Transmission of *Xanthomonas manihotis* in Seed of Cassava (*Manihot esculenta*)

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ABSTRACT

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Transmission of *Xanthomonas manihotis* in botanical seeds of cassava was studied by direct immunofluorescence and enzyme-linked immunosorbent assays. The pathogen was detected in seed embryos and in pollen but not in other tissues of cassava seeds. Healthy and infected seeds showed no signs of damage due to *X. manihotis*, but approximately 10^4 cells/ml were detected in infected embryos. Seed transmission varied from 0 to 40%.

Cassava is almost exclusively propagated by planting pieces of stem, but researchers are increasingly using true or sexual seeds (10). *Xanthomonas manihotis*, the causal agent of cassava bacterial blight, is disseminated by infected vegetative planting materials (8–10), but evidence for its dissemination through seed has not been conclusive. The possibility of seed transmission of the pathogen was first suggested in 1974 (3), but no substantiating evidence was found (8,12). Persley (14) later reported that seeds from infected plants stored at 5 C for 2–51 mo carried the bacterium, but the presence of the pathogen infecting the seeds was not shown. Considering the implications of the cassava seed exchange between international and national research centers, we undertook this study to determine whether cassava bacterial blight could be transmitted through seed.

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MATERIALS AND METHODS

Seed was collected monthly from cassava plantations in Carimagua (eastern plains of Colombia), where cassava bacterial blight is endemic. The cultivars had different levels of disease susceptibility. The fruit pericarp and endocarp were removed and the seed was examined for bacterial damage. After seeds were surface-sterilized for 1 min in 80% ethanol and washed three times in sterile distilled water, the caruncle and the outer layers of the seed were removed aseptically, and the innermost layer of the seed coat was cracked to remove the cotyledon and the embryo.

Ten samples of each tissue were examined under a dissecting microscope and later macerated in 5 ml of sterile physiological saline. Each extract was poured into 250 ml of sterile Kelman's broth medium without tetrazolium chloride (7) and shake-cultured in an Orbit incubator shaker (Lab-Line Instruments Co., Melrose Park, IL) for 48 hr at 500 rpm. This was followed by centrifugation at 10,825 *g* to concentrate the bacterial cells. The resulting pellets were resuspended in 0.01 M phosphate buffered saline (PBS) pH 7.2, and stained with a *X. manihotis* antiserum conjugated with fluorescein isothiocyanate (Xm/FITC), prepared as described previously (1,4). The slides (magnified × 1,000) were examined under an UV microscope.

Similarly, bacterial pellets were resuspended in distilled water, washed twice by centrifugation (10,825 *g*). Diluted extract was subjected to enzyme-linked immunosorbent assay (ELISA) following the method of Ellens et al (5). The enzyme used was horseradish peroxidase, and the substrate was 5-amino salicylic acid. Results were visually scored against both serial-diluted standard positive samples (10^9 to 10^1 cells/ml^{-1}) and blanks. Portions of the extracts were also plated on Kelman's tetrazolium chloride agar medium (7), to isolate the bacterium. In each of these trials, a pure culture of *X. manihotis* was used as the control.

Seeds of several cultivars from infected plantations were treated with Arasan (tetramethylthiuram disulfide, Du Pont Co., Wilmington, DE) and germinated at 37 C, to determine the percentage of infected seeds. Percent germination was scored after 20 days, and the seedlings were later transplanted into sterile fine sand in a glasshouse (26 C, 85% RH) and observed daily for symptoms of cassava bacterial blight. *X. manihotis* was isolated from infected/ungerminated seeds and seedling stem sections by streaking serial dilutions of bacterial growth after inoculations with seed or stem sample enrichments of Kelman's broth medium (7). Bacterial colonies were compared morphologically and culturally with those of typical isolates of

X. manihotis, and pathogenic tests were done using the leaf-infiltration technique (14).

Free-hand stem sections of infected seedlings were examined under the light microscope. Infected and healthy stem sections and embryos were fixed in formalin acetic acid and Allen-Bouin II (16), respectively, microtomed at 8–10 μ, stained with iron hematoxylin, and counterstained with eosin. Some microtomed stem and embryonic sections were stained with Xm/FITC conjugate for UV microscopic observation to determine the relative distribution of the pathogen. Seeds free of cassava bacterial blight were used as control in each trial.

