Structural and Exposure Time Requirements for Chemical Stimulation of Germination of Uredospores of *Uromyces phaseoli*

Richard C. French,* Charles L. Graham, Anne W. Gale, and Robin K. Long

Germination of uredospores of *Uromyces phaseoli* (bean rust) was stimulated by linear, branched, saturated, and unsaturated methyl ketones, in the range of 6–9 carbons, and by cyclic unsaturated ketones, particularly β-ionone, which was used as a standard for bioassay of activity. In contrast to uredospores of *Puccinia graminis* f. *tritici* (stem rust of wheat), spores of *U. phaseoli* were not stimulated by linear or cyclic aldehydes or alcohols. Spores of *U. phaseoli* were induced to germinate in pustule by β-ionone at concentrations lower than 0.5 ppm when diseased plants were placed in dew chambers overnight. Spores floated on water and exposed to vapors of 10 μl of β-ionone for 20 s germinated 95% at the end of the 4-h germination period, compared to 1% for controls. Uredospores of *Puccinia graminis* f. *tritici* and *Puccinia coronata* (crown rust of oats) responded similarly to vapors of nonanal.

More than 60 compounds have been shown to stimulate the germination of uredospores of *Puccinia graminis* Pers. f. *tritici* Eriks. & E. Henn (stem rust of wheat). These chemicals that stimulate germination range in length from 5 to 12 carbon atoms, and include alcohols, aldehydes, ketones, amines, cyclic and noncyclic derivatives, and sulfur derivatives (French, 1961; French and Gallimore, 1971). Two stimulatory compounds, nonanal and 6-methyl-5-hepten-2-one (methylheptenone), were first found in uredospores of *P. graminis* (French and Weintraub, 1957; Rines et al., 1974); nonanal was found also in spores of *Uromyces phaseoli* (Reben.) Wint., but did not stimulate their germination. Spores of *U. phaseoli* have not been analyzed for content of methylheptenone, but this compound stimulated their germination (French et al., 1975a).

In this research, the chemical structural requirements for compounds to stimulate germination of spores of *U. phaseoli* were determined and compared with those for compounds previously shown to stimulate germination of spores of *P. graminis*. The response of spores of *U. phaseoli* to brief exposures of β-ionone was compared with that of *P. graminis* and *Puccinia coronata* Cda. (crown rust of oats) to nonanal.

MATERIALS AND METHODS

Uredospores were grown in the greenhouse on young potted plants (*Uromyces phaseoli* on bean, *Phaseolus vulgaris* L. var. Top Crop; *Puccinia graminis* on wheat, *Triticum aestivum* L. C.V. Baart; and *Puccinia coronata* on oats, *Avena sativa* L. C.V. Clinton) and were harvested a few minutes before use. Spores (approximately 2.0 mg ± 0.06 standard error) were dispensed from a capillary tube, either directly to the surface of 0.5 ml of distilled water (alone or containing volatile test chemicals) in microdishes placed in the center of Conway diffusion cells, or after suspension on the surface of a minimum volume of distilled water in a 5-ml beaker; dishes and cells were then covered with a ground glass plate, the whole unit serving as an individual isolation chamber. With the exceptions of piperitone (85% pure) and 2,6-dimethyl-4-heptanone (80% pure), all active compounds tested exceeded 95% purity as determined by gas chromatography on diethylene glycol succinate columns.

Chemicals were added by Hamilton microsyringe to 2.0 ml of distilled water to give a final concentration from 10 to 1000 ppm in the center of the cell, and 0.5 ml was transferred to microdishes also placed in the center. Tests were carried out in the dark at room temperature, 20–25 °C, for 4 h. Formaldehyde was added to stop germination, and loops of spores were transferred to glass slides for observation. Germination percentages were based on 400 spores counted by 2 observers, and reported ± standard error. The various compounds were rated against the most effective compound, β-ionone at 25 ppm. Ratings were calculated as follows: test compound at its most active concentration (parts per million/volume) was compared with that of the most active compound, β-ionone, at 25 ppm:

$$\frac{\%\text{ germination (treated)} - \%\text{ germination (water control)}}{\%\text{ germination (}\beta\text{-ionone)} - \%\text{ germination (water control)}} \times 100 = \text{rating}$$

