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Effects of Ophiobolin A on Ion Leakage and Hexose Uptake by Maize Roots[1]

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ABSTRACT

Ophiobolin A, a sesterterpene metabolite of *Helminthosporium maydis*, Nisikado and Miyake, stimulates net leakage of electrolytes and glucose from maize (*Zea mays* L.) seedling roots. Treatment of the roots with ophiobolin A at a concentration of 10 μg/ml (25 μM) inhibits uptake of 10 mM 2-deoxyglucose by 50% and of 0.5 mM 2-deoxyglucose by 85%. Compartmental analysis of the efflux of 3-O-methylglucose failed to show a similar effect of ophiobolin A on the rate of efflux of hexose. The inhibition of uptake is not reversible by washing. There is no difference in the effects on roots from cytoplasmic male sterile or normal cytoplasm plants, and exposure of carrot (*Daucus carota* L.) root discs to ophiobolin A also causes inhibition of 2-deoxyglucose uptake by this tissue.

It has been reported (4) that partially purified preparations of *Helminthosporium maydis* race T toxin, in addition to causing a host-specific stimulation of ion leakage from maize seedling roots and leaves, caused a stimulation of carbohydrate leakage that was not host-specific but occurred with tissues of both cmsT[2] and normal plants. Further investigation suggests that the carbohydrate leakage can be ascribed to the presence, in crude toxin preparations, of ophn A.

Ophiobolin A (Fig. 1) is a sesterterpene compound originally isolated from *Helminthosporium oryzae* (*Cochliobolus miyabeanus*) (11, 15) and also produced by several related fungal species including *H. maydis* (6, 19). The chemical properties have been investigated thoroughly (ref. 6 and references therein), but the phytotoxicity has been characterized incompletely. Orsenigo (15) showed that ophn A at concentrations as low as 30 μg/ml inhibited the elongation of rice coleoptiles and seedling roots and of oat coleoptiles. Treatment of beet root slices with ophn A caused leakage of anthocyanin. In a later report (16), leakage of phosphate and organic compounds from corn seedling roots and from potato tuber discs was mentioned. The inhibition of rice seedling growth was confirmed by Ohkawa and Tamura (13) and it was shown by Oku (14) that ophn A could be isolated from infected rice leaves, suggesting that ophiobolin toxicity may play a role in disease development.

In this paper, we report that ophn A is an effective inhibitor, at low concentrations, of hexose uptake in maize and carrot roots and stimulates ion leakage from these tissues.

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[2] Abbreviations used: cmsT: cytoplasmic male sterile; ophn A: ophiobolin A; 2-deG: 2-deoxyglucose; 3-MGL: 3-O-methylglucose.

MATERIALS AND METHODS

Equipment. Nuclear magnetic resonance spectra were recorded on Hitachi Perkin-Elmer R-20B or Varian Associates HA-100 instruments with tetramethylsilane used as the internal reference. Low resolution mass spectra were obtained on an Atlas CH4 mass spectrometer. Radioactivity measurements were in Bray's solution (1) using a Packard Inst. Co. (Downers Grove, Ill.) model 2425 liquid scintillation spectrometer. Quenching was corrected with an external standard. Conductivity measurements were with a model 31 conductivity bridge, Yellow Springs Inst. Co., Yellow Springs, Ohio. Carbohydrate determinations were carried out with a Technicon Auto-Analyzer with orcinol-sulfuric acid (8) and a maltose hydrate standard.

Chemicals. 2-Deoxyglucose and 3-O-methylglucose were obtained from Aldrich Chemical Co., Milwaukee, Wis. and 3-O-methyl-D-glucose (methyl-^{3}H), 3.62 Ci/mmol, from New England Nuclear. Silica Gel GF_{254} was from Brinkmann Inst. Inc., Westbury, N.Y. All other chemicals were reagent grade.

