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USE OF POLYETHYLENE GLYCOL AND GLYCEROL AS CARRIERS OF ANTIBIOTICS FOR REDUCTION OF COMMON BLIGHT BACTERIA IN BEAN SEEDS

By

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ABSTRACT

USE OF POLYETHYLENE GLYCOL AND GLYCEROL AS CARRIERS OF ANTIBIOTICS FOR REDUCTION OF COMMON BLIGHT BACTERIA IN BEAN SEEDS

By

Li Zhe Liang

Common bacterial blight of beans caused by Xanthomonas phaseoli is an important disease in Michigan and on a world wide basis. Internally and externally contaminated seeds are the main sources of primary inoculum for the disease. There is no known acceptable method for eradication of internally borne blight bacteria. The objectives of this study were to: 1) determine if "seed priming" with polyethylene glycol (PEG) or glycerol solutions can be used to introduce antibiotics into bean seeds without major reduction in seed quality, and 2) determine if introduced antibiotics can effectively control internally seed-borne bacteria. Non infected and internally infected bean seeds were soaked in solutions of PEG and glycerol, as well as in solutions of these materials containing the antibiotics streptomycin, tetracycline and chlorotetracycline. These seeds were evaluated for germination percentage, seedling vigor, and survival of bacteria.

Prolonged soaking (up to 4 days) in either 25% PEG or 60% glycerol solutions did not diminish germination while seedling vigor was slightly reduced. PEG solutions were more effective than glycerol solutions for introduction of antibiotics into seeds. Tetracycline and chlorotetracycline in PEG solutions effectively reduced X. phaseoli, but had severe phytotoxic effects. PEG solutions containing streptomycin reduced, but did not eradicate internally seed-borne X. phaseoli and caused few phytotoxic effects. Thus, treatment of bean seeds internally infected with X. phaseoli with solutions of PEG and streptomycin provides an effective means of reducing, but not eradicating primary inoculum of this bacterium. Additional studies are needed to assure: 1) that the suggested treatments will not reduce the production potential of non infected seeds, 2) that the treatments can effectively reduce disease development in the field , and 3) that the treatments do not provide rapid selection for antibiotic resistant strains of the bacteria.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND LITERATURE REVIEW

Common bacterial blight is one of the most serious seed-borne diseases of dry, edible beans (Phaseolus vulgaris L.) throughout the world (Saettler, 1989; Sutton and Wallen, 1970; Zaunmeyer and Thomas, 1957). The major sources of primary inoculum for the disease are internally and externally contaminated seeds (Saettler and Perry, 1972). Certification programs for the production and testing of pathogen-free seed are helpful; however, blight outbreaks persist because many growers plant seed sources other than those considered to be pathogen-free. Effective treatments to eradicate internally borne blight bacteria from bean seed would contribute greatly to an integrated strategy for disease management.

Causal agent of disease

Common blight incited by Xanthomonas phaseoli (E. F. Smith) Dowson and fuscous blight incited by Xanthomonas phaseoli var. fuscans (Burkh.) Starr and Burkh. are important diseases of most commercial bean (Phaseolus vulgaris L.) cultivars. Common blight was first reported by Beach in 1892. At about the same time, Halsted (1892) reported a similar

disease of bean pods and seeds. Smith described the etiology of common blight in 1897 and named the causal bacterium Bacillus phaseoli E. F. Smith (1898, 1901). Smith (1905) further characterized the pathogen and transferred it into the genus Pseudomonas in 1901, then into the genus Bacterium in 1905. Bergey's Manual ed. II (1925) renamed the blight bacteria Phytomonas phaseoli (E. F. Smith) Bergey et al.. In 1938 Dowson placed the bacterium in the currently accepted genus Xanthomonas.

Fuscous blight was discovered by Burkholder in 1924 on an undetermined bean cultivar from Switzerland. Disease symptoms were identical to common blight and the only means of distinguishing the diseases was by culturing the causal bacterium. Xanthomonas phaseoli var. fuscans was initially known as Phytomonas phaseoli var. fuscans but later was placed in the currently recognized genus Xanthomonas.

Xanthomonas phaseoli and X. phaseoli var. fuscans are obligately aerobic, gram negative, straight rods, which produce a yellow, nondiffusible pigment and are motile by a single polar flagellum. Both bacteria produce hydrogen sulfide, liquefy gelatin, proteolize milk, hydrolyze starch and Tween 80 and produce an alkaline reaction in phenol red dextrose agar (Buchanan and Gibbons, 1974). The bacteria are differentiated by production of a brown diffusible pigment produced by X. phaseoli var. fuscans (Basu and Wallen, 1967; Basu, 1974; Hayward and Waterston, 1965a, 1965b), but are

otherwise physiologically and biochemically identical. Bergey's Manual 8th ed. currently recognizes X. phaseoli and X. phaseoli var. fuscans as nomen species of Xanthomonas campestris (Buchanan and Gibbons, 1974).

Disease description

Xanthomonas phaseoli and X. phaseoli var. fuscans produce visible disease symptoms on all plant parts except the roots; symptomatology of the two diseases is essentially identical. However, Zaumeyer and Thomas (1957) reported that X. phaseoli var. fuscans may cause a slight hypertrophy in tissue around a stem lesion and, in seedlings, a darkening of the stem around the point of inoculation.

Leaf symptoms are the most diagnostic features of the blights. Disease expression is first visible as minute water-soaked spots on the abaxial surface of the leaf with yellow discoloration opposite the spot on the adaxial side. Lesions then enlarge irregularly, dry out and become brown and brittle; occasionally a slight crust of bacterial exudate is present. The necrotic area is surrounded by a distinctive yellowish halo-like zone. Several lesions on a leaf may coalesce, and eventually occupy most of the leaf area and cause premature leaf drop (Burkholder, 1930; Zaumeyer, 1932).

Stem lesions begin as water-soaked dark green spots which eventually become dry, sunken and reddish brown in color. The

lesions usually extend longitudinally up the stem but show little downward movement. Stem symptoms are most common on seedlings and less evident as the plant matures (Burkholder, 1930; Wallen and Jackson, 1975).

Pod symptoms appear as dark green, water-soaked spots which later become dry and sunken. Drying begins at the outer edge of a lesion and extends inward; a yellow crustation of bacterial exudate may cover the lesion (Burkholder, 1930). Pod infection can result in seed infection. On white-seeded bean cultivars infection is indicated by a shiny yellow spotting of the seed coat, the extent of which varies from small blotches to complete seed discoloration. Infection of dark seeded bean cultivars is harder to detect due to seed coat pigmentation; symptoms appear as a darkening of the seed coat. Seeds which are infected early in development may be completely shrivelled or badly wrinkled, whereas those infected near pod maturity are darkened only at the hilum (Burkholder, 1930). Seed infection is most probable when the dorsal pod suture is infected. The bacteria invade the funiculus, pass through the raphe, and finally into the seed coat (Zaumeyer, 1929). Seeds also may be infected through the micropyle if the bacteria invade the pod cavity (Zaumeyer 1930).

