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DETECTION AND CHARACTERIZATION OF XANTHOMONAS PHASEOLI (E. F. SM) IN COMMON BEAN (*Phaseolus vulgaris* L) SEEDS COLLECTED IN ZIMBABWE.

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Abstract

Laboratory and glasshouse experiments were conducted to detect and characterize *Xanthomonas phaseoli* (E. F. SM) in seedlots collected from commercial seed dealers and farmers who retain common bean (*Phaseolus vulgaris* L) seeds for future planting. Even though the "Direct Plating" method did not detect *X. phaseoli*, the pathogen was however detected by the "Growing-On" and "Grind-and-Soak" methods. The "Grind-and-Soak" method revealed that seedlots of retained seeds e.g. Mazoe Citrus Estates (5.88×10^5 cfu/ml) had significantly higher bacterial population levels ($p < 0.05$) when compared to seedlots from commercial dealers e.g. Mutomba Supermarket (1.426×10^2 cfu/ml). The "Grind-and-Soak" method yielded some pathogenic *X. phaseoli* and non-pathogenic xanthomonad species on Michigan C20. Epiphytic population sizes detected at four weeks after crop emergence by the "Growing-On" procedure was significantly higher on Michigan C20 than on Natal Sugar, Cranberry and Red Kidney varieties. The experiments confirmed the presence of seedborne *X. phaseoli* in both retained and certified seedlots in Zimbabwe, with the former having significantly higher bacterial population levels. Farmers are advised not to retain seeds over long periods as this results in pathogen build-up. If farmers decide to retain seed, they should retain seeds of tolerant varieties like Red Kidney.

Key words: *Xanthomonas phaseoli*, common bean, seedlot, retained seed, seedborne, pathogen, Zimbabwe.

Introduction

Bacterial common blight disease caused by *Xanthomonas campestris* pv *phaseoli* (synonym *Xanthomonas phaseoli*) is one of the major constraints to common bean (*Phaseolus vulgaris* L) production all over the world (Mabagala and Saettler, 1992; Opio *et al*., 1996). The disease causes both quantitative and qualitative yield losses ranging between 10 and 40%, depending on bean cultivar susceptibility and environmental conditions (Saettler, 1989). Common beans are rich in proteins (6-22%), and are increasingly being consumed as an alternative to animal protein by low-income families in developing countries (Iranga *et al*., 1985). It can be consumed as immature green pods or as dry pulses.

Xanthomonas phaseoli is seed-borne (Leben, 1981; Schaad, 1982), and survives as long as the seed remains viable (Hirano and Upper, 1983). Seed transmission is the primary means by which the pathogen is disseminated (Cafati and Saettler, 1980). Both internally infected and externally infested seeds are important sources of primary inocula for *X. phaseoli* (Hall, 1994).

Seed quality and source have an effect on the outbreak of bacterial common blight. Most resource-poor farmers retain bean seed for future planting. Such seed does not usually receive any treatment to reduce pathogen levels. There is therefore a strong likelihood that such seed may act as sources of primary inocula for seedborne diseases like common blight halo blight (*Pseudomonas syringae* pv *phaseolicola*). This is particularly so for resource-poor farmers who retain seed for up to ten seasons, and may not have money to buy chemicals to dress the seed before planting out.

Besides insect pests, common blight has been reported in most bean-producing districts of Zimbabwe as one of the major constraints to bean production (Giga, 1989). The following experiments were carried out to detect and characterize *X. phaseoli* in bean seeds collected in Zimbabwe.

Materials and Methods

Seed lots were obtained from smallholder bean producers in Chinyika Resettlement Area of Manicaland Province while other seed lots were obtained from Mazoe Citrus Estates and commercial seed dealers like Farm and City Centre, TM and Mutomba Supermarkets in Harare. The seeds from Chinyika Resettlement Area were mainly seed mixtures and were named based on cluster areas/villages from which they were obtained. The seeds from commercial dealers were named after supplier and bean variety. Table 1 summarizes the seed lots, sources and varieties used for the assays:

Table 1: Seed lots, sources and bean varieties used for the assays

Seed lot	Seed Source (s)	Bean Variety
Chinyudze	Chinyika	Red Kidney
Nemaire	Chinyika	Seed Mixture
Pfumoiguru	Chinyika	Seed mixture
Bingagururu	Chinyika	Seed Mixture
Gowakowa	Chinyika	Seed Mixture
Mazoe	Mazoe Citrus Estates	Seed Mixture
Commercial	TM, Farm and City, Mutomba	Michigan C20, Red Kidney, Cranberry, Natal Sugar, Mkuzi

Experiment 1: The Seed Assays

A working sample of 400 bean seeds was used for every experiment in line International Seed Testing Association (ISTA) standards (Draper, 1995). This sample

was obtained initially by taking small portions from a given seed lot (two kilograms) and mixing them to get a composite sample, then randomly counting 400 seeds.