Ophiobolin A. *H. maydis* Nisikado and Miyake race T was grown in modified Fries medium (10) with the addition of 0.02 g $FeSO_4 \cdot 7H_2O$ and 1 g yeast extract/l; pH of the medium was 5.6. Spore suspensions were used to inoculate 200-ml portions of medium in 2,800-ml Fernbach flasks, and the flasks were incubated at 25 ± 1 C in a growth chamber with approximately 1200 ft-c of continuous light.

After 10 days, the growth medium was separated from the mycelial mats by straining through four layers of cheesecloth and filtering through Whatman No. 1 filter paper. The combined filtrate (12 liters) was transferred to a liquid-liquid extractor and extracted with diethyl ether for a total of 12 hr. The ether extract was dried with sodium sulfate and taken to an orange, nondrying oil by evaporation under vacuum at 45 C. The oil was dissolved in methylene chloride and crystallized by the addition of heptane. Washing with cold diethyl ether yielded 283 mg of white, needle-shaped crystals. Progress of the purification was monitored by TLC on Silica Gel GF_{254} plates developed with chloroform-methanol (100:2, v/v), and visualized by spraying with 1% phosphomolybdic acid in methanol followed by heating at 100 C for 2 min. After repeated recrystallization, TLC revealed a major component with an R_F (0.5) identical to that of authentic ophn A and a small amount of a second component with higher mobility. Nuclear magnetic resonance and low resolution mass spectra of this material corresponded to published data (6, 12).

Plant Material. Corn seeds (*Zea mays* L., W64A, N or T cytoplasm) were washed with water and placed in 20% H_2O_2 for 30 min. The seeds were washed three times with sterile distilled H_2O and transferred aseptically into sterile Petri dishes lined with two filter paper discs and moistened with 5 ml of 0.1 mM $CaCl_2$. Ten seeds were placed in each dish. The seeds were incubated for 3 days at 30 C in the dark. The root tips (approximately 2.5 cm) of the germinated seeds were removed for use in

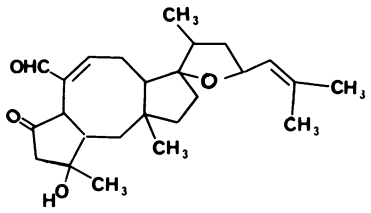

FIG. 1. Ophiobolin A.

the hexose leakage and absorption experiments. For ion leakage experiments, the seeds were not surface-sterilized and were germinated on paper towels moistened with 0.1 mM $CaCl_2$ for 5 days at 30 C in the dark.

Carrot roots (*Daucus carota* L.), obtained from a local grocery, were washed with distilled H_2O and pierced longitudinally with a 5-mm cork borer. The cylinders of tissue obtained were sliced into 2-mm lengths by using a device consisting of a series of razor blades clamped together with 2-mm spacers between the blades. The discs obtained were highly uniform, weighing 1.165 ± 0.015 g each.

Carbohydrate Leakage. Samples of 20 to 30 corn roots or carrot root discs were weighed and washed in distilled H_2O for 30 min. The samples were blotted dry and placed in test tubes containing 15 ml of 0.1 mM $CaCl_2$ in 5% acetone or 15 ml of ophn A solution (50 μg/ml) in 5% acetone containing 0.1 mM $CaCl_2$. These tubes were incubated at 30 C with aeration for 2 hr, after which the bathing solutions were decanted and the carbohydrate content was determined. To identify the carbohydrate, a portion of the bathing solution was concentrated 15-fold on a rotary evaporator and analyzed by two-dimensional paper chromatography on Whatman No. 3MM paper. In the first dimension, the chromatogram was developed three times by the ascending method in 1-butanol-pyridine-water (6:4:3, v/v) at 60 C. Development in the second dimension was descending with nitromethane-acetic acid-ethanol-water saturated with boric acid (8:1:1:1, v/v) at 40 C. The sugars were visualized by a modification of the silver nitrate dip technique (17).

