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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

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Full Length Research Paper

Crude protein electrophoresis of *Ludwigia* (L.) species in Nigeria and its taxonomic implications

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Leaves of nine species of *Ludwigia* were collected from Southwestern Nigeria and the crude proteins were extracted and analysed by electrophoretic fractionation. The results shows that *Ludwigia octovalvis* var *linearis*, *Ludwigia octovalvis* var *brevisepala*, *Ludwigia hyssopifolia* and *Ludwigia abyssinica* are more closely related based on the position of protein bands. Also, *Ludwigia decurrens* A and *Ludwigia adscendens* subsp. *diffusa* are more closely related based on the number of band and the position of band. The band at 2.1 is taxonomic for all the species of *Ludwigia* studied. Similarly, the band at 1.0 delimits *L. leptocarpa* from the other species. Protein abundance sequence of the samples is in the order *L. hyssopifolia* > *L. octovalvis* var *linearis* > *L. abyssinica* > *L. octovalvis* var *brevisepala* > *L. erecta* > *L. decurrens* B > *L. leptocarpa* > *L. decurrens* A > *L. adscendens* subsp. *diffusa*. The occurrence of a new band in *Ludwigia decurrence* B separates it from *Ludwigia decurrence* A, a new hypothetical name is suggested for the delimitation of the two species. The protein bands are taxonomically distinct as no two species have the same band distribution; diagnostic bands which could be employed for the identification of each species are also reported.

Key words: *Ludwigia,* protein bands distribution, taxonomic implication, Nigeria.

INTRODUCTION

Ludwigia species have been classified among the 200 most aggressive world plant invaders (Cronk and Fuller, 1995). This is a genus of considerable economic importance; *Ludwigia octovalvis* as a traditional herbal medicine, has been used to treat gastrointestinal disorders such as diarrhoea and dysentery, a poultice of an entire plant is externally applied to heal dermatitis, boil, ulcer, impetigo and pimple (Kadum et al., 2012). The leaves of *L. abyssinica* A. Rich. are edible and used for dyeing of straw and fibres, and it is used in medicine applied for enhancing memory. The leaves of *L. erecta* (L.) H. Hara are edible as vegetables and used for treating fevers. *Ludwigia hyssopifolia* (G. Don) Exell is valuable as green manure and the leaves can be used to treat wounds (Kadiri and Olowokudejo, 2010).

Fluctuations in the taxonomic classifications of

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Table 1. A list of *Ludwigia* species used for the study and their locations.

Species	Location	Coordinates
L. abyssinica A. Rich.	Conference Centre, O.A.U, Ile-Ife	N 07° 31.424, E 004° 31.836, 267 m
L. abyssinica A. Rich.	Infinite Grace, O.A.U, Ile- Ife	N 07° 31.613, E 004° 31.898, 272 m
L. abyssinica A. Rich.	Along Anatomy Dept. O.A.U,Ile-Ife	N 07° 31.097, E 004° 32.546, 284 m
L. adscendens subsp. *diffusa* (Forssk)	Oke-Ooye, Ilesa	N 07° 37.068, E 004° 44.756, 382 m
L. decurrens variety A Walter	Along Botany Dept., O.A.U, Ile-Ife	N 07° 31.251, E 004° 31.594, 532 m
L. decurrens variety A Walter	Along Botany Dept., O.A.U, Ile-Ife	N 07° 31.251, E 004° 31.594, 620 m
L. decurrens variety A Walter	Along Anatomy Dept.,O.A.U, Ile-Ife	N 07° 28.261, E 004° 31.594, 542 m
L. decurrens variety B Walter	Conference Centre, O.A.U, Ile-Ife	N 07° 31.430, E 004° 32.836, 269 m
L. decurrens variety B Walter	O.A.U Ile-Ife Teaching Hospital	N 07° 31.412, E 004° 34.483, 264 m
L. erecta (Linn.) Hara	Oke-Ooye, Ilesa	N 07° 37.068, E 004° 44.767, 379 m
L. erecta (Linn.) Hara	Iloko, Ijesha	N 07° 38.924, E 004° 48.965, 386 m
L. erecta (Linn.) Hara	O.A.U Ile-Ife Teaching Hospital	N 07° 31.412, E 004° 34.493, 244 m
L. erecta (Linn.) Hara	Behind GT Bank O.A.U, Ile-Ife	N 07° 31.212, E 004° 31.493, 244 m
L. hyssopifolia (G.Don) Exell	Oke-Ooye, Ilesa	N 07° 37.068, E 004° 44.767, 379 m
L. hyssopifolia (G.Don) Exell	O.A.U Ile-Ife Teaching Hospital	N 07° 31.412, E 004° 34.483, 264 m
L. hyssopifolia (G.Don) Exell	Along Anatomy Dept. O.A.U,Ile-Ife	N 07° 28.261, E 004° 31.594, 542 m
L. leptocarpa (Nutt.)	Along Road 7 Area, O.A.U, Ile-Ife	N 07° 30.787, E 004° 32.924, 261 m
L. leptocarpa (Nutt.)	Along Road 7 Area, O.A.U, Ile-Ife	N 07° 30.787, E, 004° 32.924 255 m
L. leptocarpa (Nutt.)	Along Road 7 Area, O.A.U, Ile-Ife	N 07° 30.787, E 004° 32.924, 243 m
L. octovalvis variety *brevisepala* (Jacq.) Raven	Along Anatomy Dept.,O.A.U, Ile-Ife	N 07° 31.097, E 004° 31.546, 280 m
L. octovalvis variety *brevisepala* (Jacq.) Raven	Infinite Grace, O.A.U, Ile- Ife	N 07° 31.613, E 004° 31.897, 271 m
L. octovalvis variety *brevisepala* (Jacq.) Raven	O.A.U Ile-Ife Teaching Hospital	N 07° 31.417, E 004° 34.483, 269 m
L. octovalvis variety *linearis* Raven	Along Botany Dept., O.A.U, Ile-Ife	N 07° 31.251, E 004° 31.594, 620 m
L. octovalvis variety *linearis* Raven	Oke-Ooye, Ilesa	N 07° 37.381, E 004° 47.224, 392 m
L. octovalvis variety *linearis* Raven	Ede Road, Ile-Ife	N 07° 101.139, E 004° 44.83, 377 m

Ludwigia species have been reported (Dutartre et al., 2004). These have been attributed to their phenotypic plasticity; in other words, their growth forms vary under different environmental conditions which often complicate species identification. Most classification works on the genus has been hinged on evidences from leaf morphology, palynology, and flower and seed morphology. Similarly, Folorunso et al. (2014) reported the use of foliar and stem anatomical characters in the identification of *Ludwigia* species in Nigeria.

Electrophoretic techniques for identification and classification have become a very important tool in systematic research. This technique is particularly of taxonomic importance in separating varieties of plants (Folorunso et al., 2012) and also a useful tool in studies of genetic variability in plants (Oladipo et al., 2008). Electrophoretic technique has been employed on a number of plant groups to show that many isoenzymes or polymorphic proteins are widely distributed in higher plants and also to compare protein distribution of the wild relative of plants to the cultivated ones (Folorunso et al., 2012; Oladipo and Illoh, 2012).

This study therefore compares the electrophoretic pattern of protein bands distribution in the *Ludwigia* species with the aim of providing useful information on classification and identification of *Ludwigia* species and identifies both intraspecific and interspecific variations that exist among them and the highest protein richness in them.

MATERIALS AND METHODS

Fresh leaves of nine (9) species of *Ludwigia* common to Nigeria in the family Onagraceae were collected from the Southwestern Nigeria (Table 1). The altitudes and geographical coordinates of the localities were taken using a GPS device. Species identification and confirmation was done with the assistance of the curators at the Herbarium of Obafemi Awolowo University Campus (IFE) and Forestry Research Institute of Nigeria Herbarium (FHI), Ibadan. The reagents used include acrylamide, monobasic sodium phosphate, dibasic sodium phosphate, tetramethylenediamine (TEMED), sodium dodecyl sulphate (SDS), ammonium persulphate (APS), bromophenol blue, 2-mercaptoethanol, glycerol, distilled water, Coomasic brilliant blue, methanol, glacial acetic acid.

Protein extraction was carried out by homogenizing 0.5 g of the species leaf sample in a porcelain mortar in 5 ml of 0.85% NaCl. The leaves extracts were centrifuged for 15 min at 3,000 revolutions per minute. The supernatant was removed after the precipitate had settled down and poured in sampling bottles, this

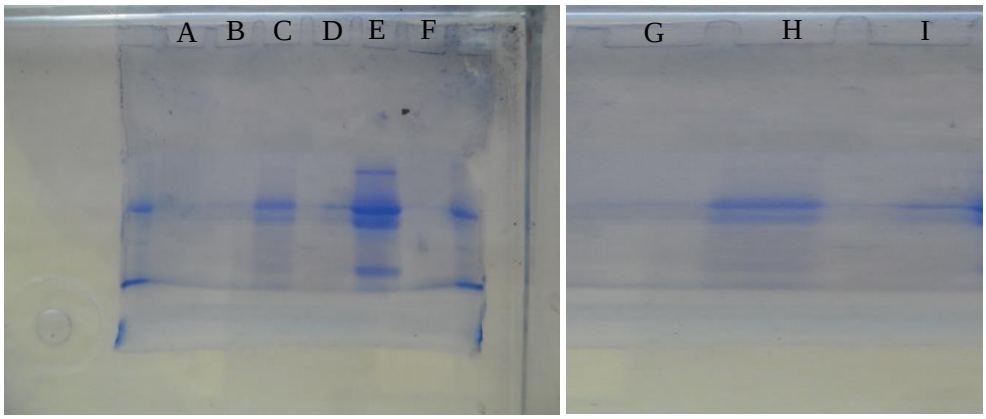

Figure 1. Pattern of protein distribution in the leaves of the *Ludwigia* species. A = *Ludwigia decurrence* A, B = *Ludwigia decurrence* B, C = *Ludwigia octovalvis* var *linearis,* D = *Ludwigia octovalvis* var *brevisepala,* E = *Ludwigia hysopifolia*, F = *Ludwigia leptocarpa,* G = *Ludwigia adscendens* subsp. *diffusa,* H = *Ludwigia erecta,* I = *Ludwigia abyssinica*.

was kept in the refrigerator till use.

Four to six (4 – 6) drops of the sample buffer was added to 10 – 12 drops of the protein sample, 0.2% bromophenol blue was added, and the sample was heated in a boiling water bath for 5 min. After boiling, the samples were introduced into each gel tube. 10% 2-mercaptoethanol was added to the sample to reduce the disulphide bonds in the protein so as to expose the protein to SDS action. The 5% SDS in an anionic detergent that distrupts nearly all non-covalent interactions in native proteins, thus the SDS forms a rod-like material with proteins that gives them uniform movement along the electrophoretic column according to their molecular weights. The glycerol added makes the protein sample denser so that it does not mix within the running buffer. The buffers are employed to maintain polyionic character of protein. Bromophenol blue was added to act as a tracer. The level of similarity of protein profile of the species was used to construct the dendrogram showing the coefficient of similarity.

RESULTS

The pattern of protein distribution in the species of *Ludwigia* studied and their diagrammatic representation are as shown in Figures 1 and 2, respectively. A keen examination of the bands shows that there are different patterns of band distribution in the genus. Marked differences were recorded for number, combination of bands and intensity of bands between species. The relationship between all the species of *Ludwigia* on the basis of band distribution is as shown in Table 2. The bands range from one to four (Figure 2). Most of the bands were found to be fast moving bands (2.0 to 3.5 cm), followed by the intermediate moving bands (1.0 to 1.9 cm), and slow moving bands (0.0 to 0.9 cm), respectively. Table 3 shows the common band relationship between all the *Ludwigia* species studied. *L. octovalvis* var *linearis* and *Ludwigia hysopifolia* have similar bands with the same intensity at 1.6 cm; *L. octovalvis* var *linearis*, *Ludwigia hysopifolia* and *Ludwigia abysssinica* have similar bands at 0.5 cm but with varying intensity.

There are diagnostic bands peculiar to each of the species which makes individuals different from one another: *Ludwigia decurrence* B shows band at 0.7 cm, *L. octovalvis* var *linearis* at 0.8 cm, *Ludwigia leptocarpa* at 1.0 cm, *Ludwigia octovalvis* var *brevisepala* at 1.8 cm, *Ludwigia abysssinica* at 2.5 cm, *Ludwigia erecta*, at 2.8 cm and *Ludwigia hysopifolia* at 3.0 cm. The band at 2.1 cm was found to be common to all the species and occur at different intensities. Inter specific bands were observed between pairs of species in the genus. *L. octovalvis* var *linearis* and *Ludwigia hysopifolia* were found to have the highest number of bands (four), followed by *Ludwigia octovalvis* var *brevisepala* and *Ludwigia abysssinica* with three bands. *Ludwigia decurrence* B, *Ludwigia leptocarpa* and *Ludwigia erecta* have two bands while *Ludwigia decurrence* A and *Ludwigia adscendens* have one band (Figure 2).

The dendrogram depicts the similarity coefficient for the *Ludwigia* species based on protein banding patterns (Figure 3). Three major clusters were formed, the first cluster comprises of *L. hyssopifolia*, *L. decurrence* A, *L. adscendens* and *L. erecta* at 1.0 similarity level. The second cluster comprises *L. decurrence* B and *L. abyssinica* at 0.85 similarity level. These two clusters are similar at 0.7 similarity level. The third cluster comprises of *L. leptocarpa* and *L. octovalvis brevisepala*, they are similar to the first two clusters at 0.45 similarity level. *L.octovalvis linearis* is delimited from the other species of *Ludwigia* studied at -0.4 similarity level similarity level. An artificial key for the identification of the *Ludwigia* species studied based on their band relationships is as shown in Table 4.

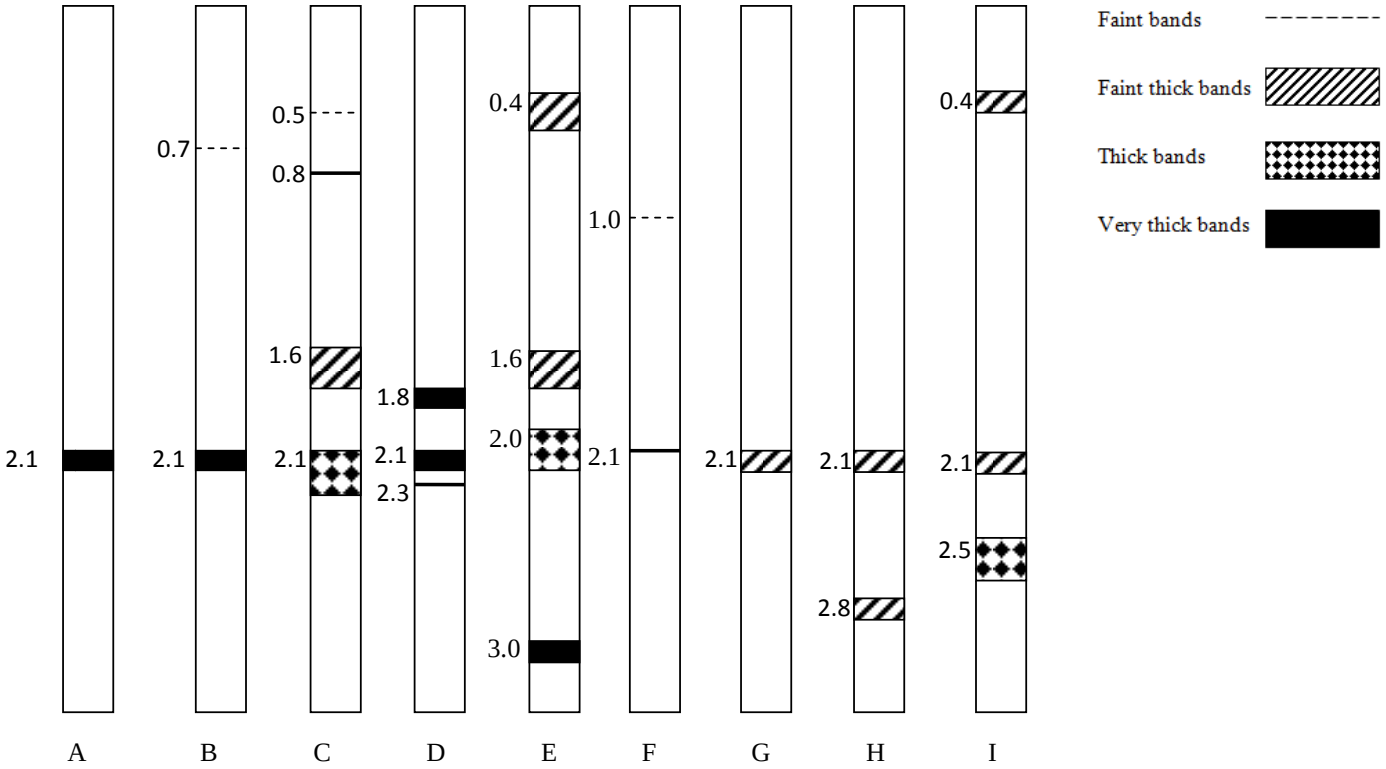

Figure 2. Diagramatic explanation of protein – bands of extrcated protein in polyacrylamide-bisacrylamide gel. A = *Ludwigia decurrence* A, B = *Ludwigia decurrence* B, C = *Ludwigia octovalvis* var *linearis,* D = *Ludwigia octovalvis* var *brevisepala,* E = *Ludwigia hysopifolia*, F = *Ludwigia leptocarpa,* G = *Ludwigia adscendens* subsp. *diffusa,* H = *Ludwigia erecta,* I = *Ludwigia abyssinica*.

Table 2. The relationship between all the *Ludwigia* species studied on the basis of band distribution.

Name of Species		Total number of bands	Higher Band	Intermediate Band	Lower Band
			2.0 - 3.5	1.0 - 1.9	0 - 0.9
A	*Ludwigia decurrence* A	1	1	-	-
B	*Ludwigia decurrence* B	2	1	-	1
C	*Ludwigia octovalvis* var *linearis*	4	1	1	2
D	*Ludwigia octovalvis* var *brevisepala*	3	2	1	-
E	*Ludwigia hysopifolia*	4	2	1	1
F	*Ludwigia leptocarpa*	2	1	1	-
G	*Ludwigia adscendens*	1	1	-	-
H	*Ludwigia erecta*	2	2	-	-
I	*Ludwigia abysssinica*	3	2	-	1
	Total	22	13	4	5

DISCUSSION

The result shows that no two species of *Ludwigia* have the same number and intensity of protein band. This affirms the morphological identification of the species studied, that is, the protein banding pattern is a reflection of their morphological characteristics. Protein variation in the species of *Ludwigia* studied is an indication of protein polymorphism; this depicts the genetic divergence in them and at the same time forms the basis of the

Table 3. Common band relationships between all the *Ludwigia* species studied.

