

Movement of Penicillin into Soybean Seeds Using Dichloromethane

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ABSTRACT

Antibiotic activity was detected in soybean (*Glycine max* 'Wells') seeds after soaking them for 0.5, 1.5, 4, or 24 hours in various concentrations of potassium penicillin G (PG) in dichloromethane (methylene chloride). Zones of inhibition around treated seeds increased in size with increase in soaking time and increase in concentration of PG. Germination and seedling vigor, immediately after treatment, was not adversely affected.

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Additional key words: seed treatment.

The effect of antibiotics on internally seedborne bacteria and seed germination is usually studied by applying the compounds in aqueous solutions (2). Soybean seed coats rapidly imbibe water, which makes the mechanical planting of such seed very difficult. Various organic solvents have been used to introduce chemicals into dry seeds, but many have adverse effects on germination (4). Meyer and Mayer (4) reported that anhydrous dichloromethane (DCM) could be used to introduce chemicals into dry seeds without apparent effects on germination or respiration. The effectiveness of DCM in introducing compounds into seeds is presumably due to its miscibility with polar and apolar solvents and its limited solubility in water (3, 4). We report on the effect of: (i) DCM on soybean seed germination and (ii) soaking time and concentration of potassium penicillin G (PG) in DCM on the uptake of PG by embryo tissues of dry soybean seeds.

MATERIALS AND METHODS.—Soybean [*Glycine max* (L.) Merr. 'Wells'] seeds were used. Approximately 500 g of seeds was either nontreated or treated with one of the following concentrations of potassium penicillin G (Squibb and Sons, Inc., New York) (PG) in dichloromethane (methylene chloride) (DCM) (Fisher Scientific Co., Fair Lawn, New Jersey): 25, 50, 100, 200, 400, 800, 1,600, or 3,200 $\mu\text{g/ml}$. Seeds were soaked in any single mixtures for 0.5, 1.5, 4.0, or 24 hours and allowed to air dry.

Antibiotic activity in the seeds was measured using Difco pennassay agar (B270) seeded with *Bacillus subtilis* spores. A standard Difco *B. subtilis* spore suspension (1 ml) was added to 100 ml of warm (50 C) liquid agar.

Bacillus subtilis-seeded agar (BSA) (5 ml) was added to 100 \times 15 mm culture plates, and stored at 4 C until used. Treated seeds were washed fifteen seconds to remove penicillin from the outside of the seed coat, then cut in half at right angles to the embryo axis, and the cut side placed down on the agar surface. Assay plates were

stored at 4 C for 12 hours, then incubated at 35 C for 25 hours. Zones of inhibition were measured from the edge of the plated tissue to the widest point of the zone. There were three replications of 10 seeds per treatment.

The seed coats were removed from 10 seeds treated for 30 minutes with 100 $\mu\text{g/ml}$ PG in DCM. The embryo was washed for 30 seconds in running distilled water and assayed for antibiotic activity as previously described. The experiment was done four times. Nontreated embryos served as controls.

In a second experiment, 10 g of dry soybean seeds were either placed in a plastic bag containing 0.5 g PG and mixed until the seeds were covered with the antibiotic or soaked in a 500 $\mu\text{g/ml}$ mixture of PG plus DCM. After 2 hours, seeds from both treatments were washed for 1 minute in running distilled water. The seed coats were removed from 50 seeds of each treatment and one cotyledon from each seed was plated on BSA. The remaining cotyledons were washed again for 1 minute and plated on BSA. Seeds were assayed for antibiotic activity as previously described. Nontreated seeds served as controls. The experiment was done three times.

To test the effect of the PG + DCM mixture on germination, seeds were: (i) nontreated, (ii) soaked for 3 hours in either 200 or 400 $\mu\text{g/ml}$ PG + DCM mixture, or (iii) soaked for 3 hours in DCM alone. The seeds were plated (100 seeds/treatment) onto cellulose pads (Kimpak) in 150-mm diameter culture plates containing 50 cc distilled water. There were four replications of 25 seeds each. The plates were incubated at 39 C under 24 hours of light (87.01 $\mu\text{w/cm}^2$). After 5 days, the percentage germination and radicle lengths were recorded. A seed was considered germinated if all parts of the young seedling were present and the radicle length was at least 10 mm.

Extracts for chromatographic analysis of the antibiotic in treated seeds (400 $\mu\text{g/ml}$ penicillin G + DCM) were prepared by washing the seeds in tap water for 15 seconds, blotting dry on cellulose pads and finally grinding in 50 ml of ethyl acetate using a mortar and pestle. The suspension was filtered twice through No. 1 Whatman filter paper and 20 μl of the filtrate was spotted on either Eastman 6061 silica gel or 13255 cellulose (6064) thin-layer chromatographic plates without fluorescent indicator. Twenty microliters of an extract from 10 g nontreated seeds and a 50 $\mu\text{g/ml}$ solution of PG served as controls. The Eastman 6061 plates were run in a mixture of isopropanol and methanol (30:70, v/v) and the Eastman 6064 plates were run in water saturated with *n*-butanol. Chromatographs were run three times in each system. When the solvent fronts reached 12-14 cm, the plates were dried and developed using the bioautography technique of Peterson et al. (5). Pennassay agar seeded with *B. subtilis* spores was sprayed onto the plates, which were incubated in a moist chamber for 18 hours at 35 C. Zones of inhibition were noted as clear areas where *B. subtilis* did not grow due to the presence of penicillin. The data was analyzed by analysis of variance.