Common and fuscous blights are major bacterial diseases of dry beans (Saettler, 1989). This disease is found in most countries where beans are grown (Hayward and Waterston, 1965a) and yield losses can be heavy. In 1967, at least 75% of

Michigan's 650,000 acres of navy beans were damaged by common blight, with 10-20% yield reduction (Anonymous, 1971). Wallen and Jackson (1975) reported a 38% yield loss in Ontario, Canada due to common blight in two years of field trials. Aerial infrared photographic surveys suggested that losses for the bean crop grown in Ontario ranged from 1252 tons in 1970 to 218 tons in 1972 (Jackson and Wallen, 1975; Wallen and Jackson, 1975). Yield losses estimated at 22% and 45% have been obtained by natural and artificial infections, respectively, in Colombia (Yoshii et al., 1976). Economic surveys, based upon field observations in the same region, estimated yield losses of 13% due to common blight bacteria (Pinstrup-Andersen et al., 1976).

Establishment of the disease in crops usually originates from infected seeds in which the bacteria can survive for many years. Thus, seed treatments to eradicate common blight bacteria could provide important means of disease control, provided such treatments are effective and ecologically sound.

Disease control

Strategies to control common blight bacteria on beans include, chemical control in the field, genetic control using resistant and tolerant cultivars, cultivation practices, and use of pathogen-free seed obtained either by eradicative treatment or certification schemes.

Various chemical controls that have been found effective include: sprays of 50% copper hydroxide and 40% potassium (hydroxymethyl) methyl dithiocarbamate, Bordeaux mixture, 5% puratized spray and cuprox dust (copper oxychloride), and sprays of 1000 ppm streptomycin sulfate (Burke and Starr, 1948; Edgerton and Moreland, 1913; Weller and Saettler, 1976). However, Marlatt (1955), found that streptomycin failed to control common blight when used as a spray. The following year Gray (1956) demonstrated that the addition of a wetting agent, i.e., glycerol, greatly increased the effectiveness of streptomycin sprays in controlling common blight in greenhouse plants. Generally, chemical field treatments for the control of the disease have not proven to be sufficiently effective to be commercially useful.

Long term hope for controlling bean blights rests in the development of resistant and tolerant commercial cultivars of Phaseolus spp. (Ekpo, 1975). Some such cultivars do exist, however, their wide-spread use is by no means assured, especially with reports that current tolerant and resistant cultivars support the epiphytic growth of the bacteria on plant parts and may produce infected seed (Cafati and Saettler, 1980a, 1980b; Schuster et al., 1979).

Cultural practices such as remaining out of bean fields when plants are wet, and proper sanitation of farm machinery, are helpful in minimizing the spread of the disease. Crop rotation, weed control, and plowing refuse under after harvest

are recommended practices (Andersen et al., 1970; Hart and Saettler, 1981).

Planting of pathogen-free seed is widely recognized as being of fundamental importance in the control of many seed-borne diseases (Baker and Smith, 1966; Neergaard, 1979). The principle of controlling bean blight through the use of pathogen-free seed has been recognized and applied in many production areas of the United States and Canada (Copeland et al., 1975; Sheppard, 1983; Sheppard, 1983).

Bacterial plant pathogens have been demonstrated to survive in various ways: in plant residues, associated with perennial hosts, as epiphytes on nonhosts, in the soil, or in association with insects (Leben, 1974, 1981; Schuster and Coyne, 1974, 1975). However, in the case of X. phaseoli in Michigan, contaminated seeds are the major source of primary inoculum (Weller and Saettler, 1980).

The inability of X. phaseoli to survive in plant residues to any significant degree has been demonstrated in New York (Burkhold, 1930), Ontario (Wallen and Galway, 1979) and Australia (Wimalajeewa and Nancarrow, 1980). Saettler and coworkers (1986) provided evidence that the primary inoculum of common blight in Michigan is contaminated seed. Several attempts were made to recover 20 isolates of X. phaseoli from ten different bean genotypes ranging in susceptibility from resistant to susceptible, over a 10-year period. Infected bean plant samples were either left standing, were buried in the

soil, or were left on the soil surface at several sites in Michigan over several different winters. Bean blight bacteria never were recovered in any of the 191 trials. X. phaseoli has, however, been shown to survive from one growing season to the next in the field in some regions with less severe winters (Schuster, 1955; Thomas and Graham, 1952).

The inability of foliar pathogens to survive long periods in the absence of a living host is often explained by a low "competitive saprophytic ability" (Garrett, 1950), i.e., the lack of physiological attributes that enable successful colonization of dead organic substances (Gray, 1976; Hirano and Upper, 1983; Lenon, 1981). Also, reduced pathogenicity and lowering of numbers of recoverable antibiotic-resistant X. phaseoli were demonstrated when infested leaves were repeatedly exposed to freezing and thawing (Lopez, 1984). No explanation as to why this occurred was provided.

Pathogen eradication from seeds

External contamination with X. phaseoli on bean seeds is easily controlled with streptomycin sulfate (Saettler and Anderson, 1978) and sodium hypochlorite (Weller, 1978). However there are no known acceptable methods for eradicating internally borne blight bacteria from the seed. Thus, improved seed treatments are needed to reduce or eradicate this pathogen internally. Among the important conditions in

development of eradication protocols are 1) localization of blight bacteria, i.e internal or external, 2) physiological state of the bacteria, i.e. hypobiotic resting state or actively growing and 3) the sensitivity of the seeds, in terms of germination and vigor, to potential treatment. If indeed, blight bacteria within the seeds are in a hypobiotic state, they may be resistant to treatments normally effective on viable growing bacteria.

Treatments to eliminate the pathogen from seed have historically been useful (Adimihardja, 1981; Marlatt, 1955; Neergaard, 1979). A slurry treatment containing streptomycin sulfate is routinely used on many Michigan seedlots (Saettler and Anderson 1978). It is generally thought that this slurry treatment is helpful against external bacterial pathogens. The most widely used method to surface disinfest seeds is submersion in 1-5% NaOCl followed by rinsing with sterile water (Abdul-Baki, 1974). Weller (1978) demonstrated that shaking infested seeds in 1:1 (v:v) commercial bleach: distilled water solution for 30 seconds consistently eliminated all surface-borne blight bacteria, and 45 seconds usually eliminated all surface microorganisms.

Seed treatment that completely eradicates internal blight bacteria without significantly affecting seedling emergence, is not available. Still there are many seed treatments that are helpful. Dihydrostreptomycin has been shown to act similarly to streptomycin sulfate. These two antibiotics were

absorbed by the stem of bean seedlings and translocated upward to the primary leaves. Within three to four days the chemical accumulated in sufficient amounts to inhibit the growth and development of halo and common blight organisms in seeds (Mitchell et al., 1952, 1953, 1954). Kreitlow (1940) found that sequential immersion in four solutions reduced blight incidence in bean seeds from 23.6% to 0.2%: 1) 1:500 mercury bichloride in di-ethyl ether 2) 1:20,000 brilliant green in 50% ethyl alcohol plus 3% acetic acid 3) 1:500 mercury bichloride in 70% ethyl alcohol plus 3% acetic acid 4) 1:20,000 gentian violet in 50% ethyl alcohol plus 3% acetic acid. Person and Edgerton (1939) found that seeds treated for 12-14 minutes in a solution of 1:500 $HgCl_2$ in 70% ethyl alcohol plus 2% acetic acid provided the best eradicative treatment for blight. Treatment of seed with dry heat at 80^0C for 35 minutes followed by New Improved Ceresan for 24 hours was helpful in reducing the number of diseased plants (Burke and Starr, 1948). Adimihardja (1981) demonstrated that treatment with tetracycline (800 ppm) in methanol under partial vacuum eradicated seed-borne blight bacteria completely from bean seed. The treatment also reduced germination and field emergence. Seed immersion in 800 ppm tetracycline and chlortetracycline in methanol for 30 minutes can help by reducing numbers of bacteria in the seeds. Soaking longer than 30 minutes reduced germination as a result of phytotoxicity due to the antibiotics.

