Other reproductive structures in tissue in vivo, in infected callus, and in axenic culture showed similar variations in size. Size of sporangia increased as the fungus changed its habitat from in vivo to in vitro, to axenic culture (15.5 by 21.0 μ, to 19.0 by 26.5 μ, to 20.0 by 29.0 μ) respectively. However, the size of oogonia was reduced in this sequence, the diameters being: 41.5 to 37.5 to 30.5 respectively. The differences in measurements were statistically significant. No oospores were noticed in the axenic culture.

The sudden emergence of saprophytic growth of *S. graminicola* from infected host callus tissues on artificial media, and, subsequently, the growth of two subcultures on the same medium still cannot be explained. The stepwise appearance from infected callus tissues of a saprophytic mycelium in the case of *Gymnosporangium juniperi-virginianae* Schw. by Cutter was explained as perhaps being due to mutation (*5*). Another possible explanation for the growth of *S. graminicola* is that the fungus adapted itself through gradual but persistent infection of host callus tissues to grow on the nutritive medium which supported good growth of the host tissues. The fungus growth obtained on the nutritive medium was limited in character, and proper nutritional requirements of the fungus need to be worked out to obtain continuous copious growth.

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Sweetclover-Weevil Feeding Deterrent B: Isolation and Identification

Abstract. *Deterrent B, a compound apparently involved in the resistance of* Melilotus infesta *to the sweetclover weevil* Sitona cylindricollis, *has been isolated from leaves by a combination of preparative paper chromatography, sublimation, and crystallization. The compound has been identified as ammonium nitrate. Isolated deterrent B and ammonium nitrate have identical feeding deterrent activities. Although the deterrent principle was isolated as the ammonium salt, nitrate ion is probably responsible for the deterrent activity in vivo.*

The sweetclover weevil *Sitona cylindricollis* (Fåhraeous) is the major insect pest of sweetclover (*Melilotus* spp.) in the northern Great Plains of the United States. The adult insects feed on sweetclover leaves, leaving characteristic crescent-shaped notches. If present in large numbers, the weevils can completely defoliate young plants. One sweetclover species, *M. infesta* Guss., is resistant to weevil feeding (*1*).

A bioassay has been developed for the detection of factors which influence feeding by the sweetclover weevil (*2*). In the bioassay, washed disks of sweetclover root tissue are impregnated with the extracts to be tested and offered to adult weevils for feeding. The extent of feeding is measured by determining the percentage of disk area consumed during a given feeding period. In a bioassay of various extracts of leaves from the weevil-resistant *M. infesta* and a susceptible species, *M. officinalis* (L.) Lam., feeding was strongly stimulated by chloroform-soluble substances from both species. Also, water-methanol or water extracts of *M. officinalis* leaves stimulated extensive feeding in the bioassay system, but little feeding was observed on bioassay disks treated with corresponding extracts of *M. infesta* leaves (*2*, *3*).

Bioassay of leaf extracts separated by chromatography has shown that *M. infesta* leaves contain at least three water-soluble substances which influence weevil feeding (*4*). Two of these substances, stimulant A and deterrent A, apparently occur in both *M. officinalis* and *M. infesta*. The third, deterrent B, was detected only in *M. infesta* extracts. Available evidence indicates that deterrent B is involved significantly in resistance of *M. infesta* to weevil feeding. Deterrent B has now been isolated and identified as ammonium nitrate.

Young *M. infesta* leaves were autoclaved in water as described (*3*, *4*) to provide an extract of which 5 ml corresponded to 1 g of dry tissue. A 100-ml sample of crude extract was first subjected to preparative paper chromatography on Whatman No. 3 MM filter paper with ascending solvents. Solvents, in order of use, were: (I) isopropyl alcohol–concentrated ammonium hydroxide–water (8 : 1 : 2); (II) isopropyl alcohol–glacial acetic acid–water (8 : 1 : 3); and (III) methanol. Water was used to elute the deterrent B band (location determined by bioassay) after each chromatographic step. After chromatography with methanol, the eluate containing deterrent B was lyophylized to dryness.