RESULTS AND DISCUSSION.—Zones of inhibition around soybean seeds treated with the PG + DCM mixture tended to increase in size with increased concentration, but this tendency was not consistent, particularly between 0.5 and 1.5 hours (Table 1). There

TABLE 1. Inhibition zones of *Bacillus subtilis* on Difco assay agar from soybean (*Glycine max*) seed halves treated at four time periods with various concentrations of potassium penicillium G (PG) in dichloromethane (DCM)

Treatment PG + DCM ($\mu\text{g/ml}$)	Inhibition zones (mm) around seeds soaked for (time in hours) ^a				LSD	
	0.5	1.5	4	24	$P = 0.05$	$P = 0.01$
25	5.8	4.6	6.8	7.9	2.4	3.6
50	7.1	5.4	7.9	8.8	2.3	3.5
100	7.9	7.5	7.8	10.2	0.9	1.4
200	7.9	8.7	8.6	9.7	1.1	1.7
400	10.3	9.9	10.3	11.4	1.9	2.8
800	10.6	10.1	11.0	12.0	0.8	1.2
1,600	10.7	11.2	12.3	12.8	1.4	2.1
3,200	12.9	11.0	12.9	12.9	2.5	3.7
LSD _{0.05} =	1.8	1.3	1.7	0.8		
LSD _{0.01} =	2.6	1.9	2.4	1.1		

^aMeans of measurements (mm) of inhibition zones in agar plates of 10 seeds per each of three replications.

was also no significant increase in the size of the zone in inhibition around seeds treated with 3,200 $\mu\text{g/ml}$ of the mixture with increase in time. No zones of inhibition were recorded around nontreated seeds.

Zones of inhibition were observed around all embryos of seeds treated with either 100 or 500 $\mu\text{g/ml}$ of PG + DCM mixture. There were no zones of inhibition around embryos of nontreated seeds or seeds treated with dry PG alone. DCM facilitated the movement of the antibiotic through the seed coats and into the embryos of treated seeds.

Seeds treated with either 200 or 400 $\mu\text{g/ml}$ PG + DCM had a percentage germination of 99 and 98, respectively. Nontreated seeds and seeds treated with DCM alone had a percentage germination of 87 and 93, respectively. The mean radicle length (in millimeters) for nontreated seeds, seeds treated with 200 or 400 $\mu\text{g/ml}$ PG + DCM, and

DCM alone were 21, 26, 24, and 22, respectively. These results agree with those of Meyer and Mayer (4), who reported that DCM would be used to introduce chemicals into dry seeds of other crops without apparent effects on germination or respiration when measured immediately after treatment.

Zones of inhibition were observed on bioautographs for penicillin G and extracts from PG + DCM-treated seeds at R_f .94 and .55 on the 6064 and 6061 TLC plates, respectively. There were no zones of inhibition produced by extracts from control seeds.

Our method introduces the antibiotic through the seed coat and into the tissues of the embryo while the soybean seed is dry and dormant. When imbibition occurs the antibiotic is present in the seed coat and embryo where it could control bacterial growth. The mode of action of penicillin depends upon actively dividing bacterial cells (1, 6, 7). Thus, the introduction of an antibiotic into dry soybean seeds using DCM may have potential as a method of controlling seedborne bacteria. The technique could also be used as a research tool to study the effects of antibiotics on internally seedborne bacteria.

LITERATURE CITED

- CARPENTER, P. L. 1963. Microbiology. W. B. Saunders, Philadelphia, London. 432 p.
- MAYER, A. M., and A. POLJAHOFF-MAYBER. 1963. The germination of seeds. Pergamon, New York. 231 p.
- MERCK AND CO. 1952. Merck index of chemicals and drugs. 6th ed. Merck and Co., Rahway, New Jersey. 1,167 p.
- MEYER, H., and A. M. MAYER. 1971. Permeation of dry seeds with chemicals: use of dichloromethane. Science 171:583-585.
- PETERSON, C. A., and L. V. EDGINGTON. 1969. Quantitative estimation of the fungicide benomyl using a bioautograph technique. J. Agric. Food Chem. 17:898-899.
- PRATT, R., and J. DUFRENOY. 1949. Antibiotics. J. B. Lippincott Co., Philadelphia. 341 p.
- WELCH, H. 1954. Antibiotic therapy. Medical Encyclopedia, Inc. New York. 256 p.