For example:

5-Methyl-2-hexanone	250 ppm	92.5% germination
β-Ionone	25 ppm	95.5% germination
Water control		0.75% germination

$$\frac{92.5 - 0.75}{95.5 - 0.75} = \frac{91.75}{94.75} = 96.8 \text{ rating}$$

Exposure time experiments were carried out with spores completely covering the surface of 2.0 ml of distilled water in the center of Conway diffusion cells. For *U. phaseoli*, 10 μl of undiluted β-ionone was placed on ground glass lids, and placed over the cells for exposures of 1, 2, 5, 10, 20, 30, 60, 120, and 300 s and then removed and covered with a clean lid. Germination was counted in the usual manner after 4 h. For spores of *P. graminis* and *P. coronata*, the same procedure was followed with 10 μl of *n*-nonanal and a germination time of 90 min.

In rinsing experiments, water was withdrawn by pipet from floating spores of *U. phaseoli* exposed for 20 s to β-ionone vapor and replaced with 2.0 ml of fresh distilled water. Other exposed spores were rinsed 5 times with 2.0 ml of distilled water to remove dissolved β-ionone. Dry spores (2.0 mg) placed in a thin layer on plastic petri dish lids were also exposed for 20 s to vapor of β-ionone (10 μl) in Conway cells. Spores were then tapped from the lids to the surface of 2.0 ml of distilled water in Conway cells,

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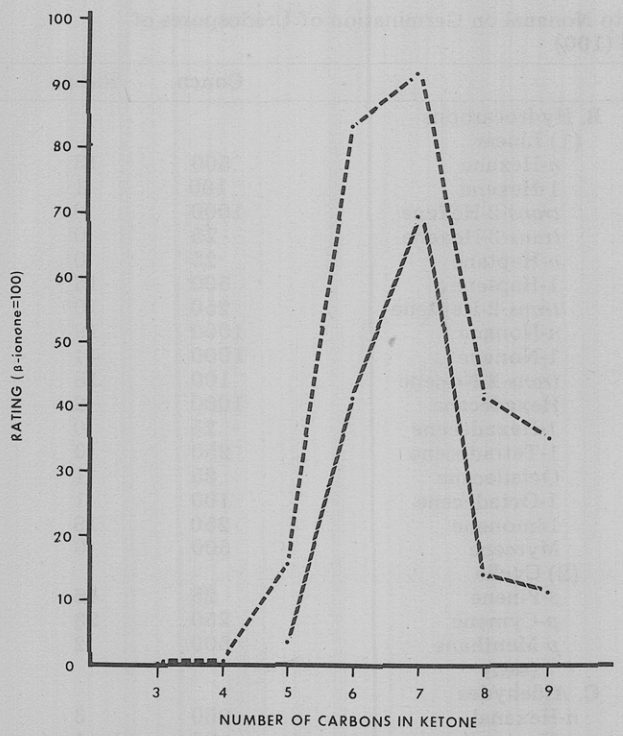

Figure 1. Comparative activity of 2- and 3-ketones on germination of uredospores of *U. phaseoli*: (upper curve) 2-ketones; (lower curve) 3-ketones.

covered, and allowed to germinate.

Germination of *U. phaseoli* spores in pustules was studied by placing diseased bean plants in a 220-l. dew chamber; 0.05–0.1 ml of undiluted β-ionone was placed on a 10-cm disk of filter paper and suspended on a 100-ml graduated cylinder in the center of the chamber overnight at 22 °C.

EXPERIMENTAL RESULTS

Of the linear 3–9 carbon ketones studied, with β-ionone as the standard, 2-heptanone was rated most active at 91 (Figure 1), followed by 2-hexanone at 83. Ketones with less than 6 carbons showed little activity. The carbonyl was more active in the 2 position than in the 3, and the carbonyl in the 3 position was more active than that in the 4. All the heptanones tested were more active than the octanones or nonanones examined (Table I). Activity in 8- and 9-carbon compounds was confined to compounds with the carbonyl in the number 2 position.

