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RESEARCH ARTICLE

EVALUATION OF *XANTHOMONAS AXONOPODIS* PV. *MANIHOTIS* POPULATION IN INFECTED STEMS OF CASSAVA VARIETIES AND THE IMPACT ON NEW SPROUTS

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Abstract

A prerequisite for a healthy cassava plantation is the use of non-infected planting material. Therefore, the distribution of *Xanthomonas axonopodis* pv. *manihotis* in cassava stems of four genotypes was determined with the aim to develop recommendations for the selection of healthy stem material for planting to avoid cassava bacterial blight. *X. axonopodis* pv. *manihotis* was detected in stems of the susceptible varieties Ben86052 and Fétonégbodji, in a discontinuous colonization pattern and not restricted to any part of the stem, with higher numbers in the upper parts of the stem (10^7 cfu/g for Ben86052 and 10^6 cfu/g for Fétonégbodji), than in the middle and basal parts (10^3 to 10^4 cfu/g). Although 90-100% and 50-90% of cuttings of varieties Ben86052 and Fétonégbodji, respectively, harbored the pathogen, only 40-50% and 20-40%, respectively, of emerging sprouts were infected. From most of the cuttings in which the bacterium was not detected, healthy sprouts emerged. No bacterial blight symptoms occurred on the known relatively resistant genotypes TMS30572 and Ggazékouté in the field, and *X. axonopodis* pv. *manihotis* was not found in any part of the plants, nor did any of the new sprouts from the planted cuttings show disease symptoms. Thus, symptomless plants of the two genotypes can be considered free of bacteria and therefore can be recommended to farmers as suitable planting material to reduce disease incidence.

Introduction:-

Cassava is an important staple crop in the tropics, and Africa produces more cassava than the rest of the world (FAO, 1998), but it is affected by a wide range of virus, bacterial, fungal, and nematode diseases, among which cassava bacterial blight (CBB), caused by *Xanthomonas axonopodis* pv. *manihotis* (Vauterin et al., 1995), former *X. campestris* pv. *manihotis* (Bondar, 1915), is the second most important disease of cassava in Africa (Hillocks and Wydra, 2002). In Togo CBB was the second most important disease of cassava and was country wide distributed (Banito et al., 2007). Annual yield losses due to cassava bacterial blight in Africa were estimated up to 7.5 million tons (CIAT, 1996).

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Cassava is propagated vegetatively, and stem cuttings are used by farmers to establish a new plantation. Dissemination of CBB from one area to another and the carry-over of the pathogen from one growing season to the next are largely due to the use of infected planting materials (Lozano, 1986; Boher et al., 1996). Symptoms of cassava bacterial blight include angular leaf spots, blighting, wilting, vascular necrosis of the stem, production of exudates and dieback (Wydra et al., 2007). In a later stage of infection, the pathogen invades the plant systemically resulting in often symptomless stems where it can survive for over one year (Lozano and Laberry, 1982; Boher and Verdier, 1994). As part of an integrated control of cassava bacterial blight careful selection of CBB-free planting material is important (Lozano, 1986; Pacumbaba, 1987). For instance, after use of control measures including careful selection of planting material from only the most lignified – basal – portion of the stem, CBB severity was reduced, and cassava production in Cuba increased from 7-8 t/ha to 20 t/ha (Cock, 1985). Recently, negative correlations were found between some CBB symptom types and the cassava root yield (Wydra et al., 2007). The basal stem part was suggested to be the most resistant to cassava bacterial blight, because of lignifications and high accumulation of pectin and cellulose (Cock, 1985). During the systemic infection of the vascular tissue, barriers such as gels and tyloses block the xylem vessels and reduce water movement in the xylem, and antimicrobial compounds accumulate to inhibitory concentrations in the infected vessels. Differences in these structural features, physiological activities, and morphological modifications between resistant and susceptible cultivars were observed, and dead *X. axonopodis* pv. *manihotis* cells were found close to tyloses in tissues of infected, CBB-resistant cassava plants (Kpémoua et al., 1996). Bactericidal activity of phenolics in *Xanthomonas*-infected plants such as cotton, rice, and cabbage was reported (Jalali et al., 1976; Reimers and Leach, 1991; Nmasivayam et al., 1971).

However, *X. axonopodis* pv. *manihotis* was found to invade the cassava stem down to the basal part above ground level also in resistant and intermediate-resistant genotypes after inoculation of leaves (Lozano and Laberry, 1982; Fanou, 1999). Pruning most of the above ground portion of infected plants or cutting off diseased leaves to delay spread of the disease and secondary infections was reported to reduce CBB severity. Also heat-treated plantlets derived from meristem cultures were reported as a successful means of producing bacteria-free cuttings for propagation (Lozano, 1986; Fanou, 1999).