The powder obtained was next sublimed at 0.2 mm-Hg and 100°C. The sublimate was washed from the cold finger with a small quantity of methanol, and the resultant solution was transferred to a graduated centrifuge tube. Deterrent B was precipitated from the solution by the addition of five volumes of ethyl ether. After centrifugation (2000*g*, 5 minutes), the supernatant was decanted and the precipitate was taken up in a small volume of methanol. To

Table 1. Feeding deterrent activity of various levels of isolated deterrent B and ammonium nitrate. Quantities shown were mixed with a quantity of stimulant A extracted from 8 mg (dry weight) of young *M. officinalis* leaves in a total of 0.15 ml of H_2O. The resultant solution was applied to five bioassay disks. A single experiment consisted of five disks of each treatment offered to a population of two adult weevils per disk in a 145-mm petri dish as previously described (*2*). The percentages are means (± S.E.) based on five such experiments.

Treatment		Disk area consumed (%)
Quantity (mg)	Substance	
	Stimulant A (control)	56.1 ± 3.1
0.05	Deterrent B	36.6 ± 2.8
0.05	NH_4NO_3	35.9 ± 2.4
0.10	Deterrent B	17.0 ± 2.5
0.10	NH_4NO_3	16.8 ± 1.5
0.20	Deterrent B	7.6 ± 1.4
0.20	NH_4NO_3	6.6 ± 1.1
0.40	Deterrent B	1.4 ± 0.5
0.40	NH_4NO_3	2.1 ± 1.0
0.80	Deterrent B	0.0 ± 0.0
0.80	NH_4NO_3	0.0 ± 0.0

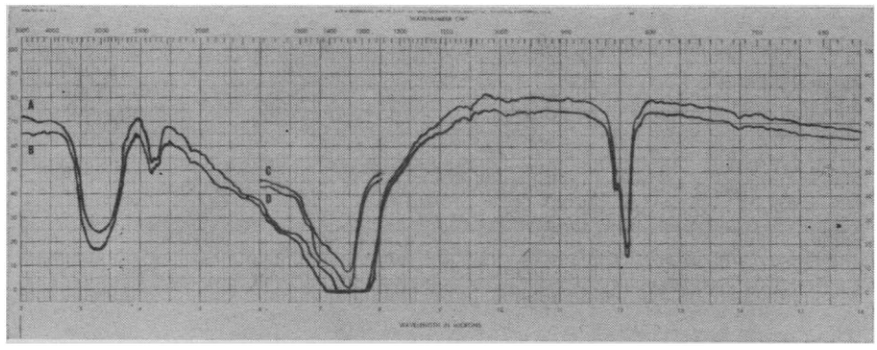

Fig. 1. Infrared spectra (determined with a Beckman model IR-5 spectrophotometer) of feeding deterrent B isolated from *M. infesta* and of ammonium nitrate in KBr pellet. (*A*) Deterrent B, 5 mg; (*B*) ammonium nitrate, 5 mg; (*C*) deterrent B, 2 mg; and (*D*) ammonium nitrate, 2 mg.

the methanolic solution, *n*-butyl alcohol (0.1 volume) was added, and the solution was allowed to stand at room temperature. Upon standing, methanol evaporated from the solution and colorless, needle-like crystals formed. The crystals were further purified by two more crystallizations from the methanol–butyl alcohol system, after which they were washed once with butyl alcohol and twice with ethyl ether. Washed crystals were dried in a vacuum (1 mm-Hg) at 40°C for 30 minutes to remove traces of ether. The yield of crystals from 100 ml of crude leaf extract was 48 mg.