Direct Plating

Seed assays were conducted on all seed lots by plating out sampled seeds on nutrient agar (NA) and MXP (a semi-selective medium), as described by Saettler *et al.* (1995) and Schaad (1988). The seeds were first washed in running water to remove soil and other debris. They were then sterilized by dipping in a 1% sodium hypochlorite solution for 30 seconds and rinsing the seeds in sterile distilled water (SDW) for three minutes to remove traces of sodium hypochlorite. The seeds were then placed on sterile laboratory paper to dry.

Fifteen sterilized seeds were plated in each Petri dish with the hilum of each seed in direct contact with the plating media. Each seed sample was replicated four times. After three days, the plated seeds were visually assessed for the presence of *X. phaseoli* colonies under a stereomicroscope.

The "Grind and Soak" Method

From each seed lot, 15 randomly selected seeds per working sample were sterilized in 1% sodium hypochlorite for 30 seconds, rinsed in SDW for 2-3 minutes and then dried on multiwipe paper. The dried seeds were ground in a blender, and then soaked in 50ml of phosphate buffer saline (PBS), pH 7.4, for three hours. The resultant supernatant collected after filtering through cotton wool was used to prepare serial dilutions as described below.

One millilitre of each working seed sample supernatant was serially diluted ten-fold by first transferring into a bottle holding 9ml SDW to obtain a one-tenth dilution of the original supernatant. One millilitre of the one-tenth dilution was transferred to another

bottle containing 9ml of SDW to obtain a one-hundredth dilution. The above process was repeated until a 1/10 000 dilution was obtained. One millilitre of the 1/1 000 and 1/10 000 were plated onto NA and MXP and incubated at 25^0C for two days.

The colonies that developed were subjected to a number of tests which included the Gram Reaction, Oxidase Reaction, Nitrate Reduction, Starch Hydrolysis tests and pathogenicity on Michigan C20 bean plants. Pathogenicity characterization was done two days after incubation when pathogen cells were still in the expontial growth phase.

Experiment 2: The "Growing-On" bacterial detection procedure

A glasshouse pot experiment was conducted to determine the presence of *X. phaseoli* in seedlots through the "Growing-On" detection technique by observing symptoms on growing plants. Five bean varieties, Michigan C20, Natal Sugar, Cranberry, Mkuzi and Red Kidney were planted in pots that contained heat-sterilized sandy loam soil.

Individual plastic pots containing 2.6kg sterile soil were fertilized with Compound D (8% N, 14% P_2O_5, 7% K_2O) fertilizer at a rate of 800 kg/ha. Three seeds were planted in each pot in five replicates of each bean variety at a depth of 10mm. The seed lots were assessed for emergence success. The seedlings were thinned down to one plant per pot. Dimethoate 40 EC (40% a.i. mass/volume) was used to control aphids and other leaf-suckers.

The pots were covered with a clear polythene sheet at 14 days after crop emergence. A hand-held laboratory sprayer was used to atomize distilled water to create a fine mist for maintaining a high humidity environment inside the polythene sheet conducive for blight development.

After a 10 to 14-day incubation period, the polythene plastic was removed, and the leaves and stems assessed for *X. phaseoli*-induced symptoms. The plants were kept in the glasshouse for further observations on symptom development up to pod filling. Symptomless leaves were assayed for epiphytic pathogen population levels of the pathogen after the two-week incubation period.

The trial was laid out as a complete randomized design with five replications. Five bean varieties (Michigan C20, Red Kidney, Cranberry, Natal Sugar and Mkuzi) were planted out. The results on epiphytic survival were analyzed as for the "Grind-and-Soak" method. Means were separated using Fischer's least significance difference (lsd) at 5% level.

Experiment 3: Pathogen Characterization

Besides colony morphology, and growth characteristics on MXP and NA, *Xanthomonas* colonies were characterized by Gram Reaction, Nitrate Reduction test, Oxidase Reaction and Isolates' pathogenicity on beans.

