Bacterial Blight of Soybean: Seedling Disease Control

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ABSTRACT

A seedling assay method was used to determine if chemicals or antagonistic bacteria applied to soybean seed reduced the seedling disease incited by *Pseudomonas glycinea*. The method also was used for assaying seed produced in the greenhouse and in the field. One antibiotic, oxytetracycline hydrochloride, and an antagonistic bacterium derived from the seed coat, reduced disease. Plants from pathogen-bearing seed produced "bacterial blight-free" seed in the greenhouse; this seed, planted in limited isolation in the field produced healthy plants which yielded seed that did not carry the pathogen as determined by the assay.

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With leaf-spotting bacterial diseases of a number of annual plants, the seed is an important mode for survival and dispersal of the pathogen. Consequently, practices aimed at preventing the association of the pathogen with the seed, or where the association already is established, practices for inhibiting the pathogen, warrant investigation. Model studies with soybean (*Glycine max* L.) seed and the pathogen *Pseudomonas glycinea* are described in this paper. The aim of the work was to control the seedling phase of bacterial blight, which may initiate epidemics (3, 4). Chemical and bacterial seed treatments were studied. Also, evidence is presented indicating that the pathogen-seed association may be broken by producing seed in the greenhouse, as previously suggested (7, 13). A short account of part of the work has been published (10).

MATERIALS AND METHODS.—*Seed lots.*—Seed naturally bearing *P. glycinea* was used. Soybean cultivar Harosoy 63 from two farm sources was employed for most seed treatment tests. Source 87 seed was treated with pentachloronitrobenzene ('Terra-coat', Olin), and source 83 seed was not treated with a fungicide. These lots usually produced > 50% heavily diseased seedlings, when assayed as described below. An experiment usually consisted of 10-16 lots of seed treated with candidate chemicals or antagonistic bacteria, plus two control lots (no chemicals or bacteria were used, but seed and seedlings were otherwise subjected to the same conditions). A lot was 100 g and contained approximately 500 seeds.

How *P. glycinea* was associated with this seed is not known. It probably was on the seed surface. It was presumed also to be within the seed, because no disease control was obtained when seed was rolled as described below with a 5.25% solution of sodium hypochlorite (three tests with source 83 seed). *Pseudomonas glycinea* commonly is within seed as well as on the surface (12).

Agar media.—"SBM" was made by autoclaving 100 g of soybean seed 0.5 hours in 1 liter of water; 20 g sucrose and 20 g of agar were added to the supernatant, which was made to 1 liter before autoclaving. "NSA" contained (g/liter): nutrient agar (Bacto), 23; sucrose, 10. The media "TTCC" and "M71" were described previously (8). Both contained cycloheximide (a fungicide) and a tetrazolium salt (an aid in recognizing *P. glycinea* colonies on agar). In addition, M71 contained boric acid, which usually inhibits the pathogen less than other bacteria commonly associated with the pathogen in nature.

Seedling assay for detecting Pseudomonas glycinea.—The detection method previously described (13) was used to assay seed for the pathogen. The method depends on exposing germinating seed to conditions favoring the formation of typical water-soaked lesions on seedling cotyledons. A 100-g seed lot was treated with

candidate chemical or bacteria, as described below. Seed was planted in water-saturated vermiculite for 2 or 3 days (2 days for the soaking method, 3 days for the rolling method–see below). Seedlings then were removed from the vermiculite, wounded by shaking in water containing sand, and replanted in water-saturated vermiculite. Seedlings emerged into water-saturated air in a lighted chamber. Numbers of plants bearing lesioned cotyledons and estimated numbers of cotyledon lesions were recorded 8-10 days after seedlings were replanted.

The assay method may be subject to artifact when used in seed treatment tests, because toxicants or antagonistic bacteria probably are redistributed on seedlings during the wounding process. This appears to be an unavoidable limitation of the test, however, because cotyledon lesions are not produced unless seedlings are wounded (13).

Treating seed with chemicals.—In the initial test of a chemical, 100 mg (0.5 ml if liquid) was put into a cylindrical jar (speciman jar) 14 cm × 5 cm in diameter. One milliliter of water was added, and the chemical was crushed with a stirring rod if necessary. Two milliliters of dichloromethane was added [to increase permeation of the chemical (11)] and stirred, and the mixture was then distributed on the walls of the closed jar by brief hand rolling. A seed lot (100 g) was added quickly, and the jar placed on a rolling machine (40 rpm) for 15 minutes. Treated seed was dried overnight in the laboratory and usually assayed for *P. glycinea* in a day or two. Chemicals seemed to be well distributed on seed by this method.