Species	***Ludwigia decurrence* A**	***Ludwigia decurrenc* B**	***Ludwigia octovalvis* var *linearis***	***Ludwigia octovalvis* var *brevisepala***	***Ludwigia hysopifolia***	***Ludwigia leptocarpa***	***Ludwigia adscendens***	***Ludwigia erecta***	***Ludwigia abysssinica***
Ludwigia decurrence A	-								
Ludwigia decurrence B	1	-							
Ludwigia var *octovalvis linearis*	1	1	-						
Ludwigia var *octovalvis brevisepala*	1	1	2	-					
Ludwigia hysopifolia	1	1	3	1	-				
Ludwigia leptocarpa	1	1	1	1	1	-			
Ludwigia adscendens	1	1	1	1	1	1	-		
Ludwigia erecta	1	1	1	1	1	1	1	-	
Ludwigia abysssinica	1	1	2	1	2	1	1	1	-

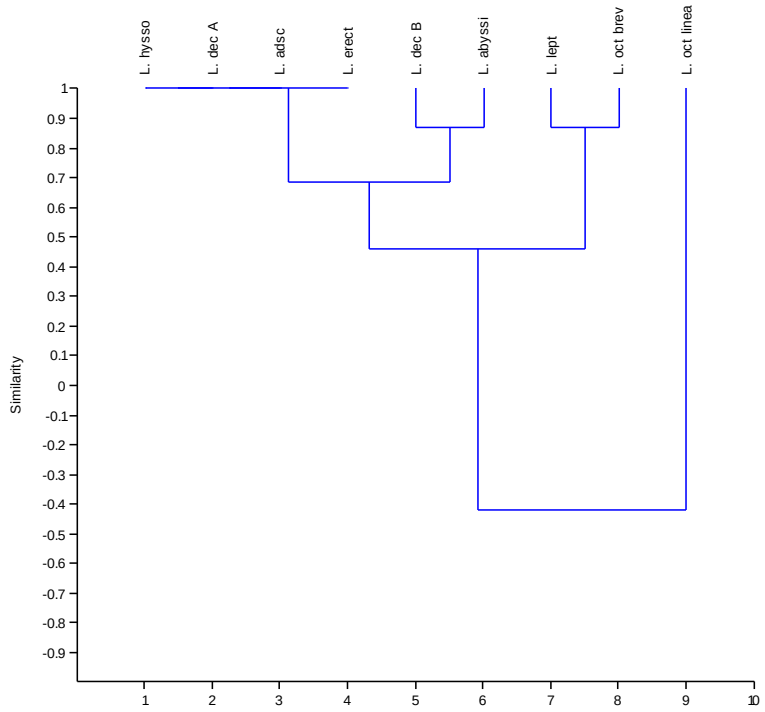

Figure 3. Single linkage cluster analysis (SCLA) dendrogram of relative mobility (Rm) values for leaf protein in the species of *Ludwigia* studied.

Table 4. An artificial key for the *Ludwigia* species studied based on their band relationships.

Band relationships	***Ludwigia* species**
Ludwigia species with more than two bands in their gel	*L. octovalvis* var *linearis*, *L. octovalvis* var *brevisepala*, *L. hyssopifolia* and *L. abyssinica*
Thick band present at 0.4 cm	*L. hyssopifolia* and *L. abyssinica*
Total number of bands in gel is four	*L. hyssopifolia*
Total number of bands in gel is three	*L. abyssinica*
Thick band absent at 0.4 cm	*L. octovalvis* var *linearis* and *L. octovalvis* var *brevisepala*
Bands present at 0.5 and 0.8 cm	*L. octovalvis* var *linearis*
Bands absent at 0.5 and 0.8 cm	*L. octovalvis* var *brevisepala*
Ludwigia species with two bands or less in their gel	*L. decurrens* A, *L. decurrens* B, *L. leptocarpa*, *L. adscendens* subsp. *diffusa* and *L. erecta*
Total number of band in gel is one	*L. decurrens* A and *L. adscendens* subsp. *diffusa*
Very thick band present	*L. decurrens* A
Very thick band absent	*L. adscendens* subsp. *diffusa*
Total number of band in gel is two	*L. decurrens* B, *L.leptocarpa* and *L. erecta*
Very thick band present at 2.1 cm	*L. decurrens* B and *L.leptocarpa*
Faint band present at 1.0 cm	*L. leptocarpa*
Faint band absent at 1.0 cm	*L. decurrens* B
Very thick band present at 2.1 cm	*L. erecta*

separation of individuals in a particular population into different taxa (Ladizinsky, 1983). This genetic divergence is reported for *Ludwigia decurrence* A and B, with *Ludwigia decurrence* B a novel band in its gel which was not reported for *Ludwigia decurrence* A. The presence of this new band in *Ludwigia decurrence* B therefore separates it from *Ludwigia decurrence* A and a new hypothetical name may be given to delimit the species. Similarly, the diagnostic bands that separate *Ludwigia octovalvis* varieties are reported, these are basically the bands at 0.5 and 0.8. The Single Linkage Cluster Analysis reveals the true similarity position of the *Ludwigia* species studied.

Bands with identical electrophoretic mobilities represent proteins with identical amino acid sequences and are therefore potentially homologous in their derivations (Scogin, 1972). *L. hyssopifolia* and *L. abyssinica* have proteins with higher molecular weights which informs why they have slow moving band at 0.4. This band therefore delimits them from the other species; other diagnostic bands for the identification of each species are reported in the results.

The protein bands are taxonomically distinct as no two species have the same band distribution. This agrees with the opinion of Olsson (1967) as reported by Folorunso et al. (2012) that biogenetic relationships can best be indicated by quantitative results using chemo-taxonomic methods. The band at 2.1 is taxonomic for all the species of *Ludwigia* studied; this shows evidence of common evolutionary origin in them. Coming from the same parental stock, their evolution is convergent thereby making it possible for character traits to be shared in common. This support the assertion of Gottlieb (1971) that when a band appears in all individuals in a population, it is assumed that the gene which codes enzyme or protein does not vary. Based on the position of protein bands *L. octovalvis* var *linearis*, *L. octovalvis* var *brevisepala*, *L. hyssopifolia* and *L. abyssinica* are more closely related. The band at 1.0 delimits *L. leptocarpa* from the other species. *L. decurrens* A and *L. adscendens* subsp. *diffusa* are more closely related based on the number of band and the position of band.

Conclusion

Taxonomic bands for *Ludwigia* species studied have been reported together with the diagnostic bands for their identification. From the Dendrogram, interspecific relationships as well as their intraspecific relationships based on protein bands were reported. An artificial key was generated for the identification of the *Ludwigia* species studied based on their protein band relationship.

Conflict of interest

The authors have not declared any conflict of interest.

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Management of plant parasitic nematodes with fulan in mechanized yam production

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The potential of Fulan (2,5-dimethoxytetrahydrofuran), a synthetic nematicide applied at 10 g /stand and two plant bed types; ridging and mounding to manage plant parasitic nematodes and increase the yield of yam was investigated at Ejura during 2011 and 2012 at three levels of N-P_20_5-K_50 (NPK). The factorial experiment was mounted on randomized complete block design and replicated three times. Ridged bed type + the highest level of fertilizer applied (60, 60, 80 kg/ha N-P_20_5-K_20) + Fulan application interacted positively ($P < 0.05$) resulting in significant yields (32 and 30%) over the control treatments in mound and ridged bed types, respectively. The same treatment resulted in the highest marketable weight of yam which was 61% over ridged bed type + fertilizer applied at 60, 60, 80 kg/ha N-P_20_5-K_20 without Fulan application. Cracks were significantly low in Fulan treated tubers. The outstanding reduction in cracks was recorded in mound + F4 + Fulan which were 92% less than mound bed type only (control) tubers. Additionally, Fulan application suppressed nematode densities. The remarkable suppression in *Meloidogyne incognita* and *Pratylenchus coffeae* occurred in ridged bed type + fertilizer applied at 45, 45, 60 kg/ha N-P_20_5-K_20 + Fulan treatment which was 93% over mound bed type without fertilizer and Fulan application and 97% over mound bed type + fertilizer applied at 60, 60, 80 kg/ha N-P_20_5-K_20 without Fulan application, respectively. Ridged bed type appeared to be the better plant bed type alternative since the highest yield and nematode suppression were recorded in that option; besides, ridging presents drudgery saving intervention which farmers could exploit.

Key words: *Dioscorea* species, drudgery saving intervention, Fulan, plant bed type.

INTRODUCTION

Yams (*Dioscorea* sp., family Dioscoreacea) constitute a major carbohydrate food source in West Africa (Osunde and Yisa, 2003). The tubers have organoleptic qualities which make them the preferred carbohydrate staple and can contribute up to 350 dietary calories per person each day (Asiedu et al., 2001). The plant has a tremendous sink capacity to store food reserves and individual tubers may weigh as much as 20-30 kg (Fuccillo et al., 2007).

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Yam therefore has immense potential as an "insurance crop" in Africa where it is heavily depended upon, because it plays a vital role in food security both at household and national levels. Yams are utilized in different ways in West Africa, a known centre of yam diversity and production. In Ghana for instance, yams may be boiled, mashed, fried or roasted. Pounded yam or "fufu" eaten with soup is a very popular form of yam consumption. Ghana is the leading exporter of yam in Africa with yam contributing to 16% of the Agricultural GDP (FAOSTAT, 2006). Besides, tubers are processed into flour, noodles, chips, and dry slices (Zheng, 2011).

Being a popular food product, yam has a surprising number of alternative uses. In the pharmaceutical industry, diosgenin, a steroidal saponin is extracted from the root of wild yam (*D. villosa* L.) with the potential to minimize post-menopausal symptoms (Benghuzzi et al., 2003) while dioscorin is extracted from the tuber of Chinese yam (*Dioscorea batatas* Decne) with sufficient antioxidant potential which confers on it tremendous health benefits (Wen-Chi et al., 2001).

In the hedonistic world, (*Dioscorea opposita* Thunb.) yam has been successfully used for the preparation of beer (Xu, 2007). The general significance of yams in the lives of a people cannot be over emphasized. However, the production of this tuber crop is constrained by both biotic and abiotic factors. Plant parasitic nematodes constitute a major biotic factor that constrains the production of yams. Those known to cause serious damage are the yam nematode (*Scutellonema bradys* Steiner and LeHew), the lesion nematode (*Pratylenchus coffeae* Zimmermann) and the root knot nematodes (*Meloidogyne* Goeldi spp). The yam nematode, *S. bradys* causes a decay of tubers known as "dry rot disease". *P. coffeae* cause deep cracks on tubers leading to a corky appearance and diseased tubers become spongy (Bridge et al., 2005). *Meloidogyne* species cause galling of roots and tubers which appear warty. Nematode infestation reduces the market value of yam tubers which negatively affects the farmers' profit margin.

For sustainable yam production therefore, the nematode menace should be managed. Synthetic agro-pesticides (nematicides) appli-cation is the single most effective management strategy against nematodes (Noling, 2012). It is universally acknowledged that synthetic agro-pesticides usage presents environmental problems (Bell, 2000). However, recommended agro-pesticides applied at the recom-mended dosages could be successfully used for food production without endangering man and the environ-ment. Planting on ridges has been shown to increase root and tuber yields (Ennin et al., 2009). The seed bed type has also been reported to interact with fertilizer application with planting on ridges resulting in higher yam tuber yield response to fertilizer than the traditional method of planting on mounds (Ennin et al., 2013). The two-fold objectives of this study therefore, were to use a recommended nematicide, Fulan (a non-fumigant with 90 days waiting period and dimethoxytetrahydrofuran) in managing plant parasitic nematodes population, and to investigate nematode control interactions with seed bed type and fertilizer application on yam production.

MATERIALS AND METHODS

Study site

The field trial was conducted in 2011 and 2012 during the major rainy seasons at Ejura in the Ejura Sekyedumasi District of the Ashanti region. Ejura is located on 07º 24′N 01º 21′W in the forest-savanna transitional zone of Ghana. It experiences a bimodal rainfall pattern. The soil type is a "Amantin series" Chromic Lixisol.

Experimental set up and treatments

The experimental field had been cropped with two varieties of yam (Dente and Pona) for the previous two years and plant parasitic nematodes population was therefore perceived to be very high. The field was ploughed, harrowed and eight (8) of the 16 plots in a replication were ridged (mechanized) while the remaining eight were mounded. The three factor experiment had plant bed type (mounding or ridging) as the main plot; fertilizer application (F1= 0 (control); F2 = 45, 45, 60; F3 = 60, 60, 60; F4 = 60, 60, 80) kg/ha N-P_20_5-K_20 as sub plot and Fulan (2, 5-dimethoxytetrahydrofuran from Sigma-Aldrich) a nematicide applied at (0 (control) and 10 g /stand) as sub-sub plot. The fertilizer was 50% split and applied in bands at 4 and 12 weeks after planting. Treatments were replicated three times. A plot measured 12 x 4.8 m of 40 mounds or four ridges of 10 stands per ridge resulted in 40 stands. Spacing on both ridges and mounds was 1.2 x 1.2 m. One variety of yam, Dente was used in planting.

Sett treatment before planting

Disease-free yams were cut into 350 g sections with a sharp kitchen knife. These were neatly packed into cane basket and nested in a 15 l plastic receptacle containing-a mixture of ordinary wood ash, Dursban (chlorpyrifos at 80 ml) an insecticide and Mancozeb, a fungicide (dithiocarbamate 80%; 120 g) in 15 l of water. The preparation was well stirred with the aid of a metal rod. The chemical solution completely covered all the setts in the cane basket before removal and air-dried under tree shade for 24 h before planting. The purpose was to control insect pests and fungal infections respectively. Fulan,10 g/stand was placed in the planting hole, covered with a little soil and the yam sett placed in it with the cut surface directed upwards and properly covered with soil. Hand weeding was done three times before harvesting of yam.

Sampling and extraction

Soil samples were collected at two time periods; at the start of the trial (April) before planting of yam and at harvest of the crop (December) with a 5 cm wide soil auger to a depth of 20 cm. The soil samples, 200 cm^3 per treatment were extracted using the modified Baermann funnel method. After 24 h of extraction,

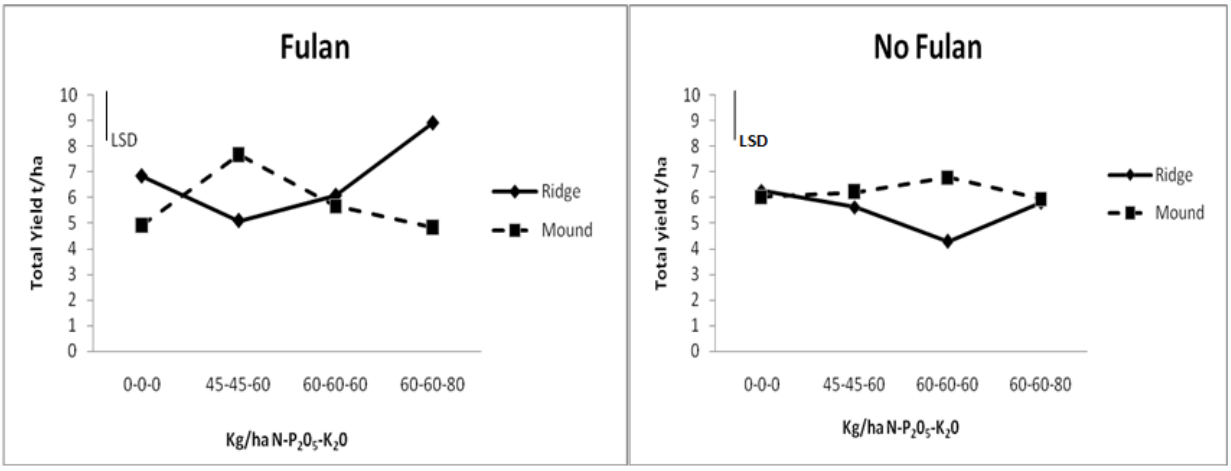

Figure 1. Effect of seedbed, fertilizer and fulan on yield of yam.

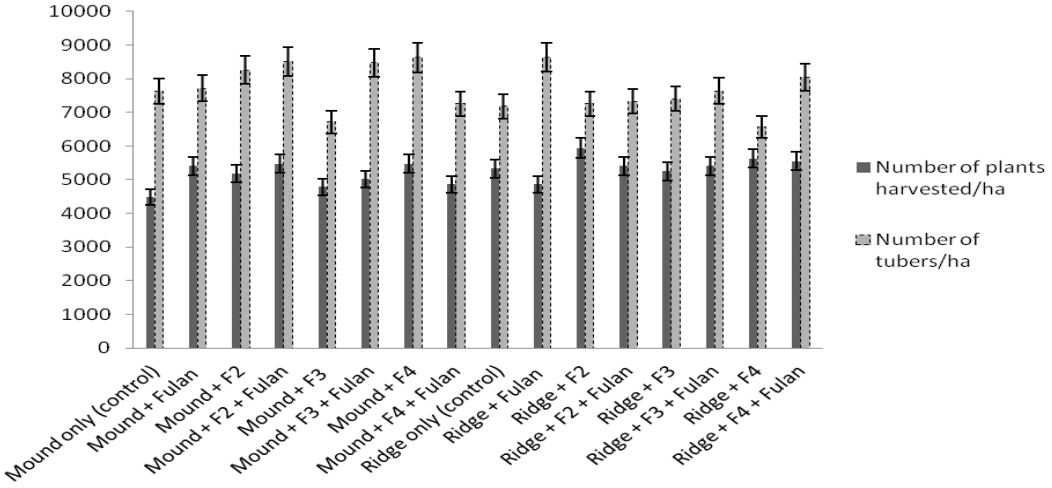

Figure 2. Number of plants and tubers harvested.

nematodes were relaxed in water at (60ºC) for 3 min and fixed with 40: 1: 89 (formalin: glacial acetic acid: distilled water). Second, third and fourth stage nematodes were mounted on aluminium double-cover glass slides and specimens were identified (CIH, 1978) by morphology under the stereo microscope at 100x while root-knot nematodes were identified through perineal pattern (Jepson, 1987). Tuber galling index (TGI) based on Zeck's 0-10 scale (Sikora and Fernandez, 2005) was recorded at harvest.

Data analysis

The two years data were pooled and analyzed once using the mixed model (REML) approach. Nematode count and indices based data were log (ln (x +1)) and square root √(1+0.5) transformed to improve homogeneity of variance before analysis using GenStat 8.1 (Lawes Agricultural Trust, VSN International). Significant mean separation was determined with Fisher's Protected Least Significant Difference (LSD) Test at $\alpha \leq 0.05$.

RESULTS

Significant ($p < 0.05$) differences were observed amongst treatments in yield of yam. Ridged bed type + the highest level of fertilizer applied (60-60-80 kg/ha) N-P_2O_5-K_2O + Fulan application interacted positively ($p < 0.05$) resulting in the highest yields which were 32% and 30% over the control treatments on mound and ridged bed type respectively (Figure 1).

The same treatment resulted in the highest total yield of yam which was 61% over ridged bed type + fertilizer applied at 60-60-60 kg/ha N-P_2O_5-K_2O without Fulan application (Figure 1). However, no differences were observed in both the number of plants and tubers harvested amongst treatments (Figure 2). All treatments were equally affected by anthracnose and virus infections

Table 1. Effect of treatments on anthracnose and virus severity and cracks on tubers

Treatment	Anthracnose severity	Virus severity	Cracks
Mound only (control)	3.2a	2.6a	3.7a
Mound + Fulan	3.2a	3.0a	1.3c
Mound + F2	3.7a	3.3a	2.7b
Mound + F2 + Fulan	3.4a	2.6a	0.7c
Mound + F3	3.8a	2.6a	1.0c
Mound + F3 + Fulan	3.6a	3.3a	2.0c
Mound + F4	3.6a	2.6a	0.7c
Mound + F4 + Fulan	3.3a	3.1a	0.3d
Ridge only	3.3a	2.4a	0.3d
Ridge + Fulan	3.7a	2.3a	2.0c
Ridge + F2	3.5a	3.1a	2.6b
Ridge + F2 + Fulan	3.6a	3.3a	2.0c
Ridge + F3	3.8a	2.9a	2.3c
Ridge + F3 + Fulan	3.3a	3.2a	1.0c
Ridge + F4	3.6a	2.8a	1.7c
Ridge + F4 + Fulan	3.0a	2.8a	1.7c
Lsd	0.6NS	0.6NS	2.4

Data are means of three replications. Within the same column, mean values followed by the same letter are not significantly different at α = 5%.