Elemental analysis of the crystalline deterrent B disclosed the following percentages: H, 5.31; O, 59.31; N, 32.30; C, trace (< 1); and ash, trace. This composition corresponds reasonably well to that of ammonium nitrate (calculated percentages: H, 5.04; O, 59.96; and N, 35.00). The isolated material melted at 169° to 170°C (uncorrected, aluminum block) while known ammonium nitrate melted at 169.6°C. The ultraviolet absorption spectra of aqueous solutions of deterrent B and ammonium nitrate were identical, with molar extinction coefficients of approximately 15,400 and 7.0, respectively, at the 204 nm and 303 nm absorption maxima. The infrared spectrum of deterrent B also coincided with that of ammonium nitrate (Fig. 1). The R_F values for deterrent B and ammonium nitrate, respectively, in the three solvent systems used in the initial purification steps were as follows (15-cm solvent ascent): solvent I—0.56, 0.55; solvent II—0.55, 0.53; and solvent III—0.60, 0.55. Both deterrent B and ammonium nitrate were detected on the chromatograms as absorbing spots under short-wavelength ultraviolet light (peak near 254 nm). The feeding deterrent activity of the isolated compound was quantitatively similar to that of reagent grade ammonium nitrate at all levels of treatment (Table 1). Thus, the activity of isolated deterrent B appears to be due to ammonium nitrate rather than to impurities which might be present in trace amounts.

On the basis of preliminary tests, the feeding deterrent activities of potassium nitrate and sodium nitrate appear to be very similar to that of ammonium nitrate. In addition, nitrate assays of young *M. infesta* leaves indicate a level five to ten times as high as that in comparable *M. officinalis* leaves. Therefore, the hypothesis is advanced that in young, intact leaves of *M. infesta* and in crude aqueous extracts of such leaves, nitrate ion is the feeding deterrent. The ammonium salt was probably formed during the first chromatographic purification, inasmuch as this step employed an ammoniacal solvent.

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β-1,3-Glucanase of Sea Urchin Eggs: Release from Particles at Fertilization

Abstract. *β-1,3-Glucanase is a latent enzyme found in large and small particles in unfertilized eggs. At fertilization, the enzyme in the large particles is released into the surrounding perivitelline space. The enzyme may be involved in transformations of extracellular glycoproteins.*

In eggs of most species, contact with sperm results in the breakdown of a peripheral ring of cortical granules 1 to 2 μ in diameter (*1*). In the sea urchin egg, substances released from such granules are involved in the elevation and hardening of the fertilization membrane and in establishing the blockage of polyspermy (*1, 2*). Acidic glycoproteins contained within these cortical granules are also transformed into the 2 to 3 μ thick hyaline layer (*3*) necessary for cell adhesion and blastula formation (*4, 5*). Little is known about the chemical basis of these "cortical reactions." The major constituents of cortical granules have not been characterized, nor have enzymes capable of metabolizing such constituents been identified.

We have found a β-1,3-glucanohydrolase (β-1,3-glucanase) (E.C. 3.2.1.-39) in the eggs of the sea urchin, *Strongylocentrotus purpuratus* (*6*). This enzyme is particulate in the unfertilized egg and exhibits little activity unless released from the particle (that is, the enzyme is latent). After fertilization, a major portion of the activity is released rapidly from the egg into the perivitelline space and surrounding seawater. These properties and behavior suggest that the enzyme is associated with cortical granules and lead us to speculate that the β-1,3-glucanase may be involved in the "cortical reactions."

Enzyme activity is assayed as the release of free glucose from the algal β-1,3-glucan, laminarin (*7*), by means of the glucose oxidase (*8*) or Nelson-Somogyi (*9*) methods. Properties of this enzyme, determined with a 200-fold purified preparation, are described elsewhere (*10*). Briefly, the enzyme's properties are unlike those described for most β-1,3-glucanases (*11*), but are similar to a recently described fungal enzyme (*12*). The sea urchin enzyme is an exohydrolase specific for the β-1,3-glucosidic linkage. Its reac-