Uptake of 2-deG. Samples of 20 to 30 corn roots or carrot root discs were preincubated in 15 ml of 0.1 mM $CaCl_2$ or ophn A in 0.1 mM $CaCl_2$ at 30 C for 30 min with aeration. The samples were then removed and placed in 15 ml of 2-deG solution and the incubation continued at 30 C with aeration. After 2 to 3 hr, the samples were transferred to running distilled H_2O and washed for 30 min. The samples were then extracted by grinding with a small amount of sand in a mortar with three 10-ml portions of 80% ethanol at 65 C. The extracts were filtered through cheesecloth and centrifuged to remove debris. The content of 2-deG in the extracts was estimated by a modification of the malonaldehyde-thiobarbituric acid method (21) in which the concentrations of periodic acid and arsenite reagents were increased to 0.075 N and 4.5%, respectively.

Leakage of 3-MG. Sterile roots from W64A N seeds were washed 2 hr in running distilled H_2O and then transferred to 1 mM 3-MG in 0.1 mM $CaCl_2$ with 18,000 cpm/μmol 3-MG (methyl-^{3}H). The samples were incubated at 30 C with aeration for 18 hr. The roots were removed, blotted dry, divided into approximately 1-g samples, and weighed. The samples were placed in separate 20-ml plastic syringes, the outlets of which had been fitted with rubber tubes clamped off with pinch clamps. The roots in each syringe were then covered with 10-ml of unlabeled 1 mM 3-MG in 0.1 mM $CaCl_2$ and aerated. At measured time intervals, the solutions were drained from the syringes and replaced with new 10-ml aliquots of the same composition. Samples on which the effect of ophn A was being tested were washed during the first 30 min with solutions containing 10 μg/ml ophn A in addition to the 3-MG and $CaCl_2$.

A portion (approximately 0.5 g) of the 3-MG (methyl-^{3}H)-loaded roots was set aside before the washout, weighed, and ground in a mortar and pestle with 80% ethanol at 65 C. The extract, filtered through Pyrex wool into a 25-ml volumetric flask and diluted to the mark, was used to estimate the amount of labeled 3-MG taken up by the roots before the washout began.

RESULTS

Glucose, and a trace of fructose, identified by two-dimensional paper chromatography, were the only carbohydrates detectable in the residue remaining from evaporation of an ophn A solution in which maize seedling roots had been soaked. Quantitative measurements of the carbohydrate leached by water or ophn A solutions (Table I) show that there is no difference in the effect on roots from cmsT and normal plants; furthermore, the leaching of carbohydrates from carrot root discs is stimulated to about the same extent as from corn roots. Ion leakage also was stimulated by ophn A, with no significant difference between cmsT and normal roots (Table II). The ophn A in the leakage experiments was dissolved in acetone and added to the bathing solution so the final concentration of acetone was 5%. Control experiments showed that acetone at this concentration had no effect on leakage of either carbohydrates or electrolytes.

Table I. Stimulation of Net Leakage of Carbohydrate from Root Tissues by Ophiobolin A.

Tissue	Ophn A 50 μg/ml	μg carbohydrate/ g fresh wt of tissue/ min
Maize, T cytoplasm	-	8.3
Maize, T cytoplasm	+	20
Maize, N cytoplasm	-	8.3
Maize, N cytoplasm	+	19
Carrot	-	29
Carrot	+	86

Table II. Stimulation of Ion Leakage from Maize Roots by Ophiobolin A.

Five-day-old seedling roots were bathed in 0.1 mM $CaCl_2$ in 5% acetone or 50 μg/ml ophn A in 0.1 mM $CaCl_2$ in 5% acetone. Conductivity was measured over a 2.5 hr period as described by Halloin et al. (4). Results are based on fresh weight of tissue.