A chemical that gave good disease control in the initial test was retested at the same and at lower concentrations. Chemicals which partly controlled the disease were retested at twice the initial concentration. If seed germination was inhibited by the initial concentration, the concentration was halved in successive tests, or until germination was sufficient for a disease reading to be made. As other trials continued, dichloromethane was omitted from the treating mixture (2 ml of water were substituted) to determine if this substance was necessary for control. A chemical showing promise also was dispersed in 0.5 ml of a 70% superior oil ('Sun Spray 7E', Sun Oil Company) instead of the aqueous mixture.

Selecting antagonistic bacteria.—Bacteria antagonistic to *P. glycinea* in an agar diffusion test were used to treat seed, as described below. Antagonists were obtained from soybean seed coats in the following series of steps at 24 C: (i) Ten seeds from a given seed source were placed for 2 days on a piece of sterile filter paper in a petri dish containing 7 ml of sterile water (in some tests one seed and 3.5 ml of water were used). (ii) Seed coats were removed and placed in 10 ml of sterile water in a test tube, which was agitated 10-15 seconds in a swirl mixer. (iii) Four, 12.7 mm diameter sterile blotting paper disks were dipped in the resultant suspension, drained briefly, and two disks placed on the surface of an agar medium in each of two petri plates. In about one fourth of the tests the TTCC medium was used and in the remainder, SBM. (iv) Plates were incubated 2 days and the agar surface sprayed with a cell suspension of *P. glycinea* containing approximately 10^7 cells per milliliter. (Cells were derived from NGA slants incubated 2 days. An atomizer fitted with an air filter was used for spraying.) (v) Plates were incubated 2 days. Suspensions from some seed coats inhibited *P. glycinea* in large circular zones around disks; other suspensions produced no zones. Bacterial colonies were common on and at the edges of disks. (vi) To isolate the bacterium responsible for a large zone, a disk was agitated in water, the resulting suspension was streaked on TTCC, and a pure culture was made from the predominant colony type. (vii) Predominant bacteria were tested for antagonism against *P. glycinea* as described above. Isolates producing large zones were used in seed treatment tests.

Treating seed with antagonistic bacteria.—In the initial test with an antagonist, the soaking method of application was used. The antagonist was grown for 1-2 days at 24 C on a slant of NGA. The surface growth was suspended in water and made to 200 ml (approximately 10^7-10^8 cells per milliliter). A seed lot (100 g) was soaked 2-3 hours in this suspension, planted in vermiculite, and the seedlings then handled as described in the seedling assay.

Antagonists reducing disease in two or more tests also were applied to seed by the rolling method. An isolate was grown on three agar slants of NGA for 1-2 days. Surface growth was suspended in 3 ml of water. A seed lot was rolled in the viscous suspension as described above for chemicals. All of the liquid was absorbed during the rolling period. Seed dressed with bacteria was dried overnight and stored at 10 C until assayed for *P. glycinea*.

Seed produced in the greenhouse and field.—Seed bearing *P. glycinea* was planted in the greenhouse in early spring, 1973. Care was taken not to wet the foliage or flowers of the growing plants. Seeds from these plants were hand-harvested, assayed for *P. glycinea* by the seedling assay, and used to plant field plots in June, 1973. Each seed lot was planted in two rows 70 m long (15 seeds per meter); rows were 3 m apart and at least 3 m from the field margin or other plantings. This distance was considered sufficient to prevent spread of *P. glycinea* between rows, except during unusually violent storms (3). Such storms did not occur in 1973. Throughout the season a weed-inhibiting chemical and cultivation virtually eliminated weeds within 3 m of the plants.

RESULTS.—*Chemicals as seed dressings.*—Most of the miscellaneous group of 67 chemicals tested were selected on the basis of the agar diffusion tests for toxicity described previously (8). Chemicals used were "generally toxic", i.e., they inhibited *P. glycinea* and the other bacteria > 10 mm from the chemically impregnated paper strips used in those tests.

One chemical, oxytetracycline hydrochloride (Pfizer Co., Brooklyn, N.Y.), reduced disease consistently. This antibiotic, commercially known as "Terramycin" and here designated "OTH", is soluble in water at the concentrations used. The related compound, tetracycline hydrochloride, also reduced disease and probably would prove suitable with more study. Dichloromethane applied with the OTH did not improve disease control, so it was not used in later tests. OTH dispersed in the light oil gave good disease control; contrary to expectation (17), tetracycline base in the oil was not so effective. At the present time, dispersal of OTH in the oil seems to be the best method for general application. The oil did not reduce seed germination.

