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Malformin in *Aspergillus niger*-Infected Onion Bulbs (*Allium cepa*)[1]

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Malformin was identified, by its biological activity and chromatography, in acetone extracts of the outer scales of onion bulbs infected with *Aspergillus niger*. Malformin was not detected in tissue underlying the infected areas or in the central portions of the bulbs, nor was malformein liberated from extracts or extracted tissues after reduction with zinc in acetic acid. This is the first report of naturally occurring malformin.

Black mold is a storage disease caused by *Aspergillus niger* on both colored and white cultivars of onions (*Allium cepa*). Although signs of the pathogen are most obvious on the outer scales, the disease is not confined to the exterior portion of the bulb (12). We recently obtained a supply of black molded onions and examined them for the presence of malformins, a small family of cyclic pentapeptides produced by *A. niger* (1, 11) and several other members of the *A. niger* group of *Aspergillaceae* (4). In addition to causing serious disturbances in the growth of higher plants (2, 6), malformin is antibiotic to a variety of bacteria (9).

We report the first example of the natural occurrence of malformin.

MATERIALS AND METHODS

Source and preparation of samples. Onion bulbs (*A. cepa* cv. Spartan Banner) were submitted to the Plant Disease Diagnostic Laboratory (Botany and Plant Pathology, Purdue University) in February 1974 for disease identification. There were irregular masses of black powdery spores generally restricted to two or three outermost scales. Internal scales lacking fungal sporulation appeared normal. Outer scales of severely infected onions were slightly desiccated and easily separated from inner scales. The onions were grown in northern Indiana on muck soil during the summer of 1973. Because of dry soil conditions, onion foliage dried prematurely during mid-August, and foliar fungicide sprays, normally applied weekly until harvest, were terminated 2 to 3 weeks prior to harvest in late August. Bulbs were field-cured before harvest and placed in conventional storage. Disease was not apparent at harvest. Onions were removed from storage in early February and processed by normal topping, sorting, and packaging. Onions showing disease were culled during sorting and either destroyed or delivered to our laboratory for diagnosis. Grower losses were negligible.

Three portions of the bulbs were extracted and analyzed for the presence of malformin. The outer, visibly infected scales were separated from healthy appearing tissue. After removal of infected scales, the bulbs were washed in tap water to remove surface contaminating conidia. A layer one scale in thickness was removed and termed the underlying tissue. The remainder of the bulb is referred to as the central area.

Extraction. Infected outer scales (638 g fresh weight), underlying scales (1,140 g), and central areas (1,280 g) were macerated and steeped separately for 24 to 48 h in acetone (2 liters). The extraction was repeated three times with fresh acetone. The extracts were combined, filtered through cheese cloth, and evaporated to dryness on a steam bath, and portions of the residues were examined for the presence of malformin by bioassay.

Bioassay. Bean seedling malformations and corn root curvatures were used to assess weighed portions of the acetone-soluble compounds for malformin (3). Fractions (1.0 g) of the residue were dissolved in water (20 ml), diluted with water containing Tween 20 (0.1%), and applied to the apical bud of *Phaseolus vulgaris* cv. Harvester, and the seedlings were observed for malformations after 10 days in the greenhouse. Similar dilutions, without Tween 20, were assayed by the root curvature method in which seeds of *Zea mays* WF9 × 38-11 were germinated for 3 days at 28 C on filter paper, moistened with 3.0 ml of test solution, in petri dishes. The amount of malformin present was estimated from standard curves prepared from root curvature bioassays using authentic malformin A isolated from culture filtrates of *A. niger* strain 58-883 (10).

Chromatography. A portion (4.0 g) of the acetone-soluble residue was dissolved in water (150 ml) containing an excess of $NaHCO_3$ (pH 8.0) and extracted four times with ethyl ether (150 ml). The ether, in which malformin is soluble, was evaporated in a hood, and the residue was dissolved in ethyl acetate (30 ml). Strips (2.5 by 40 cm) of Whatman no.

[1] Journal Paper no. 5514 of the Purdue Agricultural Experiment Station.

