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The Myth and Reality of Internal and External Seed Transmission of Cowpea Bacterial Blight *Xanthomonas axonopodis Pv. Vignicola* (Burkholder) Dye

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Abstract

Believe over the years were that when the pathogen was systemically transmitted to seed, it was termed internal seed-borne. Furthermore, delineating pathogens into internal or external seed borne was based on the experimental procedure to isolate pathogen from seed samples. Pathogen isolated by washing assay was termed external seed-borne while that isolated by seed destruction assay was termed internal seed-borne. Therefore, it was desirable to further investigate the part(s) of seed usually colonize by the pathogen *Xanthomonas axonopodis* pv. *vignicola (Xav*). In order to study the parts of seed where the pathogen usually colonize, three methods were used (seed washing and seed plating) were used to detect the external borne pathogens while the third (separation of seed into components and maceration) were used to detect the internally borne pathogen. The results showed that hilum was the most colonized part of the seeds. The embryo colonization was low and there was no *Xav* detected on cotyledons. The result of this work shows the external parts of seeds (hilum and seed coat) were the most infected/infested by *Xav*. Bacterial pathogens that stick to seeds during harvesting or post harvest do so through biofilm formation. This could be by firmbrial (flagella, pilli) and non fimbrial (production of extracellular polymeric substance) and could responsible for recalcitrant nature of seed-borne pathogens which might easily misunderstood as internal seed-borne

Keywords: Pathogen, Seed-Born, Isolation, External, Internal and Biofilm

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Introduction

Seeds are regarded as a highly effective means for transporting plant pathogens over a long distance. Numerous examples exist in agricultural literatures for the international spread of plant disease as a result of the importation of seeds that were infected or contaminated with pathogens (Agrawal and Sinclair, 1996). Since the 1900s, consumer demand for new plant products gave opportunity for many plant pathogens to disseminate to new areas on imported seeds. New markets for plant commodities encouraged plant breeders to begin collecting seed stocks from abroad. The birth of new seed companies extends their markets to new area (Elmer, 2011).The seed borne pathogen may result in loss of germination, discoloration and shriveling, development in plant diseases and toxin production in infected seeds (Fraedrick, 1996; Agrawal and Sinclair 1996; Elmer, 2011). Cowpea seed consists of three basic parts: (a) embryo, (b) storage tissue / cotyledons and (c) seed coat. The embryo has a reproductive function; being capable of initiating cellular division and growth. It is an axis that originates growth in two directions with objectives of originating a root and a shoot (Schaad *et al*., 2001). It consists of a radicle, plumule, two cotyledons, and hypocotyle. The episperm (seed cover) consists of two layers; the testa, hilum (scar or point of attachment of the seed to the funiculus), and microphyle (the point upon the seeds at which the orifice of the ovule through which the pollen tube enters). The establishment of a pathogen in any part of the seed described above is referred to as seed infection. The myths over the years were that when the pathogen was systemically transmitted to seed, it was termed internal seed-borne (Cafati and Seattler, 1980; Barak *et al*., 2002). Delineating pathogens internal or external seed borne was based on the experimental procedure to isolate pathogen from seed samples (Mortesen, 1997; Berg *et al*., 2005). Seed washing assay was predominantly used to isolate externally carried pathogen on the surface of the seed while seed destruction assay was used to isolate internally carried pathogen within internal parts of seed (Mortesen, 1997). The process of seed infection is influenced by the conditions under which the crop grows (Darrasse *et al*., 2010). Seed-borne inoculum is important in the survival and dissemination of plant pathogenic bacteria.

Most seed-borne bacteria survive as long as the seed remains viable (Cafati and Saettler, 1980). Seed transmission is the primary means for dissemination of the pathogen. Attributes of seed transmitted organisms are: ability to gain access to seed, ability to survive commercial processes such as harvest, cleaning, treatment and storage, ability to establish on emerging seedlings Primary or secondary transmission. The pathogen may thus, be carried with the seeds in three ways: (a) Admixture: pathogens are independent of seeds but accompany them. *Ergot sclerotia* are mixed with healthy seeds during threshing. (b) External: the pathogen may be present on seed surface. (c) Internal: pathogens establish within the seed with definite relationship with seed parts (Agrawal Sinclair, 1996; Elmer, 2011). Bacterial adherence to seeds is a process that strongly influences rhizosphere colonization. The role of adhesion in virulence was demonstrated for plant pathogens (Astua-Monge *et al*., 2005; Feil *et al*., 2007). Bacteria interact with plant tissues through adhesins; including polysaccharides and surface proteins, with initial contact often mediated by active mortality (Danhorn and Fugua, 2007). The bacterial surface structures important for adhesion cover a broad group of fimbrial and non fimbrial structures commonly known as adhesins (Barnhart and Chapman, 2006; Latasa *et al*., 2006). Attachment is required for biofilm formation (i e., aggregation of cells to each other after cells had attached to a surface). Biofilms are notoriously resistant to washing and other common antibacterial treatments (Ramey *et al*., 2004). The development of quarantine programs for seeds requires biological and ecological information about seed-borne pathogens, the ability to detect their presence, knowledge about the inoculum type and its location on seeds, are effective means for control strategies. Although it is understood that *Xav* is seed transmitted, and that seed-borne inoculums play a significant role in the disease epidemiology, the nature of seed transmission (location in and on seed) has not been fully elucidated. Therefore, it was desirable to further investigate the part(s) of seed usually colonize by the pathogen *Xanthomonas axonopodis* pv. *vignicola* to shed more light on internal and external seed-borne transmission of pathogens.