(Table 1). Treatments did not either inhibit or increase the severity of anthracnose and virus infections. However, Fulan application resulted in reduced tuber cracks. Mound + F4 + Fulan recorded 92% less cracks compared with mound bed type only (control). Fulan application suppressed nematode population compared with untreated plots in the three nematode genera encountered; however, nematode suppression was significant only in the root-knot nematode, *M. incognita* and the lesion nematode, *P. coffeae*. The lowest *M. incognita* and *P. coffeae* population was recorded in ridged bed type + fertilizer applied at 45-45-60 kg/ha N-P_20_5-K_20 + Fulan treatment which was 93% over mound bed type without Fulan and fertilizer application and 97% over mound bed type + fertilizer applied at 60-60-80 kg/ha N-P_20_5-K_20 without Fulan application respectively (Table 2). However, population of the reniform nematode, *R. reniformis* was not affected by treatments. Similarly, there was no difference amongst treatments in tuber galling index (Table 2).

DISCUSSION

The use of synthetic pesticides in crop production has been seriously criticized by environmentalists on grounds of environmental degradation and consumer health. However, easy accessibility, sure and quick action against target pests, will continue to render synthetic chemical control the first choice of farmers (Sabir, 2013). It is important to note that judicious use of synthetic agro-products is essential to sustainable productivity. Lack of fertilizer application in farming systems has been associated with lower yields (Ennin and Dapaah, 2008) whilst optimum fertilizer application has been reported to increase yield (Wang et al., 2012). In the present study, the combination of chemical fertilizer (NPK) and synthetic nematicide, Fulan resulted in the highest yield of yam. It is therefore important that farmers are taught the basics of good agricultural practices (GAP) that address environmental, economic and social sustainability for on-farm processes which result in safe and quality food and non-food agricultural products (Anonymous, 2003) to use synthetic chemicals appropriately.

Treatments were not different in the number of plants harvested and also the number of tubers harvested. Since one variety of yams was used, the implication was that treatments did not have any effect on either the sprouting of yam or multiple tubers.

Treatments inability to effect any changes in the anthracnose and virus incidence and severity was expected. Indeed, plant bed type, fertilizer and nematicide application have never been documented to manage fungal and viral infections in crop production.

Interestingly, populations of root-knot nematodes, *M. incognita* and the lesion nematode, *P. coffeae* were suppressed by Fulan application irrespective of the bed type or fertilizer application. In both nematode species,

Table 2. Effect of treatments on population density of plant parasitic nematodes.

Treatment	Meloi	Praty	Roty	TGI
Mound only (control)	183 (2.3)*a	119 (2.0) a	70 (1.8) a	0.7 a
Mound + Fulan	36 (1.1) b	24 (1.0) c	44 (1.2) a	0.3 a
Mound + F2	111 (1.4) ab	137 (2.1) a	74 (1.8) a	1.0 a
Mound + F 2 + Fulan	44 (1.2) c	30 (1.1) c	58 (1.6) a	1.3 a
Mound + F3	134 (2.1) a	105 (1.9) ab	69 (1.8) a	0.8 a
Mound + F3 + Fulan	30 (1.1) c	16 (0.6) d	46 (1.2) a	0.7 a
Mound + F4	172 (2.2) a	256 (2.4) a	110 (2.0) a	1.0 a
Mound + F4 + Fulan	35 (1.1) b	25 (1.0) c	52 (1.3) a	0.7 a
Ridge only (control)	145 (1.5) ab	153 (2.2) a	79 (1.8) a	0.4 a
Ridge + Fulan	43 (1.2) b	49 (1.2) c	65 (1.8) a	0.3 a
Ridge + F2	57 (1.3) b	238 (2.3) a	114 (2.0) a	0.8 a
Ridge + F2 + Fulan	13 (0.5) c	7 (0.4) d	50 (1.3) a	1.0 a
Ridge + F3	178 (2.3) a	224 (2.3) a	114 (2.0) a	0.8 a
Ridge + F3 + Fulan	30 (1.1) b	87 (1.7) b	88 (1.8) a	1.0 a
Ridge + F4	160 (2.2) a	217 (2.3) a	123 (2.0) a	0.7 a
Ridge + F4 + Fulan	22 (1.0) b	27(1.1) c	74(1.8)a	1.0 a
Lsd	(1.0)	(0.5)	(0.9)NS	2.0 NS

Data are means of three replications. *Log (ln (x +1)) transformed data used in analysis in parenthesis. Meloi = *Meloidogyne incognita,* Praty = *Pratylenchus coffeae*, Roty = *Rotylenchulus reniformis*. TGI = Tuber gall index. Within the same column, mean values followed by the same letter are not significantly different at α = 5%.

Reductions of more than 90% were significant. Additionally, the potential of Fulan manifested in the development of insignificant cracks in Fulan treated tubers while untreated tubers suffered major cracks. Though, Fulan suppressed *R. reniformis* populations, such reductions when compared with untreated plots were not significant. Similarly, no difference existed between tuber galling from Fulan treated samples and untreated samples. The highest yield and nematode suppression were recorded in ridged method of planting. Ridging was effective in reducing nematode density because in ridging, nematodes and their eggs are more exposed to sunshine which kills them compared with mounding, and also resulted in a more efficient use of Fulan and fertilizer. Therefore, farmers' incentive in adopting ridging over mounding in addition to the foregoing is the fact that ridging represents drudgery saving intervention.

Conclusion

This study has revealed that the recommended nematicide Fulan, applied at 10 g/stand effectively managed plant parasitic nematodes irrespective of the other components of the factorial regime. Application of Fulan increased the response of yam tuber yield to fertilizer rate especially on ridges, with 60-60-80 kg/ha N-P_20_5-K_20 resulting in highest yields. Finally, ridging was a better alternative to mounding as yield was not compromised and the method also lends itself to mechanization. Fulan, the non-fumigant with a 90 day resting period is safe for yam production since yam matures at 210 days on the average.

The efficacy of synthetic agro products would continue to attract farmers, their implications on the environment notwithstanding. Pragmatic policies should therefore be put in place by governments to ensure their sustainable use by farmers.

Conflict of interest

The authors have not declared any conflict of interest.

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Full Length Research Paper

A method to use very small size potato (*Solanum tuberosum* L.) tubers as seed

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Potato, being a vegetatively propagated crop, tubers of 30-60 g size is used as seed. Whereas, < 20 g size potato tubers are left in the soil itself after harvesting, as they are not suitable either for consumption or seed purpose. Seed pelleting technique is used to increase the tuber size and provide additional nutrient support. Two cultivars of potato (*Solanum tuberosum* L.) with five different treatments using left over under sized tubers (having radius of around 3.98 to 5.57 mm) were used for this experiment. During storage, weight loss was minimum in *Acacia* treatment (8.33%) and sprouting was not affected by *Acacia* and salicylic acid treatments. At the end of storage, phenol was high in *Acacia* treated tubers. These tubers were planted in the field. The time taken for 50% germination was similar in control (22.33 days), *Acacia* (22.33 days) and salicylic acid (22.67 days) treatments. Varietal differences were observed with the type of chemical used for pelleting. In general the yield obtained with *Acacia* leaf powder pelleting was more in both the cultivars.

Key words: Seed potato, pelleting, *Acacia nilotica* and sprout.

INTRODUCTION

Traditionally, potato (*Solanum tuberosum* L.) is being propagated vegetatively by all over the world. The proportion of cost involved towards seed tubers in potato seed production is alarming to an extent of 40% (Sarjeet Singh and Naik, 1993) to 70% (Almekinders et al., 1996). In India, the seed rate is 2.5 t ha^{-1} (Jagpal Singh, 1993) because tubers are sold in kilograms not by numbers. This prejudices to find a suitable alternate method to reduce the cost of seed material. Few available methods are using true potato seed (TPS), cut potatoes, sprouts, sprout cuts, single node cuts, micro and mini tubers. TPS technology is added with many advantages like cost effective, easy transport, less disease incidence etc but the adoption by farmers is less (Chilver et al., 2005; Rowell et al., 1986; Vander Zaag et al., 1989; Benz et al, 1995; Sikka et al., 1994; Pangaribuan, 1994; Adhikari, 2005; Gupta et al., 2004) particularly where land availability is more for potato cultivation. The other technique, using “cut potato” but, poor performance is reported by Nielson et al. (1989); Rykbost and Lockeil (1999) and chances of virus multiplication, which may in turn lead to economic loss to the grower. Potato is infected with nearly 40 species of viruses (Valkonen, 2007). Potato is a vulnerable crop for many viruses;

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many of them are transmitted mechanically except few like PLRV (Mike Mayo et al., 2000). Cuts transmit more viruses, hence adaption is difficult. The other options are microtubers and minitubers. Microtubers are small potato tubers produced *in vitro* by plant lets in controlled condition inside the test tube or jar. Long term storage and its performance in the field when directly planted is questionable (Paolo Ranalli, 2007). Minitubers are the produce of *in vitro* plantlets in the field. These tubers are also small tubers range from 0.1 to 10 g or more (Struik, 2007). Here, the cost per tuber is much higher than the conventional one due to its procedural cost.

Making use of conventionally produced, small size left over tubers which are of no use otherwise is another way, if the size of the tuber is increased to acceptable planting tuber size and addition of nutrients is made to give a proper kick start to plant growth under field condition. Seed pelleting, is a technique for coating seed used to increase the size of very small horticultural seeds which provides improved planting features and is a potential alternative for improving sowing efficiency. Pelleting allows the seeds not only to gain both weight and volume but also standardizes seed size. The materials used for coating seeds are classified into two groups: binder materials and bulking or coating materials with or without micronutrients or growth regulators for the invigoration of the planting material. Furthermore, the pellet's external surface is smooth and not deformed, facilitating its planting by seed drills if it is used for mechanical planting. Pelleting is mainly used for seeds which have a high market value, such as tobacco, eucalyptus, some vegetables and ornamental plants, but is uncommon for smaller seeds, such as grasses, which have a lower market value, and high volume high value crops like potato. This has been successfully practiced in many crops like chilli, groundnut, soybean etc., but, information on their effect on seed potato is scanty. Keeping the above facts in mind, a trial was initiated in two cultivars of potato (Kufri Swarna and Kufri Jyoti) during the year 2009 and 2010. Pelleting was chosen because of its advantage over priming as reported by Murata et al., (2008) in groundnut. To our knowledge, this is the first attempt which indicates the possibility of pelleting the potato tubers. *Acacia nilotica* leaf powder is used as a base coating material for pelleting. The use of *Acacia* leaf powder as pelleting agent was reported by Nargis et al (1999). *Acacia* species are used to treat diseases of livestock as reported by Dafallah and Al-Mustapha (1996), Shah et al. (1997), Amos et al. (1999), Gilani et al. (1999) and Hussein et al. (2000). Hence, we attempted to use the *Acacia* in our study.

MATERIALS AND METHODS

Experimental site and material details

The trials were conducted at Central Potato Research Station, Muthorai, Tamil Nadu, India located at an elevation of 2140 m above MSL at 11° 24' North latitude and 74° 4' East longitude. The normal rainfall of the location is 1300 mm, received in 85 rainy days and it is well distributed during both South West and North East monsoons. The mean maximum and minimum temperatures of the region range between 15 and 23°C and 6 and 13° C, respectively. The climate of the region is moist sub humid type (Manorama K, 2004.). The soil type of experimental site is sandy clay loam with a pH range of 4.7 to 5.0, 0.1 dSm^{-1} EC and high in available nitrogen, low in available phosphorus and high in available potassium. The very small undersize seed tubers, left over from foundation seed multiplication plots of cultivars Kufri Swarna and Kufri Jyoti were collected manually at Central Potato Research Station, Muthorai (PO), The Nilgiris, India constituted the study material for the present investigation. The collected tubers were stored for 15days at room temperature in an average temperature of 20°C with a relative humidity of 85% for curing. The average size of tubers was 3.98 to 5.57 mm radius with 13.78 g of weight. The radius was measured with a help of vernier calipers.

The yield potential of Kufri Swarna and Kufri Jyoti is 25 t ha^{-1}. The experiment was conducted with tubers collected from summer harvest of 2009 and 2010 with five treatments. Ten tubers were used for each treatment for field experiment and five tubers for further analysis. The treatments were T1- Dried Leaf powder of *Acacia nilotica* (AN) alone, T2- Dried Leaf powder of *Acacia nilotica* + Calcium Sulphate (CS), T3 - Dried Leaf powder of *Acacia nilotica* + Salicylic acid(SA), T4 - Dried Leaf powder of *Acacia nilotica* + Tri-iodo benzoic acid (TIBA) and T5 - No pelleting (Control).

Pelleting material

The matured green leaves were collected from the *Acacia nilotica* plants from nearby Mettupalayam forest and shade dried. Well dried leaves were kept under oven at 50^0C for 2 hours and powdered with the help of a grinder. Fine powder was collected through 300 mm sieve trays. Maida, a finely milled and refined flour of wheat using a fine mesh of 600 meshes per square inch was used as adhesive. Calcium sulphate (Brand:CDH), Salicylic acid (Brand :Merck) and TIBA (Brand: Merck) were purchased from a commercial supplier.

Pelleting methodology

Bulking (or coating) material used was *Acacia nilotica* leaf powder with respective chemicals and 2% Mancozeb (Indofil company) as antifungal as per treatments. The binder was prepared by Maida flour of 20 g into 80 ml of distilled water until a smooth paste is achieved by light heating and brought to the normal temperature before use. The binder should not be too thick or too drippy. The well mixed bulking material (*Acacia nilotica* leaf powder) was taken in a separate bowl and tuber placed into it; then the binder was added slowly in to the bowl containing tubers by gentle rotation. Repeated coating was performed by intermittent shade drying till the desirable size. An average of 13.79 g of bulking material along with binder was used for a tuber. The tubers were dried for an hour on the paper towel before stored.

Pelleted tubers were stored at normal storage with a temperature range of 5.7 to 18.5°C and the RH range of 49.2 to 81.3% for 150 days. Observations were taken at 15 day intervals throughout the experiment. Sprouting rate, weight loss and length of sprouts were recorded.

***Sprouting rate*:** At the end of every 15 days of storage, the numbers of sprouted tubers were counted. Sprouted tuber percentage was worked out in each treatment and expressed in percentage.

$$\text{Sprouted tubers (\%)} = \frac{\text{Sprouted tuber number at the end of 15 days of storage}}{\text{Sprouted tuber number at the beginning of 15 days of storage}} \times 100$$

***Tuber weight loss*:** Tuber weights (10 tubers) were recorded prior to storage, and after every 15 days of storage. Weight loss was expressed as percentage: [(tuber weight prior to storage - tuber weight after every 15 of storage) / tuber weight prior to storage] x 100.

***Length of the sprout*:** Tuber sprout length (10 tubers) was measured at the end of storage period in each treatment with the help of vernier caliper and expressed in millimeter.

At the end of storage, tubers were taken for field experiment. Randomised block design Design (RBD) with three replications. One row of 10 tubers each, with a spacing of 40 x 20 cm were planted. Farmyard manure and fertilizers were applied as per recommendation. One per cent urea spray was given at 45^{th} and 60^{th} days after planting on the foliage.

After maturity, tuber yield and number was recorded in three different grades (<30, 30-60 and >60 g) by grading from the row and was expressed in kg per 10 plants and number per 10 plants.

***Bio chemical analysis*:** Reducing sugars was estimated by using the method of Nelson (1944), Total free amino acids by Roe et al., (1990) and Phenols by Sadasivam and Manickam (1992). For this purpose, three tubers were selected randomly and the analysis was carried out.

Field potential of pelleted seed tubers

Tuber yield in different grades

Tuber yield was recorded in three different grades (<30 g (small size), 30-60 g (seed size) and >60 g (large size)) by manual grading and the weight was expressed in kilogram.

Tuber number in different grades

Tuber number was recorded in three grades (<30 g (small size), 30-60g (seed size) and >60g (large size)) and it was expressed in numbers.

Statistical analysis

Data were analyzed using Analysis of Variance with mean separation by LSD Test using WASP 2, a web based software developed by ICAR research complex, Goa, India. Percentage data was arc-sin transformed before analysis.

RESULTS

***Weight loss (%)*:** Tuber weight loss was recorded from the day of storage till 150 days at 15 days interval. In control, out of the two varieties tried, the per cent weight loss of stored tubers was more in K. Swarna (16.02%) than that of K. Jyoti (14.92%). In K. Swarna, weight loss started after 15 days of storage in CS, TIBA and control whereas it started at 45 days in SA and at 50 days after storage in AN treatments. The same trend was observed in K. Jyoti cultivar except in AN treatment where weight loss has started after 75 days of storage (Figure 1b and 1e). Loss of weight in storage is expected to reduce the vigour of the tubers when planted in main field. The delay in weight loss was observed in SA and AN treatments, and the delay was more spectacular in AN treatment in both the varieties. Even at 150 days after storage the weight loss in AN treatment was nearly 50% only in K. Swarna and it was up to 65% only in K. Jyoti when compared with control. This indicates that AN was effective in reducing the tuber weight loss in storage.

***Sprouting (%)*:** All Pelleted tubers sprouted well after 150 days of storage in both the cultivars. In control sprouting started at 45 days in both the varieties. But the AN treated tubers started sprouting only after 90 days in K. Jyoti and at 75 days in K. Swarna (Figure 1a). All the tubers sprouted within 120 days in control in both the varieties. Hundred per cent sprouting of tubers was recorded at 120 days in TIBA in both the varieties. In K Jyoti CS treatment recorded cent per cent sprouting at 150 days and in K Swarna at 135 days. (Figure 1d). However, in CS treatment sprouting started at 15 days and it progressed slowly. The rate of sprouting could be controlled greatly by AN treatment in both the varieties and more effectively in K Jyoti.

***Sprouting length (mm)*:** Pelleting significantly influenced the length of sprouts during storage of potatoes in both the cultivars. The highest sprout length of 56.29 mm was recorded at 150 days in K. Swarna in CS treatment and it was 47.30 mm in K. Jyoti at 150 days under the same treatment. Minimal length of longest sprout was recorded in AN (8.02, 9.24 mm) followed by SA (9.56, 12.63 mm) in K. Swarna and K. Jyoti, respectively at 150 days achieved in K. Swarna followed by K. Jyoti (47.30) (Figure 1c and 1f). In K. Swarna the per cent reduction in longest sprout length has been to the tune of 17% in comparison with the control and it was 26% in K. Jyoti in AN treated tubers at 150 days. Among the different pelleting treatments AN has proved to be more effective in reducing the sprout length in both the varieties.

***Biochemical analysis*:** Higher content of reducing sugars was recorded in SA (294.89) treatment in K. Swarna and it is 320.50 in K. Jyoti under AN treatment. Control recorded the lowest values for both the varieties. Higher total free amino acids were recorded in CS treatment in both the varieties and the lowest values were observed in control. Higher phenol content was recorded in AN treatment for both the varieties (Figures 2 and 3) and the lowest were recorded in control.