The distribution of *X. axonopodis* pv. *manihotis* in infected stems of field plants was reported for some varieties (Fanou, 1999), but never established in detail for varieties frequently grown in Togo. Also the incidence of infected sprouts deriving from infected cuttings has not been studied. To develop sanitation measures in areas with a high pressure of cassava bacterial blight, the role of infected cuttings in disease dissemination has to be known. Therefore, the aim of the present study was to determine (i) the distribution of *X. axonopodis* pv. *manihotis* in different parts of stems of cassava varieties from Togo, and (ii) the incidence of infected sprouts, in order to develop recommendations for the selection of stem cuttings.

Materials and Methods:-

Cuttings of the local, susceptible and resistant varieties Fétonégbodji and Gbazékouté and the improved highly susceptible and resistant varieties Ben86052 and TMS30572, respectively, (Boher and Agbobli, 1992; Banito et al., 2008) were planted in the field in the forest savanna transition zone at the Institut Togolais de Recherche Agronomique (ITRA) station, Lomé, Togo. One month old plants were inoculated three times with a bacterial suspension of 10^7 cfu/ml of a 48-hour old culture of a virulent *X. axonopodis* pv. *manihotis* strain from Togo (X27 received from ITRA) at intervals of three weeks. Ten stems per variety from 14 months old plants of the inoculated once were sampled at random in the field and CBB symptoms described on each selected plant. Detection followed the method described by Fanou (1999) with few modifications. The plants were divided into upper, middle and basal part, and surface-disinfected with 70% ethanol. For each part, a cutting of 40 cm length was used and 10 cm of both sides were cut off, weighed, cut into small pieces, crushed using a mixer blender and suspended in 0.01 M $MgSO_4$ for one hour. The suspension was filtered through cheesecloth and centrifuged for 20 min at 4,000 x g. The pellet was suspended in 5 ml of sterile 0.01 M $MgSO_4$ and serial dilutions were performed. Fifty µl of each dilution were plated on GYCA (glucose 5 g/l, yeast 5 g/l, CaCO3 10 g/l, agar 15 g/l) (Dye, 1962) medium supplemented with Cycloheximide (Sigma, Germany) (250 mg/l GYCA medium) and incubated at 30 °C. After 48 to 72 hours, *X. axonopodis* pv. *manihotis* colonies were counted.

To check symptom development on sprouts, cassava stem cuttings of 20 cm length from the 40 cm sample of each stem part were planted at a spacing of 0.5 m x 0.5 m on well prepared flat ground in the field. Weeding and watering were applied when necessary. Evaluation of CBB symptom development started five days after planting and was followed up every five days up to 40 days.

Results:-

X. axonopodis pv. *manihotis* was detected in stem cuttings of the local, improved variety Ben86052 from Benin and the local, susceptible variety Fétonégbodji from Togo (Table 2), while no bacteria were found in stems of the improved variety TMS30572, and the local variety Gbazékouté from Togo, which also did not show CBB symptoms on the selected plants in the field.

All the plants of the susceptible variety Ben86052 selected for *X. axonopodis* pv. *manihotis* detection showed CBB symptoms in the field, but no dieback. Exudates were observed on the tips of four plants and five plants showed wilt symptoms, while three plants showed no systemic symptoms (Table 1). Ninety percent of the cuttings from the upper and basal parts, and all the cuttings from the middle part were infected with average bacteria numbers of 10^7, $1.9*10^4$ and $4.3*10^3$, respectively (Table 2). The average bacterial number was significantly higher in the upper part than in the middle and basal parts ($p = 0.02$). Infected sprouts developed from 40-50% of the cuttings planted. Also sprouts derived from cuttings with low numbers of bacteria or no detection of bacteria developed symptoms, while cuttings with high bacteria numbers did not always develop infected sprouts.

Seven plants of the variety Fétonégbodji selected for *X. axonopodis* pv. *manihotis* detection showed dieback in the field, exudates were observed on six plants. Only one plant did not show systemic symptoms (Table 1). Ninety percent of the cuttings from the upper, 70% from the middle and 50% from the basal part were infected with up to 6.1×10^6 cfu/g, 4.1×10^3 cfu/g, and 2.3×10^3 cfu/g, respectively (Table 2). Differences in the average bacterial numbers between stems parts were not significant ($p = 0.1$). *X. axonopodis* pv. *manihotis* was found in all 3 parts of the stem in four, and not continuously detected in six plants, one of them being the plant without systemic symptoms. Also from a cutting without bacterial detection, a wilted sprout developed. Infected sprouts emerged from 20% of cuttings from the basal and upper parts, and from 40% of the middle part, although 50, 90 and 70% of the cuttings, respectively, harboured *X. axonopodis* pv. *manihotis* (Table 2).

