Bergholz, H.; Erttmann, R. R.; Damm, K. H. *Experientia* **1980**, *36*, 333.
Corry, J. E. L.; Sharma, M. R.; Bates, M. L. *J. Appl. Bacteriol. Tech. Ser.* **1983**, *18*, 349.
English, P. B. *Vet. Rec.* **1965**, *77*, 810.
Jaksch, W. *Dtsch. Tieraertzl. Wochenschr.* **1961**, *68*, 466.
Mercer, H. D.; Rollins, L. D.; Garth, M. A.; Carter, G. C. *J. Am. Vet. Med. Assoc.* **1971a**, *158*, 776.
Mercer, D.; Righter, H. F.; Carter, G. C. *J. Am. Vet. Med. Assoc.* **1971b**, *159*, 61.
Mercer, H. D.; Teske, R. H.; Long, P. E.; Showalter, D. H.; Bryant, H. H. *J. Vet. Pharmacol. Ther.* **1978**, *1*, 253.
Moats, W. A. *J. Chromatogr.* **1984**, *317*, 311.
Moreno, B.; Calles, A. *An. Bromatol.* **1980**, *32*, 22.
Nouws, J. F. M.; Ziv, G. *Tijdschr. Diergeneesk.* **1977**, *102*, 1173.
Rieve, D.; Wernerssen, H., Zimmerman, T. *Arch. Lebensmittelh* **1974**, *23*, 264.
Rolinski, Z.; Fidecka, H. *Med. Wtryn.* **1962**, *18*, 654.
Smither, R. *J. Appl. Bacteriol.* **1978**, *45*, 267.
Teske, R. H.; Rollins, L. D.; Carter, G. C. *J. Am. Vet. Med. Assoc.* **1972**, *160*, 873.
Vilim, A. B.; Larocque, L. *J. Assoc. Off. Anal. Chem.* **1983**, *66*, 176.

Received for review June 25, 1985. Revised manuscript received December 19, 1985. Accepted February 24, 1986. Mention of specific products or trade names is for identification purposes only and does not imply endorsement by the U.S. Department of Agriculture over other similar products not mentioned specifically by name.

Isolation of Coumarin in Snap Beans and Its Effect on Uredospore Germination

Filmore I. Meredith,* Charles A. Thomas, and Robert J. Horvat

Coumarin was isolated and identified for the first time from leaves and pods of five cultivars of snap beans (*Phaseolus vulgaris* L.). Isolation of coumarin was by thin-layer chromatography and identification by thin-layer cochromatography, ultraviolet spectroscopy, and mass spectrometry. The maximum concentration of coumarin in all leaf and pod tissues analyzed never exceeded 3.0 μg/g fresh weight. Results of uredospore germination test are presented, showing that coumarin concentration in the leaf and pod are below the threshold necessary to stimulate uredospore germination.

INTRODUCTION

Most cultivars of snap beans (*Phaseolus vulgaris* L.) are susceptible to a rust disease incited by *Uromyces appendiculatus* (Pers.) Unger var. appendiculatus. It it well-known that fungal uredospores of dense populations germinate at a very reduced rate or not at all. Inhibition of uredospores germination was believed to be due to chemical constituents, either endogenous or exogenous. Water extracts of *Puccinia graminis* f. sp. *tritici* (wheat rust) demonstrated the presence of this inhibiting component of germination (Allen, 1955), which was later identified from bean rust uredospores as methyl 3,4-dimethoxycinnamate (Macko et al., 1970). Allen (1972) determined that only *cis*-methyl 3,4-dimethoxycinnamate would inhibit the germination of the uredospores, suggesting stereochemical specificity. Investigations of uredospore germination and the inhibitor have been reviewed by Allen and Dunkle (1971), Macko et al. (1976), Allen (1976), and Staples and Yaniv (1976).