Many reports indicate that soaking seeds in polyethylene glycol (PEG) solutions does not impair germination and can increase seedling resistance to adverse effects of environment (Fu et al., 1988; Heydecker, 1973/1974; Khan et al., 1978; Muhyaddin and Wiebe, 1989). This treatment called osmotic conditioning may thus be an efficient means to introduce antibacterial materials into bean seeds infected with pathogenic bacteria.

Some research has been devoted to using osmotic conditioning as an aid in eradication of microorganisms from the seeds. Saettler (unpublished) showed that seeds incubated up to 5 days in a 65% glycerol solution containing 800 ppm chlorotetracycline retained normal viability. Antibiotic activity was found inside the seeds using standard bioassay procedures. Hepperly and Sinclair (1977) used PEG solutions to introduce penicillin G and streptomycin into soybean seeds. Antibiotic activity was detected in all seed parts after 15 weeks of storage. Thus, simultaneous osmotic conditioning of seeds and application of bactericides may provide a means for delivery of the bactericides into seeds without deleteriously affecting germination potential.

This study assesses the potential of osmotic conditioning as an aid in eradicating or reducing the incidence of X. phaseoli from bean seeds. The objectives are to: (1) assess the impact of concentration and time of immersion in PEG and glycerol solutions on navy bean seed germination, (2) test the

efficacy of PEG and glycerol solutions for introducing antibiotics into seeds, (3) test whether PEG or glycerol can cause phytotoxicity when mixed with antibiotics, and (4) determine if these treatments are effective in eliminating common blight bacteria from internally contaminated seeds.

MATERIALS AND METHODS

Seeds

Seeds of commercial cultivar C-20, a white-seeded navy bean (*Phaseolus vulgaris* L.), were obtained from the Michigan Foundation Seed Association. Seeds were stored dry at room temperature for 9 months before use in this study.

Media

All chemicals, unless stated otherwise, were purchased from Sigma Chemical Corporation, St. Louis, MO. Media were autoclaved for 20 minutes before pouring into 9 cm diameter disposable plastic petri plates. Agar plates were allowed to cool to room temperature overnight before use.

Bacto yeast extract (BYE) agar: BYE agar was prepared by dissolving 10 grams of Bacto yeast extract and 15 grams of Bacto agar in 1000 ml of 0.01 M sodium phosphate buffer (pH 7.2), and autoclaving. BYE agar seeded with *X. phaseoli* was used for assaying of antibiotic activity in seeds.

Bacto yeast extract (BYE) liquid medium: BYE liquid media was prepared by dissolving 1 gram Bacto yeast extract in 1000

ml of 0.01 M sodium phosphate buffer (pH 7.2), and autoclaving. This medium was used for increasing X. phaseoli for the production of artificially infected seeds.

Yeast extract calcium carbonate agar (YCA): YCA was used as a standard medium for the recovery of X. phaseoli. It contained 10.0 g yeast extract, 2.5 g calcium carbonate, and 15.0 g Bacto-agar per liter of distilled water. Colonies of X. phaseoli on YCA were not visible until 24 hours of incubation at 27^{0}C. X. phaseoli colonies first appeared as small yellow, circular and convex colonies with entire margins. After 3 days of incubation, isolates of X. phaseoli var. fuscans were differentiated by the presence of a brown, water soluble diffusible pigment on this medium.

Semi-selective medium (SSM): SSM was used to isolate and confirm the identity X. phaseoli. The SSM was prepared by dissolving 1.0 g of yeast extract, 15.0 g Bacto-agar, and 8.0 g soluble potato starch in 970 ml 0.01 M sodium phosphate buffer (pH 7.2). Six ul of a 1% aqueous solution of methyl green and three ul of a 1% solution of methyl violet 2B in 20% ethanol were added. The protocols for preparation of stock solutions were as outlined by Trujillo and Saettler (1980). Stock solutions of antibiotics were filter-sterilized and stored at 4^{0}C. the final amount of antibiotics per liter of SSM were: 25.0 mg cycloheximide, 2.0 mg nitrofurantoin, 1.0 mg nalidixic acid, and 0.5 mg gentamycin sulfate. Some non X. phaseoli bacteria grow on SSM, however, the X. phaseoli are

clearly differentiated on the basis of colony morphology. Colonies of X. phaseoli and X. phaseoli var. fuscans appeared as light yellow, convex, slimy colonies with entire margins and a zone of starch hydrolysis. With further incubation the regions of starch hydrolysis expand and coalesce. Isolates that are fuscans variants do not produce the brown pigment in SSM.

Liquid semi-selective medium (LSSM): LSSM was used for selectively enhancing growth of X. phaseoli and X. phaseoli var. fuscans while inhibiting the growth of contaminating bacteria. LSSM was prepared by dissolving 1.0 g yeast extract in 970 ml 0.01 M sodium phosphate buffer (pH 7.2), sterilizing by autoclaving 20 minutes, allowing to cool to 25^{0}C, and aseptically adding following antibiotics: 25 mg cycloheximide, 2 mg nitrofurantoin, 1.0 mg nalidixic acid, and 0.5 mg gentamicin. Preparation of stock solutions of the four inhibitors was outlined by Trujillo and Saettler (1980). LSSM was dispensed aseptically into sterilized test tubes for recovery of X. phaseoli from contaminated seeds.

Effects of PEG and glycerol treatments on germination

Polyethylene glycol 8000 powder (Formerly PEG 6000) was dissolved in distilled water at room temperature. Samples of 400 hundred seeds were soaked in 100 ml solutions with 10%, 15%, 20%, 25% and 30% PEG, or in solutions containing 50%,

60%, and 70% glycerol. The seeds were soaked at 20^{0}C for one to five days (Fermentation occurs when seeds are soaked for up to 4 days at 25^{0}C). After soaking, seeds were washed for 20 seconds in tap water and dried in a laminar flow hood for 16 hours at 25^{0}C. Germination tests were performed by using the between paper (BP) method according to the International Rules for Seed Testing (ISTA, 1985). Seeds from each treatment were divided into four equal portions and placed in rolled towels. The towels were wrapped in plastic bags and kept in an upright position at a constant temperature of 25^{0}C. Germination percentage and fresh weights of seedlings were determined after 7 days. Numbers of normal and abnormal seedlings were noted during the counting (ISTA, 1979). Nontreated seeds were germinated in the same manner as controls.

Effects of antibiotics in PEG and glycerol solutions on germination

Samples of 400 seeds were soaked in 100 ml solutions of 25% PEG or 60% glycerol mixed with 400 ppm or 800 ppm streptomycin sulfate (Sigma Chemical Co., ST Louis, MO 63178), 400 ppm or 800 ppm chlortetracycline hydrochloride (United States Biochemical Co., Cleveland, Ohio 44128), or 400 ppm or 800 ppm tetracycline hydrochloride (United States Biochemical Co., Cleveland, Ohio 44128) for one, three or five days. After soaking, seeds were washed in tap water, dried in a laminar

flow hood for 16 hours at 25^{0}C and germinated as described previously. For testing antibiotic-induced reduction in seedling vigor, both the germination percentages and fresh weights of seedlings were recorded on the seventh day of germination. Seeds soaked only in 25% PEG or 60% glycerol were used as controls.