Cytoplasm	Ophn A	μmhos/g.hr ± Std. dev.
T	-	22.4 ± 4.8
T	+	123.6 ± 24
N	-	25.2 ± 2.8
N	+	93.2 ± 5.6

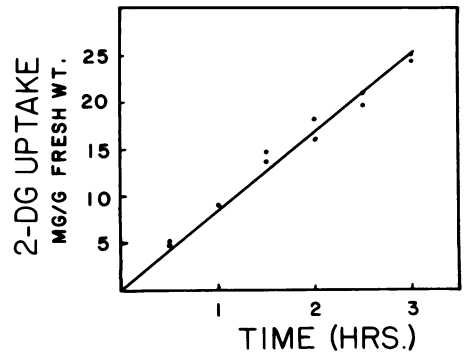

FIG. 2. Uptake of 2-DG by excised maize roots is linear with time.

The rate of leakage of phosphate was very low, and in contrast to the report of Orsenigo (16), there was no detectable stimulation of phosphate ion leakage by ophn A (data not shown).

To test the possibility that the stimulation of glucose leakage by ophn A was due to an inhibition of reabsorption of glucose that had leaked out, measurements of the rate of uptake of the nonmetabolizable glucose analogue 2-deG in the presence and absence of ophn A were made. Initial velocities were estimated from uptake in a 2-hr period. Uptake was linear with time for at least 3 hr at the highest concentration of 2-deG used (Fig. 2) and Sammler and Ehwald (18) have shown that at lower concentrations uptake of 2-deG by maize seedling roots is linear with time for at least 5 hr. Uptake of 2-deG by maize seedling roots exhibited Michaelis-Menten kinetics in each of the two concentration ranges tested, 0.05 to 1 mM and 3 to 40 mM. Hexose uptake by potato slices exhibits a similar behavior, with four phases detected (9), and multiphasic absorption of amino acids has also been reported (5). Ophn A concentrations as low as 2 μg/ml (5 μM) inhibited 2-deG uptake significantly (Fig. 3). The effect of 10 μg/ml ophn A on the maximum rate of uptake and K_m in two 2-deG concentration ranges is shown in Table III. In the higher concentration range, the maximum rate of uptake was not affected by ophn A while the K_m was increased 3-fold. The effect of ophn A was not eliminated by washing the roots between exposure to ophn A and beginning the uptake of 2-deG (data not shown). In the lower concentration range, both the maximum rate and the K_m were affected (Table IV) and this inhibition also was not reversed by washing. Inhibition of 2-deG uptake could result from interference with ATP synthesis by Ophn A rather than from a direct effect on the transport system. The kinetic data are not consistent with this explanation, however. Lowered ATP levels might lead to lowered maximum rates but they should have no effect on the K_m for 2-deG. The observed effect in the high concentration range is on K_m rather than the maximum rate. In the lower 2-deG concentration range, the maximum rate is lowered, but it is difficult to imagine that the cause is lowered ATP levels if the ATP supply was sufficient to maintain the much higher rate of uptake in the higher 2-deG concentration range. Uptake of 2-deG by carrot root slices was measured at only two substrate concentrations, within the low and high concentration ranges used with maize roots. At both concentrations, uptake was inhibited appreciably (Table IV).

To observe the effect of ophn A on the rate of efflux of glucose

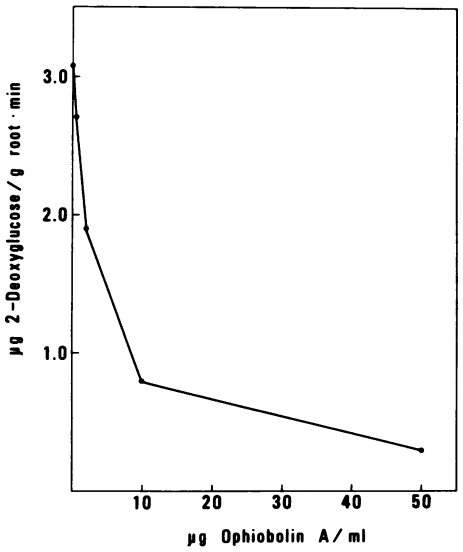

FIG. 3. Inhibition by ophiobolin A of 2-DG uptake into excised maize roots.