Unwashed bean rust uredospores floated on the surface of distilled water had only 0.2% germination while those placed on aqueous leachate from snap bean leaves had 26%. Uredospores washed with distilled water and then placed on the aqueous leachate had over 70% germination (Thomas and Meiners, 1977). Increased germination of the unwashed uredospores placed on the snap bean leachate compared to the uredospores placed on distilled water indicates that one or more biologically active chemical components are present either in or on snap bean leaves. These active chemical compounds are capable of counteracting the effect of the germination inhibitor in uredospores, thus increasing uredospore germination. Studies on *Uromyces phaseoli* (bean rust) showed that chemical structures such as linear, branched, saturated, and unsaturated methyl ketones of six to nine carbons and cyclic ketones greatly stimulate uredospore germination (French et al., 1977). An investigation by van Sumere et al. (1957) demonstrated that coumarin extracted from wheat rust uredosphores at concentrations as low as 10 μg/mL was extremely effective in promoting the germination of uredospores of *P. graminis* f. sp. *tritici*.

Preliminary experiments conducted on the aqueous leachate from snap bean leaves that had increased uredospore germination showed that coumarin was not present at a detectable level. Coumarin was also below the detectable level in condensate water collected from snap bean leaves grown in a dew chamber (Thomas, unpublished data).

As far as we determined in the literature, coumarin has not been isolated from snap bean tissue. Coumarin has been reported to be a hepatotoxic compound in test animals and is listed as a suspected carcinogenic compound (Dickens and Jones, 1965; Cohen, 1979; Evans et al., 1979). Due to the ability of coumarin to counteract the germination inhibitor of uredospores from wheat rust and its importance as a natural toxin in plants, a study was conducted to determine whether coumarin is present in leaf and pod tissue of snap beans and to determine the coumarin concentration required to stimulate maximum bean rust uredospore germination.

MATERIALS AND METHODS

Plant Samples. Five cultivars of snap beans, Eagle, Topcrop, Tender Crop, Mountaineer White Half Runner, and Bush BlueLake 274, were grown at Beltsville, MD. All of the cultivars were susceptible in the field and greenhouse

Richard B. Russell Agricultural Research Center, USDA-ARS, Athens, Georgia 30613 (F.I.M., R.J.H.), and Beltsville Agricultural Research Center, USDA-ARS, Beltsville, Maryland 20705 (C.A.T.).

at Beltsville, MD, to one or more races of the pathogens (collection 73-16, 73-23, 73-32; Stavley, 1984). Leaf and pod tissues were harvested when the pods were at their prime marketable maturity. The plant tissue was frozen until chemically analyzed.

Chemical Reagents. All of the chemicals were of reagent grade. Coumarin was purchased from Aldrich Chemical Co., Milwaukee, WI. Thin-layer chromatography (TLC) plates were silica gel G (20 × 20 cm, Fisher Scientific, Pittsburg, PA). The TLC plates used were washed with methanol and dried in an oven at 30 °C overnight.

Plant Extraction. The plant tissue (leaves or pods, 1.5 kg) was chopped into 1 cm or smaller pieces and was extracted with boiling methanol (1500 mL) for 5 min. The extract was filtered through Whatman number 1 filter paper and the volume reduced to 750 mL by evaporation of the methanol at reduced pressure at 25 °C. Water was added to the methanol extract to make a volume corresponding to 1 mL/g of fresh tissue. Methylene chloride (500 mL) was then added to the water–methanol solution, the mixture shaken, and the water–methanol phase was discarded. The methylene chloride extract was evaporated by reduced pressure at 25 °C to 20 mL.

Chromatography. Solvent systems used in developing the TLC plates were methylene chloride–methanol (99.5:0.5, v/v), R_f 0.65; petroleum ether–ethyl ether–ethyl acetate (90:5:5, v/v/v), R_f 0.20; petroleum ether–ethyl acetate (80:20, v/v), R_f 0.50. After development, each TLC plate was exposed to long-wavelength ultraviolet (366 nm) to detect the coumarin. The desired band, after being isolated on the TLC plate, was removed by scraping off the silica gel and eluting it with methylene chloride. If difficulty was encountered in distinguishing the band, a small strip along the side of the TLC plate was sprayed with 2 N NaOH and coumarin was detected by long-wavelength ultraviolet. Under alkaline conditions the lactone ring of coumarin opens, producing a compound that fluoresces bright yellow with long-wavelength ultraviolet radiation.