Gram Reaction

Grease on the slide was removed by flaming it several times. A small drop of water was placed on the middle of the slide. A small amount of the yellow pigment colonies was removed from the culture using a sterile wire loop and placed in the water drop. After mixing the cells with the water on the slide, the smear was dried by holding the slide over the flame. When the slide had cooled, crystal violet was pipetted onto the surface and left for one minute. The stain was poured off the slide, washed with 70% alcohol, and iodine solution added for one minute before being rinsed off with water. Safranin was then applied for three minutes. This was then rinsed off with water before

drying over the flame. The mounted specimen was examined under a stereomicroscope with a ×100 lens using oil immersion with no cover slip.

Nitrate Reduction Test

Two-day-old yellow pigmented cultures were used to inoculate separate test tubes of nitrate broth (NB). These were then incubated for seven days at 37^0C. After incubation, the media in each tube was tested for the presence of nitrates by placing a drop on a spot plate and adding Gries-Illosvay A and a drop of GriesIllosvay B. The presence of nitrites is manifested by a bright red colour, indicating reduction of a nitrate to nitrite.

Oxidase Reaction Test

Yellow-pigmented colonies were cultured on King's Medium B at 26^0C for 24 hours. Some colonies were picked off the medium using a platinum loop and then rubbed on filter paper impregnated with 1% aqueous tetra-p-phenylenediamine dihydrochloride solution. The production of a purple colour within 10-60 seconds indicated a positive Oxidase Test.

Pathogenicity Tests with Isolates of X. phaseoli and other xanthomonads

Bean plants grown in sterilized soil in 2.6kg plastic pots in the glasshouse were inoculated with some isolates obtained from the Seed Assay experiments. After subculturing, single colonies of *X. phaseoli* were isolated on NA and MXP, then transferred onto NB and incubated on a shaker for 24 hours at 25^0C. Each strain was inoculated onto the adaxial surface of a half to three-quarter size trifoliate leaf of a 14 day-old plant by the carborundum cotton swab inoculation method. Distilled water was sprayed onto the plants using a hand-held laboratory sprayer to create high humidity ideal

for common blight development. The plants were assessed for blight symptoms from seven days after inoculation

Results

The Seed Assay

Direct Plating

Neither *X. phaseoli* nor xanthomonads developed from all the seeds directly plated on NA and MXP.

The "Grind and Soak" Method

All the seed lots were infected with *X. phaseoli*, except those of the Red Kidney variety. The seed lot from Mazoe Citrus Estates had the highest bacterial population of 5.78×10^5 cfu/ml, while the Cranberry seed from Mutomba Supermarket had the lowest *X phaseoli* population (Table 2). There were no significant differences in the bacterial population levels of the seed batches from Mazoe Citrus Estates and Chinyika Resettlement Area ($p<0.05$). Seed lots from commercial dealers had significantly lower pathogen population levels when compared to those from Chinyika Resettlement Area and Mazoe Citrus Estates ($p<0.05$).

Table 2: Bacterial population in seedlots

Seed Source	Bacterial Population (cfu/ml)
Mazoe Citrus Estates	5.762[a]
Chinyudze[1]	4.919[a]
Nemaire[1]	4.860[a]
Bingaguru[1]	4.858[a]
Pfumoiguru[1]	4.833[a]
Gowakowa	4.817[a]
Farm and City- N.S[2]	2.752[b]
Farm and City- Mich[2]	2.701[b]
TM-Mkuzi	2.640[b]
Farm andCity- Mkuzi	2.591[b]
TM- Cranberry	2.591[b]
Farm and City- Cranberry	2.544[b]

Mutomba- Mich[2]	2.477^b
Mutomba- Mkuzi	2.427^b
Mutomba- Cranberry	2.154^b
Farm and City- Red Kidney	0.000^c
Chinyudze- Red Kidney	0.000^c
N	68
CV (%)	39.66
DMRT $LSD_{0.05}$	2.078
s_x	0.729