Of the branched and/or unsaturated ketones studied (Table I), 5-methyl-2-hexanone was almost as active (a 96 rating at 250 ppm) as the standard. The ketone endogenous to spores of *P. graminis*, 6-methyl-5-hepten-2-one, at 50 ppm had activity equal to 64% of that of the standard. 5-Hexen-2-one had 42% activity at 250 ppm.

Of the unsaturated cyclic ketones studied, α-ionone 93 (almost as active as β-ionone), carvone 88, and piperitone 51 were the most active. The ketone, 4-phenyl-3-buten-2-one, is structurally related to β-ionone, but the ring is benzenoid and unsubstituted, and was completely inactive, as was 1-phenyl-2-butanone. The saturated cyclic ketones, menthone and fenchone, were also inactive or slightly inhibitory.

The hydrocarbons studied, ranging from 6 to 18 carbons, had little activity. 1-Nonene was the most active at 1000 ppm, 44% of the standard. The following compounds had minimal activity (less than 20% of standard): *n*-nonane, *trans*-2-nonene, *n*-hexane, 1-heptene, *trans*-2-heptene, and limonene.

Figure 2. Rusted bean plants after an overnight exposure at 22 °C to dew ± 0.5 ppm of β-ionone: (left) control; (right) treated, showing cottony, white masses of germ tubes.

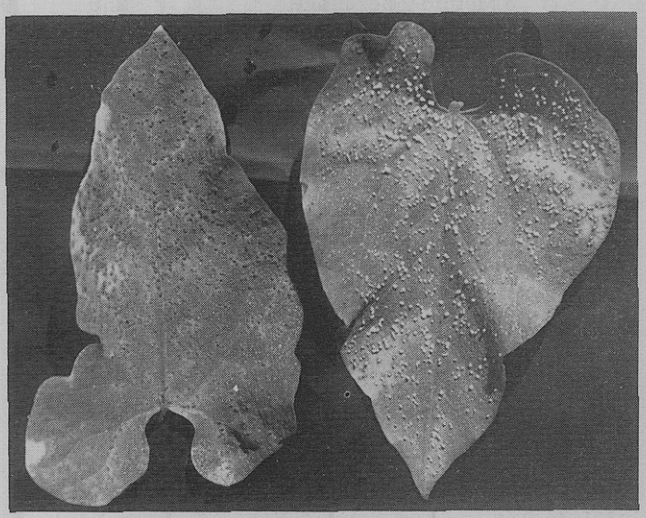

Figure 3. Leaves from bean plants treated in dew chambers; (left) control; (right) β-ionone.

When compared with the responses of *P. graminis*, the lack of activity of *U. phaseoli* to a wide assortment of linear, cyclic, saturated, and unsaturated alcohols and aldehydes was particularly striking. Nitrogen and sulfur derivatives also were virtually inactive. Methyl ketones were by far the most active class of compounds. β-Ionone, 5-methyl-2-hexanone, α-ionone, and 2-heptanone were most effective, in that order.

Of the stimulatory compounds tested, only β-ionone stimulated germination of spores of *U. phaseoli* in the pustules. Up to 95% of the pustules of diseased plants exposed to as little as 0.5 ppm of this compound showed as cottony white spots, instead of the usual dark brown of the ungerminated spores (Figures 2 and 3). In in vitro studies, spores floated on water solutions of 25 ppm of β-ionone at 22 °C overnight also germinated to form masses of cottony white germ tubes (Figure 4). Germination of spores floated on water solutions was stimulated by β-ionone at concentrations as low as 0.1 ppm (Figure 5).