3 paper were streaked with small volumes (0.1 ml) of the solution, developed in a variety of solvents by descending chromatography, dried, and cut into 2.0-cm sections beginning 1.0 cm behind the origin. Each section was heated in water (20 ml) for 5 min on a steam table, and the solution was examined for malformin by the root curvature method. To locate malformin, similar chromatograms were streaked with fractions of the test solution containing [^{14}C]malformin A (ca. 800 to 900 dpm) prepared by biosynthesis as described by Ciarlante and Curtis (Proc. 8th. Int. Conf. Plant Growth Substances, Tokyo, 1973, in press). These strips were developed, dried, sectioned, and analyzed for ^{14}C by liquid scintillation counting.

RESULTS

Identification of pathogen. Five fungal isolates were obtained by streaking spores from onion tissues onto potato dextrose agar. Cultures transferred to Czapek agar were all identified as *A. niger* as described by Raper and Fennell (7).

Bioassay. Bean seedlings treated with the acetone-soluble residue from the central and underlying tissues of infected onions at three concentrations (20, 2, 0.2 mg/ml) grew normally. Similar preparations from infected exterior scales induced severe malformations on 100% of the seedlings (20 mg/ml), moderate malformations on 70% (2 mg/ml), and slight malformations on 7% of the seedlings (0.2 mg/ml). The malformations were identical to those induced by authentic malformin (Fig. 1).

No evidence for the presence of malformin in the acetone-soluble residue from the central and underlying tissues was obtained by the root curvature bioassay (Table 1), but a typical malformin dosage-response relationship was obtained with extracts of the infected outer scales. These root curvatures were identical to those induced by authentic malformin (Fig. 2). By comparison with standard curves obtained with malformin, we estimated that infected tissues contained the malformin equivalent of 3 to 9 mg/kg (fresh weight). Malformin was not detected in extracts from any portion of disease-free bulbs.

The apparent absence of malformin-like activity in extracts of the central and underlying tissues is not proof for the absence of malformin in either the tissues or the extracts. Malformin reacts with thiol compounds to form 1:1 addition products (5), and when [^{14}C]malformin is supplied to *P. vulgaris* a substantial portion is bound to a cell wall fraction and can not be extracted with organic solvents (D. Ciarlante and R. Curtis, in press). However, much of the

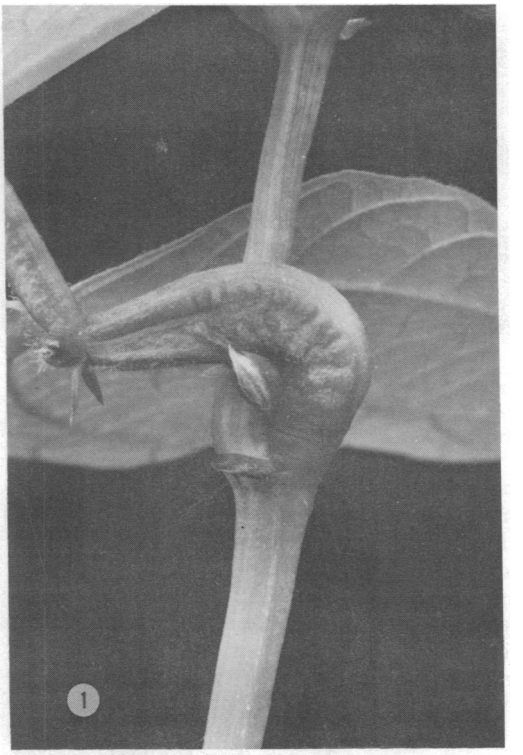

FIG. 1. *Malformed bean seedling produced by treatment with extract of A. niger-infected onion scales.*

TABLE 1. *Root curvature bioassay for malformin in acetone-soluble compounds from portions of onion bulbs infected by A. niger*

Concn of residue[a] (mg/ml)	Roots with >90° curvature (%)		
	1[b]	2	3
50	0	7.7	15.4
25	0	10.0	3.2
12.5	3.2	13.3	10.0
6.2	13.3	13.3	10.0
3.1	0	3.2	46.6
1.5	6.7	13.3	100.0
0.7	10.0	6.7	100.0[c]
0.3	3.2	6.7	100.0
0.1	10.0	0	93.5
0.05	6.7	3.2	81.0

[a] Control was water (4.0% of the roots had >90° curvature).