***Field performance*:** The time taken for 50% germination was similar in Control (22.33 days), AN (22.33 days) and

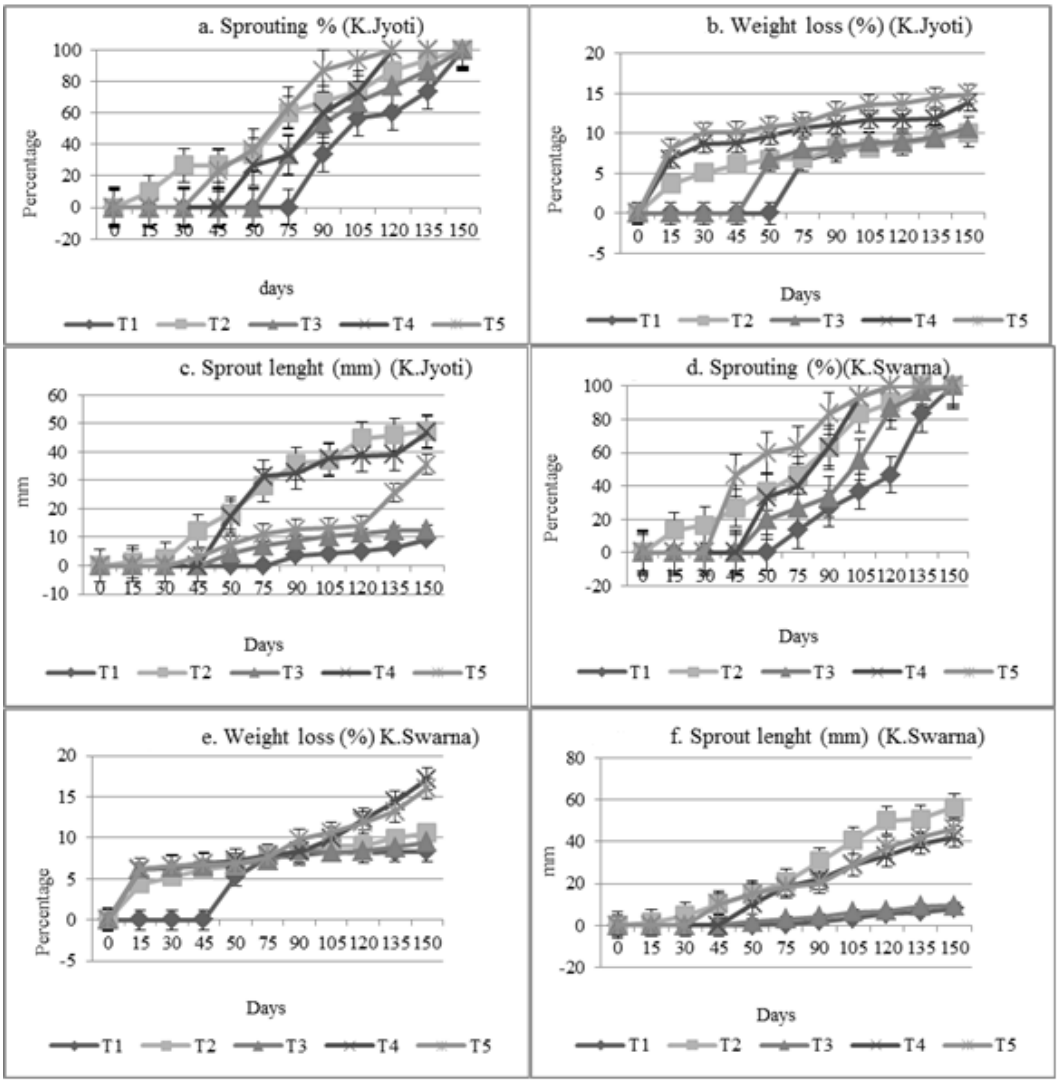

Figure 1. Effect of pelleting on sprouting (%), weight loss (%) and sprout length (mm) in K. Jyoti and K. Swarna.

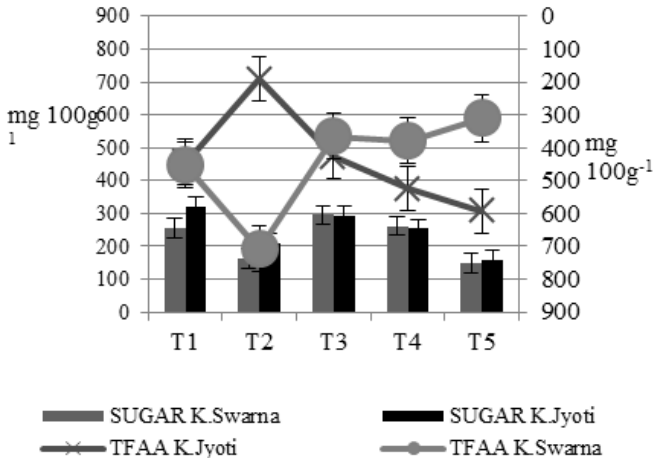

Figure 2. Efffect of pelleting on free sugars (mg 100 g^{-1}) and total free amino acid (TFAA) after storage.

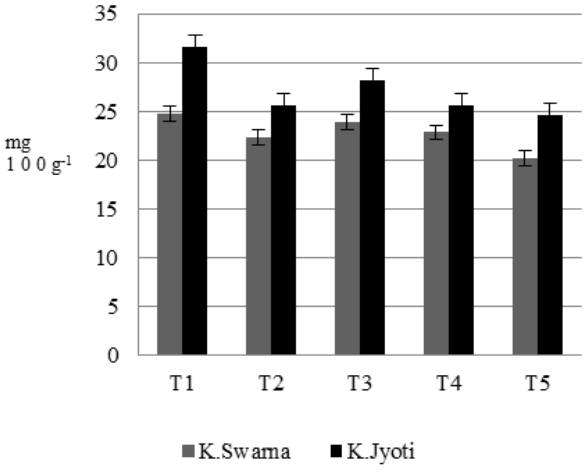

Figure 3. Efffect of pelleting on phenol (mg 100 g^{-1}) after storage.

Table 1. Field performance of pelleted potato seed tubers.

Cultivar	Treatment (T)	Numbers of tubers (numbers 10 plants^{-1})				Weight of tuber (Kg 10 plants^{-1})			
		<25 g	25-75g	>75g	Total	<25g	25-75g	>75g	Total
V1	AN	16	163	7	186	0.30	0.98	0.90	2.18
	CS	9	125	3	137	0.17	0.84	0.25	1.25
	SA	12	156	5	173	0.16	0.98	0.45	1.59
	TIBA	14	155	4	173	0.21	1.06	0.38	1.65
	Control	9	115	1	125	0.19	0.69	0.10	0.98
	Mean	12	143	4	159	0.20	0.91	0.42	1.53
V2	AN	11	211	7	229	0.17	0.91	0.93	2.01
	CS	10	200	2	212	0.14	1.10	0.20	1.44
	SA	11	204	5	220	0.22	1.24	0.45	1.91
	TIBA	11	195	5	211	0.17	1.16	0.54	1.87
	Control	4	122	0	126	0.09	0.82	0.00	0.90
	Mean	9	186	4	200	0.16	1.05	0.42	1.62
		CD(P≤ 0.05)	CD(P≤ 0.05)	CD(P≤ 0.05)	CD(P≤ 0.05)	CD(P≤ 0.05)	CD(P≤ 0.05)	CD(P≤ 0.05)	CD(P≤ 0.05)
v		1.08	9.84	NS	20.93	0.02	0.05	NS	0.95
t		1.71	15.56	0.42	40.25	0.03	0.09	0.05	1.50
v x t		2.42	22.01	0.60	NS	0.05	0.12	0.07	NS

V1 = K. Swarna V2 = K. Jyoti NS = Non-Significant.

SA (22.67 days) treatments. But in due course of time, all the treatments achieved cent percent germination. Significant differences were noticed in field performance of different pelleting treatments in both the varieties tested. In both the varieties, seed sized tuber number (163, 211) as well as yield (0.98, 0.91) and total tuber number (186, 229) and yield (2.18, 2.01) (for K Swarna and K. Jyoti, respectively) were more in AN treatment. The increase in tuber number (49%) was highly significant in K. Swarna which is a shy bearing with reference to tuber number. In terms of yield, the increase is more in K. Swarna as it produced almost double the yield than the control (Table 1).

DISCUSSION

The rationale for seed pelleting is to make the seed perfect for planting by increasing the size as well as making the nutrition sufficient enough to give growth in the field. Selection of binder materials and bulking or coating materials is important for pelleting. Pellet forming is an artistic method combined with science.

In our experiment, vigor enhancement by way of early sprouting was noticed in SA treatments in both the cultivars by nearly 30 days over other treatments in both the cultivars. Salicylic acid (SA) is known for its defense reactions induced endogenously (Gaffney et al., 1993; Clarke et al., 2000) but effect on germination is not well defined because in some crops it inhibited the germination namely, maize (Guan and Scandalios, 1995) and caused vigour enhancement in wheat (Shakirova et al., 2003) and pea (McCue et al., 2000). Vigor enhancement by the incorporation of growth regulators in pelleting might be due to increased cell division and hormonal balancing system which caused an increase in sprout growth. Many basic works have been done in Arabdiobsis to understand the role of SA on seed vigour and found that SA plays a major role from seed storage protein mobilization to protein translation which in turn increase the seed vigour. Finally, the proteomic data revealed a close interplay between abscisic (ABA) signaling and SA elicitation of seed vigor.

Abscisic acid (ABA) reduction is believed to be important for dormancy breaking which in turn leads to sprout initiation in potato. Still, this phenomenon is controversial (Ume Sonnewald, 2001) as decline of ABA did not correlate with the sprouting behavior of the tubers, ruling out the possibility that the decline of ABA content below a certain threshold level is responsible for the break of dormancy (Biemelt et al., 2000). Although, in our experiment, early sprouting has indicated that SA as growth regulator might have played some role in altering the growth promotor (GA and cytokines) and inhibition (ABA and ethylene) ratio to promote the sprouting.

In general, calcium is amended as a nutrient in the soil to enhance the quality of potato because of its direct correlation with post-harvest quality and to enhance the drought tolerance in potato cultivation. Calcium is important for the growth of sprout in potato and to have the apical

Table 2. Phytochemical Components of *A. nilotica* ethanolic leaf extract

Secondary metabolites	Ethanolic leaf extract
Saponon	+
Saponin glycosides	+
Volatile oil	+
Hydrolysable tannin	+
Steroids	0
Triterpenoid	+
Tannin	+
Flavonoids	+
Phenol	+
Alkaloid	+

+ = Presence, 0 = absence (Solomon and Shittu, 2010).

dominance, which arrests the lateral branches. Dark storage creates calcium deficiency and causes sub-apical necrosis followed by death (Dyson and Digby, 1975(a)). In pelleting, the tubers are covered by pelleting material and a dark condition is created for the tuber. Calcium Sulphate was added in one of the treatments to understand and to avoid the formation of sub-apical necrosis.

Calcium sulphate pelleted tubers were able to sprout within 60 days after storage in both the cultivars when compared with control (45 days) showing its ability to overcome the sub-apical necrosis. In all the treatments sub-apical necrosis was not noticed. The sprouting was delayed by 15 days in CS when compared with control. This shows that some physiological activities either dormancy breaking or sprout initiation was modified. Arrest of physiological ageing through calcium application is tested by Dyson and Digby (1975b). Further, Booth (1963) showed that lateral branching is due to poor interaction between auxin and gibberellin.

Tri-iodo-benzoic acid (TIBA) was added to the experiment to understand the role of an inhibitor in the pelleting process. TIBA was identified as auxin inhibitor by Kuse (1953) when he found that auxin moving from the leaf did not pass the point where the TIBA was applied in a band of lanolin paste on the petiole. In this paper, these deductions have been tested by direct methods. Further this was confirmed by Muller (1999) in wild type peas. Ana Rincon et al. (2001) hypothesized that TIBA prevented fungal IAA transport towards the root *Laccaria bicolor*. In our experiment, tubers pelleted with TIBA took 60-75 days for sprouting when it is only 45 days in control. Sprout growth was inhibited by TIBA for 15 to 30 days. This might be due to the alteration in the auxins level. Though auxins did not play a direct role in the sprouting but played a major role in the dormancy breaking mechanism which is a pre request for the sprout initiation.

The sprouting percent was affected by *Acacia* leaf powder pelleting. It took 90 days to start sprouting which is 45 days later than control. The highest total extractable phenolics and total extractable tannin values were recorded in *A. nilotica* (168.36 and 176.15 mg g^{-1} DM, respectively) (Mtui Dorah, 2008). Pelleted with acacia leaf powder delayed germination might be due to the less accessibility to oxygen by the sprouts.

Many phenolic compounds are reported in the potato tubers (Uppal and Verma, 1982; Ghanekar et al., 1984). These compounds are the substrates of polyphenoloxidase enzyme and are involved in enzymatic browning of peeled potatoes (Mapson et al., 1963), in resistance of potatoes to diseases (Tripathi and Verma, 1975) and further reported that a decrease in the content of total phenols in the peels and pulp of two varieties of potatoes stored under ambient conditions. In the present investigation, the there is significant change in the phenol content among the treatments and between varieties. The chemistry of *A. nilotica* leaf extracts has shown the presence of phenol (Table 2) which played a role when it is coated over the tubers especially giving good health in the field by avoiding the diseases. Though there is a significant difference among the treatments in the free sugars and total free amino acids, a definite correlation could not be arrived.

The early achievement of 50% germination in the field by control (without coating) which is one of the vigour indicating factors may be due to non-hindrance by coating. Reduced emergence due to pelleting is possible when clay is applied over sugar beet seeds (Durant and Loads, 1986) but macronutrient solution immersed seeds yielded with more in cereals (Woomer et al., 2003) and nutrient pelleted seeds accelerated growth and development (Konstantinov, 1983) which has not happened in our experiment with potato. There is a significant difference between *A. nilotica* pelleting alone with that of added nutrients treatments. The performance of pelleted seed with other growth regulators might have affected the chemical transformation in the plant system. Further, Razzaque et al. (2004) reported that the application of TIBA had reduced the height of tomato plants. The increase in the tuber number and the total yield by *A. nilotica* coating is due to its antimicrobial activity, disease resistance is due to high phenol content and non-hindrance with other nutrients during growth period which facilitated the good source to sink movement during the bulking stage, a crucial period in potato growth.

Conclusion

Potato seed tuber which acts as a mother, supplies the food to growing sprout till it starts photosynthesis. This feeding results in vigorous and rapid crop growth during early growth stages, which probably leads to higher tuber yields. The vigorous sprout growth is due to the availability of external nutrients applied to the acacia leaf powder and suppression of tannins and phenols available in the acacia leaf powder. Acacia leaf powder alone is having the potential to prolong the shelf life of very small

left over tubers in the field and to give little lesser than the normal potato yield when it is planted in the field without much extra care. Hence, very small potato seeds which or otherwise waste in the field can be used as potato seed by modifying its size by pelleting where the seed cost is very high depending upon the labour availability.

Conflict of interest

The authors have not declared any conflict of interest.

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Characterization of fructans from *Agave durangensis*

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Agave **plants are members of the** ***Agavaceae*** **family and utilize crassulacean acid metabolism (CAM) for CO_2 fixation. Fructans are the main photosynthetic products produced by** ***Agave*** **plants, and are their principal source of storage carbohydrates. The aim of this work was to determine the chemical and molecular characterization of fructans from** ***Agave durangensis*****. Fructans were extracted from 10 year old** ***A. durangensis*** **plants. Trimethylsilyl derivatization was employed to determine the monomer composition. The linkage types in these carbohydrates were determined by methylation followed by reduction and O-acetylation, and finally analysis by gas chromatography-mass spectrometry (GC-MS). Samples were shown to contain** ***t*****-β-D-Fru*****f****,** ***t*****-α-D-Glu*****p****,** ***i*****-α-D-6-Glu*****p*** **and 1,6-di-β-D-Fru*****f*** **linkages. The analysis of the degree of polymerization (DP) was confirmed by MALDI-TOF-MS, showing a wide DP ranging from 2 to 29 units. The analyses performed revealed that fructans from** ***A. durangensis*** **are formed of 97.11% fructose and 2.89% glucose, and are a complex mixture of fructooligosaccharides of the neo-fructan type containing principally β(2-1) and β(2-6) linkages, with branch moieties.**

Key words: Degree of polymerization (DP), GC-MS, MALDI-TOF-MS.

INTRODUCTION

Mexico has been considered the center of origin and biodiversity of the *Agave* genus, due to the taxonomic diversity found within its borders. Of the 310 species reported, about 272 can be found in this country. Members of the Agavaceae family are distributed throughout Mexico, and are well adapted to, both arid and semiarid regions (García-Mendoza and Galván, 1995). They have undergone both morphological and

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Abbreviations: DMSO, Dimethyl sulfoxide; **TFA,** trifluoroacetic acid; **EtOH,** ethanol; **HMDS,** hexamethyldisilazane; **NaOH,** sodium hydroxide; **CH_3I,** iodomethane; **$NaBD_4$,** sodium borodeuteride; **NH_4OH,** ammonium hydroxide; **N_2,** nitrogen; **CO_2,** carbon dioxide; **H_2O,** water; **CAM,** crassulacean acid metabolism; **PAAMs,** partially methylated alditol acetates; **WSC,** water soluble carbohydrates; **DP,** degree of polymerization; **GC-MS,** gas chromatography-mass spectrometry; **MALDI-TOF-MS,** matrix-assisted laser desorption time-of-flight mass spectrometry.

physiological adaptations to survive in such adverse conditions (López et al., 2003). One such physiological adaptation of the plant is the use of crassulacean acid metabolism (CAM), which serves to minimize water loss (Santamaría et al., 1995) by opening stomata at night when the temperature is cooler (Nobel and Linton, 1997). The principal photosynthetic products of CAM in *Agave* plants are fructans (Sánchez-Marroquín and Hope, 1953), which are synthesized and stored in the stems, and whose primary function in such plants is storage. Nevertheless, agave plants also are source of saponins and polyphenols, which are compounds considered agents with several activities such as anticancer, antifungal, anti-inflammatory, antidiabetic, anti-inflammatory, among others (Santos-Zea et al., 2012). Fructans may also act as osmo-protectants during drought (Wang and Nobel, 1998), which represents a secondary physiological adaptation. In plants, ~ 15% of higher species contain fructans, and in certain species fructans constitute the plant's sole reserve of carbohydrates. Fructans are polymers or oligomers of fructofuranosyl residues, are commonly water soluble, and are synthesized from sucrose accumulated in the vacuole of the plant (Vijn and Smeekens, 1999). Agave fructans have been used as ingredients of a granola bar, resulting in a product which has a moderate glycemic index (Zamora-Gasga et al., 2014). Indigestible fraction of this granola bar showed potential prebiotic activity, since it affected anaerobic batch cultures inoculated with human gut flora, demonstrating that agave ingredients are good sources of fermentable dietary fiber (Zamora-Gasga et al., 2015). Another work (Crispín-Isidro et al., 2015) reported that agave fructans enhanced sensory attributes of a reduced milk-fat yogurt.

According to the manner that the β-fructofuranosyl units are linked, five main types of fructan can be identified: (i) linear inulins with β(2-1)-fructofuranosyl linkages, (ii) levans with β(2-6) linkages, (iii) graminans, which are mixed fructans containing type (i) and (ii) linkages, (iv) neoseries inulins, which contains a glucose residue between two fructofuranosyl units containing β(2-1) linkages, and (v) neoseries levans, formed by β(2-1) and β(2-6)-fructofuranosyl linkages (Mancilla-Margalli and López, 2006; Sims et al., 2001). Fructans are usually present in plants as a heterogeneous mixture with varying degrees of polymerization (DP) and structure as a result of both environmental conditions and the developmental stage of the plant (Sims, 2003).