Spores were exposed to vapors of β-ionone to determine how brief an exposure could induce maximum percent germination. Germination was stimulated after an exposure time of only 1 s. Exposures for 20 s increased germination from 1% in controls to over 95% (Figure 6). Comparable experiments with 10 μl of *n*-nonanal on germination of uredospores of *P. graminis* and *P. coronata*

Table I. Stimulatory Action of Terpenes and Chemicals Related to Nonanal on Germination of Uredospores of *Uromyces phaseoli*, Compared to β-Ionone at 25 ppm as Standard (100)

	Concn	Rating
A. Ketones		
(1) Linear, saturated		
2-Propanone (acetone)	500	0
2-Butanone	1000	1
2-Pentanone	1000	15
3-Pentanone	1000	3
2-Hexanone	500	83
3-Hexanone	100	41
2-Heptanone	100	91
3-Heptanone	250	69
4-Heptanone	100	44
2-Octanone	50	41
3-Octanone	250	14
2-Nonanone	100	35
3-Nonanone	25	11
4-Nonanone	25	1
5-Nonanone	100	1
(2) Unsaturated, branched		
5-Methyl-2-hexanone	250	96
6-Methyl-5-hepten-2-one	50	64
5-Hexen-2-one	250	42
2,6-Dimethyl-4-heptanone	1000	31
4-Methyl-3-penten-2-one	500	9
3-Methyl-2-butanone	250	8
(3) Cyclic		
α-Ionone	25	93
Carvone	50	88
(+)-α-Carvone	25	72
(−)-α-Carvone	25	23
Piperitone	25	51
Menthone (sat.)	25	7
Fenchone (sat.)	100	−7
1-Phenyl-2-butanone	10	1
4-Phenyl-3-buten-2-one	10	1
2-Furyl methyl ketone	100	1
1-(2-Furyl)-1,3-butanedione	500	0
B. Hydrocarbons		
(1) Linear		
n-Hexane	500	13
1-Hexene	100	1
trans-2-Hexene	1000	0
trans-3-Hexene	25	0
n-Heptane	25	0
1-Heptene	500	16
trans-2-Heptene	250	10
n-Nonane	1000	19
1-Nonene	1000	44
trans-2-Nonene	100	18
Hexadecane	1000	2
1-Hexadecene	25	0
1-Tetradecene	250	0
Octadecane	25	1
1-Octadecene	100	1
Limonene	250	16
Myrcene	500	0
(2) Cyclic		
β-Pinene	25	46
p-Cymene	250	23
p-Menthane (sat.)	500	2
C. Aldehydes		
n-Hexanal	250	3
n-Heptanal	25	−1
n-Octanal	25	0
n-Nonanal	50	0
Dodecylaldehyde	500	1
Citral	25	5
o-Tolualdehyde	500	0
m-Tolualdehyde	1000	0
Cumenic aldehyde	25	4
Cinnamaldehyde	25	8
D. Alcohols		
1-Nonanol	25	0
1-Nonen-3-ol	25	0
4-Methyl-3-heptanol	50	2
Cedrenol	250	3
Terpineol	25	0
Isopulegol	25	0
Carveol	25	−4
E. N, S, and Cl Derivatives		
Octyl cyanide	100	0
Octyl thiocyanate	50	15
Octyl sulfide	250	9

indicated the same type of germination response (Figure 6). Dry uredospores of *P. graminis* did not respond to nonanal vapor (French and Gallimore, 1972a). Although nonanol has been shown to be a more effective stimulator than nonanal when *P. graminis* spores are placed on water solutions of these compounds (French and Gallimore, 1971), nonanal was much more effective than nonanol in these experiments because it is more volatile (Buttery et al., 1969).

Spores of *U. phaseoli* on 2.0 ml of water exposed to vapor for 20 s showed no reduction in percent germination when the water was replaced with 2.0 ml of fresh water immediately after exposure. Replacement of the water medium by five successive 2.0-ml portions of fresh water also showed no reduction in germination. Dry spores exposed on plastic lids to β-ionone vapor for 20 s and transferred to distilled water also were stimulated, indicating that the stimulator did not have to be present throughout the germination period.