Stems of variety Ben86052 harboured higher average bacterial numbers in all three parts than variety Fétonégbodji. In both varieties, the bacterial concentration decreased from the upper to the basal part of the stems, though not significantly in variety Fétonégbodji, with higher numbers in the upper part of Ben86052, but similar numbers in basal parts of both varieties. An average of 43% of sprouts including the basal, middle and upper parts from Ben86052 and 27% from Fétonégbodji were infected. Symptoms on sprouts occurred generally earlier in variety Fétonégbodji than on sprouts of Ben86052 (Table 2).

Discussion:-

Cassava bacterial blight is an important and world-wide occurring disease of cassava that is subjected to international phytosanitary quarantine. Infected cassava stems are largely responsible for the carry-over of *X. axonopodis* pv. *manihotis* from one growing season to the next, and for dissemination to different areas. Therefore, recommendations to farmers on the choice of suitable planting material are necessary. The study was designed to investigate the colonization and distribution of *X. axonopodis* pv. *manihotis* in different parts of the stem of infected cassava plants of two locally important cassava varieties, Gbazékouté and Fétonégbodji, compared to the standard susceptible and resistant varieties Ben86052 and TMS30572, respectively.

The basal, middle and upper stem parts of the varieties Ben86052 and Fétonégbodji were colonized by *X. axonopodis* pv. *manihotis*, continuously or discontinuously, with more bacteria-free cuttings in variety Fétonégbodji. Also Fanou (1999) and Daniel and Boher (1985) found partly discontinuous colonization of cassava plant stems. *X. axonopodis* pv. *manihotis* invaded cassava stems downwards until five centimeters above ground level on resistant and intermediate-resistant genotypes after leaf-inoculation (Lozano and Laberry, 1982). But, these authors did not relate the bacterial number in stems to the symptoms on the plant in the field and to latent infection, nor was the relation of infected stems to infected sprouts studied. In both susceptible varieties, the average *X. axonopodis* pv. *manihotis* concentration was by a factor of 10^3 higher in the upper part than in the middle and basal parts. Although some plants of the susceptible varieties had only shown leaf symptoms when sampling, bacteria were generally found in all parts of the stems, indicating that the pathogen invaded the stem from the infected leaves, but stayed in a latent phase in stems. Nevertheless, infected sprouts emerged from stem parts in which no bacteria were detected. Bacteria still existing inside stems in low numbers might not have been detected due to methodological limits in sampling and bacterial detection, since the parts used for planting could not be used for bacterial detection, but only small portions on the ends of the planted cuttings were tested for bacteria, and low bacterial numbers may not

always be detected by the agar plating method. Daniel and Boher (1981) reported that the classical techniques of isolation on agar may fail to detect low levels of pathogen populations in seeds. Nevertheless, isolation is still considered as among the most sensitive method for detection of bacteria.

X. axonopodis pv. *manihotis* was not found in the CBB-resistant genotype TMS30572 and the local genotype Gbazékouté, which also had not shown symptoms on mother plants of cuttings in spite of several inoculations, nor on emerging sprouts. The variety Gbazékouté was among the highly resistant varieties, whereas Fétonégbodji was susceptible after stem-inoculation with four highly virulent *X. axonopodis* pv. *manihotis* strains from different African origins (Banito et al., 2010). Nevertheless, symptoms had been observed in previous field trials in all the tested genotypes, and for the 4 genotypes, the following order of CBB severity calculated as area under severity index progress curve (AUSiPC) was established: Gbazékouté > Ben86052 > TMS30572 > Fétonégbodji (unpublished data). Among them, Gbazékouté showed resistance to CBB after stem-inoculation (Banito et al., 2010), but was susceptible under field conditions. Also Fanou (1999) detected no *X. axonopodis* pv. *manihotis* in stems of variety TMS30572 deriving from symptomless stems, but only in stems of plants showing CBB symptoms. Zinsou (2001) found lower *X. axonopodis* pv. *manihotis* numbers in stems and leaves of some plants of the resistant variety TMS30572 than in the susceptible Ben86052 after leaf-inoculation under greenhouse conditions, and after leaf-infiltration of low inoculum concentrations, no CBB symptoms occurred on variety TMS30572.