The presence of fructans in *Agave* was first reported in 1888 (Suzuki, 1993), with *Agave veracruz* and *Agave americana* being the most studied species (Aspinall and Gupta, 1959; Bathia and Nandra, 1979). More recently, Sims et al. (2001) reported the presence of a similar fructan structure in members of the *Asparagales* order, in which the *Agavaceae* family is included. However, in *Agave* species more than one fructan structure has been reported. Sánchez-Marroquín and Hope (1953) and Bathia and Nandra (1979) reported inulins the principal storage carbohydrate in *Agave tequilana* and *Agave americana*, respectively. Meanwhile, reports (Aspinall and Gupta, 1959; Dorland et al., 1977) on *Agave veracruz* showed the presence of a complex mixture of highly branched fructans with an internal glucose and containing both *β*(2-1) and *β*(2-6) linkages. More recently, Wang and Nobel (1998) reported the presence of a DP5 in *Agave deserti*, primarily in the vascular tissue. Therefore, different agaves contain fructans with a wide variety of structures, so it is necessary to characterize the fructans of each species of agave. Thus, the aim of this work was to determine the chemical and molecular characterization of fructans from *Agave durangensis*.

MATERIALS AND METHODS

Standard material

1-Kestose and Nystose standards (inulin series DP3 and DP4, respectively) were supplied by Sigma; 1, 1, 1-Kestopentaose (inulin DP5) was from Megazyme. Fructans from *Agave durangensis* were extracted and derivatized to trimethylsilyl (TMS) oximes as described below. Derivatization reagents were supplied by Sigma.

Plant materials

Ten year old *Agave durangensis* plants were harvested in the wild, in the zone of Nombre de Dios, Durango, Mexico.

Extraction of *Agave* fructans

The pines of *Agave* were cut off, the cuts were small and uniform (2×2×2 cm). Five kilograms of pine produced from mature *A. durangensis* heads were placed in a container with 10 L of distilled water at 75°C and heated for 3 h to extract the fructans content. The obtained juice was then filtered through a filter paper Whatman No. 4 (non.sterile) and stored at -20°C until further analysis (Waleckx et al., 2007).

Isolation of fructans

Four fructan fractions were obtained from the supernatant and four from the pellet by precipitation of individual samples with different amounts of EtOH (final concentrations: 100% v/v, 80% v/v, 60% v/v and 40% v/v) at 4°C overnight. The fructan fractions were collected by centrifugation (5000 g; 10 min), washed twice with the respective EtOH concentration and freeze-dried to give a white product (Wack and Blaschek, 2006).

TMS Derivatization

A test tube containing 500 µL of extract, 57 µL of 2 M acetic acid and 20 µL of inositol (as internal standard) was placed on a heating block for 45 min at 75°C and the solvent evaporated to dryness with a stream of dry nitrogen. Sugars were initially converted to their oximes by the addition of 500 µL of methoxyamine hydrochloride (25 mg/mL in pyridine) and heated for 30 min at 70°C. Sugars were then trimethylsilylated with a mixture of 900 µL HMDS (hexamethyldisilazane) and 100 µL TFA (trifluoroacetic acid), and

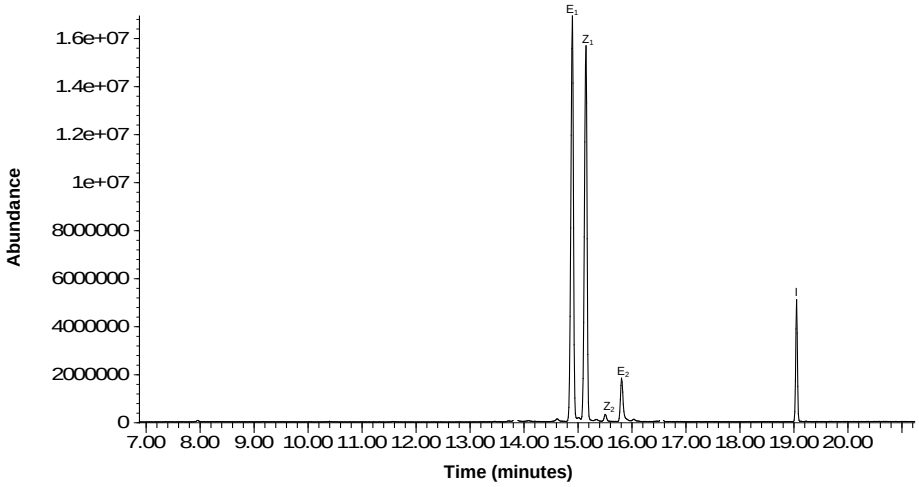

Figure 1. Capillary GC-MS separation of oxime-TMS derivatives of fructans from *Agave durangensis*. Peak identification; E_1: *syn* isomer of fructose; Z_1: *anti*-oxime isomer of fructose; E_2: *syn* isomer of glucose; Z_2: *anti*-oxime isomer of glucose, and I: inositol as internal standard.

the resultant mixture was heated for 1 h at 100°C (5, 8, 12). One microliter was injected on a gas chromatograph and separated on a 15 m x 0.25 mm x 0.25 µm DB-1 column (Hewlett-Packard) with an initial temperature of 150°C for 4 min followed by a temperature program: 4°C/min until 192°C for 0.5 min, 10°C/min until 240°C for 7 min, and then 10 °C/min until 300°C held for 10 min. The injector and detector temperatures were 260 and 310°C, respectively (Total elution time: 42.80 min).

Standard substances (1 mg/mL): fructose, glucose, inositol, sorbitol, mannitol, mannose, xylose and galactose (Sigma). The ionization spectra of all compounds were compared with those from derivatized standards.

Glycosyl linkage analysis by methylation

One milligram of *Agave durangensis* fructans were dissolved in 20 drops of DMSO, stirred on low speed overnight or until complete dissolution. Derivatization to PAAMs was carried out using the method of Ciucanu and Kerek with some modifications (Ciucanu and Kerek, 1984). Methylation was carried out by subsequent additions of NaOH (prepared from 50% aqueous NaOH in DMSO by sonication and washing twice the precipitate with DMSO) and CH_3I (2 M stabilized by copper, Sigma). Permethylated carbohydrates were extracted once with methylene chloride, washed with water, and dried under a stream of nitrogen. Those derivatives were hydrolyzed under acidic conditions with 2 M TFA at 121°C for 2 h. Reduction was carried out with $NaBD_4$ dissolved in 1 M NH_4OH at room temperature for 3 h. Excess borate was neutralized with acetic acid, and the products were taken to complete dryness with repeated addition of 9:1 acetic acid in a methanolic solution. Acetylation was performed at 50°C for 20 min using 250 µL of acetic anhydride and 250 µL of concentrate TFA. The products were extracted with methylene chloride; the organic phases were washed with water and dried under a stream of N_2. The derivatized carbohydrates were separated and identified by GC-MS. Samples were dissolved in 100 µL of methylene chloride, and 1 µL was injected into the GC-MS. Derivatized monosaccharides were separated on a 30 m x 0.25 mm i.d. x 0.25 µm SP-2330 column (Supelco, Bellefonte, PA), using helium as the carrier gas at 2.5 mL/min. The oven temperature was 80°C for 2 min and then ramped at a rate of 30°C/min to 170°C and then at 4°C/min to 240°C and held for 20 min. Injector and detector temperatures were 300°C, and column head pressure was kept at 5 psi.

MALDI-TOF-MS Analysis

Five hundred microliters of fructans from agave were dissolved in 200 µL of DMSO, purged with dry nitrogen, and sonicated for 10 min. After this time, 300 µL of NaOH and 150 µL of CH_3I were added to the sample, stirred and sonicated again for 15 min. The products were extracted with methylene chloride; the organic phases were washed with water and dried under a stream of N_2. Permethylated glycans were dissolved in 25 µL of 100% methanol, and the matrix was 2,5-dihydroxybenzoic acid; sample mixtures from 0.5 to 1 µL were applied onto the plate and quickly dried under N_2. The sample solution was serially dried with matrix to obtain optimal sensitivity. A mixture of oligosaccharides was used as the calibration standard. MALDI-TOF-MS measurement was performed using a Hewlett-Packard (Cupertino, CA) LDI AOOXP MS in the positive ion mode. The instrument was operated at an accelerating voltage of 30 kV and an extractor voltage of 9 kV. The pressure was ~2.1 x 10^{-6} Torr (Stahl et al., 1997).

RESULTS AND DISCUSSION

Composition of Fructans from *Agave durangensis*

The content of fructose and glucose was determined by gas chromatography coupled to mass (GC-MSof their TMS derivatives Carbohydrates are commonly analyzed by gas chromatography (GC) as their trimethylsilyl (TMS) derivatives. Because tautomeric forms of reducing sugars can produce multiple peaks, approaches have been taken in order to suppress the anomeric center before silylation, the most popular being the formation of oximes from the carbonyl group. The major peak, which always eluted first (Figure 1) was assigned to *syn* (*E*) isomer and

Table 1. Quantification of glucose and fructose presents in the different fractions from fructans of *A. durangensis*.

Sample	% of Fructose	% of Glucose
Fructan raw	98.63 ± 0.30	1.37 ± 0.31
Fructan in 80% of EtOH-H_2O (supernatant)	95.79 ± 1.20	4.86 ± 3.22
Fructan in 60% of EtOH-H_2O (supernatant)	97.05 ± 1.91	2.94 ± 1.68
Fructan in 80% of EtOH-H_2O (pellet)	96.96 ± 1.32	3.03 ± 1.08

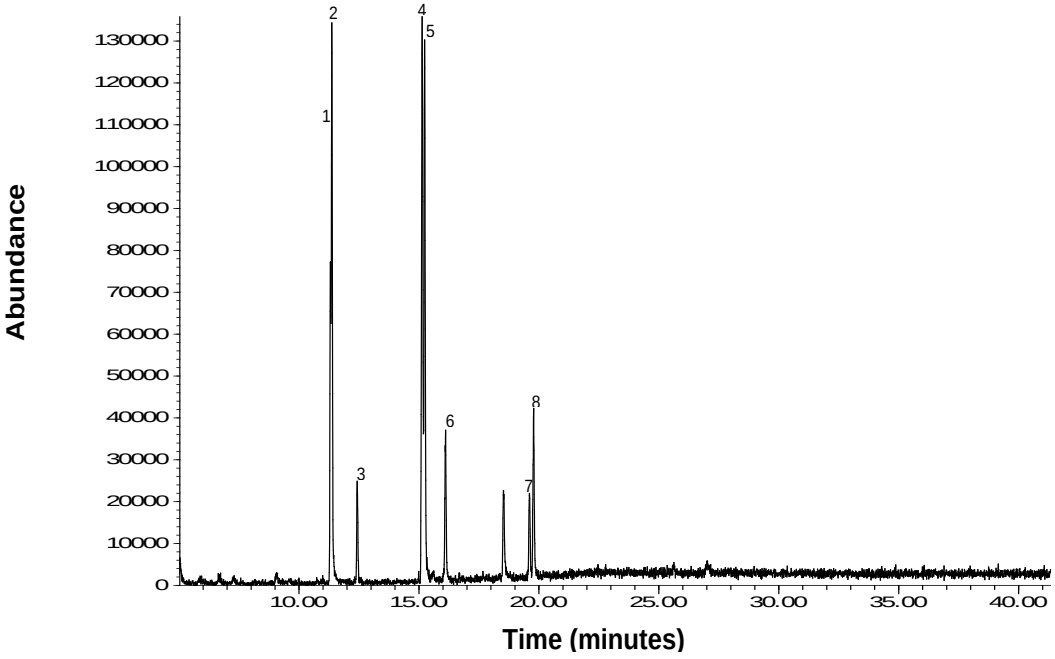

Figure 2. Chromatographic profile of derivatization products of fructans from *Agave durangensis*. Numbered peaks correspond to elution order, and they were identified as indicated in Table 2.

the minor to the *anti*-oxime (*Z*) isomer, according to the results found by Sanz et al. (2003). The *syn* (*E*) and *anti*-oxime (*Z*) isomers produced in the reaction can be separated by GC.

This method has been used to analyze different monosaccharides (Scanlon and Willis, 1985; Willis, 1983). Although several disaccharides have been analyzed as their TMS oximes, GC retention data for these derivatives are relatively scarce. This methodology was used to analyze the four fractions of fructans (100, 80, 60 and 40% in EtOH-H_2O), obtained from the supernatant, pellet and fructan raw extract. The analysis revealed only the presence of glucose and fructose.

The chromatograms of certain fractions showed only the presence of fructose and were discarded from the analysis. These included the 40 and 100% EtOH fractions of the supernatant, and the 40, 60, and 100% fractions of the pellet. The percentages of glucose and fructose in the remaining fractions are shown in Table 1, these results show that fructans from *A. durangensis* contain fructose in a proportion higher than 90%. This table also shows that the fraction of raw fructan, showed the highest content of fructose (98.63 ± 0.30%), because this fraction did not undergo any modification, unlike the rest, which were precipitated with EtOH at different concentrations. Figure 1 shows the different sugars present in the samples, and the fructose and glucose each yielded two peaks, possibly α and β isomers (Chapman and Horvat, 1989), in a 1:1 ratio for fructose and 6:1 for glucose.

Glycosyl linkage types

The results of the methylation analysis (Ciucano and Kerek, 1989) of agave fructans provided highly valuable information on the linkage types presents in *A. durangensis*. Figure 2 shows a typical chromatogram of the reductive cleavage of agave fructans. A good separation of all methylated compounds can be

Table 2. Glycosyl linkage types identified in fructans from *Agave durangensis.*

Peak[a]	t_R[b]	Derivative compound	Fragmentation pattern[c]	Linkage type[d]
1	11.31	2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-*D*-mannitol	129-**162**-**161**-87-101-102-75-145-72-146-205	*t*-β-D-Fru*f*
2	11.36	2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-*D*-glucitol	129-**162**-**161**-87-101-102-75-72-145-146	*t*-β-D-Fru*f*
3	12.42	1,5-di-*O*-acetyl-(1-deuterio)-2,3,4,6-tetra-*O*-methylglucitol	102-129-118-101-145-71-72-87-162-161-**205**	*t*-α-D-Glu*p*
4	15.12	2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methylglucitol 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylglucitol	129-87-**161**-190-162-101-100-71-72-75- 118-**189**	*(2-1)/(2-6)*-β-D-Fru*f*
5	15.24	1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylmannitol	129-87-161-**190**-101-100-71-72-75-145	*(2-1)*-β-D-Fru*f*
6	16.10	1,5,6-tri-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methylglucitol	102-118-129-87-101-**162**-71-189-145-233	*i*-α-D-6-Glu*p*
7 y 8	19.60 19.78	1,2,5,6-tetra-*O*-acetyl-(2-deuterio)-3,4-di-*O*-methylhexitol	129-87-**190**-**189**-100-99-60-71-72	1,6-di-β-D-Fru*f*

[a]Peak numbers correspond to the elution order shown in Figure 3. [b]Retention time (minutes) in the SP-2330 column. [c]Values in black color are the fragmentation primary patterns. [d]*t*, terminal; *i*, internal.

observed.

Table 2 shows all derivatized compounds found. 2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-D-mannitol (Peak 1) and 2,5-di-*O*-acetyl-(2-deuterio)-1,3,4,6-tetra-*O*-methyl-D-glucitol (Peak 2) were the products of a terminal fructose (*t*-β-D-Fru*f*). 1,5-di-*O*-acetyl-(1-deuterio)-2,3,4,6-tetra-*O*-methylglucitol (Peak 3) resulted from the presence of a terminal glucose (*t*-α-D-Glu*p*). The compound 2,5,6-tri-*O*-acetyl-(2-deuterio)-1,3,4-tri-*O*-methylglucitol and 1,2,5-tri-*O*-acetyl-(2-deuterio)-3,4,6-tri-*O*-methylglucitol (Peak 4) indicated the existence of branches in the fructans, while, the presence of internal glucose was confirmed by the compound 1,5,6-tri-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methylglucitol (Peak 6). Finally the compound 1,2,5,6-tetra-*O*-acetyl-(2-deuterio)-3,4-di-*O*-methylhexitol (Peaks 7 and 8) was due to the presence of a 1,6-di-β-D-fructofuranose. The derivatization products of *Agave durangensis* were compared with those from well-studied *Agave tequilana* Weber var. *azul* (López et al., 2003; Mancilla-Margalli and López, 2006). The fructan structural characteristics determined for *A. durangensis* coincided with those reported for other *Agave* species; the linkages, the fragmentation patterns and degree of polymerization were similar. The identity of each compound derivative was determined by comparison with standards and fragmentation patterns of spectra generated by gas chromatography coupled to mass spectrometry, and can be seen in Table 2.

In this study the fractions of fructans (80, 60 and 40% from the supernatant, and 80% from the pellet) analyzed that contained some glucose all had the same number of peaks (eight peaks), and each had the same retention time compared with other fractions, the differences were only in the abundance of each peak.

Reduced fructose produces mannitol and glucitol epimers, in the case of the terminal β-D-fructofuranose (*t*-β-D-Fru*f*), both epimers were resolved well in the column used (SP-2330) and correspond to peaks 1 and 2 (Figure 2), indicating the presence of short chain fructans [~DP3-10 (14)]. These molecules are characterized by the presence of a doublet at m/z 161 and 162 as primary fragments and doublets at m/z 145 and 146, and m/z 101 and 102 as secondary fragments, which can be observed in the spectra of Figure 4.

The elution of peak 3 corresponds to the terminal α-D-glucopyranoside (*t*-α-D-Glc*p*) with a primary fragment at m/z 205. (2-1)-β-D-fructofuranosyl and (2-6)-β-D-fructofuranosyl linkages were found in peak 4, with primary fragments at m/z 161 and 189 respectively, these linkages indicate the presence in *A. durangensis* of the Neo-fructans class.

The fragmentation pattern of an aditional peak (Peak 6) indicates the presence of internal α-D-6-glucopyranose (*i*-

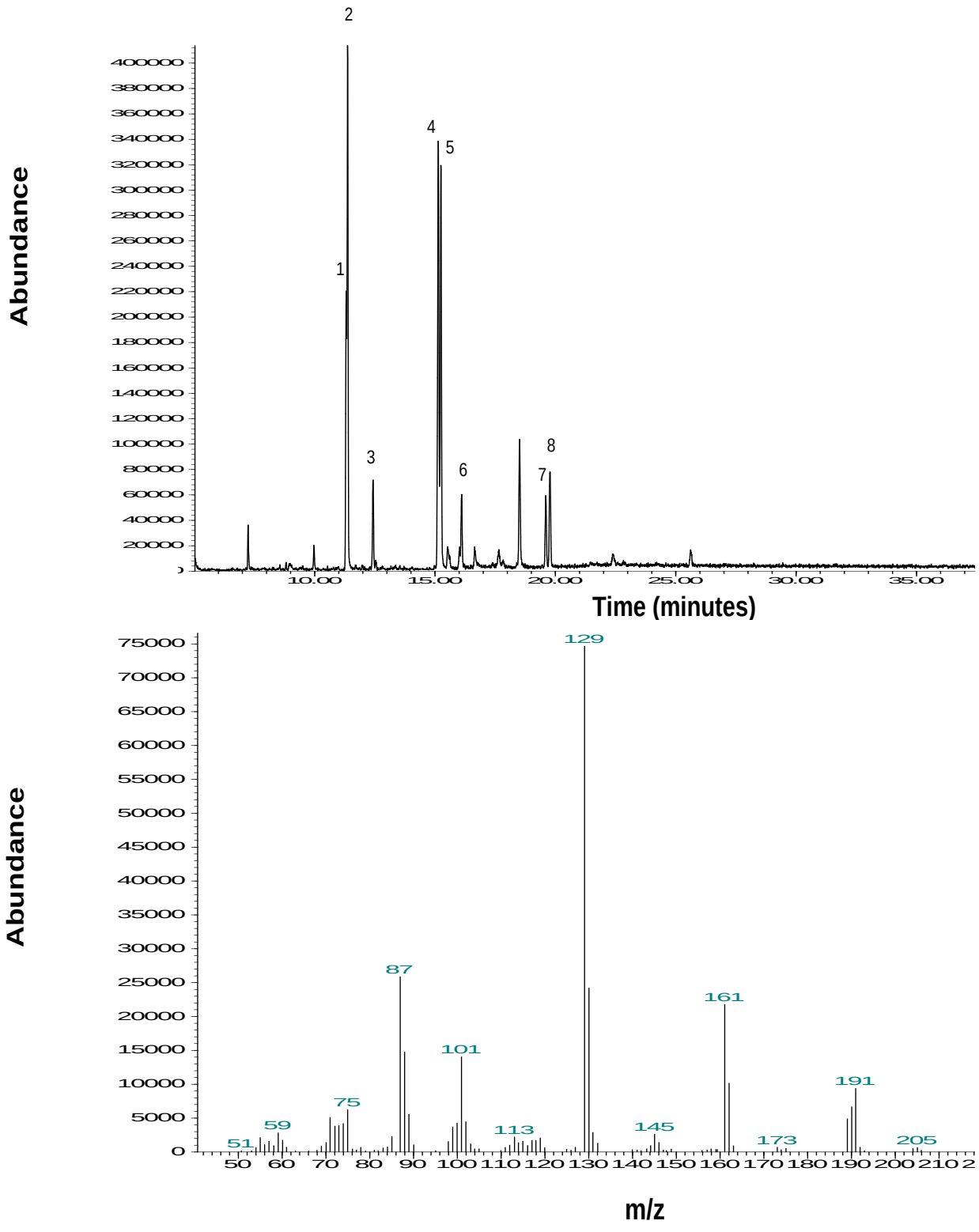

Figure 3. Separation by GC-MS of derivatives of fructans from *Agave durangensis* (a); fragmentation primary patterns (b).