Assay of antibiotics in seeds

Antibiotic activity in seeds was measured by using a BYE agar. Petri plates of BYE agar seeded with X. phaseoli were prepared by mixing 900 ml BYE agar at 40^{0}C with 100 ml of 10^9 cells per ml of X. phaseoli suspension in 0.01 M phosphate buffer, pH 7.2, giving a final concentration of 10^8 bacterial cells per ml. Twenty ml of the seeded medium were pipetted into 90 mm diameter petri plates, which then were stored at 4^{0}C prior to use. Seeds soaked for 1, 3, 5, 7, 24, and 72 hours respectively in PEG or glycerol, mixed with either 400 ppm streptomycin sulphate, 400 ppm chlortetracycline hydrochloride or 400 ppm tetracycline hydrochloride were assayed. Treated seeds were rinsed three times in distilled water, halved longitudinally, and seed coats were removed. Cotyledons and embryos were separated, dried overnight in a laminar flow hood, and placed on bacteria-seeded BYE agar plates. Plates were placed in a 28^{0}C incubator for 24 hours and zones of inhibition were measured from the edge of the

assayed piece to the farthest point of clearing. Cotyledons and embryos of seeds which were soaked only in PEG or glycerol served as controls. Forty eight seeds were used for each treatment.

Production of naturally infected seeds

Naturally infected seeds were produced in field plots at the Botany and Plant Pathology Farm, Michigan State University, East Lansing, Michigan. Two isolates of Xanthomonas phaseoli and two isolates of X. phaseoli var. fuscans were used to inoculate navy bean plants. X. phaseoli-susceptible cultivar (C-20) of navy bean was used for the experiment. A bacterial suspension containing about 10^5 colony forming units per ml was prepared by washing a 24 hour-old culture of X. phaseoli from YCA plates and sprayed onto foliage at 15, 30 and 45 days after planting. One hundred pounds of seeds were harvested mechanically and assayed for the presence of internally seed-borne blight bacteria. Seeds with visible symptoms were selected manually to provide a sample with 24% internal infection. This sample was used for the eradication treatments throughout this study.

Production of artificially infected seeds

To produce large quantities of seed internally infected with Xanthomonas phaseoli, seeds were inoculated by vacuum

infiltration as described by Goth (1966), with the following modifications. An isolate of X. phaseoli var. fuscans was grown on YCA plates for 48 hours. Bacteria then were transferred into BYE liquid medium, with 18 hours shaking at 25^{0}C. The bacterial suspension used for inoculation was adjusted to an optical density 0.2 at 620 nm, and contained approximately 10^6 colony forming units per ml. Seeds were surface-disinfected by rinsing in a 1:1 dilution of commercial bleach (2.6% NaOCl) for two minutes, rinsed three times in sterile distilled water, blotted with sterile paper towels, and dried at room temperature in laminar flow hood for 24 hours. Seeds then were submerged in the bacterial suspension and exposed to a vacuum of approximately 500 mm of Hg for two minutes. Air bubbles were allowed to escape from the seeds by shaking the suction flask lightly. At the end of the vacuum infiltration period the suction was released abruptly, and the seeds were allowed stand for an additional 1 minute. Seeds then were blotted dry with sterile paper toweling and left in trays in a laminar flow hood for 4 hours, and dried at 25^{0}C for another 48 hours before use. Seeds that appeared to have broken or cracked seed coats were removed. This procedure produced seeds with 43% internal infection, which were used for eradication treatments in this study.

Assay for the presence of internally seed-bone, X. phaseoli

Assays for internally seed-borne X. phaseoli were done as follows. Seeds were surface disinfected by shaking in a 1:1 commercial bleach-distilled water solution for 1 minute. This method has been demonstrated to eliminate all surface-borne blight bacteria (Weller, 1978). Seeds then were rinsed in sterile distilled water and incubated individually in test tubes with 3 ml of a liquid, semiselective medium. Seeds were placed on a shaker at 20^0C for up to ten days. Samples of liquid from tubes showing turbidity were streaked on YCA. Pale yellow colonies were transferred onto semiselective media (SSM). Pale yellow, convex, shining colonies surrounded by zones of starch hydrolysis were diagnostic for the occurrence of X. phaseoli. Pathogenicity of isolated bacteria was tested by infiltrating a bacterial suspension (10^8 cfu/ml) into 15 day-old Pinto bean seedlings. Pathogenicity was expressed by development of bacterial lesions within 10 days after inoculation.

Results from 46 positively identified fuscous blight bacteria isolations showed that the production of brown pigmentation on YCA accompanied by a pale yellow colony was 100% correlated with a positive reaction on semiselective media and with pathogenicity. This showed that accurate identification of fuscous blight isolates can be obtained by only examining them on YCA. Results from 154 positively

identified non fuscous blight bacteria isolations showed that use of YCA yielded 22% false positives. The chances for positive identification using both semiselective media and the leaf pathogenicity test were 100% with both groups of bacteria. Thus, accurate identification of non fuscous blight bacteria can be achieved by examining them both on YCA and on SSM. Based upon the above observation, the use of YCA for identifying fuscous isolates and the use of YCA together with SSM for identifying non fuscous isolates were applied throughout this study.

The effect of antibiotic residue on bacterial growth

To determine if antibiotic residue in the seeds can affect bacterial growth in semiselective liquid media, seeds were soaked in 25% PEG mixed with 800 ppm streptomycin, 800 ppm chlorotetracycline and 800 ppm tetracycline respectively for 3 days. Seeds then were rinsed in sterile distilled water and incubated individually in test tubes with 1, 2, 3, 4 or 5 ml of semiselective liquid medium. Seeds then were soaked for 1 day at 5^0C to allow antibiotic to diffuse into the liquid medium. Then, 5 ul of a suspension of blight bacteria with a concentration of 10^2 cfu/ml was delivered into each tube. These tubes were incubated for 7 days on a shaker at 25^0C. Samples of liquid from each tube were streaked on YCA and SSM. Blight bacteria were detected in all tubes, except that those

tubes that contain only 1 ml of liquid medium showed less bacterial colony forming units (Less than 0.7 x 10^2/ml). Tubes which contained from 2 to 5 ml of liquid medium all had more than 0.7 x 10^3 colony forming units (Appendix 1). Use of tubes with 3 ml of liquid medium was selected as a standard throughout the study.

Efficacy of eradication of common blight bacteria

For eradication treatment, artificially and naturally infected seeds were soaked for one and three days at 20^{0}C in solutions containing 25% PEG or 60% glycerol mixed with 400 ppm or 800 ppm streptomycin, 400 ppm or 800 ppm chlorotetracycline and 400 ppm or 800 ppm tetracycline separately for one and three days at 20^{0}C. After soaking seeds were rinsed in sterile distilled water for 15 seconds and dried at room temperature in a laminar flow hood for 24 hours. Treated seeds were incubated individually in test tubes with 3 ml of semiselective liquid medium on a shaker for 10 days. Treated seeds were surface-disinfected with 1:10 commercial bleach-distilled water for 1 minute immediately before the incubation to avoid decreased X. phaseoli recovery due to interference by saprophytic bacteria. Turbid tubes were noted and liquid samples were streaked on YCA and SSM plates. Four hundred seeds were tested in each treatment for artificially

infected seeds. Eight hundred seeds were tested in each treatment for naturally infected seeds.