Multiple resistance factors are induced in resistant plants after inoculation (Nicholson and Hammerschmidt, 1992), and defence mechanisms in CBB-resistant cassava plants were demonstrated (Kpémoua et al., 1996). Cassava cultivars may vary in their resistance to *X. axonopodis* pv. *manihotis* due to toxin concentrations (Perreaux et al., 1985), some of which were found to be too low in susceptible cultivars (Cooper et al., 1995). In few cases bacteria were not detected although exudates had been observed on stems of susceptible genotypes. This might be due to the discontinuous distribution of *X. axonopodis* pv. *manihotis* in stems and the destructive sampling method which did not allow to detect *X. axonopodis* pv. *manihotis* in the whole stem.

Surprisingly, 50-80% of the planted cuttings from the stem parts of Ben86052 and Fétonégbodji did not develop CBB symptoms on the new sprouts, although the stems harboured the pathogen. Although sprout infection was less in Fétonégbodji, sprout symptoms generally developed faster than in Ben86052. These observations could be generally due to the discontinuity of the colonization of *X. axonopodis* pv. *manihotis* or to differences in vascular connections between the xylem of new sprouts and the one of the old cuttings. New shoots generate a new system of xylem apart from the old vessels of the mother stem. Most of the new xylem was found to be unconnected to the old xylem as demonstrated by dye uptake and standard light microscopy. Additionally, old xylem was heavily occluded by tylosis. Thus, *X. axonopodis* pv. *manihotis* is unlikely to cross easily between old and new xylem because living parenchyma cells separate them. However, occasionally a connection was found, and *X. axonopodis* pv. *manihotis* could cross over from the xylem of the old stem to the new shoots, but this seems to be an infrequent event (R. Cooper, University of Bath UK, unpublished data) and may depend on storage conditions of the cuttings and environmental conditions after planting (K. Wydra, University of Hannover, Germany, personal communication).

Since the pathogen was not found in any part of the 10 tested plants of varieties TMS30572 and Gbazékouté, nor did any of the new shoots from the planted cuttings show CBB symptoms, symptomless plants of both cultivars could be considered free of *X. axonopodis* pv. *manihotis*. The stems of these two genotypes could be infested by the bacteria but the systemic infection of the vascular tissue did not occurred due to the plant barriers such as structural features, physiological activities, and morphological modifications (Reimers and Leach, 1991; Kpémoua et al., 1996). But, in case of field plants with symptoms, also these varieties may harbour the pathogen in their cuttings as reported by Fanou (1999). Therefore, a careful selection of cuttings from plants of resistant varieties without symptoms is recommended to farmers to receive CBB-free cassava planting material. A latent infection of symptomless cuttings of any stem part has always to be considered when cuttings come from an infected field. Therefore, also the basal part of the stems should not, contrary to recommendations of Cock (1985), be used to receive healthy planting material. Further on, breeders should consider differences between varieties in restriction of systemic infection, latent infection of stems and restriction of sprout symptoms as additional characteristics in selection of varieties for resistance.

Table 1:- Status of cassava stems collected from the field for *X. axonopodis* pv. *manihotis*

Ben86052						
	Stems	spot	blight	wilt	exudates	dieback
	1	+	+	-	-	-
	2	-	+	-	-	-
	3	-	+	-	-	-
	4	+	+	+	-	-
	5	-	+	+	+	-
	6	+	-	-	+	-
	7	+	+	+	+	-
	8	+	+	+	-	-
	9	+	-	-	+	-
	10	+	+	+	-	-
Fétonégbodji						
	Stems	spot	blight	wilt	exudates	dieback
	1	+	+	-	-	-
	2	-	+	+	+	+
	3	+	+	-	+	+
	4	+	-	+	-	+
	5	-	-	+	+	-
	6	+	+	-	-	+
	7	+	+	-	-	+
	8	-	+	-	+	+
	9	+	-	+	+	-
	10	-	+	-	+	+

+ = presence of CBB symptoms; - = absence of CBB symptoms

Table 2:- Detection of *X. axonopodis* pv. *manihotis* in stems of 14-month old cassava plants and the symptoms on new sprouts

Ben86052			
Plant parts	Mean cfu/g	Infected stems (%)	Infected sprouts (%)
Basal	$4.3*10^3$b	90	40
Middle	$1.9*10^4$b	100	40
Upper	$1*10^7$a[1]	90	50
Fétonégbodji			
Plant parts	Mean cfu/g	Infected stems (%)	Infected sprouts (%)
Basal	$8.9*10^2$a	50	20
Middle	$1.4*10^3$a	70	40
Upper	$1.4*10^6$a	90	20

[1]significant differences in means of cfu/g between stem parts at $p < 0.05$.

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