α-D-6-Glu*p*), with a fragment at m/z 162, indicative of an additional acetyl group in the C_6 position (Figure 3). Finally, 1-6-di-β-D-fructofuranosyl linkage was identified in peak 7 and 8, meaning the presence of branched fructans. The six different linkages above were found in all fructan fractions differing only in the abundance of

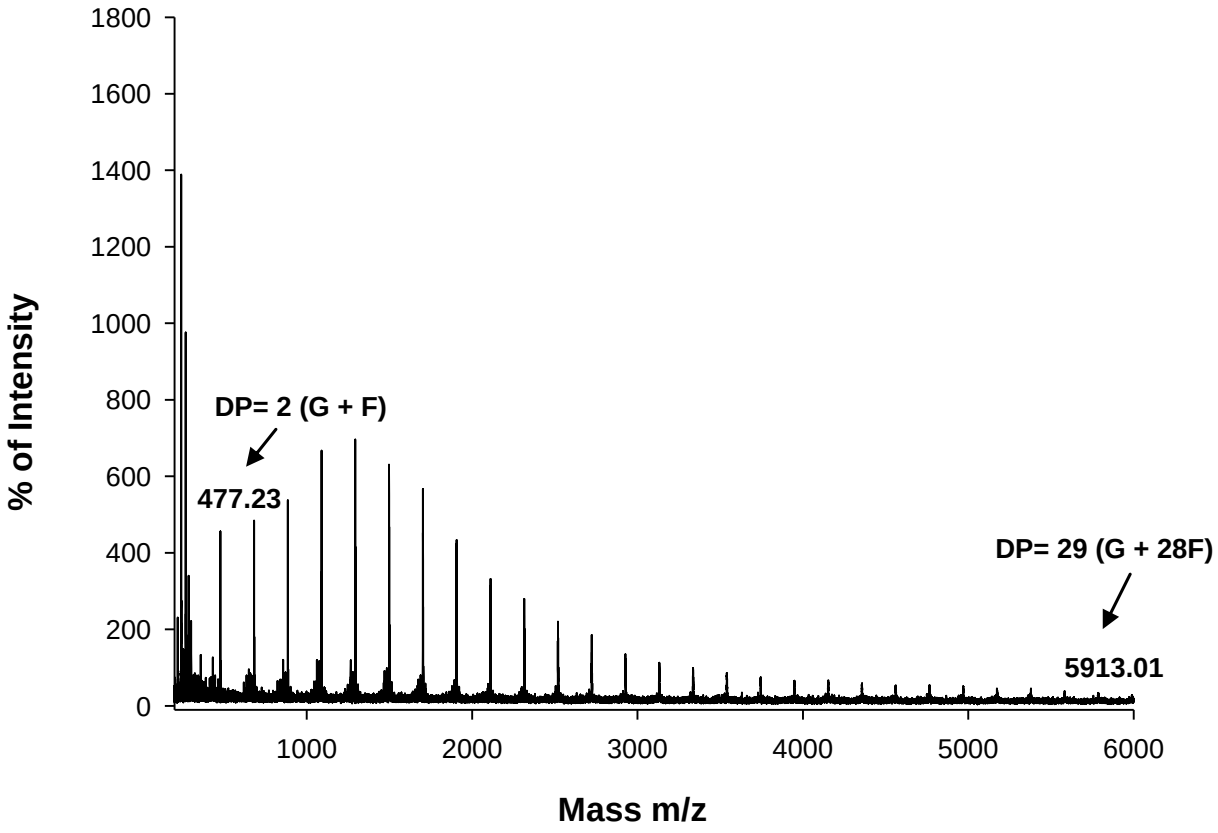

Figure 4. MALDI-TOF-MS mass spectrum of fructans from *Agave durangensis* recorded with 2,5-dihydroxybenzoic acid as the matrix. Numbers in parentheses are the number of fructose units in each fructooligosaccharide.

each peak. These linkages types are characteristic of species included in the *Asparageles* order and *A. tequilana* (López et al., 2003).

Degree of polymerization (DP) of fructans

Among all of the different analytical measurements performed with agave fructans, MALDI-TOF-MS proved to be the best option for establishing the DP distribution of these types of carbohydrates. Since 1991, MALDI has also been successfully applied to glycan analysis and is a superior technique if complex mixtures of oligo- or polysaccharides are to be analyzed as such (Stahl et al., 1997). The spectrum of masses of *A. durangensis* fructans is shown in Figure 4. It can clearly be seen that the extract displayed a complex mixture of fructans molecules; this mixture presented a molecular weight distribution of 273-5936 Da, which corresponds to a range of degree of polymerization (DP) from 2 to 29 units.

Finally, it is important to mention that the physiological functions of fructan metabolism in agave plants need to be studied carefully, because they could point to many other relevant functions such as resistance under the adverse conditions where most agave plants grow.

Conclusions

The fructans from *A. durangensis* are constituted by: 82% of water soluble carbohydrates (WSC), only fructose (97.11 ± 1.17%) and glucose (2.89 ± 1.31%) sugars; the presence of the β(2-1) and β(2-1/2-6) linkages and a molecular weight distribution of 273-5936 Da, which corresponds to a range of degree of polymerization (DP) from 2 to 29 units. These fructans are a complex mixture of oligosaccharides from Neo-fructans type. The presence of different linkages, including β(2-1) and β(2-1/2-6), the former being the most abundant, as well as GC-MS data allowed the establishment of the fructan type present in *A. durangensis*.

Conflict of interest

The authors have not declared any conflict of interest.

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Review

Rizobacteria in the control of pest insects in agriculture

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Biological control of pest insects in agriculture is the focus of many studies, because of the risks in the continuous use of synthetic insecticides, which can cause resistant pests or the occurrence of secondary pests. The use of microorganisms like endophytic bacteria has been performed separately or combined with other forms of control in the Integrated Pest Management. Endophytic bacteria live inside host plants, without causing any apparent damage or pathogenicity symptom. Besides performing many functions important to the host, these microorganisms are potentially useful in agriculture, since they are capable of substituting chemical products. By performing biocontrol actions and/or promoting plant growth, these microorganisms are favoring environmental preservation and are thus identified as a viable alternative for ecologically and economically sustainable agricultural production systems. Given the above, this review aimed to present a panorama of the potential application of plant growth-promoting bacteria in the control of pest insects in agriculture, in view of the great biotechnological advances.

Key words: Endophytic bacteria, plant growth promotion, biological control.

INTRODUCTION

The interest in the use of microorganisms in agriculture has increased significantly in the last years, because both in plant growth promotion and insect biological control, among other applications, they are potential substitutes of chemical products, thus favoring environmental preservation (Peixoto Neto et al., 2002; Souza, 2001). These microorganisms have the important property of providing protection to plants, either by the presence of endophytic microorganisms in host plants or by the application of biocontrol agents, which can result in the elimination of important agricultural pests (Souza, 2001). Due to this, there has been a great interest in the study on occurrence, colonization potential and the use of endophytic bacteria to promote plant growth and pest biological control in agriculture.

Microbial, especially bacteria-based, insecticides have been seen since the 1970s as an excellent alternative of biological control, considering the resistance developed by insects to chemical pesticides. Many products based on these microorganisms are available in the market to control a diversity of pests (Federici et al., 2010), and these products have reached a higher level in the global

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market because of the ecological and environmental demands.

The problems caused by the use of insecticides to guarantee high yields in agriculture over time have led to important
studies in the search for alternate, safe methods in the control of insects (Machado et al., 2012). The use of plant growth-promoting microorganisms is one of the alternatives for modern agriculture to face the challenge of increasing crop yield with sustainability. Among these microorganisms, there are the endophytic bacteria, also known as plant growth-promoting bacteria (PGPB). These bacteria, which are found inside plant tissues, especially leaves, branches and stem, without causing any apparent damage to the host, have a great potential for insect biological control, and the host-plant protection promoted by these endophytes is very complex (Azevedo et al., 2000). The capacity for biocontrol and plant growth promotion of these endophytic microorganisms can come from various mechanisms, such as: biological nitrogen fixation (Huergo et al., 2008; Han et al., 2005), phosphate solubilization (Rodriguez et al., 2004; Vasselev and Vassileva, 2003; Vassey, 2003), production of growth hormones like auxins, gibberellins and cytokinins (Donate-Correa et al., 2004; Radwan et al., 2004; Creus et al., 2004; Dobbelaere et al., 2003), synthesis of siderophores (Vessey, 2003), among others.

However, the role of these endophytes microorganisms in plants has been much discussed but little understanding and studies have been developed to further understand PGPB potentialities for the biological control of insects. Therefore, the use of PGPB will probably be one of the most important tactics in the current world, due to the emerging demand to decrease the dependence on chemical products and to the need for the development of a sustainable and productive agriculture (Moreira and Siqueira, 2006).

ENDOPHYTIC BACTERIA ASSOCIATED WITH PLANTS

The bacteria that colonize internal plant tissues without causing damages to the host are called endophytic (Schulz and Boyle, 2006; Strobel et al., 2004). This definition includes all bacteria living inside a plant in at least one period of the life cycle, and can be found in the rhizosphere, root surface or even inside root tissues, stems and leaves of plants (Berg et al., 2005; Okunishi et al., 2005; Sessitsch et al., 2002). Some of these bacteria have positive effects on plant development, producing substances that favor plant growth and/or avoid the development of pathogenic organisms (Pavlo et al., 2011; Hallmann, 2006; Ping and Boland, 2004).

Endophytic bacteria can develop their entire cycle in a host plant, depending on it for development and reproduction, in this case called obligatory endophytes, or develop part of their cycle outside a host plant, being called facultative endophytes. The division of the term into facultative and obligatory endophyte was proposed to distinguish, respectively, strains capable of colonizing both the surface and the inside of roots and able to survive in soil, from the ones that do not survive in the soil, but colonize the inside and the shoots of plant tissues without causing pathogenicity symptoms (Baldani et al., 1997).

The capacity to colonize the interior of a plant can provide endophytic bacteria with ecological advantages over the others, because internal plant tissues provide a more uniform and protected environment for micro-organisms compared with the surface, where they are exposed to extreme environmental conditions of temperature, ultraviolet radiation and microbial competition, which are the most limiting factors for the survival of bacteria over time (Cocking, 2003).

Despite systematically colonizing plants, endophytic bacteria have a preference to colonize certain tissues. Kuklinsky-Sobral et al. (2004) observed, in soybean, that the density and diversity of endophytic bacteria vary according to tissue, plant development stage, seasonal changes and host genotype, where the observed bacterial density was higher in roots in lower in leaves. Endophytic bacteria can change physiological and morphological conditions in the host, besides acting in other microorganisms living inside plants (Andreote et al., 2006).

The intimate plant-endophyte relationship has shown interesting characteristics for biotechnological and agricultural applications, like the promotion of plant growth, because both organisms benefit from it, that is, bacteria can provide nutrients to plants, through biological nitrogen fixation for instance, while plants provide carbon-rich exudates to bacteria. The plant-endophyte relationship is not fully comprehended yet, but apparently neutral and beneficial relationships are known to exist (Melo, 1998).

BIOTECHNOLOGICAL POTENTIAL OF ENDOPHYTIC BACTERIA

Endophytic microorganisms were first mentioned in the beginning of the 19th century, but Bary (1866) was the one who first outlined a possible distinction between them and plant pathogens. Defined as asymptomatic, they remained almost forgotten until the end of the 1970s, when, for a number of reasons, they started to draw attention. At this time, it was verified that, far from mere inhabitants of plant interior, they had properties of interest, such as providing protection against pest insects (Azevedo et al., 2000), which made evident their biotechnological potential.

Endophytic microorganisms are potentially useful to agriculture and industry, particularly to food and pharmaceutical sectors; many selected endophyte species have the potential to be used in agrochemical industries, besides being used as genetic vectors (Souza et al., 2004). These microorganisms can produce toxins, antibiotics and other pharmaceuticals, besides performing other functions important to the host, such as providing higher resistance to stress conditions, changing physiological properties and producing phytohormones (Azevedo et al., 2000).

The systematic resistance induced against a broad spectrum of pests is acquired after appropriate stimulation and has been reported as the explanation for the control of pest insects in agriculture. The modification in the structure of cell walls (deposition of lignin) and the biological and physiological changes lead to the synthesis of proteins and chemical substances involved in plant defense mechanisms.

The potential to use PGPB in the control of pest insects has been related to stimuli generated in the plant itself, through the action in different metabolic routes (salicylic acid, jasmonic acid and ethylene). These compounds act as elicitors to induce defense and/or resistance, which are kept inactive in their absence. This process, called resistance induction, causes plant to produce or increase the production of proteinase-inhibitor compounds (pathogens produce extracellular proteinases and, in response to their action, plants synthesize inhibitors like serine, cysteine and aspartate), glycoalkaloids, polyphenols etc.

In addition, the response induced by the plant can involve other mechanisms like the accumulation of secondary metabolytes (synthesis of siderophores, phytoalexins and phenylpropanoids) and biosynthesis of pathogenesis-related proteins (PR-protein). Phytoalexins, considered as a microbial property, accumulate in the infection site and around it. Phenylpropanoids catalyze the formation of trans-cinnamic acid – precursor of various plant defense compounds. Pathogenesis-relate proteins (peroxidase of phenols) are associated with processes related to cell wall and lignification of plant cells during the defense reaction against the pathogenic agent – the formation of papilla and hypersensitive response –, making difficult pathogen entrance, establishment and development in the host plant.

The elucidation of the ecological functions of these endophytic microorganisms can bring benefits, especially to the exploration of their biotechnological potential as plant-growth promoting agents (Peixoto Neto et al., 2002), and the characteristics of the growth-promoting potential can be analyzed through molecular techniques, with the quantification of genes involved in the desired characteristics. The biochemical tests are also useful in the search of microorganisms with the capacity to produce indoleacetic acid (IAA), phosphate solubilization, nitrogen fixation (Kuklinsky-Sobral et al., 2004), resistance induction, biological control of pests and diseases (Ramamoorthy et al., 2001) and production of siderophores (Compant et al., 2005).

BIOLOGICAL CONTROL AS A MECHANISM OF PLANT GROWTH PROMOTION

Chemical pesticides have been used in agriculture for a long time (Grigoletti Junior et al., 2000); however, besides their risks to human health, they also cause strong environmental imbalances, destructing the natural enemies of the different crop pests where they are applied. Chemical control can cause damage to the microbiota that is beneficial to plants, besides frequently leaving residues in the environment (Ethur et al., 2007) and in foods. Thus, the use of bacteria as an action of biocontrol and/or promotion of growth has been identified as a viable alternative for ecologically and economically sustainable agricultural production systems (Sousa et al., 2009; Compant et al., 2005), and the biological control through antagonists has allowed viable solutions for many pests considered difficult to control.

Bacteria, especially the genus *Bacillus*, have significant participation among the commercialized biological control products. Up to 50% of these products are bacterial formulations, from various species of *Bacillus.* The identification of different action mechanisms can lead to the combination of isolates to control a broad spectrum of pests (Lutz et al., 2004). Biotechnology can contribute to biological control, transforming microorganisms, so that they express more than one gene responsible for the desired characteristics, combining different action mechanisms (Timms-Wilson et al., 2000).

ENDOPHYTIC BACTERIA IN THE CONTROL OF PEST INSECTS IN AGRICULTURE

In Brazil and worldwide, many studies have described the use of different endophytic and/or plant growth-promoting bacteria in the control of pest insects references. In the study of Bong and Sikorowski (1991), it was found alteration in larval growth and reduction in the emergence of adults of *Helicoverpa zea* caused by *Pseudomonas maltophila*.

In 2006, Thuler et al. (2006) verified that the isolates EN4 of *Kluyvera ascorbata* and EN5 of *Alcaligenes piechaudii*, little reported in the literature in studies on insects, reduced the viability of *Plutella xylostella* in about 80 to 50%, respectively, indicating a broad field of research to explore the potentials of endophytic bacteria. Selecting and characterizing Brazilian strains of *Bacillus thuringiensis* toxic to *P. xylostella*, Praça (2012) found that the *B. thuringiensis* strains S1905 and S2122 caused

100% of mortality in caterpillars in the 3º instar of *Plutella xylostella* in evaluation performed 48 h after caterpillars had been exposed to selective bioassays, whereas S2124 caused 58.33% of mortality after the same period and 98.33% of mortality after 96 h. In these cases, CL_{50} values ranged from 2.33 to 4.84 ng/mL, with results of toxicity similar to the control. Viana et al. (2009) found that, from the 58 isolates of *B. thuringiensis* tested in caterpillars of *Plutella xylostella*, 12 caused 100% of mortality within 24-48 h: 3A.140, T3A.259, T08.024, E1, E26, 2.7L, 1.7L, E22, 22.7L, 49.19A and E2. Castelo Branco et al. (2003) observed 100% of mortality for larvae of *Plutella xylostella* in the 2º instar with the application of *B. thuringiensis.*

Recently, Macedo et al. (2012), selecting and characterizing native strains of *B. thuringiensis* toxic to *Diatraea saccharalis* (Lepidoptera: Crambidae), observed that the strains causing more than 75% of mortality after dilution of 50 times were: S602, S1264 and S1301. From these strains, the most toxic to *D. saccharalis* were S602 and S1264, with statistically similar CL_{50} values, but different from S1301. Melatti et al. (2010), selecting strains of *B. thuringiensis* for the control of cotton aphid (*Aphis gossypii*), found that the strains S29, S40, S616, S1576 (*Bacillus aizawai*) and S1168(*Bacillus kurstaki*) were the most toxic to *Aphis gossypii*, causing mortality higher than 50%. Among the analyzed strains, S29 and S1168 were the most effective in the selective bioassay, causing mortalities of 76 and 73% against *A. gossypii*, respectively.