Materials and Methods

The occurrence of *Xav* on the surface of the seeds were determined by randomly selecting 400 cowpea seeds from each of the seed lots from nine cowpea producing states and three cowpea seed producing companies. These were put into a 250 ml flask containing sterile distilled water (SDW) and shake vigorously using electric shaker. After mechanical stirring for 5 minutes, the seed suspension was filtered through layers of Cheese-cloth. The filtrate was dispensed in two 50 ml centrifuge tubes and centrifuge at 12,000 g for 10 minutes (approx. 10,000 rpm) to pellet bacteria. Extract of 1.0 ml was introduced into a McCartney bottle containing 9 ml of SDW, from which a serial dilution of up to 10^{-5} were prepared. Ten micro-liter of the serial dilution were introduced in Petri- dishes containing 20 ml of Yeast Dextrose Carbonate Agar (YDCA) and spread using glass spreaders (Chiarappa, 1988; Mortensen, 1997). The treatments were incubated at 28 ^{0}C for 48 h. Colony enumeration from the incubated Petri dishes were done after 48 h. The experiment was laid out using a completely randomized design CRD with five replications. Colony forming units (cfu) were taken after 48-72 h. The trial was repeated twice.

Nine seed lots from nine states and three cowpea seed companies were collected as described previously, from which 400 seeds from each seed lots were washed with sterile distilled water. Afterwards, 10 seeds were plated on YDCA and replicated ten times with the hilum upward, and downwards, in an alternating manner (Opio *et al*. 1993). Seeds on which there was *Xav* growth were recorded as positive and those on which there was no *Xav* growth were recorded as negative. Colonies that hydrolyze starch on YDCA were assumed to be *Xav*. Colonies with typical characteristics of *Xav* were selected for pathogenicity testing as described in section 3.1.2. Data collected were analyzed statistically using analysis of variance (ANOVA) and means separated using NDMRT. The trial was repeated once.

Four hundred seeds from each seed lots were randomly selected and washed in a conical flask containing 250 ml of SDW for 5 minutes. The seeds were gently pressed using sterilized scalpel and pin to remove the seed coat, embryo

from the cotyledons and the helium. The cotyledons, embryo, hilum and the seed coats were separated and put into 5 % sodium hypochlorite solution for 5 minutes and rinsed with SDW (Youcef- Benkada *et al.*, 1994). Care was taken to avoid contaminations. After surface sterilization, the samples (seed coat, embryo, hilum and cotyledons) each were put into a conical flask containing 100 ml of SDW for 2 h; after which 1 ml of each of the samples were introduced into 3 McCartney bottles containing 9 ml of SDW, from which a serial dilution of up to 10^{-3} were made. 10 µl of the serial dilutions were introduced into the Petri-dishes containing 20 ml of YDCA and spread thoroughly using sterilized glass spreader. The treatments were incubated at 28 ^{0}C and colony enumeration was done after 48-72 h. The experiments were laid out using CRD with five replications. Data collected were analyzed statistically using analysis of variance (Anova), and means were separated using NDMRT. The trial was repeated once.

Results and Discussion

The population of *Xav* on seed samples collected from Kogi, Nassarawa and Niger States were the highest followed by Kaduna State and were not statistically different from samples collected from other states and the seed companies. The lowest *Xav* populations were recorded on samples collected from the seed companies though not statistically different from samples collected from other states except Niger, Nassarawa and Kogi. In planting the seeds hilum up (Table 2), however, there was no significance difference between *Xav* detected owing to low incidence on the part of the seed, while there was high significant difference in *Xav* detected on seeds plated hilum down. The seed samples collected from seed companies had lowest incidence of *Xav* (0.00- 0.01 %) though not statistically different from seeds samples collected from Borno, Gombe, Adamawa Sokoto, and Kebbi states. Seed samples collected from Kogi had the hightest incidence (0.12 %) and was statistically different from samples collected from Niger, Nassarawa, and Kaduna States. Table 3 shows relative distribution of *Xav* in internal parts of seeds. There was no significant difference in the population of *Xav* isolated from seed testa across the seed lots tested. Similar results were also recorded on seed hilum and embryo. There was no *Xav* detected on the cotyledons. The hilum, however, had the highest population of *Xav* (10^3) compared to testa and embryo which had 10^1 cfu.

