this study, the resumption of growth by *H. carbonum* eliminated that hypothesis.

The results of this study suggest that germ tube growth of *S. botryosum* is inhibited by a transitory effect on the hyphal apex. Such a brief delay in a radial mycelial growth bioassay would not be detectable if elongation, once resumed, rapidly reached the rate of the controls. This transitory effect also is detectable by decreased rates of oxygen (3) and glucose uptake and by increased leakage of organic compounds from the hyphae. As the same effect on elongation occurs with *H. carbonum*, which is inhibited in mycelial growth bioassays, the question arises whether there is a second longer-term effect of maackiain that causes decreased rates of mycelial growth by *H. carbonum* but does not affect *S. botryosum*. Detailed studies of the growth of *H. carbonum* in the presence of maackiain will be required to clarify this point.

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