Despite the various studies involving the lepidopteran *Spodoptera frugiperda* and its control, it is difficult to find bacterial isolates pathogenic to this species. This claim is confirmed in the study of Polanczyky et al. (2003), using 58 subspecies of *Bacillus thuringiensis* in *S. frugiperda*, which showed that only the *Bacillus thuringiensis morrisoni* caused 80% of mortality in caterpillars. Also, Berlitz et al. (2003), testing 24 isolates of *Bacillus thuringiensis* from many rice-growing regions of Rio Grande do Sul, Brazil, in the control of *S. frigiperda*, obtained the best mortality rates between 31.6 and 100% with only five isolates. Campanini et al. (2012), studying the pathogenicity of isolates of *B. thuringiensis* over *Spodoptera frigiperda* and *Sphenophorus levis* found that the isolates IB17.3 and IB8.2 are highly efficient in the control of caterpillars of *S. frugiperda*, and that the isolate IB26.2 is the most efficient in the control of larvae of *S. Levis*, all of them with average mortality rates higher than 75%.

However, the fact that an isolate causes mortality to caterpillars does not mean that they will be active in the insect when the toxic proteins are purified. The CL_{50} of Cry proteins of *Bacillus thuringiensis aizawai* for caterpillars of *S. frugiperda* in the 3º instar was determined by Lucho (2004). The obtained results indicated CL_{50} of 2.22; 0.41 and 0.18 µg/mL for 2, 3 and 4 days after treatment application, respectively, and revealed that the proteins Cry1Aa, Cry1Ab, Cry1C and Cry1D, synthesized by *Bacillus thuringiensis aizawai*, are highly toxic to *Spodoptera frugiperda*. For the same species, data from Knaak et al. (2007) show that the toxicity of the proteins Cry1Ab and Cry1Ac, synthesized by *Bacillus thuringiensis aizawai* 407 and *Bacillus thuringiensis kurstaki* HD73, respectively, revealed a CL_{50} of 9.29 and 1.79 µg/cm^2 to caterpillars in the 1º instar.

Analyses of mortality of caterpillars of *Anticarsia gemmatalis* caused by isolates of *Bacillus thuringiensis*, performed by Azambuja and Fiuza (2003), showed 37 and 50% of corrected mortality against velvetbean caterpillar using two natural isolates of *Bacillus thuringiensis* from rice-growing regions of Rio Grande do Sul, Brazil. In the pathogenicity evaluations performed with primitive isolates, Silva and coworkers (2004) revealed high numbers of isolates pathogenic to the same insect order. Praça et al. (2004) verified that, among the 300 tested strains of *Bacillus thuringiensis*, only S234 and S997 were simultaneously effective in the control of larvae of *Spodoptera frugiperda, Anticarsia gemmatalis, Anthonomus grandis, Aedes aegypti* and *Culex quinquefasciatus*.

For *Oryzophagus oryzae*, little data is reported in the literature with respect to the action of proteins of *Bacillus thuringiensis*. Pinto and coworkers 2003 selected 6 isolates of *Bacillus thuringiensis* with the presence of genes from the class *cry*3 or *cry*7, which synthesize proteins insecticidal to coleopterous, and evaluated their insecticide activity to *O. oryzae*. From the tested isolates, two caused corrected mortality of 100%, three between 59 and 67% and one around 50%. Steffens et al. (2001) obtained 53.41% of mortality of larvae of *O. oryzae* with an isolate of *B. thuringiensis* containing *cry3* genes, specific to coleopterous. Results obtained by the different authors confirmed the prediciton of the insecticide action of *B. thuringiensis*, possibly due to the presence of the *cry3* and *cry7* genes, which codify proteins specific to coleopterous.

References on endophytic bacteria against isopterous are restricted, with little data available, such as Castilhos-Fortes et al. (2002). Considering the pathogenicity of *Bacillus thuringiensis* for *Nasutitermes ehrhardti*, these authors tested 57 strains of this bacteria, and found the seven most effective: *B. thuringiensis sooncheon* (Bts) and *B. thuringiensis roskildiensis* (Btr) with a mortality of 100%; followed by the isolates *B. thuringiensis yunnanensis* (Bty) with 71.4%; *B. thuringiensis huazhongensis* (Bth) with 57.1%; *B. thuringiensis brasiliensis* (Btb) with 52.3%; *B. thuringiensis colmeri* (Btc) with 42.85% and *B. thuringiensis kurstaki* (Btk) with 28.57% of mortality at the 7th day after treatment application. For the determination of CL_{50} of *B. thuringiensis*, these authors used the isolates *B. thuringiensis sooncheon* and *B. thuringiensis roskildiensis*,

which caused 100% of mortality in the pre-selective assays. The observed CL_{50} values of *B. thuringiensis sooncheon* were 46.98×10^8, 66.19×10^6 and 5.14×10^5 spores/mL, at 3, 5 and 7 days after treatment application. For *B. thuringiensis roskildiensis*, the values were 30.78×10^5, 48.40×10^6 and 16.80×10^4 spores/mL at 3, 5 and 7 days, respectively.

Besides all the above mentioned information, endophytic and/or plant growth-promoting bacteria can be used in combination with other microorganisms in the control of pest insects in agriculture. In this context, Broderick et al. (2000) identified an increase of 35% in the mortality of the lepidopterous *Lymantria dispar* (L.) when using *B. thuringiensis* and zwittermycin A of *Bacillus cereus*, which is responsible for the synergetic effect of the microorganisms. Results from Wraight and Ramos (2005) also show synergism of 35.2, 33.8, and 21.1% when commercial products based on *B. thuringiensis* and on the fungus *Beauveria bassiana* were simultaneously used in *Leptinotarsa decemlineata*. These authors reveal that the interaction may have resulted from the intoxication caused by entomopathogen, inhibiting insect feeding, thus causing stress, and physiological effects, which facilitated fungus penetration in the insect. Similar effects were also observed by Ma et al. (2008), when the Cry 1Ac protein of *B. thuringiensis* was used with *B. bassiana*. These authors observed deleterious effects in the mortality of larvae of *Ostrinia furnacalis* (Lepidoptera: Crambidae), besides the decrease in the formation of pupae and emergence of the adult insects.

These data indicate that studies on PGPB are increasingly important, since chemical insecticide application results in large impacts on the ecosystem, because they not only affect the natural enemies of the insects, but also contaminate soil and underground waters.

CONCLUSIONS

Given the information and the promising results already obtained with respect to the interaction between bacteria and host plants, the study on endophytic bacteria as biocontrol agents for inumerous pests and as plant growth promoters has been gaining special attention. The practical applications of these microorganisms tend to increase, as the aspects of this interaction become better understood. Therefore, it is essential to know the diversity of these endophytes, their presence, frequence and functions, because this understanding will allow expanding the spectrum of use of endophytes as a biotechnological tool, aiming to increase yields and decrease the use of agrochemicals, besides providing an efficient, economic and ecological alternative for the solution of damages caused by pest insects in agriculture.

Conflict of Interest

The authors have not declared any conflict of interest.

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Review

Soya bean glycine max (l.) Merr. genetic resources in Tanzania, 1905-2013

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Soya beans, first grown in what is now Tanzania early in the twentieth century, have always been a minor crop in area planted and total production. German East Africa introductions before 1918 were from Japan, China and Rhodesia. Introductions by the British Trusteeship of Tanganyika in 1938 and 1939 were from India, South Africa, the USA and the Far East. Hernon 237 was introduced in 1950 to replace the American variety Dixie. Experiments at Nachingwea by the Overseas Food Corporation resulted in development of high-yielding and locally adapted varieties in the 1950s and 1960s. Research at Kilosa in the 1970s, based on Nachingwea varieties, emphasized intercropping, fertilization and promiscuous nodulation. The international agricultural research institutions was assisted with introductions and expertise during the 1980s. Soya research has shifted among research centres several times but in the twenty-first century, only Uyole in the southern highlands, with limited human, financial and material resources, works on soya breeding. Two strains from this centre plus the Bossier variety are the only types registered by the national plant certification authority. Smallholder farmers do their own research and adaptive trials with Zambian material. Some large scale farmers grow soya as an alternative crop and to supply protein to the animal feed industry. In spite of oft-expressed intentions by government to support soya and publication of a national soya development strategy in 2010, there has been little increase in area planted and in output. Producers continue to face problems in seed supply, technical advice and marketing.

Key words: Biodiversity, plant breeding, varietal trials, promiscuous varieties, *Rhizobium*, nodulation.

INTRODUCTION

Much of Tanzania in terms of rainfall, temperature and soil type is suitable for soya bean *Glycine max* (L.) Merr. cultivation. Cultivated area and total production are insignificant, however, compared to the major cereal staples (maize, millet and rice) and grain legumes (groundnuts, common beans, sesame and pigeon pea), such that soya is, and always has been, a minor crop in Tanzania. Soya bean contributes, nonetheless, to national and household food supply, provides income, adds diversity to arable systems and fixes nitrogen that

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improves soil fertility and condition (MAFC, 2010). Much of Tanzania is suitable for soya cultivation and the bean is indeed grown in most areas. Favoured areas, however, are the Southern Highlands (Iringa, Mbeya, Rukwa and Ruvuma Regions), Morogoro Region, the southern Lindi and Mtwara Regions and the northern Arusha, Kilimanjaro and Manyara Regions (Malema, 2005; 2007).

Production of soya bean is mainly for the domestic market but supply is much less than demand. Formal imports to overcome the deficit are mainly from India and neighbouring countries.

There are also "informal" imports from Tanzania's contiguous states. Most soya is grown by smallholder farmers under rainfed conditions in small plots using traditional husbandry methods and sowing local or "nondescript" varieties that are produced from home saved seed (Chianu et al., 2008). Some larger mechanized farms in the so-called Ihemi Cluster of the Southern Agricultural Growth Corridor of Tanzania (SAGCOT) already produce, or have well advanced plans to produce, soya bean: these are or will be partially vertically integrated operations with some degree of processing and organized marketing (SAGCOT, 2012). Soya bean contributes much less than 1 per cent to Agricultural Gross Domestic Product (GDP) at present but has great potential to bestow much more. In the 50-year period from 1961 to 2011 the area cultivated to soya varied from as much as 16,000 ha to as little as 2000 ha with the greatest area grown in the early 1960s and the smallest in the late 1980s and early 1990s (FAO, 2012). (The larger area planted in the early 1960s is largely due to the 6000 ha grown by the then Tanganyika Agricultural Corporation (TAC) – successor to the notorious Overseas Food Corporation or "Groundnut Scheme" – on its 18 farms around Nachingwea in Mtwara Region (DoA, 1958, 1962; Auckland, 1970): the author of this paper was responsible for the management of two of these farms in this period and grew about 1000 ha of soya). One source quotes annual production of grain soya in the early twenty-first century as being in the range of 3000-5000 tonnes from a cultivated area of 5000-6000 ha (Malema, 2005). Another source, based on Ministry of Agriculture sample surveys, indicates average production over the two years 2003/2004 and 2004/2005 of 1000 tonnes of grain from under 2000 ha equivalent to a yield of 727 kg/ha (ICRISAT, 2011).

This paper reviews the import, export, development and use of soya bean varieties in Tanzania (formerly German East Africa and Tanganyika) over a period in excess of 100 years.

RESEARCH, DEVELOPMENT AND USE OF SOYA BEAN GENETIC RESOURCES

It is reported anecdotally that soya beans were first introduced to what is now Tanzania in 1907. This date is given by innumerable secondary sources but there is no firm reference to support the claim nor is there any indication of who imported them, whence they came and by what route. The first reliable and documented source (Greenway, 1945) states that there were introductions from the USA in 1909 and further introductions from Japan, China and South Africa in 1911 (Table 1). These last introductions, seven from Japan, one from China and three from South Africa, are referred to in more detail in a paper - which is the probable source of the information provided by Greenway (1945) - published by the then German Biological Agricultural Research Institute in Amani (Biologisch Landwirtschaftlichen Institut Amani) (Eichenger, 1912). This latter paper details seven varieties from Yokohama in Japan, five – Natsu-Mame, Shiro Daizu, Ao Mame, Gogatsumame and Daizu – being white and two -- Kuro Teppo and Kuro Mame – bring black, one from China – Chinese white type – and three unspecified varieties from Rhodesia (Eichenger, 1912). There is no reference in this authoritative paper to any earlier introduction to German East Africa nor is there in two earlier papers from the same institute, the one devoted to soya beans in British South Africa (Anon., 1910) (the paper did, however, suggest that soya might shortly become an export crop as a Lever Brothers soap factory intended to buy soya beans produced locally for making oil and soap.) the other being a more general paper on soya and its uses (Reiter, 1910). Another unsubstantiated statement refers to soya beans being introduced to the Masasi and Nachingwea Districts in Mtwara Region (in southern Tanzania) prior to 1918 (MAFC, 2010), that is, prior to German East Africa becoming the League of Nations Territory of Tanganyika under the administration of the Government of the United Kingdom of Great Britain and Northern Ireland (Greenway was working at Amani when he published his papers). No more introductions appear to have been made until 1938 and 1939 when 64 cultivars were established at Amani via direct imports from India, South Africa and the USA together with a large number of American and Far Eastern cultivars arriving from Rwanda (Auckland, 1970). Soya beans were grown in the Bukoba area during the period of the Second World War and, depending on the source of the information did well (Auckland, 1970) or did badly (Mmbaga, 1975).

The Overseas Food Corporation (OFC, colloquially known as the "Groundnut Scheme") presumably accepting that it would get little oil from its eponymous crop started soya bean cultivation in Nachingwea in 1947 but the cultivars used produced low yields and proved unsuitable to the conditions of southern Tanzania. OFC then employed a breeder to improve the country's gene pool of the time. Hernon 237 was introduced from Rhodesia (Zimbabwe) to replace the Dixie variety (Auckland, 1970), which was possibly from the USA

Table 1. Summary of soya bean genetic resources used in Tanzania, 1907-2013.

Time period	Location	Variety
< 1915	Amani	USA – unspecified (1909); China – 1 variety Chinese white type (2); Japan (Yokohama) – Natsu-Mame (white), Shiro Daizu (white), Kuro Teppo (black), Kuro Mame (black), Ao Mame (white), Gogatsumame (white), Daizu (white); South Africa – unspecified
1938-1939	Amani	64 cultivars USA, India and South Africa and via Rwanda from "America" and Far East
1950	Nachingwea	Hernon 237 introduced from Rhodesia [Zimbabwe] to replace Dixie variety (presumably USA variety from 1938-1939 imports)
1957-1963	Nachingwea	New adapted varieties for lowland conditions included IH/192 ("Tanzania's standard line"), 3H/1, 3H/101, 7H/149/1: Hernon 237 x Light Speckled, Hernon 237 x R184 and Benares x Light Speckled produced highest-yielding and most agronomically desirable strains: over 5-year period HLS [Hernon Light Speckled] 219 out yielded parents Hernon 237 by 40% and Light Speckled by 37%
mid 1970s	Miwaleni (Pemba)	Missay, Belgian Congo
1978	???	Recommended varieties were Bossier (from Bossier City, Louisiana, USA) and 3H/1
1982	Sokoine Agricultural University	F_6 lines (IH/192 x Bossier), Caribe, Orba, TCM249-4-6, 245-4-OTRS, Calland, Tunia, Tracey, Hardeel S, CS90, IH/192, Improved Pelican, CH-3; F_7 and F_8 lines to be tested in future
1981-1982	Ilonga and Kilosa area	79S0047 F4, GPM L-2, SI-4, 59S0057 F4-1, TGM 622, TGM 737 x 80, S3-2, 79S0018 F4, GPM L-4-2 and 3H/l
1992	Selian	Bossier, Bossier IL, Duiker, Sable, EAI 3715, Still, Delma, SAB/7, PERY 41
1997-2000	Various locations	AGS292, AGS329, AGS338 and AGS339 lines from AVRDC Arusha Centre provided to research organizations for testing
2002-20013	Uyole	Uyole Soya 1 (SH1), Uyole Soya 2 (SH2)
2005	Ilonga	TGx 1876-4E, TGx 1895-4F, TGx 1895-33F, TGx 1895-49F (IITA varieties developed under TLII project)
2012	Ihemi Cluster	Songea (long season, nodulates with local bean Rhizobium), Squire (good rust resistance but poor germination), SB8, SB19, Solitaire, Dina, Pan
Continuing	Farmer fields	Soyalishe, TGx 1805-8E, Songea, Safari, Bossier, Karea, Kaleya, Ndogo, landraces described on morphology (seed size and colour, hymen colour)
Likely future	Station and farmer fields	WF-L19, Maksoy 2N, Namsoy 4N x Uganda (for release by Uyole); Namsoy, TGx 1740-2F, TGx 1987-64F (from N2Africa/Kenya); Squire, Safari, Spike, Semeki (from SeedCo, Arusha)

Source: Compiled by the author from citations and references in the text.

import in 1938. Breeding new varieties adapted to the lowland conditions of southern Tanzania continued from 1956 to 1963 and several cultivars were released during this period. This included IH/192 (described as

Figure 1. A strong crop of Hernon 237 x Light Speckled soya bean on Farm 4 Nachingwea in March 1965 (Photo: R Trevor Wilson).

"Tanganyika's standard line"), 3H/1, 3H/101, 7H/149/1. Hybrids such as Hernon 237 x light speckled (Figure 1), Hernon 237 x R184 and Benares x Light Speckled produced the highest-yielding and most agronomically desirable strains: over a 5-year period Hernon Light Speckled (HLS) 219 out-yielded its parent Hernon 237 by 40% and its Light Speckled parent by 37% (Auckland, 1966; 1967). The Tanganyika Agricultural Corporation successor to the OFC also grew soya beans at its Urambo site in western Tanzania and at its Rufiji Basin site where it mainly tested the effects of rotations and fertilizer application on a range of oil seed and cereal crops (TAC, 1958; 1960).

Agronomic and health studies continued throughout the 1960s and 1970s and into the early 1980s. At Ilonga to the west of Morogoro attention was given to the use of insecticides for the control of soya bean pests using varieties that had been developed at Nachingwea (Robertson, 1969). Trials to find soya bean varieties adapted to the middle altitudes (as opposed to the lowland coastal and highland southern and northern areas) were carried out in the Morogoro area in the early 1970s (Uriyo, 1974) but no practical outcome appears to have been achieved. A programme was initiated at Sokoine University of Agriculture also in the Morogoro area in the late 1970s and early 1980s in attempts to breed varieties with enhanced nitrogen fixation capability: no such varieties were bred but strong responses to inoculation by extant varieties were observed (Chowdhury, 1977; Chowdhury and Dottu, 1982; Chowdhury et al., 1983). Additional experiments at Sokoine included the varieties Caribe, Orba, TCM249-4-6, 245-4-OTRS, Calland, Tunia, Tracey, Hardeel S, CS90, IH/192, Improved Pelican, CH-3 and of F_6 lines of IH/192 x Bossier with F_7 and F_8 lines to be tested in future (Doto, 1986).

Studies at Lyamungu on the lower slopes of Kilimanjaro in northen Tanzania also showed the benefits of inoculation but best yields were obtained when inoculation was combined with a dressing of 40 kg/ha of phosphorus and without nitrogen (Sachansky, 1977; Ndakidemi et al., 2006). In further experiments it was demonstrated that cultivars bred and selected in Tanzania were compatible with native *Rhizobium* spp. whereas the USA variety Bossier did not nodulate unless it was inoculated. The local cultivars were promiscuous and recognized *Rhizobium* species that were ineffective on USA material but they had low yield potential in contrast to the USA cultivars. The latter were agronomically superior but required inoculation with *R. japonicum* to realize their yield potential and it was suggested that a breeding programme based on transferring the promiscuous character of local cultivars to improved USA material could produce varieties that did not require inoculation and still produce high yield (Pulver et al., 1982; 1985).