Most of the studies with OTH were made with seed lot 83. For example, in four tests 2.5 mg of OTH per 100 g lot

of seed resulted in 2, 2, 0, and 0% diseased seedlings, as compared with 60, 50, > 90, and 80% diseased seedlings in the respective controls (in the first two tests, the OTH was dissolved in water; in the remaining two tests, it was dispersed in the oil). In another test, 10 mg in water resulted in 1% diseased seedlings, in comparison with 50% in the control. With seed lot 87 and 10 mg of OTH in the oil, there were no diseased seedlings (the control was 77% diseased); at 2.5 mg, there was 4% disease as compared with > 90% in the control. Three additional pathogen-bearing lots of seed were tested with 10 mg of OTH dispersed in oil. Disease was 0, 0.5, and 0% as compared with 14, 43, and 10% in the respective controls.

The above figures actually understate the reduction of disease by OTH. This is because seedlings from treated seeds usually had only one to two small *P. glycinea* lesions on one cotyledon, whereas control seedlings had many larger lesions–it was not unusual for 5-10% of the outer surface of the cotyledons to be covered. Thus, seed dressing with OTH would not only reduce numbers of diseased plants, but more importantly, it would greatly reduce the amount of secondary inoculum.

The cotyledons of seedlings from seed treated with 2.5 or 10 mg of OTH/seed lot were yellowed, but germination and seedling growth were not impaired. At 20 mg, seedlings were yellow and germination was reduced. Consequently, the 10 mg rate (approximately 1/6 oz/100 lb of seed) is suggested for further tests. However, at this rate disease was not completely eliminated in some tests.

Some of the other chemicals examined afforded partial disease control, and some were toxic to the seedlings. Compounds of interest that were very active in diffusion tests, but failed to provide disease control, were neomycin, polymixin, and streptomycin sulfates, sodium novobiocin, chloromycetin, and naladixic acid.

Bacteria associated with the soybean seed coat.—A chance observation of the surface of seed coats from seed that had germinated 2 days revealed large populations of bacteria. Some of these organisms were used as seed dressings, as described in the next section.

Seeds harvested for commercial purposes were germinated in petri dishes as described in Materials and Methods, and the seed coat was examined for viable bacteria by dilution plating on the surface of TTCC or by direct observation. A seed coat (15-30 mg, wet weight) usually carried 10^6-10^8 viable propagules. When seed coats were bleached and stained (2), bacteria were readily observed on the outer surface. Diversity of types and large numbers were notable. Large and small rods predominated; cocci were present also. Separated bacterial cells were common, but most often they were in groups, masses, and "rivers" (masses that were linearly aligned). That these organisms were associated with the seed coat was demonstrated in tests in which the coats were separated from dry seed, moistened, and the surface inspected at various times. No bacteria were seen when the coats were first moistened; at 19 hours individual bacteria and groups were found, and at 48 hours there was a mosaic of a profuse bacterial development.

When suspensions made from seed coats were plated on TTCC or other media, many types of bacterial colonies were evident. Moreover, the predominant colony types from seed coats from different seeds, even in the same seed lot, varied. The antagonistic properties of predominant isolates likewise differed, as was readily demonstrated in agar diffusion tests. We did not attempt to characterize bacteria from the 100 lots of seed examined, but the range of variation was large and a sizable effort will be required to characterize even the most commonly observed types. This was beyond the scope of the present studies.

Antagonistic bacteria as seed dressings.—The agar diffusion technique described in Materials and Methods was used to select antagonistic isolates from 100 seed sources, representing a number of cultivars grown in different areas. This survey resulted in the selection of 25 isolates that produce sizable inhibition zones of *P. glycinea*. Inhibition zones 30-40 mm in diameter were common.

These 25 isolates first were applied to seed by the soaking method in attempts to control the disease. Seed germination and seedling growth were not inhibited by antagonists. Three isolates were tested two or more times. One, isolate SC735, was selected for further study.

Using isolate SC735 and the soaking application method in three tests, 7, 17, and 17% of the seedlings were diseased, as compared with 46, 87, and 98% diseased seedlings in the respective controls (one test with seed source 87, two with source 83. In tests in which the bacterium was applied by the rolling method to source 87 seed, 4 and 3% of the seedlings were diseased in comparison with 65 and 65% in the respective controls.