Prevention of phytotoxicity

Seeds treated with tetracycline and chlorotetracycline in this study showed signs of phytotoxicity, both etiolated seedlings and reduced seedling fresh weights. Humaydan et al. (1979) demonstrated that phytotoxicity caused by oxytetracycline and chlorotetracycline could be overcome by resoaking seeds 30 minutes in 0.5% NaOCl in the case of Brassica. The method thus was applied for prevention of phytotoxicity in bean seeds. Seeds that were soaked in PEG solution containing tetracycline for one day were rinsed in tap water, dried, and immediately resoaked in a freshly prepared solution of 1:1 and 1:10 commercial bleach (2.6% NaOCl) for various times. After the final soak, seeds were rinsed in running tap water and dried in a laminar flow hood for 24 hours before germination tests.

RESULTS

Effects of PEG and glycerol treatments on germination

The effect of PEG concentration and soaking time on navy bean seed germination percentage is presented in Figure 1. The untreated control seeds had a germination percentage of 85%. The germination percentage of treated seeds increased with increasing PEG content of the soaking solution, but decreased with increasing soaking times. Seeds soaked in 25% and 30% PEG solutions for one to four days had germination percentages equal to or greater than that of the control. Seeds soaked longer than one day in solutions with 20% or lower PEG content had lower germination percentages than the control.

Figure 2 shows the effect of glycerol concentration and soaking time on navy bean seed germination. Seeds soaked in 50% glycerol had a lower germination percentage than the control, and germination percentage decreased with increased soaking time. Seeds soaked in 60% or 70% glycerol solutions had germination percentages comparable with the control (85% germination) except for those soaked in 60% glycerol solution for five days.

Figure 1. Germination percentages of navy bean seeds soaked in different concentrations of polyethelene glycol for up to 5 days. Non soaked seeds were used as controls. Four hundred seeds were tested in each treatment.

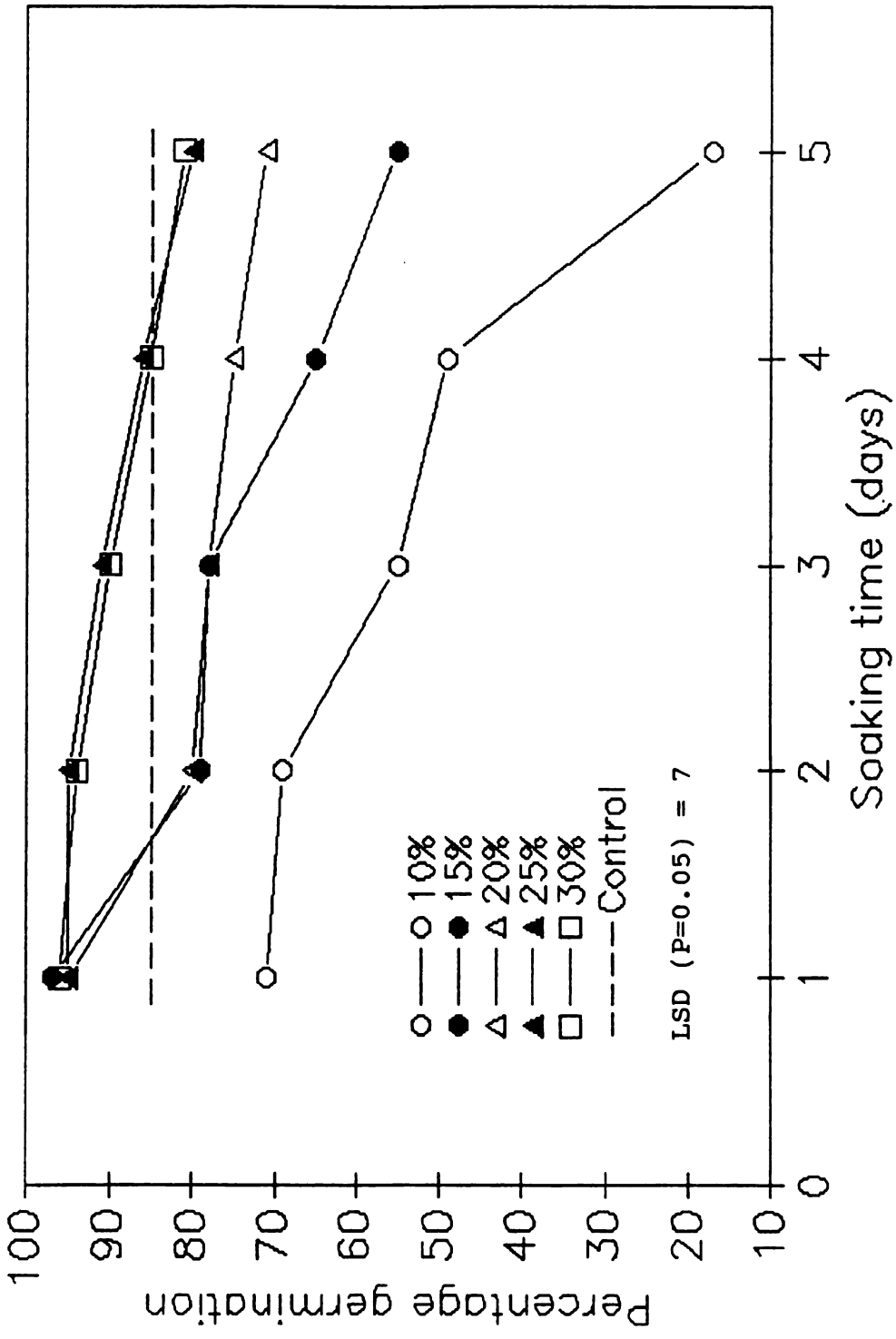
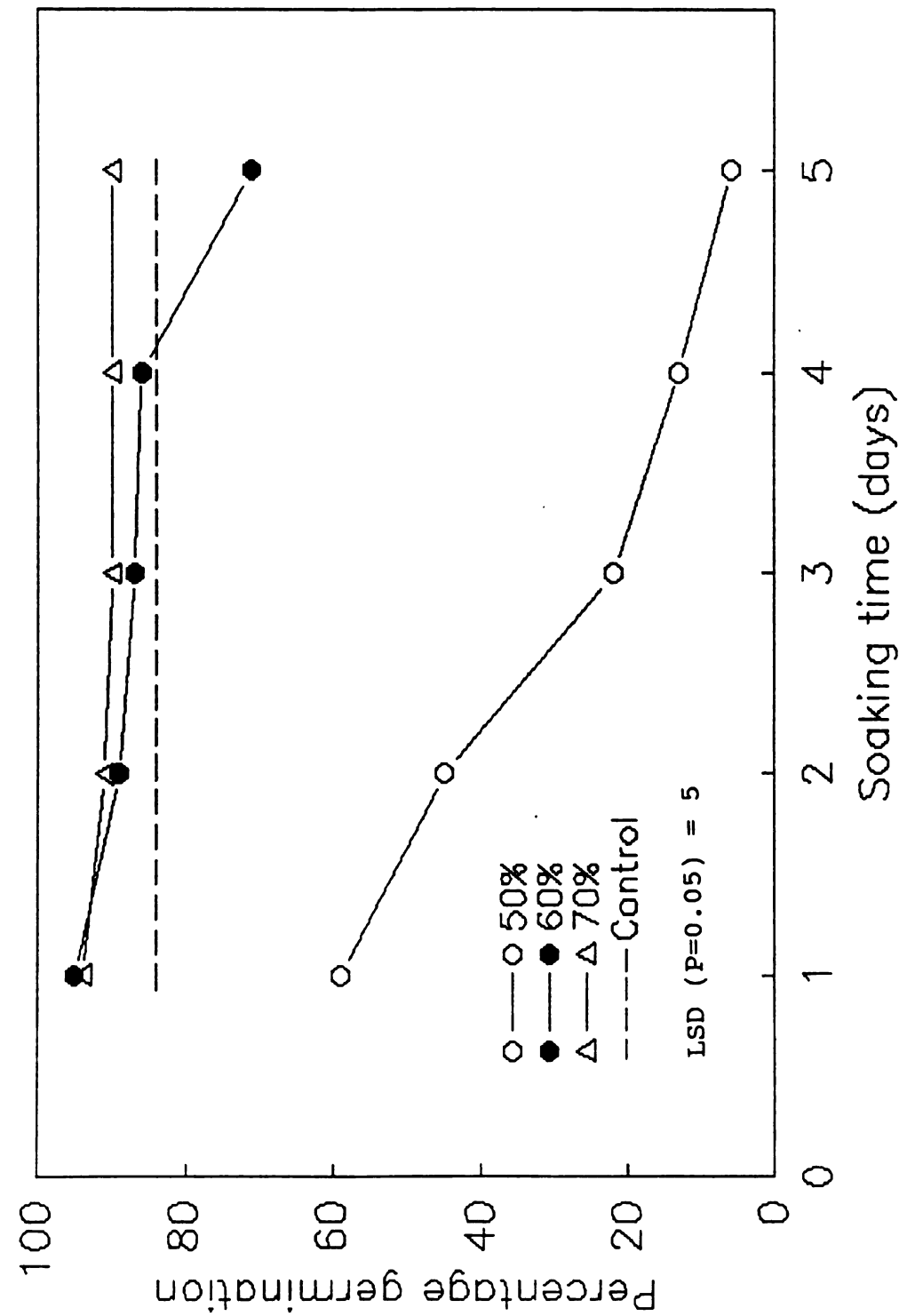