[b] Location of tissue: 1, center; 2, underlying; 3, infected exterior scales.

[c] Taken as equivalent to the response of corn roots to the optimal concentration of malformin (0.1 μg/ml) as determined from standard curves prepared from assays using authentic malformin A. Curvatures on less than 15% of roots not considered significant.

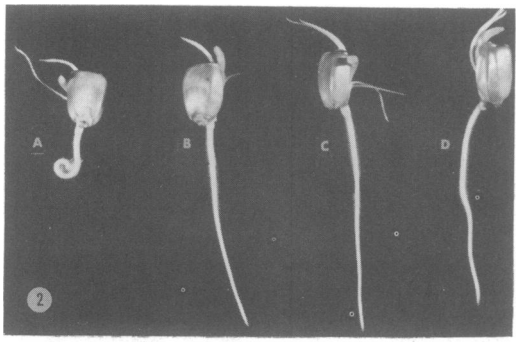

FIG. 2. *Zea mays germinated in the presence of extracts from (a) infected, (b) underlying, and (c) central portions of A. niger-infected onions, and (d) water.*

bound malformin is released as [^{14}C]malformein (dithiol malformin) after reduction with zinc in boiling acetic acid. Consequently, acetone extract residues and the tissues remaining after extraction were reduced, filtered, evaporated to dryness, and extracted with ether, and the ether-soluble fraction was examined for malformein by the root curvature assay. No evidence for the presence of malformein in the central and underlying tissue was obtained. Furthermore, after reduction the biological activity of acetone-soluble residues from infected scales decreased by approximately 90%, which also occurs when malformin is similarly converted to malformein.

Chromatography. The chromatographic behavior of the active substance extracted from infected onion scales was identical with that of [^{14}C]malformin A (Table 2). No other biologically active compounds were detected on the chromatograms, and we concluded that a malformin was present in *A. niger*-infected onion bulbs.

TABLE 2. *R_f values of malformin extracted from A. niger-infected onion bulbs and authentic [^{14}C]malformin A*

Solvent	R_f	
	Onion-extracted malformin	[^{14}C] malformin A
N-heptane	0.00	0.00
Chloroform	0.86	0.86
N-heptane-chloroform (9:1, vol/vol)	0.00	0.00
N-heptane-chloroform (1:1, vol/vol)	0.05	0.05
N-heptane-chloroform (4:6, vol/vol)	0.09	0.09
N-heptane-chloroform (3:7, vol/vol)	0.22	0.22
Isopropanol-NH_3-water (10:1:1, vol/vol/vol)	0.91	0.91
Isopentanol-pyridine-water (10:10:9, vol/vol/vol)	0.93	0.92
Toluene-acetic acid (1:1, vol/vol)	0.93	0.93
Water	0.76	0.76

DISCUSSION

When compared with authentic malformin, the similar solubility properties, biological activity, chromatographic behavior, and partial loss of activity after reduction indicate that the active substance extracted from *A. niger*-infected onion bulbs is a member of the malformin group. No attempt was made to determine which of the malformins was present, and the various types cannot be differentiated by paper chromatography. The presence of malformin in only the infected, external scales indicates little or no diffusion to the inner portions of the bulb. Reaction of malformin with thiol groups in the tissues may preclude or hinder movement to other portions.

Little information is available concerning the toxicity of malformin to animals. The compound is quite toxic to mice (mean lethal dose = 0.72 mg/kg, intraperitoneally) and is cytostatic in vitro to P-815 mastocytoma cells (mean effective dose = 0.059 μg/ml) (H. P. Sigg, Sandoz Ltd., personal communication). Among aspergilli grown on wheat and soybeans and fed to mice and chicks, *A. niger*, *A. ficuum*, and *A. phoenicis* were toxic and/or growth stunting (8). Although not implicated in these studies, malformins are produced by each of these species (4). The presence of malformin in *A. niger*-infected onions warrants an awareness for this compound in other foods molded by this ubiquitous species.

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