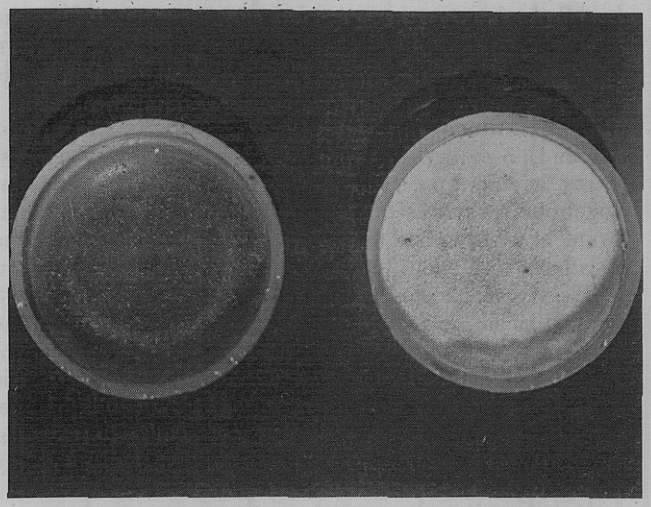

Figure 4. Uredospores of *U. phaseoli* on distilled water (0.5 ml) (left) and on 25 ppm of β-ionone (right), after an overnight period in Conway diffusion cells at 22 °C.

DISCUSSION

Nonanal was one of the first endogenous organic compounds reported to stimulate fungal spore germination (French and Weintraub, 1957). Many different compounds (fatty aldehydes, alcohols, ketones, terpenes, volatile flavor compounds, etc.) including β-ionone (French, 1961) have

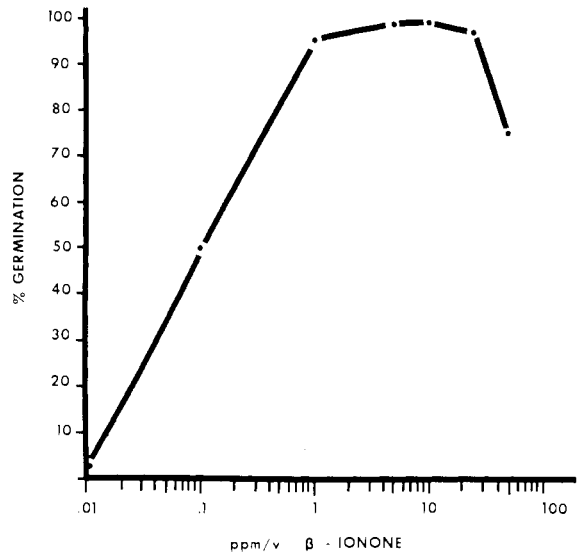

Figure 5. Percent germination of uredospores of *U. phaseoli* on water suspensions of 0.01-100 ppm/v β-ionone, in Conway cells after 4 h; controls = 0.8%.

since been shown to stimulate spores of *P. graminis* f. *tritici* (French and Gallimore, 1971). The stimulation of germination of spores of *Urocystis tritici* Koern. by benzaldehyde and related compounds (Noble, 1924) was recently called to our attention. Benzaldehyde was also active on spores of *P. graminis* f. *tritici* (French and Gallimore, 1971). Recent reports have shown that the germination of 19 species of rust and smut spores (in five genera) could be chemically stimulated by nonanal, nonanol, methylheptenone, β-ionone, or other compounds (French et al., 1975a,b). Among the stimulated species, a degree of specificity for certain chemical structures was noted. Nonanal or nonanol stimulated 15 of the 19 species. *U. phaseoli* was the first species found to be stimulated by a compound other than nonanal. This research has examined in some detail the structural requirements for the chemical stimulation of uredospores of the bean rust organism, *U. phaseoli*. In contrast to spores of *P. graminis*, which responded to a broad range of chemical substituents, particularly aldehydes, alcohols, and ketones, the spores of *U. phaseoli* responded only to a narrow range of ketones, primarily the methyl ketones ionone (α- and β-), 2-heptanone, and 5-methyl-2-hexanone.

The chemical induction of spore germination in pustules of diseased plants has been reported for *P. graminis* f. *tritici* (French and Gallimore, 1972b), *P. coronata*, *P. sorghi* Schw. (common corn rust), and *P. recondita* Rob. ex Desm. (leaf rust of wheat) (French et al., 1975b), and some of those fungi showed a degree of specificity for certain chemicals. Whereas the fungal spores studied previously responded best in pustule to octyl or nonyl derivatives, the uredospores of *U. phaseoli* in pustule responded exclusively to ionone.