Identification. The isolated compound was identified by comparing its UV spectrum to authentic coumarin, by cochromatography of the isolated coumarin with authentic coumarin, and by mass spectrometry. Ultraviolet spectra were run on a Beckman 35 spectrophotometer (Palo Alto, CA). A Du Pont 21-490B mass spectrometer operated in the direct probe mode was used to obtain the mass spectrum (MS). Conditions: ion source 210 °C; scan rate 100 s per decade from 15 to 500 atomic mass units; ionizing voltage 70 eV; ion source pressure 2×10^{-6} torr.

Coumarin Levels. Coumarin concentration in the snap bean tissue was determined by TLC by comparing the size and florescent intensity by long-wavelength ultraviolet light under alkaline conditions to the size and florescent intensity of a coumarin standard. Also estimates of coumarin level in snap beans were obtained from a standard curve made by plotting the UV absorption maximum at 273 nm vs. various concentrations of coumarin.

Spore Germination. Five milliliters of water containing 0.005% Tween 20 and coumarin at concentrations of 0, 3.1, 6.2, 12.5, 25, 50, 100, and 200 μg/g of H_2O were placed in tissue culture dishes. Uredospores (0.5 mg) were placed on the surface of the water in each dish. The uredosphores were incubated at 16 °C for 16 h after which germination counts were made. The germination test was replicated three times.

RESULTS AND DISCUSSION

Cochromatography of the snap bean extract and coumarin samples yielded bands possessing identical retention

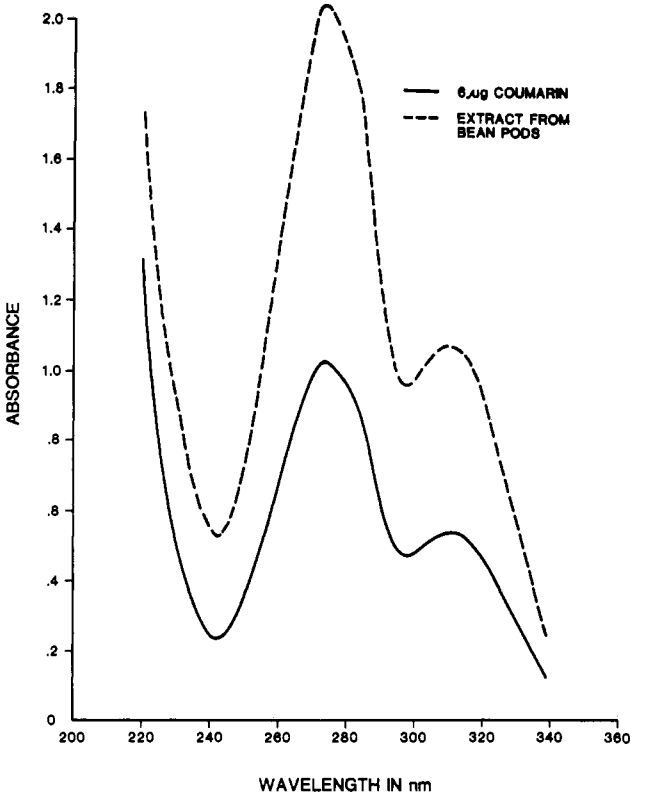

Figure 1. Absorption spectrum (in methanol) of the compound isolated with TLC and coumarin.