Percentage germination
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
Soaking time (days)
10%
15%
20%
25%
30%
Control
LSD (P=0.05) = 7

Figure 2. Germination percentages of navy bean seeds soaked in different concentrations of glycerol for up to 5 days. Non soaked seeds were used as controls. Four hundred seeds were tested in each treatment.


Percentage germination
100
80
60
40
20
0
0
1
2
3
4
5
Soaking time (days)
50%
60%
70%
Control
LSD (P=0.05) = 5

Several of the above treatments were repeated with weighing of seeds before and after soaking, to determine if the solutions were absorbed into the seeds. Results of this experiment presented in Table 1 demonstrate that the 60% glycerol solution was not taken up by seeds, whereas the other solutions were. This explains the absence of germination reduction with 60% glycerol.

Effects of antibiotics in PEG and glycerol solutions on germination and seedling vigor

No consistent reduction in germination percentages (Table 2) or seedling fresh weights (Table 3) were found between seeds treated with 60% glycerol mixed with streptomycin, chlorotetracycline or tetracycline and those soaked only in 60% glycerol. Seeds treated with PEG mixed with both chlorotetracycline and tetracycline showed significant reduction in seedling fresh weights (Table 3), but no reduction in seed germination compared with seeds soaked only in 25% PEG (Table 2). No significant difference in germination percentage (Table 2) or seedling fresh weights (Table 3) was observed between seeds soaked in PEG with streptomycin and seeds soaked only in 25% PEG. Albinism was observed on the seedlings which received treatment of PEG and tetracycline (400 ppm) or chlorotetracycline (400 ppm) when these seedlings were grown in a green house. No albinism was observed on the

Table 1. Weights of imbibed seeds and seed germination after soaking in glycerol and polyethylene glycol (PEG) solutions for 3 days.

Treatment	Imbibed seed weight (g/100 seeds)	Germination (%)
Dry seed	16	85
Glycerol 60%	16.8	87
Glycerol 50%	22.7	22
PEG 25%	24.9	91
PEG 20%	26.1	78
LSD (0.05)	2.14	5.7

Table 3. Seedling fresh weights of navy been seed soaked in 60% glycerol or 25% polyethylene glycol (PEG) mixed with streptomycin, chlorotetracycline and tetracycline for up to five days compared with seed soaked only in 60% glycerol or 25% PEG. Four hundred seeds were tested in each treatment.

Soak time (days)	Mean fresh weight of seedling (g)						
	Check	Streptomycin		Chlorotetracycline		Tetracycline	
		400ppm	800ppm	400ppm	800ppm	400ppm	800ppm
	Glycerol						
1	1.08	0.99	0.97	1.08	0.94	0.95	0.93
3	0.88	0.89	0.88	0.93	0.80	0.78	0.86
5	0.79	0.83	0.74	0.80	0.81	0.76	0.79
			LSD (0.05) = 0.116				
	PEG						
1	0.96	0.97	0.86	0.60	0.52	0.64	0.56
3	0.85	0.78	0.76	0.45	0.49	0.54	0.45
5	0.73	0.66	0.69	0.45	0.45	0.48	0.46
			LSD (0.05) = 0.103				

Table 2. Germination percentages of navy bean seeds soaked in 60% glycerol or 25% polyethylene glycol (PEG) mixed with streptomycin, chlorotetracycline and tetracycline for up to five days and seeds soaked only in 60% glycerol or 25% PEG. Four hundred seeds were tested in each treatment.

Soak time (days)	Germination						
	Check	Streptomycin		Chlorotetracycline		Tetracycline	
		400ppm	800ppm	400ppm	800ppm	400ppm	800ppm
	Glycerol						
1	88	91	91	87	90	94	88
3	83	84	83	88	84	80	84
5	68	73	74	75	81	71	79
			LSD (0.05) = 4.8				
	PEG						
1	94	98	97	95	91	98	97
3	86	92	95	84	82	95	93
5	79	86	87	76	71	72	73
			LSD (0.05) = 5.0				

seedlings which received treatment with PEG and streptomycin up to 1600 ppm) under the same conditions.

Effect of PEG and glycerol on antibiotic uptake

Antibiotic activity as determined by zones of inhibition on bacteria-seeded BYE agar was detected in cotyledons and embryos when seeds were soaked either in PEG or in glycerol solutions containing streptomycin, chlorotetracycline or tetracycline. Less antibiotic activity was detected for seeds soaked only in PEG or glycerol (Table 4). More antibiotic activity was detected in seeds soaked in PEG than in glycerol. Small zones of inhibition were observed around embryos and cotyledons soaked only in glycerol or PEG.

Effect of soaking time on antibiotic uptake

Antibiotic activity in cotyledons gradually increased between 1 and 24 hours of soaking in PEG or glycerol solutions (Table 5). There were no further increases in antibiotic activity when the seeds were soaked longer than 24 hours.

Efficacy of seed treatment in eliminating bacteria from seeds

The blight bacteria were completely eliminated from artificially infected seeds by immersion for either 1 or 3

Table 4. Zones of inhibition in Xanthomonas phaseoli seeded agar plates around the embryos and cotyledons of navy bean seeds soaked in 25% polyethylene glycol (PEG) or in 60% glycerol mixed with either 400 ppm streptomycin, 400 ppm chlorotetracycline, or 400 ppm tetracycline for 1 day.