The experiment was carried out on clean and healthy seeds (physical purity) used by the farmers for planting purposes. Bacteria are carried on seeds by contamination/infection. In this case the pathogen may be independent of seeds but accompany them. Walcott (2005) reported that seed contamination or infestation during threshing provide a possible explanation for the production of contaminated seed lots from virtually inspected fields and also may be the reason why seeds collected from the seeds companies were infected with *Xav* in this study. External contamination of seeds occurs via fruit as a consequence of contact of the seed with fruit tissue showing symptoms, or at harvest and threshing with residues carrying large bacterial populations (Maude, 1996). It is also important to note that saprophytic and antagonistic bacteria associated with bean seeds often interfere with the isolation of *Xav* on plating media such as YDCA. The number of colonies of *Xav* recoverable from various seed lots often depends upon the number of saprophytic and antagonistic bacteria present on the seeds, and this may partly account for the differential incidence and population of *Xav*. in various seed lots. Up to 50 % fewer colonies of *Xanthomonas campestris* were recovered when antagonistic bacteria were present in the seed washing (Randhawa and Schaad, 1984). Wettability and hydrophobicity also plays a key role in bacterial adhesion (Morais *et al.*, 2008). Hadas *et al*. (2001) paralleled this by reporting that population of *Erwinia carotovora* sub sp *carotovora* decline rapidly within six months in storage at 23 ^{o}C and after 8 months at 6 ^{o}C. Direct planting of infested seed results in transmission to seedling under ideal environmental condition (Walcotte, 2005). Gottwald *et al*. (2002) reported that exposed bacterium on contaminated surfaces perishes when the surface dry out.

This may account for the low *Xav* population on seed lots collected from Borno, Gombe, Adamawa, Sokoto, Kaduna, Kebbi and seed lots collected from seed companies where production took under hot and dry weather. But bacteria in biofilm are resistant to desiccation and washing (Fett and Cooke, 2003) and could also be the reason for differences in *Xav* incidence and population among the states. Bacterial pathogens that stick to seeds during harvesting or post harvest do so through biofilm formation. This could be by firmbrial (flagella, pilli) and non fimbrial (production of extracellular polymeric substance) (Wassenaar, 2009). The high percentage of *Xav* on the hilum indicated the suitability of the site for the pathogen. Contamination outside the seed's testa can produce healthy seedling, but the inoculums infects the soil and from there it can cause infections at more developed stages of the plant (Nome *et al.*, 2011). This is because hilum is a natural opening, through which the pathogen can hide (Nome *et al*. 2011). Hilum is also an intermediate between the internal pants (Microphyle) and the external parts (funiculus). The pathogen that could not enter the microphyl may remain in the hilum and feeds on the nutrient provided by the funiculus from the pod placenta (Cafati and Saettler, 1980). The invasion of hilum by pathogenic bacteria has been reported and constituted the main area of seed for the bacteria habitation (Darsonval *et al.*, 2009). Low population of *Xav* was detected on the seed embryo. The blight organisms may also enter the hilum via the vascular tissue of the pod suture (Cafati and Saettler, 1980). Embrayo can be infected only in the early stage of seed development after which it remain closed (inaccessible) until harvest (Wang and Maule, 1992). There is only a limited 'window' in the seeds developmental process during which bacteria can enter. Such a 'window' might easily differ with the physiological status of the host plant and could account in part for the commonly observed variation in incidence and population of *Xav* on the seeds samples (Wang and Maule, 1992).