Table III. Ophiobolin A Inhibition of 2-Deoxyglucose Uptake by Maize Seedling Roots.

Uptake in the higher concentration range was measured using 3, 5, 10, 20, and 40 mM 2-deG and in the lower concentration range using 0.050, 0.125, 0.250, 0.500 and 1 mM 2-deG. Kinetic constants were estimated from a weighted least-squares fit of the data as described by Siano et al. (20). N and T cytoplasm roots were used in separate experiments but the results were indistinguishable and the data were pooled. Ophn A concentration was 10 μg/ml.

Concentration Range	Ophn A	Maximum Rate of Uptake	K_m
		μmol/hr.g	mM
High	--	5.9 ± 0.3	10.5 ± 1.2
High	+	6.7 ± 0.8	33.3 ± 3.0
Low	--	1.25 ± 0.06	0.13 ± 0.02
Low	+	0.55 ± 0.11	0.9 ± 0.2

Table IV. Ophiobolin A Inhibition of 2-Deoxyglucose Uptake by Carrot Root Slices.

2-deG	2-deG Uptake -Ophn A	2-deG Uptake +Ophn A 10 μg/ml
mM	μg/g.min	
0	0.37, 0.37	0.37, 0.36
0.25	0.45, 0.46	0.36, 0.37
20.0	1.49, 1.49	1.03, 1.03

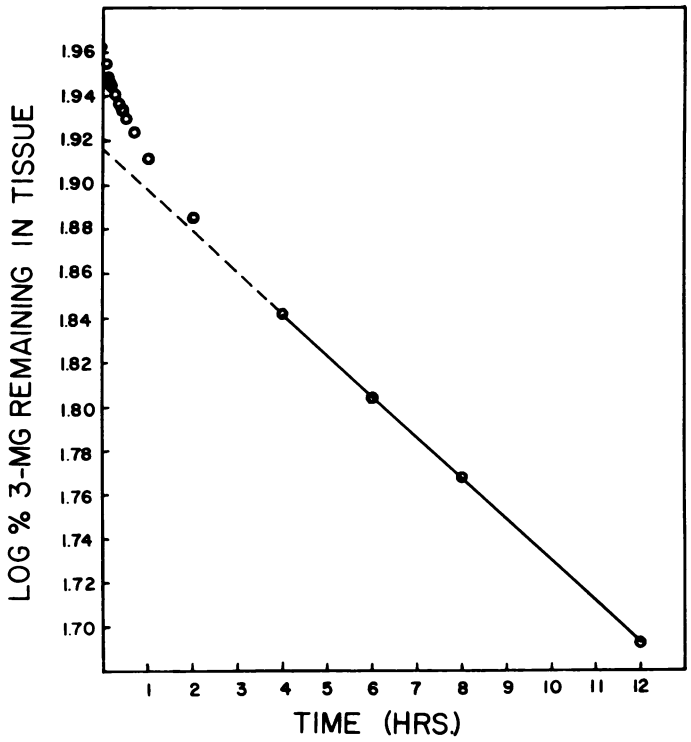

FIG. 4. Compartmental analysis of the rate of efflux of 3-O-methylglucose from excised maize roots. The line, which represents the limiting rate from the slowest compartment to empty, was fitted to the last four points by the method of least squares. The data in this figure and in Figure 5, are from the experiment labeled trial 1 in Table V and are representative of the data obtained in all of the experiments in Table V.