Although floating spores of *U. phaseoli* and *P. graminis* respond most effectively to two markedly different chemical structures, ionone and nonanal, both respond rapidly to brief exposures of vapors of these compounds, which indicates that they have a similar response mechanism. Their responses could be compared to two species of insects responding exclusively to release of two different volatile, specific insect pheromones. Uredospores of *U. phaseoli* responded to vapor of β-ionone when spores were dry and transferred immediately to water, when they were floating en masse on water in Conway diffusion cells, or when they remained in the pustules on diseased plants that were exposed in dew chambers. The response of spores to such a brief stimulus suggests a chemically triggered physical mechanism, perhaps comparable to the rapid heat

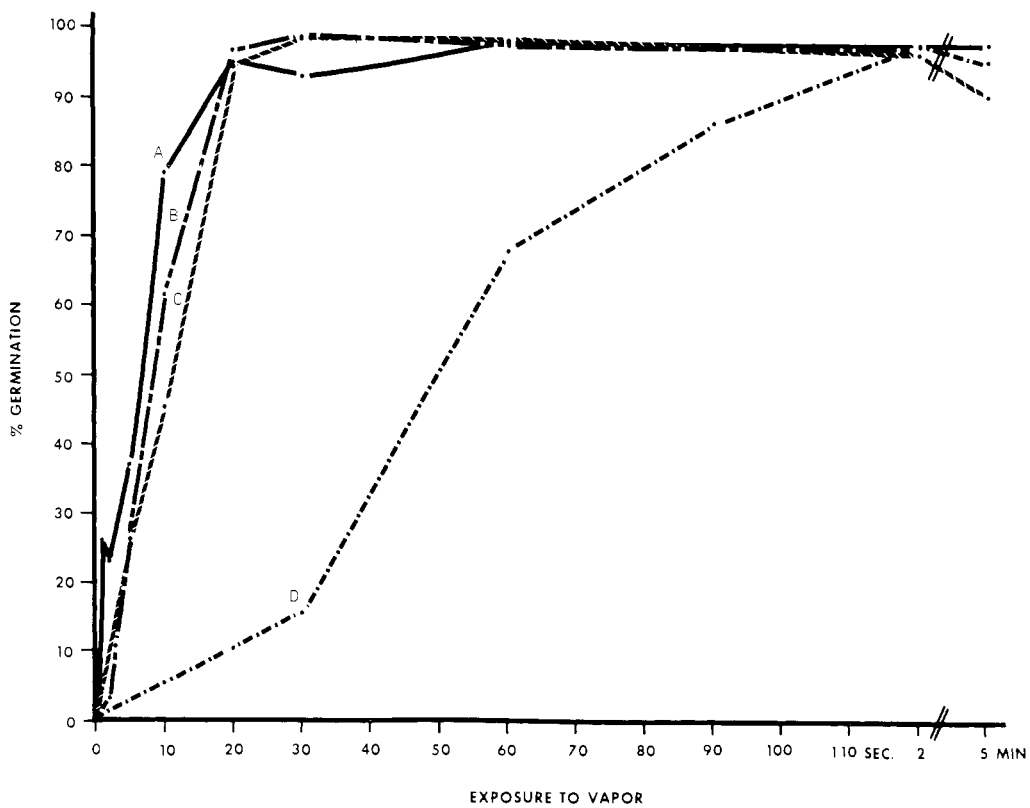

Figure 6. Percent germination of uredospores floated on 2.0 ml of water in response to brief exposures to stimulator vapors (10 μl): (A) *U. phaseoli* to β-ionone (4 h); (B) *P. graminis* f. *tritici* to *n*-nonanal (1.5 h); (C) *P. coronata* to *n*-nonanal (1.5 h); (D) *P. coronata* to *n*-nonanol (1.5 h).

activation of spores, the red-far-red induction phenomenon, or the sensing of an odor or an insect pheromone, rather than a metabolic shift in which stimulator molecules (i.e. ionone or nonanal) are continuously required as substrate for a new branch in a metabolic pathway. In simpler terms, the stimulator molecules appear to trigger a very sensitive chemical switch, rather than function as an additional energy source for a new branch of the metabolic circuit.