Treatment	Inhibition zone (mm)*	
	Embryo	Cotyledon
Untreated	0.0	0.0
PEG only	0.3	1.2
PEG+streptomycin	1.3	5.2
PEG+chlorotetracycline	1.3	5.9
PEG+tetracycline	1.3	6.1
Glycerol only	0.3	1.2
Glycerol+streptomycin	0.5	1.6
Glycerol+chlorotetracycline	0.6	1.6
Glycerol+tetracycline	0.5	1.6
LSD (0.05)	0.16	0.55

*Each value is the mean of 48 embryos or cotyledons.

Table 5. Diameters (mm) of inhibition zones in Xanthomonas phaseoli seeded agar plates around the cotyledons of navy bean seed soaked in 25% polyethylene glycol (PEG) or 60% glycerol mixed with 400 ppm streptomycin (STR), 400 ppm chlorotetracycline (CTE) or 400 ppm tetracycline (TET) for up to seventy two hours.

Treatment	Soaking time (hours)					
	1	3	5	7	24	72
PEG+STR	1.8*	2.7	3.1	4.3	5.2	5.3
PEG+CTE	2.1	2.9	4.2	5.1	6.1	6.0
PEG+TET	2.3	3.3	4.3	5.0	6.0	6.2
Glycerol+STR	0.8	1.1	1.4	1.5	1.6	1.6
Glycerol+CTE	1.0	1.2	1.4	1.5	1.6	1.7
Glycerol+TET	1.0	1.2	1.5	1.5	1.6	1.6

LSD (0.05) = 0.36

*Each value is the mean of 48 cotyledons.

days in a 25% PEG solution containing 1600 ppm streptomycin, 400 ppm tetracycline or 400 ppm chlortetracycline (Table 6). igher concentrations of streptomycin than of tetracycline or chlorotetracycline were required for elimination of blight bacteria. With all treatments, increases in either the antibiotic concentration or the soak time aided in reducing the recovery of bacteria from seeds. None of the treatments involving application of antibiotics in glycerol solutions were effective in eliminating common blight bacteria from the artificially infected seeds.

The most promising treatments from the artificially infected seeds were repeated using naturally infected seeds. Results of this experiment are summarized in Table 7. None of the treatments involving application of antibiotics with glycerol provided useful reductions in recovery of blight bacteria from naturally infected seeds as they did in artificially infected seeds. Conversely, all treatments with mixtures of antibiotics in 25% PEG solutions provided large reductions in the recovery of blight bacteria from naturally infected seeds, especially with 3 days of soaking. Because bacteria within naturally infected seeds were not eradicated by treatments with PEG and streptomycin, the experiment was partly repeated. An additional treatment was included, doubling the maximum streptomycin concentration to 3200 ppm, and a group of seeds were soaked for one to five days in 25% PEG and 1600 ppm streptomycin. Additionally, a portion of the

Table 6. Effect of application of antibiotics in 25% polyethylene glycol (PEG) and 60% glycerol on recovery of common blight bacteria from artificially infected navy bean seeds. Four hundred seeds were tested in each treatment.

Treatment	Bacterial recovery percentage	
	1 day soak	3 day soak
Untreated	43.0	43.0
Glycerol only	27.8	27.0
Glycerol+streptomycin 400*	25.6	10.4
Glycerol+streptomycin 800	24.4	8.2
Glycerol+streptomycin 1600	17.8	7.4
Glycerol+tetracycline 400	16.7	9.6
Glycerol+tetracycline 800	5.2	1.2
Glycerol+chlortetracycline 400	13.9	7.4
Glycerol+chlortetracycline 800	4.6	1.6
PEG only	20.0	19.0
PEG+streptomycin 400	18.0	3.3
PEG+streptomycin 800	8.9	0.6
PEG+streptomycin 1600	0	0
PEG+tetracycline 400	0	0
PEG+chlortetracycline 400	0	0
	LSD (0.05) = 3.18	

*Antibiotic concentrations in parts per million.

Table 7. Effect of antibiotics in 25% polyethylene glycol (PEG) and 60% glycerol on recovery of common blight bacteria from naturally infected seeds. Eight hundred seeds were tested in each treatment.

Treatment	Bacteria recovery percentage	
	1 day soak	3 day soak
Untreated	23.0	23.0
Glycerol only	22.3	21.5
Glycerol+tetracycline 800*	18.9	15.8
Glycerol+chlortetracycline 800	20.6	16.1
Glycerol+streptomycin 1600	21.8	20.6
PEG only	19.4	18.6
PEG+tetracycline 400	0.6	0.1
PEG+chlortetracycline 400	0.8	0.2
PEG+streptpmycin 1600	2.4	0.4
LSD (0.05) = 2.32		

*Antibiotic concentrations in parts per million.

seeds were germinated for fresh weight determinations. Results of these experiments are presented in Tables 8 and 9. None of these treatments eradicated the blight bacteria from naturally infected seeds. Slight reduction in seedling fresh weight was apparently caused by the highest concentrations of streptomycin.

Prevention of phytotoxicity

The efficacy of NaOCl in reducing the phytotoxic effects of tetracycline and chlorotetracycline is presented in Table 10. Seedling fresh weights improved by NaOCl soaking. Three to ten minutes resoaking in NaOCl provided the best results among the partially recovered seedlings. Seedling vigor was reduced either by prolonging the resoaking time or by increasing the concentration of NaOCl. However, seedlings from the resoaked seeds still were severely etiolated.

Table 8. Effect of streptomycin concentration in 25% polyethylene glycol on recovery of Xanthomonas phaseoli and fresh weights of seedlings from naturally infected seeds soaked for 3 days.

Streptomycin content (ppm)	Bacteria recovery (%)	Fresh weight (g)
Untreated	23.0	1.2
0	18.4	0.9
400	6.8	0.8
800	2.3	0.8
1600	0.4	0.7
3200	0.4	0.6
LSD (0.05)	1.42	0.09

Table 9. Effect of soaking time on recovery of common blight bacteria and fresh weights of seedlings from naturally infected seeds when seeds were soaked in solutions of 25% polyethylene glycol and 1600 ppm streptomycin solutions.

Soaking time (DAY)	Bacteria recovery (%)	Fresh weight (g)
0	23.0	1.2
1	2.4	0.8
2	2.2	0.8
3	0.4	0.7
4	0.6	0.7
5	0.3	0.6
LSD (0.05)	0.82	0.09

Table 10. Effects on germination percentages and seedling fresh weights of resoaking seeds in NaOCl with different soaking times following presoaking seeds in 800 ppm chlortetracycline or tetracycline or 25% polyethylene glycol for 24 hours.

Resoak time (min)	Germination (%)		Fresh weight (g)	
	CTC	TTC	CTC	TTC
1:1 NaOCl:H_2O				
0	91	91	0.50	0.50
3	79	74	0.73	0.71
10	51	32	0.61	0.60
20	36	16	0.48	0.40
30	17	12	0.45	0.41
1:10 NaOCl:H_2O				
0	91	91	0.50	0.50
3	92	94	0.74	0.72
10	89	90	0.73	0.70
20	87	90	0.66	0.68
30	83	87	0.61	0.60
LSD (0.05)	5.7		0.053	

DISCUSSION

This study showed that PEG solutions can be used as an antibiotic carrier for reduction but not eradication of internally seed-borne common blight bacteria without affecting seed germination percentage. A PEG concentration of 25% showed the best result in facilitating movement of the antibiotics into the seeds and reducing X. phaseoli within internally contaminated seeds. However, seedling vigor was expressed by fresh weights was slightly reduced in this treatment. This problem could be overcome by increasing the PEG concentration. Unfortunately, percentage reduction of X. phaseoli was impaired by increasing the concentration of PEG solution (Appendix 2). Quite probably, increased viscosity of the PEG solution resulted in less antibiotic infiltration. Another problem noticed was that PEG treatment is only good for seeds that are less than one year old. Germination percentage was reduced by 18% with seeds that were one and a half years old, and by 54% with seeds that were two years old (Appendix 3).