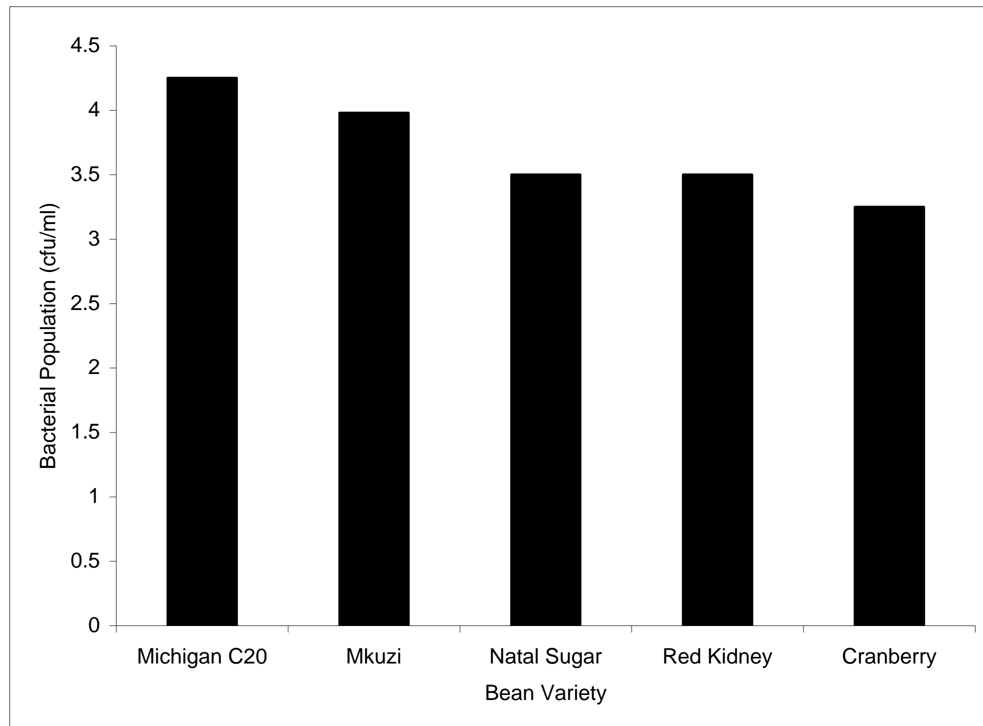
[1]Seedlots from Chinyika Resettlement Area
[2]N.S- Natal Sugar; Mich- Michigan C20
Mean populations followed by the same letter are not significantly different from each other ($p<0.05$).
The figures in the table are antilog transformed mean bacterial population.

The "Growing-On" Detection Procedure

Twenty percent pf the planted seeds failed to germinate even though they had been dressed with Captan to control damping off. No *X. phaseoli*-induced symptoms were observed on all the varieties. However, studies on bacterial epiphytic survival however revealed the presence of the pathogen on leaves. Figure 1 shows the epiphytic bacterial populations detected on the bean varieties at four weeks after crop emergence.

Even though no *X. phaseoli* was detected on the Red Kidney seeds by the Seed Assays, epiphytic populations were detected at four weeks after crop emergence. Epiphytic population size was affected by bean variety. The highest population was detected on Michigan C20 (1.524×10^4 cfu/ml), while the lowest (1.932×10^3 cfu/ml) was on Cranberry. The epiphytic population of Michigan C20 was not significantly different from that of Mkuzi, but it was different from that of the other varieties ($p<0.05$).

Figure1: Epiphytic *X. phaseoli* population on bean plants grown in the glasshouse



n = 15
$LSD_{0.05}$ = 0.35
CV (%) = 18.26

Pathogen characterization

The "Grind-and-Soak" procedure yielded some xanthomonad species which resembled *X. phaseoli* on colony colour and shape on NA and MXP. On MXP, colonies of *X. phaseoli* were pale yellow, convex and mucoid, and were surrounded by a zone of starch hydrolysis. On NA, they were yellow, convex and mucoid, whereas those of the xanthomonads were darker yellow, less mucoid and smaller than those of *X. phaseoli*. After five days of growth on NA, colonies of xanthomonads produced sectors that were light yellow and mucoid and resembled colonies of *X. phaseoli*.

Both *X. phaseoli* and the xanthomonads that developed when plates were incubated at 25^0C, were gram negative, oxidase negative, and hydrolyzed starch. The observed characteristics of *X. phaseoli* and xanthomonads are summarized in Table 3. Both did not reduce nitrates. *X. phaseoli* was however, pathogenic on Michigan C20 plants, while the other xanthomonads were nonpathogenic. Points of inoculation with

xanthomonads became necrotic, but did not enlarge. Five days after inoculation, the pathogenic *X. phaseoli* strain induced water-soaked spots on leaf underside. Each spot was encircled by a narrow zone of yellow tissue which later turned brown and necrotic.