The rapid response of spores to stimulatory volatiles suggests that brief exposures might have some practical value in inducing spores to germinate under circumstances which would preclude infection of the host and hence prevent the disease. The results of short-time exposure to stimulators, interacting with water vapor and the endogenous germination inhibitors described by Allen (1955) and identified by Macko et al. (1970–1972), are being studied in detail to determine possible applications in spore germination control and hence fungal disease control.

ACKNOWLEDGMENT

The authors are indebted to John F. Brown, The University of New England, Armidale, N.S.W., Australia for reference to stimulation of spores of *Urocystis tritici* Koern. by benzaldehyde and related compounds, reported by R. J. Noble (1924).

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Received for review May 6, 1976. Accepted August 30, 1976.

Indole Alkaloids from *Balansia epichloë* (Weese)

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Balansia epichloë (Weese) is a clavicipitaceous fungus which parasitizes pasture grasses. This class of fungi may be involved with ergot-type syndromes observed in cattle grazed on infected pastures. Three indole alkaloids were isolated from laboratory cultures of *B. epichloë* and their identities were determined by ultraviolet, infrared, nuclear magnetic resonance, and mass spectroscopy as: 4-(3-indolyl)butane-1,2,3-triol; 3-(3,3-diindolyl)propane-1,2-diol; and 3-(3-indolyl)propane-1,2,3-triol. The alkaloids were toxic to fertile leghorn eggs.

Balansia epichloë (Weese), a systemic grass pathogen, has been implicated in ergot-type syndromes observed in cattle grazed on pasture grasses (Bacon et al., 1975; Nobindro, 1934; Porter et al., 1975). Bermuda grass tremors (convulsive ergotism) and fescue foot (gangrenous ergotism) are associated with cattle grazed on *Cynodon dactylon* (L.) Pers and *Festuca arundinaceae* Schreb., respectively.

The indications that *Balansia* may be involved in the etiology of clavicipitaceous diseases in cattle prompted us to investigate the possibility of indole alkaloid production by *Balansia*. From submerged cultures of an isolate of *B. epichloë* (Bacon et al., 1975) two fractions (A and B) were isolated by preparative thin-layer chromatography (TLC). Each gave a UV spectrum (Bacon et al., 1975; Agurell, 1965) and color reaction with *p*-dimethylaminocinnamaldehyde (Stahl, 1969) characteristic for indole alkaloids. We report the isolation, chemical characterization, and toxicities of three compounds in these fractions.

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EXPERIMENTAL SECTION

Materials and Methods of Culture. Subcultures and inocula of *B. epichloë*, 200 SF, were prepared according to Bacon et al. (1975). The fungus was incubated in a 2.8-l. Fernback flask for 21 days at 24 to 28 °C, on a gyratory shaker (200 rpm, 1-cm circular orbit) in 250 ml of the following medium: soluble starch (2% solution, pH 5.8), 70 g; mannitol, 30 g; ammonium succinate, 12 g; $MgSO_4 \cdot 7H_2O$, 2 g; K_2SO_4, 0.70 g; $FeSO_4 \cdot 7H_2O$, 50 mg; $CuSO_4 \cdot 5H_2O$, 5 mg; $ZnSO_4 \cdot 7H_2O$, 5 mg; $MnSO_4 \cdot H_2O$, 10 mg; thiamin, 0.2 μg/ml; distilled water, 1000 ml; and concentrated NH_4OH added to adjust the pH to 5.6.

Chromatography. Column chromatography was performed on Porapak Q (80/100 mesh; Waters Associated Inc.) as previously described (Bacon et al., 1975). Preparative TLC was on silica gel GF 254 (Brinkman) according to reported procedures (Bacon et al., 1975; Porter et al., 1974) and the developing solvent systems were (v/v): (I) $CHCl_3$–CH_3OH (80:20); (II) C_6H_6–DMF (86.5:13.5). All solvents were analytical reagent grade and were not further purified.

Analytical Methods. Ultraviolet (UV) spectra of the