The results demonstrated that glycerol is not suitable as an antibiotic carrier for the reduction or eradication of internally seed-borne bacteria. Glycerol solutions with concentrations greater than 60% were not absorbed by the

seeds, and lower concentrations reduced seed germination. This phenomenon explains why no phytotoxicity was observed when seeds were soaked in 60% glycerol solutions containing tetracycline and chlorotetracycline, although these two antibiotics induced phytotoxicity in PEG solutions.

Tetracycline and chlorotetracycline in PEG solutions proved effective in reduction of internally seed-borne X. phaseoli. However, phytotoxicity rendered these treatments unacceptable. Streptomycin appears to be the most promising antibiotic for reduction of X. phaseoli. A concentration of 1600 ppm in a 25% PEG solution provided the most effective reduction of X. phaseoli infection in this study. Further increases in streptomycin concentration did not provide increased reduction in X. phaseoli, and seedling fresh weight was reduced. The inability to completely eliminate the bacteria from naturally infected seeds appeared to be partially due to the occurrence of seeds with hard coats. These seeds did not imbibe the PEG solution within the soaking period. Seeds with hard coats accounted for 10.2, 8.5, 6.9 5.5 and 4.5% of naturally infected seeds of cultivar C-20 which were soaked for 1, 2, 3, 4 and 5 days, respectively. Investigation showed that 72% of the surviving X. phaseoli were recovered from seeds with hard coats soaked for one day and 67% from seeds with hard coats soaked for three days (Appendix 4).

Antibiotics such as streptomycin (Klisiewiecz and Pound, 1961) and chlorotetracycline (Lockhart et al., 1976) have been used to eradicate Xanthomonas campestris from Brassica seeds, but these treatments often resulted in reduced germination and seedling vigor (Huber and Gould, 1949). Humayadan et al. (1980) overcame this disadvantage with a thirty minute soak in a solution of 0.5% sodium hypochlorite following a one hour soak in 500 ppm of either oxytetracycline or streptomycin. Seed lots that received the combined treatment were unaffected either in germination or seedling vigor. However, this method was not effective in our study. Resoaking seeds in NaOCl only partially overcame the phytotoxicity induced by tetracycline or chlorotetracycline. The seedlings nevertheless were still severely etiolated. Success in eliminating Xanthomonas campestris pv. campestris from Brassica seeds was probably due to the fact that this pathogen is an externally seed-borne bacterium on Brassica, and could be eliminated by conventional surface type treatments. For example, one hour soaking with antibiotics in water proved effective in eliminating the bacteria (Humaydan et al., 1980). However, such a short period of time did not allow the antibiotics to penetrate into the embryo and cotyledon. Thus the antibiotics on the seed surface and in the seed coat were easily oxidized by NaOCl. Failure of NaOCl to completely overcome phytotoxicity of the tetracyclines in bean seeds was probably due to the

localization of the antibiotics inside the seeds, where the oxidant failed to penetrate.

Blight bacteria in artificially infected seeds are more sensitive to antibiotic treatment than those in naturally infected seeds. Clearly, the vacuum inoculation technique did not completely duplicate field infection. Field inoculation with common blight bacteria was more realistic for providing internally infected seed for this study. Bacteria in artificially infected seeds probably are localized around the outside of the cotyledon, whereas bacteria in naturally infected seeds may be localized within the cotyledons and embryo tissues.

SUGGESTIONS AND FUTURE STUDIES

Several additional, related lines of investigation seem warranted prior to potential commercial application of methods developed in this investigation. 1) Determine if the treatment (1600 ppm streptomycin in 25% PEG solution) could have an effect on seed production and crop yield. 2) Determine if the treatment effectively reduces disease development under field conditions. 3) Ascertain whether application of the method affords the hazard of selecting for pathogenic strains of X. phaseoli that are resistant to streptomycin. One previous study demonstrated the occurrence of such strains in Michigan (Weller, 1978). 4) Determine if the antibiotic can be systemically transported by the plant following seed germination, and the time that the antibiotic persists in the plant in sufficient concentration to retard blight development. This may provide evidence on the potential effect of the antibiotic on bacteria which were not eliminated. 5) Investigate whether other antibiotics that are lethal to X. phaseoli but have no phytotoxicity on bean are available. 6) Use the same technique to eliminate other seed-borne bacteria from beans and other crops.

APPENDICES

APPENDIX 1

Effect of antibiotics on the growth of common blight bacteria.

Volume of liquid medium* in test tube	Antibiotic	Bacteria recovery (%)	Bacterial colony forming unit
1 ml	Streptomycin	100	$> 0.3 \times 10^3$
	Tetracycline	100	$< 0.7 \times 10^2$
	Chlorotetracycline	100	$< 0.7 \times 10^2$
2 to 5 ml	Streptomycin	100	$> 0.7 \times 10^3$
	Tetracycline	100	$> 0.7 \times 10^3$
	Chlorotetracycline	100	$> 0.7 \times 10^3$

*Liquid medium was prepared by dissolving 1.0 g yeast extract in 970 ml 0.01 M sodium phosphate buffer (pH 7.2), sterilizing by autoclaving 20 minutes, allowing to cool to 25^0C, and aseptically adding following antibiotics: 25 mg cycloheximide, 2 mg nitrofurantoin, 1.0 mg nalidixic acid, and 0.5 mg gentamicin.

APPENDIX 2

Effect of polyethylene glycol (PEG) concentration on seed germination, seedling fresh weight and blight bacteria recovery percentage of naturally infected seeds, when these PEG solutions were mixed with 1600 ppm streptomycin and seeds were soaked for three days.

PEG concentration	Germination percentage	Mean fresh weight (g)	% Bacteria recovery
25%	88	0.84	0.4
30%	89	1.03	5.6
35%	92	1.09	13.1
40%	96	1.12	13.8
45%	96	1.20	14.4
Untreated	86	1.20	23.0
LSD (0.05)	5.6	0.118	1.94

APPENDIX 3

Germination of navy bean seeds soaked in 25% polyethylene glycol solutions for 3 days following different times of storage after harvesting.

Seed storage time (months)	Germination percentage	
	Untreated (Check)	PEG soaking
9	85	91
18	81	75
24	72	56

LSD (0.05) = 5.8

Note: Harvested seeds were stored in polyethelene plastic bags at room temperature (ca. 25^0C).

APPENDIX 4

Common blight bacteria recovered from impermeable (hard coat) and permeable (soft coat) seeds when the naturally infected seeds were soaked for 1 and 3 days in solutions of 25% polyethylene glycol and 1600 ppm streptomycin.

Soaking time (days)	Bacteria recovery percentage	
	Hard coat	Soft coat
1	1.7	0.7
3	0.3	0.1
Untreated	23.0	

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