RESULTS AND DISCUSSION

Our results showed that *X. manihotis* is indeed a seedborne pathogen, confirming Persley's findings (13,14). The pathogen was consistently detected in seed embryos by both direct immunofluorescence and ELISA. ELISA plates examined visually gave tentative positive results on crude extracts, but after enrichment culturing, firm positive results were obtained at 10^4 cells/ml^{-1} when the reaction produced brown colors markedly darker than the PBS blanks (Table 1). Other seed tissues did not react, suggesting that they were free from the pathogen.

The bacterial cell concentration in the embryo was apparently lower than 10^4 because direct assays did not detect the pathogen. Infected and clean seeds showed no visible damage or difference in shape, and artificial inoculation of healthy tissues did not result in any visible bacterial spread, suggesting that *X. manihotis* was apparently unable to colonize these tissues. Some infected seedlings showed browning xylem vessels in free-hand sections, however. Histologically, no damage was apparent in infected embryos; the bacterial cells were generally observed by immunofluorescence assay in the plumule section of the embryo.

The results therefore suggest latent survival of the pathogen in these tissues, but more studies are needed to determine the length of time the bacterium can survive in the tissues. *X. manihotis* can apparently survive for long periods; after 3 mo of storage, the pathogen could be detected in seed embryos. Persley (14) detected the pathogen after 2–51 mo of storage. Other seedborne xanthomonads survive even longer; for example, *X. phaseoli* can survive for 15 yr in French beans (11). Also, the fact that *X. manihotis* is seed-transmitted should not be surprising since many *Xanthomonas* spp., such as *X. malvacearum* (2), *X. oryzae* (6), *X. campestris* (17), and *X. phaseoli* (15) are seedborne.

The pathogen apparently affects the germination of infected seeds (Table 2). *X. manihotis* infection of the seed embryo varied from 0 to 40% and was between 40 and 60% in ungerminated seeds.

During the dry season (less than 50 mm of rain), embryo infection averaged 10%, but in June (more than 350 mm of rain), embryo infection went up to 40%, which could be the infection trend for most of the rainy season. During April and May (the beginning of the rainy season), embryo infection was low (Table 2), probably because the inoculum potential had not built up yet.

The bacterium was detected in seeds of susceptible, tolerant, and resistant varieties (always in embryonic tissues). Similarly, the pathogen was detected in enriched cultures of macerated pollen by immunofluorescence tests, but the location of *X. manihotis* cells on or in the pollen was not determined. Consequently, seed infection could occur by a pollen-to-ovule transfer.

The presence of *X. manihotis* in seed tissues and its low survival have implications for plant quarantine regulations, which are generally less strict for true seeds than for vegetative materials. For example, the inter-African phytosanitary regulations require a phytosanitary certificate for importing vegetative materials but do not restrict cassava seed importation (18).

Most cassava material is exchanged between international and national centers; care must therefore be taken to ship only seeds (10,11) from disease-free cassava plantations. These seeds must also be treated with appropriate fungicides before shipment.

Table 1. Sensitivity of enzyme-linked immunosorbent (ELISA) and immunofluorescence (IF) assays for detection of *Xanthomonas manihotis* in enriched[a] embryonic extracts

Washed cells from enrichment (no./ml)	ELISA reaction	IF reaction
10^9	++[b]	++
10^8	++	++
10^7	++	+
10^6	++	+
10^5	+	+
10^4	+	±
10^3	±	±
10^2	±	−
10^1	−	−

[a] Enriched broth medium: 10 g of glucose, 10 g of peptone, 1 g of cassamino acids, and 1,000 ml of distilled water.
[b] ++ = Strongly positive; + = positive, ± = weakly positive, considered negative; − = negative.

Table 2. Germination and embryo infection of true cassava seeds collected from cassava bacterial blight-infected and uninfected fields

Sampling date and weather		Seeds from infected fields[a]		Seeds from uninfected fields	
		Germination (%)	Embryo[b] infection (%)	Germination (%)	Embryo infection (%)
January	(dry)	...[c]	10	...	0
February	(dry)	...	3	...	0
March	(dry)	12	5	84	0
April	(rainy)	6	2	66	0
May	(rainy)	20	0	74	0
June	(rainy)	...	40	...	0
Average		13	10	70	0

[a] Data taken from an average of 24 seeds/sample.
[b] ELISA reactions were positive in March and April samples, negative in May samples. Percentage of embryo infection was obtained by the immunofluorescence method.
[c] Germination of seeds was under controlled conditions (28 C, 80% RH) on water-soaked paper towels. ... = no sampling.

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