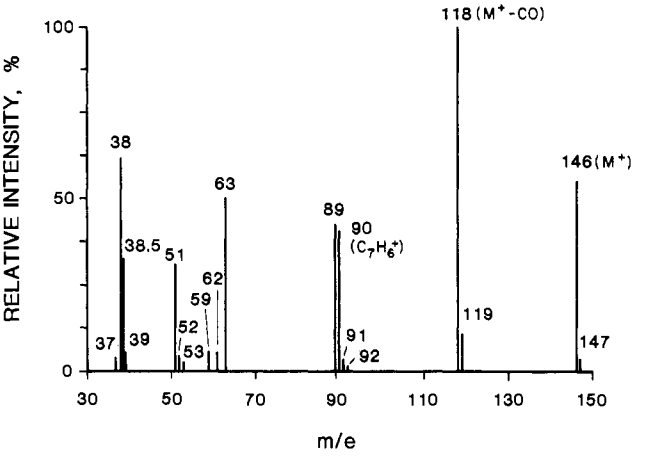

Figure 2. Mass spectrum of the compound isolated from TLC.

times using the same TLC conditions and methods of visualization previously described above. The compound isolated from the snap bean tissue gave the same ultraviolet spectrum as coumarin (Figure 1). The mass spectrum (Figure 2) matched that reported in the literature for coumarin (EPA/NIH Mass Spectral Data Base, 1978). Thus, it can be concluded that coumarin is present in snap bean leaves and pods. All the leaf and pod tissues of the cultivars tested showed the presence of coumarin. Concentrations of coumarin for leaves and pods varied from 1.3 to 3.0 μg/g (ppm) fresh weight, below the level required for uredospore germination. No attempt was made to determine the amount of "bound coumarin", *o*-coumaric acid glucoside, present.

Germination of uredospores floated on water containing coumarin at various concentrations showed that 6 μg/mL (ppm) was the lowest concentration capable of counteracting the bean rust inhibitor (Table I). Free coumarin, not detected in aqueous leachate in snap bean leaves, is

Table I. Germination (Percent ±SD) of Uredospores Floated on Water Containing Tween 20 (0.005%) and Coumarin[a]

coumarin, μg/mL (ppm)	% germin	coumarin, μg/mL (ppm)	% germin
0	0.2 ± 0.1	25.0	74.3 ± 9.0
3.1	0.9 ± 0.2	50.0	49.7 ± 2.1
6.2	6.7 ± 1.5	100.0	11.0 ± 2.1
12.5	41.7 ± 7.0	200.0	0.4 ± 0.2

[a] Reported values are means of three replications.

present in the leaf and pod tissue at concentrations too low to markedly effect snap bean rust uredosphore germination. Knowledge of the presence of coumarin in snap bean pods may be important because coumarin has been reported to be a hepatotoxic compound in test animals and is listed as a carcinogenic compound (Dickens and Jones, 1965; Cohen, 1979; Evans et al., 1979).

In summary, coumarin, a biologically active compound, was isolated for the first time from snap bean leaves and pods at concentrations no greater than 3.0 μg/g fresh weight. The concentration of coumarin in the snap bean leaves and pods is not sufficiently high to counteract the uredospore inhibitor and to initiate germination of the snap bean rust uredospores. However, leachate from snap bean leaves was capable of counteracting the uredospore germination inhibitor, indicating the presence of an unknown factor or factors causing germination of the bean rust spores. Additional studies are being conducted to determine the identity of the other chemical constituent or constituents in snap bean tissue capable of counteracting the germination inhibitor.

Registry No. Coumarin, 91-64-5.