As with the OTH treatment, a notable feature of blight control by SC735 was a reduction in lesion area as well as in number of diseased plants. Presumably the diffusible toxicant was responsible for disease control. Biological control by this antagonist or others seems possible, but field tests are needed and much additional study is required.

The greenhouse as a source of blight-free seed.—Seed of the cultivars Beeson and Harosoy 63 was planted in the greenhouse; these lots produced 66 and 63% diseased seedlings, respectively, when assayed by the seedling test. Greenhouse plants were healthy. Seed from them was assayed (no *P. glycinea* was detected in either cultivar) and used for the field planting. Plants were free of blight the entire season. Seed from field plants did not carry the pathogen, as determined by the seedling test. There was not a comparable field planting of the original seed bearing *P. glycinea;* however, bacterial blight was prevalent in adjacent fields of these cultivars.

It was concluded from these studies that the seed-pathogen association could be broken by producing seed in the greenhouse. Schmitthenner et al. (16) reported the successful use of this method to control a mung bean (*Phaseolus aureus* L.) disease incited by a strain of *Pseudomonas phaseolicola;* 3 years of additional trials have supported the earlier results (A. F. Schmitthenner, *unpublished*).

While the greenhouse method appears to be effective for producing "bacterial blight-free" seed, it is costly and may not be necessary, particularly in the seed-increase stages. Some seed lots were blight-free when assayed (13); could these be used to produce clean seed stocks in the field, as has been done successfully in Michigan for the control of bean (*Phaseolus vulgaris* L.) blights (1, 15)? To test this possibility, commercially produced seed (cultivars Beeson and Harosoy 63), indicated by the

seedling assay not to carry *P. glycinea*, was planted in a field study exactly parallel to the one described above. No blight was observed in field plants and the seed produced by them was free of the pathogen.

DISCUSSION.—It seems to be a general principle that bacterial pathogens fare well only in associations with plants (9). Thus, pathogens appear to be poorly adapted for either short- or long-term survival in the absence of living or dead plant tissue, and a number of pathogens possess a resident phase (i.e., they multiply in association with the healthy plant) as well as a pathogenic phase. True saprophytic growth probably is rare.

In this paper, three approaches were used in attempts to break the close association between the host and the surviving pathogen at a critical point–the seed. With care and isolation, the production of "blight-free" seed appears to be possible in moist as well as in dry climates. Chemical and biological control measures for treating seed are described; results suggest that bacterial pathogens within, as well as upon, seed can be controlled by seed treatment, and that the chemical and biological measures should be examined further in laboratory and field tests.

Ideally, it would be desirable to break the *P. glycinea*–seed association completely, but this probably could only be done with greenhouse seed, an impossible task where so much seed is required. A more reasonable approach would be to use (i) greenhouse seed to produce mother seed stocks, (ii) chemical and/or biological seed treatments, and (iii) isolation, field inspection, and seed assay for seed increase generations. Even though each step probably would not completely assure breaking the association, it may be possible to keep it at an acceptably low level. Outlays required to attempt to reduce soybean blight in our region by these techniques do not now appear to be warranted, but if the crop is threatened by an unexpected outbreak they should be considered.

The present studies may be helpful in devising control measures for other seed-borne bacterial pathogens. Methods aimed at breaking the pathogen-seed association would not be useful, of course, if the pathogen survived season-to-season in debris or in insects, if it possessed a resident phase on a weed, as has been recently described (5), or if the pathogen is transported by machinery, etc., into isolated plantings. In studies in 1974, not reported in detail, there was evidence of the transfer of the pathogen by the Japanese beetle (*Popillia japonica* Newman) between plots separated by 3 m; consequently, insect control measures should be employed, especially where plots are so close.

The rationale for attempting to identify diffusible toxicants for seed treatments is based on evidence suggesting that *P. glycinea* survives within the seed coat or the outer region of the cotyledon. Thus, only the outer surface of the cotyledon is lesioned (13). Protected survival sites (9) for *P. glycinea* may well be within the vessels of the seed coat and the adjacent cotyledon surface of soybean, as has been suggested with bean bacterial pathogens (14), possibly a position reached via the healthy flower (6, 12). Fast-acting diffusible toxicants thus appear to hold most promise for future studies. It should be noted that if the chemical here studied, OTH, proves effective in the field, it may not be usable because of cost or because current importance in human medicine would prohibit its use on agricultural seed.

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