When the seed germinate, if the embryo is infected, the pathogen initiates its growth together with the plant. Symptoms can show up during different stages of development (Nome *et al.*, 2011). The testa which is the seed cover consists of stony, leathery membranous layer. The nature of the surface influence the attachment of the pathogen to the surface while rough and fleshy surface attract adhesion, the stony and leathery surface was not suitable for attachment (Morais *et al*., 2008). Testas are formed by one or more layers of cells originated from the ovule integuments and

sometimes from the pericarp made from the walls of the ovary (Wang and Maule, 1992). The pathogen was not detected in the seed cotyledons. Phytopathogenic bacteria can only enter the cotyledons through breakage of the testa, establishing itself in the endopleura or the endosperm. This is because bacteria lack the capacity to penetrate the testa directly unlike the fungal pathogens which can penetrate through the testa as a result of the germination of the Teliospores or mycelium (Nome *et al*., 2011), and this may account for the non detection of *Xav* on the cotyledons. The recalcitrant nature of seed-borne pathogens was as a result of bacteria forming biofilms on the surfaces of seeds and often mistaken to mean internal infection. Biofilms are notoriously resistant to washing and other common antibacterial treatments (Ramey *et al*., 2004). Although, Opio *et al*. (1993) reported isolating *Xav* from diseased (damaged) cowpea seeds, there was no *Xav* isolated from cotyledons of seeds used in this work which was undamaged seed.

Conclusion and Recommendations

Seed borne inoculum is important in the survival an. dissemination of plant pathogenic bacteria. Believe over the years were that when the pathogen was systemically transmitted to seed, it was termed internal seed-borne. Furthermore, delineating pathogens into internal or external seed borne was based on the experimental procedure to isolate pathogen from seed samples. Pathogen isolated by washing assay was termed external seed-borne while that isolated by seed destruction assay was termed internal seed-borne. Most seed born bacteria survive as long as the seed remains viable. Three methods, seed plating. Seed washing and seed destruction assay were used. The study demonstrated that cowpea seeds used by farmers in cowpea producing areas in Nigeria were heavily infected by *Xav* both locally produced seeds and the seeds produced by the seed companies. There was low *Xav* incidence from seeds collected from Borno, Adamawa, Kebbi, Gombe and Sokoto. The highest *Xav* population was isolated from the hilum while no *Xav* was detected on the cotyledons. The reality is that, putting together, the result of this work shows the external parts of seeds were the most infected by *Xav* and cotyledon which form the internal parts of seeds were not infected by the pathogen.

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Tables

Table 1: Bacterial Population from Washing Assay (cfu) and Incidence of X*av* on Seed Surface in 2010 Seed Plating

Seed Source/Variety	**Bacterial population (cfu) in Washing assay**	**Incidence of *Xav* on seed plating (%)**	
		Hilum up	**Hilum down**
Borno (Bama)	0.33 x 10^2bc	0.00e	0.07f
Gombe (Akor)	0.30 x 10^2bc	0.00e	0.07f
Adamawa (Mubi)	0.45 x 10^2bc	0.00e	0.06g
Sokoto(Duchinkura)	0.47 x 10^2bc	0.00e	0.05h
Kaduna (Saminaka)	0.48 x 10^2bc	0.05c	0.12e
Kebbi (Jega)	0.45 x 10^2bc	0.00e	0.13d
Kogi (Idah)	1.83 x 10^2a	0.12a	0.29a
Nassarawa (Keffi)	0.50 x 10^2a	0.10b	0.18c
Niger (Mokwa)	1.67 x 10^2a	0.10b	0.25b
Alhari	0.20 x 10^2bc	0.01d	0.05h
Masalaha	0.30 x 10^2c	0.00e	0.05h
Premier	0.33 x 10^2c	0.00e	0.05h
S.E	0.25	0.33	0.42

Means with the same letter within a column are not significantly different at $p \leq 0.05$ level of significance using NDMRT test.

Table 2: Relative Distribution of *Xav* in Internal Parts of Seeds in 2010

Source/Variety	**Testa**	**Hilum**	**Embryo**
Borno (Bama)	1.0x10^1a	1.5x10^3a	0.3x10^1a
Gombe (Akor)	1.3x10^1a	2.0x10^3a	0.4x10^1a
Adamawa (Mubi)	1.1x10^1a	1.8x10^3a	0.3x10^1a
Sokoto (Duchin kura)	1.1x10^1a	1.7x10^3a	0.3x10^1a
Kaduna (Saminaka)	1.2x10^1a	2.0x10^3a	0.5x10^1a
Kebbi (Jega)	1.0x10^1a	2.2x10^3a	0.3x10^1a
Kogi (Idah)	1.4x10^1a	2.5x10^3a	0.5x10^1a
Nassarawa (Keffi)	1.3x10^1a	1.9x10^3a	0.5x10^1a
Niger (Mokwa)	1.3x10^1a	2.1x10^3a	0.5x10^1a
Alhari	1.0x10^1a	1.5x10^3a	0.5x10^1a
Masalaha	1.0x10^1a	1.4x10^3a	0.5x10^1a
Premier	1.0x10^1a	1.4x10^3a	0.4x10^1a
S.E	0.33	0.42	0.40

Figures with the same letter within a column are not significantly different at $p \leq 0.05$ level of significance using NDMRT test.