LITERATURE CITED

Allen, J. P. In "Encyclopedia of Plant Physiology New Series"; Springer-Verlag: Berlin, 1976; Vol 4, Chapter 2.
Allen, J. P. *Science (Washington, D.C.)* **1972**, *169*, 3497–3500.
Allen, J. P.; Dunkle, L. D. In "Morphological and Biochemical Events in Plant-Parasite Interactions"; The Phytopathological Society of Japan: Tokyo, 1971; pp 23–58.
Allen, J. P. *Phytopathology* **1955**, 45, 259–266.
Cohen, A. J. *Food Cosmet. Toxicol.* **1979**, *17*, 277–289.
Dickens, F.; Jones, H. E. H. *Br. J. Cancer.* **1965**, *19*, 392–403.
Evans, J. G.; Gaunt, I. F.; Lake, B. G. *Food Cosmet. Toxicol.* **1979**, *17*, 187–193.
French, R. C.; Graham, C. L.; Gale, A. W.; Long, R. K. *J. Agric. Food Chem.* **1977**, *25*, 84–88.
Heller, S. R.; Milne, G. W. A. "EPA/NIH Mass Spectral Data Base"; U.S. Department of Commerce: Washington, DC, 1978; Vol. 1, p 419, Reference Spectra 91-64-5.
Macko, V.; Staples, R. C.; Yaniv, Z.; Granados, R. R. In "The Fungal Spore: Form and Function"; Wiley: New York, 1976; pp 73–100.
Macko, V.; Staples, R. C.; Renwick, J. A. A. *Science (Washington, D.C.)* **1970**, *170*, 539–540.
Staples, R. C.; Yaniv, Z. In "Encyclopedia of Plant Physiology New Series"; Springer-Verlag: Berlin, 1976; Vol. 4, pp 88–103.
Stavley, J. R. *Plant Dis.* **1984**, *68*, 95–99.
Thomas, C. A.; Meiners, J. P. *Proc. Am. Phytopath. Soc.* **1977**, *4*, 219–220.
Thomas, C. A. unpublished data.
van Sumere, C. F.; van Sumere-de Preter, C.; Vining, L. C.; Ledingham, G. A. *Can. J. Microbiol.* **1957**, *3*, 847–862.

Received for review May 28, 1985. Revised manuscript received November 7, 1985. Accepted January 24, 1986. References to brand or firm names does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Production of Deepoxydeoxynivalenol (DOM-1), a Metabolite of Deoxynivalenol, by in Vitro Rumen Incubation

Louise-Marie Côté,[1] Jean Nicoletti, Steven P. Swanson, and William B. Buck*

A method for the production and purification of deepoxydeoxynivalenol (DOM-1), the only known metabolite of deoxynivalenol (DON), is presented. An in vitro incubation technique with rumen microorgansms was used either with an extract from highly DON-contaminated corn or with DON-contamination corn as a substrate. The incubation of 1500 mg of DON from corn extract yielded, after extraction and purification, 340 mg of DOM-1. The incubation of ground DON-contaminated corn was advantageous over the incubation of the extract from the corn because it was more time and cost effective.

Deoxynivalenol (DON, vomitoxin) is a mycotoxin produced primarily by the fungus *Fusarium graminearum*. Infection of corn and cereal grains by *F. graminearum* and concomitant DON production occur most frequently during those years when the maturation and harvest season is wet and cool. The deleterious effects of DON in swine and laboratory animals have been described (Vesonder et al., 1976; Friend et al., 1982; Chavez, 1984; Khera et al., 1984; Tryphonas et al., 1984; Trenholm et al., 1985). These effects included feed refusal, decreased feed efficiency, reduced weight gain, emesis, depletion of hepatic glycogen, and hypoglycemia. However, very few metabolism studies have been conducted to determine the fate of this toxin in animals. Yoshizawa et al. (1983) were the first to characterize a metabolite of DON, namely DOM-1, in rat urine and feces. In our laboratory we have recently found DOM-1 in milk of cows fed a diet containing DON-contaminated corn. (Cote et al., submitted for publication). Since DON is a frequent contaminant of livestock feed

Department of Veterinary Biosciences, College of Veterinary Medicine, University of Illinois, Urbana, Illinois 61801.

[1] On assignment of a Ph.D. program from: Animal Research Center, Agriculture Canada, Ottawa, Ontario, Canada.

0021-8561/86/1434-0458$01.50/0