Table 3: Characteristics of *X. phaseoli* and xanthomonads

Characteristic	*X. phaseoli* reaction	Xanthomonads
Yellow, mucoid, convex colonies on NA	+	+
Gram negative rods	+	+
Growth at 24-26^{0}C	+	+
Glucose utilization	+	+
Oxidase reaction	+	-
Starch hydrolysis	+	+
Xanthomonadin pigment	+	+
Pathogenicity on beans	+	-
Pathogenicity on beans	-	-

Key
(+): Positive identification
(-): Negative identification

Discussion

Seed assays are the most reliable methods of determining whether or not seeds are infected with seedborne pathogens (Ridout and Roberts, 1997). Infected bean seeds of tolerant varieties may show no visible external symptoms. Also, symptoms induced by *X. phaseoli* may be masked by other diseases (Leben, 1981). Even though direct plating of the bean seed did not detect the pathogen, this did not imply that the seeds were pathogen-free. Sterilization was effective in eliminating contaminant bacteria. The “Grind-and-Soak” method confirmed the presence of the pathogen in most seed lots.

The seed lots from Mazoe Citrus Estates and Chinyika Resettlement Area had higher population levels of *X. phaseoli* when compared with seed lots from commercial dealers. These seed lots consisted of seed that had been retained for at least four seasons. Since all farmers initially source their seed from commercial seed dealers, this could account for the pathogen build-up in the retained seed. There is therefore a stronger

possibility of disease outbreak with retained seed than with certified seed bought from commercial dealers. However, even under field conditions, the quantitative importance of seedborne *X. phaseoli* inoculate in disease development is a function of the interaction of several environmental factors with the inocula density per seed and the number of diseased seeds. Where seeds are heavily infected, and temperature is below 28^{0}C and relative humidity 60%, no disease develops (Weller and Saettler, 1980). On the other hand, if seeds are lightly infected but environmental conditions are ideal for disease development, common blight epidemics occur.

The number of *X. phaseoli* colonies recovered by seed assays depends on the sensitivity of the method used. Though the “Grind-and-Soak” technique for pathogenic bacteria has proved to be very useful (Webster *et al*., 1983), it is not perfect. With bean seedlots, Schaad (1982) reported that the numbers of viable cells of X. campestris are often reduced when seeds are ground and soaked. When seeds are ground, there is a possibility that this releases a chemical inhibitory to the growth of the pathogen, hence a lower pathogen count is recorded (Schaad, 1982). The chemical inhibitor has not been identified. The “Grind-and-Soak” technique has also failed to isolate *X. phaseoli* from known naturally infected seedlots. This could have been true with the Red Kidney seed lots from which the pathogen could not be isolated but detected as epiphytes by the “Growing-On” experiment.

In the “Growing-On” experiment, the fact that 100% emergence was not obtained from all the seeds planted out can be attributed to many factors which include reduced seed viability, large temperature fluctuations in the glasshouse, and the presence of seedborne pathogens. Despite the fact that all the seeds were dressed with Captan, some

Red Kidney and Cranberry seeds decomposed and so failed to emerge. One of the seedborne pathogens detected in the experiment was *X. phaseoli*.

Given that smallholder farmers retain seeds for long periods, there is a strong likelihood that the seeds planted out would have higher *X. phaseoli* population levels than in the preceding season. Hall (1994) and Weller and Saettler (1980) reported that if seed heavily infected seeds fail to germinate resulting in reduced crop stand. Seedling mortality resulting from heavy seed infection may be up to 60% (Agrios, 1988). Even if the seed emerges and the seedling does not die, the bean plant will be infected with common blight at an earlier stage in the season resulting in both quantitative and qualitative yield losses. Even when farmers improve other agronomic practices, but continue planting out retained seed, common blight will remain a threat to high quality and quantity bean production. Quantitative losses have not yet been ascertained in Zimbabwe, but qualitative losses are evident, especially where beans are being grown for export. Where farmers produce green beans, pods with lesions may be rejected by the consumer, or may be bought at low price.

Plants inoculated with *X. phaseoli* isolates and those in the "Growing-On" experiment may not have developed symptoms probably because the pathogen failed to reach the threshold necessary for disease induction. Wyman and VanEtten (1982) reported that an endophytic population threshold of between 10^6 and 10^7 bacterial cells per cm^2 leaf tissue was needed for disease induction in susceptible cultivars. In this study, only the epiphytic population was determined, and was 7.29×10^4 cfu/ml on Michigan C20, the most susceptible variety. This population was therefore lower than the threshold level needed for disease induction.

Conclusions and Recommendations

The experiments confirmed the presence of *X. phaseoli* as a seedborne pathogen in both retained and certified seed lots in Zimbabwe. Retained seed had higher levels of pathogen infection compared to certified seeds. Given that the pathogen builds up in the seed over seasons, farmers are encouraged not to retain seed over long periods e.g. four years. Where long term retention occurs, farmers ought to dress the seed. Alternatively, the tolerant varieties like Red Kidney, and avoid susceptible varieties like Michigan C20.

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