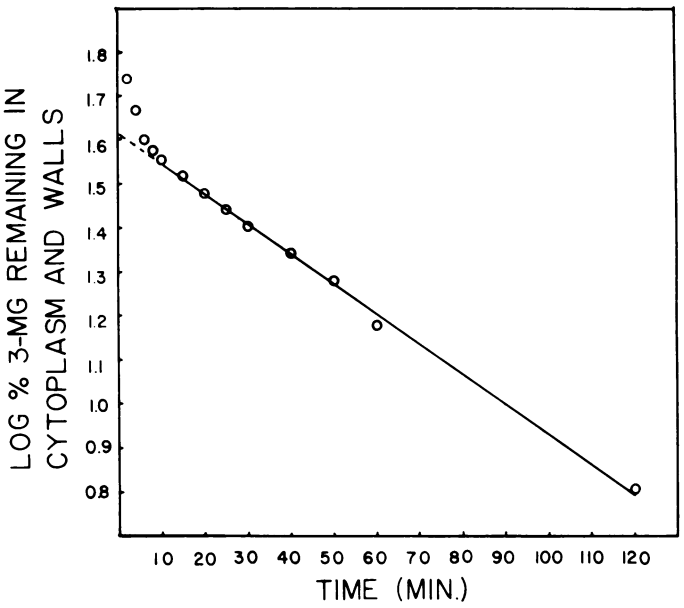

FIG. 5. Compartmental analysis of the rate of efflux of 3-O-methylglucose from excised maize roots. The line, fitted by least squares to the last seven points, gives the rate of efflux from the second slowest compartment to empty.

from maize roots, 2-deG is not a suitable analogue because it is phosphorylated within the cells (18). 3-MG, which behaves quite similarly in uptake (18) but is not phosphorylated, was used instead. Compartmental analysis (7) of the rate of leakage of 3-MG-(methyl-^{3}H) from maize seedling roots was carried out (Figs. 4 and 5) and the half-times for exit from the vacuolar and cytoplasmic compartments determined in the presence and absence of ophn A (Table V).

DISCUSSION

The effects of ophn A reported in this paper and in previous work (13-16) all relate to cellular permeability. Plant tissues shown to be affected include not only tissues from plants that are hosts for the *Helminthosporium* species that produce ophn A, but root slices from the unrelated plants, beet and carrot. Materials reported to leak from treated tissues range from inorganic electrolytes to anthocyanins. These observations suggest a general disruption of membrane structure during ophn A treatment. Most recently, a reinvestigation of the effects of ophn A on rice (*Oryza sativa* L.) and beet (*Beta vulgaris* L.) led Chattopadhyay and Samaddar (2) to the conclusion that the compound causes nonspecific damage to cell membranes. Our results suggest that ophn A is not causing general membrane disruption but is inhibiting specific membrane transport processes. Although we observed a stimulation of electrolyte leakage from maize seedling roots, we were unable to confirm Orsenigo's report of increased phosphate leakage. We are not able to reconcile our results with those of Chattopadhyay and Samaddar completely (2), but the concentrations of ophn A used in their experiments were relatively high, ranging up to 150 μg/ml, whereas the maximum concentration we could use, because of limited solubility, was 50 μg/ml.

Our results show that the increased net leakage of hexose from corn roots caused by ophn A treatment cannot be ascribed to an increased rate of efflux. A plausible explanation is suggested by the increased *Km* for hexose uptake in the presence of ophn A. This change would cause hexose, which has leaked out of the tissue at a normal rate, to be reabsorbed less effectively from the dilute exterior solution. The irreversibility of the effects of ophn A treatment and the lowering of the maximum rate of uptake in the high affinity system for hexose transport suggest a covalent modification of some membrane component related to transport. Ophn A thus might be used as a reagent for the derivatization of membrane components related to transport in plant cells.

Table V. Effect of Ophiobolin A on the Rate of Exit of 3-O-Methylglucose from Maize Seedling Roots.

Trial	Ophn A	$t_{0.5}$ ± Std. dev.[1] Vacuole	Cytoplasm
No.		Hr.	Min.
1	--	16.1±0.1	44.3±0.8
2	--	18.9±0.3	38.5±0.1
3	+	24.4±0.1	47 ±4
4	+	21.5±0.1	52 ±3

[1]Half-times and standard deviations for each trial were obtained by least-squares fit of the data.

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