Further attempts to improve yields as well as the acceptability of soya beans by local peasant farmers involved intercropping, already a traditional practice using other combinations of cereals and legumes (Finlay, 1975; Jana, 1980; Nyambo et al., 1982; Kang, 1983). One report indicated some 1800 hectares had been sown as trials in intercropping and that 2000 hectares had been sown in a "peanut" area (Freckman, 1975) (Freckman was reporting on a conference held in Ethiopia in 1974 and is thus not a primary source: if the peanut area refers

to the Nachingwea groundnut scheme then the information was clearly very much out of date. He also stated that Japan had been seeking rights to grow soya in Tanzania for export to Japan). Trials were not confined to the mainland, however, as in the mid-1970s at Miwaleni on the island of Pemba experiments involving production of two crops per year under irrigated conditions indicated that an annual yield of more than 3000 kg/ha could be achieved: the best yielding varieties in these circumstances were Missay (3100 kg/ha) and Belgian Congo (2666 kg/ha) (Sachansky, 1976).

By 1967 the research arm in Senegal was testing 23 Tanzania soya varieties for its own conditions and later released some to other West African countries (Larcher, 1980; Larcher et al., 1988). Tanzanian seeds were tested in a trial of 24 collections to examine the effects of fungal infection on subsequent germination characteristics (Paschal and Ellis, 1978). Several Tanzania lines were also tested in the Solomon Islands in the South Pacific Ocean (Holsheimer, 1960) as, probably, was the Nachingwea-bred HLS in Tonga (Anders, 1976). Conversely, 10 years later, Tanzania participated in adaptive trials undertaken in many countries which showed that the Tanzania variety Hernon 237 was one of only ten out of 400 worldwide varieties that were promiscuous in their nodulation (Pulver et al., 1985). In the mid-to late-1970s soya was tested in Tanzania as part of a wide scale international variety testing programme (Judy and Whigham, 1978; Whigham and Judy, 1978; Judy and Hill, 1979).

In an attempt “to increase Tanzanian food production” the United States Agency for International Development and the Tanzania Government allocated US $14.9 million to a seed multiplication project to be managed by a private American company in 1971. One objective was to increase Tanzania’s production of soya beans from the then annual 500 bushels (13.6 tonnes) to 3 million bushels (81 thousand tonnes) as a contribution to the 21 million bushels (567 thousand tonnes) needed to supply oil and protein needs (Soybean Digest, 1971). During this period, with continued financing from the USA, more than 600 soya bean accessions were assembled at Ilonga for studies on yield, disease and insect resistance (IITA, 1982). In 1981-1982 some ten new breeding lines, 79S0047 F4, GPM L-2, SI-4, 59S0057 F4-1, TGM 622, TGM 737 x 80, S3-2, 79S0018 F4, GPM L-4-2 and 3H/l with yields varying from 1962 to 2945 kg/ha considerably outperformed Bossier with a yield of only 1241 kg/ha.

Another source indicates that Tanzanian soya production was 4000 tonnes in 1970, an increase of one third over the 3000 tonnes of 1960-1964 (IRAT, 1972). The National Grain Legume Research Programme became operational in 1974/1975. As part of this programme soya beans returned to Nachingwea when 750 ha were planted on a parastatal and a prison farm with plans to expand large scale mechanized production to 8000 ha over 5 years coupled to the construction of an oil mill to process soya beans, sesame and groundnuts. It was also considered that soya beans were a promising cash crop for ‘ujamaa’ (Tanzania version of social collective) villages (Brockman, 1977). Public research on and government support for soya beans since the 1970s has, however, been spasmodic and inconsistent in spite of oft-repeated official statements of their importance and the need to increase output.

The Agricultural Research Institute (ARI) at Naliendele in Mtwara Region was designated and acted as the coordinating centre for the Oilseeds Research Programme (ORP) in 1978 but its main emphasis in the twenty-first century is sesame and groundnuts. Varietal research has also been undertaken at Ilonga (on at least two discrete occasions, 1960 to 1969 and from 1973 onwards) in Morogoro Region, at the Kilombero Agricultural Research and Training Institute (1968 to 1976) in Morogoro Region, at Lyamungu ARI (from 1978) in Kilimanjaro Region and at Selian ARI in Arusha Region (from 1986) and at the nearby Lambo Estate (Mwandemele and Nchimbi, 1990; Malema, 2005).

A continuation of the breeding programme in the later 1970s comprising a second phase at Ilonga and new work at Lyamungu aimed to obtain new varieties with: high yield; high protein and oil content; resistance to lodging and shattering; resistance to major diseases; good branching habit; medium plant height of 60-90 cm (traditional Tanzania varieties were 130-150 cm tall and took 210-240 days to mature); ability to set pods at 10-15 cm above the ground; and good adaptation to the local environment (Chianu et al., 2008; Malema, 2005).

A programme to find alternatives to the traditional crops was initiated at Selian in Kilimanjaro Region in 1986. Soya beans were included in the mix with variety trials using Bossier, Bossier IL, Duicker, Sable, EAI 3715, Still, Delma, and SAB/7 being carried out at Selian and also at Lambo Estate (Kilimanjaro) where PERY 41 was added to the varieties (Mwandemele and Nchimbi, 1990). Yields were generally low, ranging from 270 kg/ha for Bossier IL to 1430 kg/ha for Duicker.

At the turn of the twenty-first century the International Vegetable Research Centre (AVRDC) provided seeds of four soya lines (AGS292, AGS329, AGS338 and AGS339) to National Research Institutes from its centre in Arusha for further evaluation and future release (Chadha and Oluoch, 2001). Since the beginning of the new millennium except for a short-lived trial at Kilosa in 2005, Uyole ARI is the only public sector institution working on soya bean breeding. Uyole has released two new varieties (which have not proved successful in the real life situation of either small or large farms), these being Uyole 1 in 2004 and Uyole 2 in 2011 and continues to test new lines (Figure 2) although the human (one researcher), material and financial resources allocated to the programme are meagre. The two Uyole varieties and

Figure 2. Soya bean genetic resources in Tanzania: Line 19 under test in 2013 (left) and Uyole 1 released in 2004 (right) (Photo :R Trevor Wilson).

Bossier are the only varieties approved by the Tanzania Official Seed Certification Authority (Catherine Madata, personal communication).

Objective 7 of the Tropical Legumes II Project is "Enhancing promiscuous, multipurpose soybean productivity and production in drought-prone areas of sub-Saharan

Figure 3. Variety Dina showing (left) good nodulation and (right) strong growth at Makota Farm, Southern Highlands (Photo: R Trevor Wilson).

Africa" [Tropical Legumes-II, a project funded by the Bill and Melinda Gates Foundation, is a joint initiative of three international agricultural research centres -- International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, working on chickpea, groundnut and pigeonpea), the International Institute for Tropical Agriculture (IITA, cowpea and soybean) and CIAT (common bean)]. As part of this project several IITA varieties (TGx 1876-4E, TGx 1895-4F, TGx 1895-33F, TGx 1895-49F) along with Bossier were screened for their nodulation and seed production properties at Kilosa during 2005. Experimental plots were very small, averaging 0.28 ha for a total of 1.4 ha and yields were very low in the range of 436 to 861 kg/ha (Malema, 2007).

Some semiformal and frankly informal research and observation trials are undertaken by smallholder farmers. In this case it is clear that the country's most widely grown variety, Safari, which is not licensed in Tanzania, was first introduced in unofficial cross border transactions from Zambia by smallholders acting on their own initiative. Safari, developed by Seed Company Limited in Zimbabwe, is an indeterminate variety with high seed yield potential and is well adapted to altitudes in excess of 900 m, is resistant to lodging, has a long period during which it does not shatter and can thus be harvested with little loss of yield and has good ground clearance of the bottom pods. Seeds are of medium size with a yellow hilum and are of good quality. Other varieties grown by small farmers include Soyalishe, TGx 1805-8E, Songea, Bossier, Karea, Kaleya and Ndogo. In addition many local landraces that are referred to or described by morphology (seed size and colour, hymen colour) are grown in various areas (several smallholder farmers, personal communications). Some varieties, and notably the long-season Songea variety is well known for being promiscuous and is favoured by small farmers for this reason as most attempts to breed such varieties have had little field success and the constant search for variety-specific rhizobia has ended with similar results (Malema, 2005).

Some ("informal") observational trials are undertaken by large farmers in the Ihemi Cluster using both local varieties in addition to others developed in western Kenya and provided by the Centro Internacional de Agricultura Tropical (CIAT) through the N2Africa Programme (N2Africa is also funded by The Bill & Melinda Gates Foundation through a grant to Plant Production Systems, Wageningen University who lead the project together with CIAT-TSBF, IITA and many partners in the Democratic Republic of Congo, Ghana, Nigeria, Kenya, Malawi, Mozambique, Rwanda, Tanzania and Zimbabwe). As with smallholder farmers Songea has been shown to do well in large part because of its ability to nodulate with local bean *Rhizobium*. Excellent nodulation and strong growth are exhibited by the Dina variety (Figure 3). On the other hand Squire is a poor germinator (Figure 4) and does not grow well probably because it does not have good nodulation traits but does appear to have good resistance to rust. Other varieties being tested on large and emerging farmer scales include SB8, SB19, Solitaire and Pan (Rick Ghaui, personal communication).

The soya bean is reputedly resistant to many common legume pests and diseases and especially those of the common beans *Phaseolus vulgaris* grown in Tanzania. This does not mean, however, that it is disease free. A report of 1962 discusses soybean mosaic virus disease on soya in Tanzania (DoA, 1962). Peanut mottle virus was isolated from naturally infected soya beans in the early 1970s with *Aphis craccivora* being the vector (Bock, 1973). Soybean bacterial blight (*Pseudomonas savastonoi* pv. *glycinea*) was first noted in Tanzania in 2003 (Catherine Madata, personal communication).

Figure 4. Variety Squire with poor germination and weak growth (foreground) compared to variety SB19 (background) at Kisolanza Farm, Southern Highlands (Photo: R Trevor Wilson).

Soya Bean Rust caused by the fungus *Phakopsora pachyrhizi* was first noted on soya beans in 2008 (Catherine Madata, personal communication) and has since been the subject of some studies in 2012 to 2014 (Murithi et al., 2014) although the pathogen had been recorded in Tanzania as early as 1979 (Ebbels and Allen, 1979; Terri and Keswani, 1981). Soya Bean Rust can reduce yields by up to 90%. Safari is generally resistant to or tolerant of most soya bean pathogens but its high susceptibility to Soya Bean Rust means it may require spraying against the fungus two or even three times during the growing period. Soya Bean Rust is now the subject of a special programme being implemented by IITA in Tanzania and its neighbouring countries but its main rust-resistant variety TGx 1835-10E has yet to be made available. Other potential soya diseases that may need research in the future include Frogeye Leaf Spot, Wildfire, Downy Mildew and Red Leaf Blotch (Catherine Madata, personal communication).

DISCUSSION

In 2015, the soya bean had been cultivated in Tanzania for more than 100 years. Research on genetics and agronomy and transfer of the benefits of such activities to producers has been spasmodic. Varietal research have been carried out in widely differing areas, has usually been of short duration and has mainly depended on special or external funding. Soya bean has always been a minor crop and its contribution to individual and national income and to human food and animal feed is minimal and is still negligible compared to many countries. In the early 1980s it was said that the crop's potential had yet to be realized (Ayiseni, 1982), a premise which still holds in 2015. Research and extension services have been weak in supporting soya production, as a result of which farmers have limited knowledge of the crop's cultivation and its potential as a food crop for human consumption and as a high quality protein feed for livestock. Marketing, processing and use of the crop remain problematical (Chianu et al., 2008) in large part because the soya bean value chain is weakly developed (SAGCOT, 2012; Wilson, 2015).

The matrix "promiscuity-*Rhizobium*-inoculation" has long been a research subject. Early identification of native promiscuous varieties (Pulver et al., 1982), useful *Rhizobium* species (Chowdhury, 1972; Chowdhury and Doto, 1982; Chowdhury et al., 1983) and of the benefits of inoculation with *Rhizobium* to increase nitrogen fixation

and grain yield (Sachansky, 1977; Pulver et al, 1982) have not been pursued nor have the benefits been transferred to farmers fields. Inoculants were produced at the University of Dar es Salaam as part of an FAO project to select better strains of rhizobia (Mugabe, 1994) and Sokoine University of Agriculture developed commercial inoculants ("Nitrosua") for both soya and common bean and also undertook extension work to disseminate inoculants. As all these activities were dependent on external funding they came to a halt when the projects reached their termination date (Bala et al., 2011).

Direct Government interest in soya bean since Independence in 1961 has been limited and intermittent (URT, 2006). Some new varieties have been released and others are being developed and tested. In common with the whole research community, attempts to improve soya production and processing have endured limited funding and outmoded equipment for many years and research and development (R&D) have suffered as a result. Extension services for soya are particularly weak and devolution of these from the central Ministry and its branches to local authorities (who are even more constrained for funds than the ministry and its specialized institutions) has been a brake on expanded production. Some seeds and fertilizer and crop health inputs are available at small private outlets throughout the highlands and indeed over most of the country but these are not specific to the soya bean. The official availability of certified seed is low. The fact that only three varieties are on the approved list further reduces interest by farmers in growing the crop. Problems in expanding the approved list arise from the bureaucracy and inadequacy of the official seed certification authority and the reluctance of the international seed breeding companies to supply the country because of problems with intellectual property rights.

Actual or potential soya outlets in Tanzania include household consumption, small and medium scale food processors, small and medium-to-large scale animal feed processors and exports. Local production is dominated by small farmers who cannot benefit from economies of scale and often make "emergency" sales for immediate needs. Low or sporadic demand for soya and its products and tenuous market linkages do not encourage farmers to invest in soya production. Small traders buy at the production point and move the product to processors and consumers in a generally ill-defined chain. Small traders dealing in small amounts that are subject to physical and biological degradation do little to improve the end product. Some beans are sold in local markets to a few small scale processors for incorporation in "fortified" local foods (Laswai et al., 2006; Laswai and Mutayoba, 2007) but demand for home consumption is extremely limited.

Animal feed processors continue to use local low cost fishmeal ('dagaa') as the main protein ingredient. The associated human health risks (due to *Salmonella* contamination) and the taint ("fishy taste") imparted to poultry meat are widely accepted. 'Dagaa' is a lower quality protein than soya bean meal, a fact that is encouraging some feed manufacturers to turn to international soya markets where standardized quality beans and meal are available. Import substitution should be a further driver to increased domestic soya bean production.

Broad opportunities exist for enhancing the soya bean value chain from the research arm to the consumer. Continuing interest in the crop is not only from the Government but also from international development agencies and Non-Governmental Organizations [especially the 'Soya ni Pesa' (Soya is money) project of the Catholic Relief Services] (CRS, 2012) as well as the private sector. The bulk of the literature on soya bean produced in Tanzania in recent years has been philosophical rather than practical (Malema, 2005; 2007; Malema et al., 2007). The publication of the Tanzania Soybean Development Strategy (TADS) 2010 to 2020 (MAFC, 2010) was hailed as a milestone in public support for soya production but sets totally unrealistic physical targets rising from its baseline 5000 tonnes of soya grain from 5000 hectares in 2010 to 2.0 million tonnes from 1.4 million ha with 40% being used for processing and 35% for export by 2020. The potential for greatly increased production and for export is, nonetheless, enormous. Tanzania has natural resources suitable for soya bean production and the country is well placed geographically to supply external demand. It has so far failed to capitalize on its natural comparative advantage and there have been virtually no exports of beans or meal.

Some 40 years ago it was written that the "Future prospects for the soybean in Tanzania are absolutely bright" (Mmbaga, 1975). That statement could be true today. For it to become true, however, much more work needs to be done on breeding soya bean genetic resources adapted to local conditions, able to nodulate profusely (either promiscuously or with the assistance of inoculation), resistant to local pests and diseases and perhaps above all acceptable to farmers. Such research will need to be only the first link in a chain that extends through pertinent advice, transparent marketing, appropriate processing and purchasing by consumers.

Conflict of interest

The author declares that there are no conflicting interests.

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Comparative pathogenicity studies of the *Xanthomonas vasicola* species on maize, sugarcane and banana

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Previous biochemical and molecular sequence analyses of *Xanthomonas campestris* pathovar *musacearum*, the etiological agent of banana *Xanthomonas* wilt, suggest that it belongs within the species *Xanthomonas vasicola* (*X. vasicola* pv. *vasculorum* and *X. vasicola* pathovar *holcicola.* However, the *X. vasicola* pathovar names were considered invalid according to pathovar naming standards and placed as one *X. vasicola* species; this was also not helped by the lack of sufficient comparative pathogenicity studies. Hence the proposal to rename *X. campestris* pathovar *musacearum* was no longer further supported. This study therefore carried out large scale comparative pathogenicity trial studies on the *X. vasicola* strains and *X. campestris* pathovar *musacearum* on 112 plants for banana and maize, and 84 plants for sugarcane, to establish or support the proper *X. vasicola* pathovar designations. The study also included nine common plant pathogenic *Xanthomonas* pathovars and one non-*Xanthomonas* strain. The six strains of *X. campestris* pathovar *musacearum* used in the study caused disease in sugarcane and banana but not on maize. 2 and 4 strains of *X. vasicola* pathovar *vasculorum* and *X. vasicola* pathovar *holcicola*, respectively were not only pathogenic on maize and sugarcane but each also caused distinct symptoms on maize. *X. vasicola* pathovar *vasculorum* caused deformation of the plant while *X. vasicola* pathovar *holcicola* caused stunted growth.

Key words: Pathogenicity, *X. axonopodis* pv. *vasculorum, X.campestris* pv. *musacearum, X vasicola* pv. *holcicola, X. vasicola* pv. *vasculorum, Xanthomonas* wilt of bananas.

INTRODUCTION

Xanthomonas campestris pv. *musacearum (Xcm)* is the etiological agent of Banana *Xanthomonas* wilt and a major threat to the existence of *Musa* species (bananas and plantain) in East Africa and *Ensete ventricosum*

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(enset) in Ethiopia (Biruma et al., 2007). The disease was first discovered in Ethiopia on enset plants (Yirgou and Bradbury et al.,1968) and the first report in a major banana-growing area was in Mukono district, Uganda in 2001 (Tushemereirwe et al., 2004). It has since spread to the neighboring countries of Democratic Republic of Congo (Ndungo et al, 2006), Kenya, Tanzania and Burundi (Carter et al., 2010) and Rwanda (Reeder et al, 2007). *Xanthomonas* wilt causes severe losses to banana yields and production, thereby devastating livelihoods of millions in Uganda. Symptoms include premature ripening of the fruit, shriveling of male buds, progressive yellowing, and wilting of leaves. When the pseudostem is cut, pockets of yellow bacterial ooze are seen after 15 to 20 min, confirming the presence of the disease (Tinzaara et al., 2006). There are no known resistant cultivars of banana. The current control methods include complete destruction of infect plants/plant materials, use of sterile cutting or harvesting tools and removal of males buds (Tripathi et al., 2010; Biruma et al., 2007).

Recent characterization studies of *Xcm* isolates through Fatty Acid Methyl Ester (FAME) analysis, Rep-PCR and B gyrase sequencing (Aritua et al, 2008 and Parkinson et al., 2009) have revealed that *Xcm* is phylogenetically very closely related to strains of *X. vasicola* pathovars: *X. vasicola* pv. *vasculorum* (*Xvv*) and *X. vasicola* pv. *holcicola (Xvh*) that are pathogens on sugarcane and sorghum, respectively (Vauterin et al, 1995). Strains of *Xvv* were originally classified as *X. campestris* pv. *vasculorum* (Vauterin et al, 1995). Aritua et al. (2008) revealed that *Xcm* did not show much genetic similarity with *Xanthomonas campestris* pathovars. Aritua et al. (2008) further carried out pathogenicity studies with the *X. vasicola* pathovars on maize and banana and reported that *Xcm* not only caused disease in banana but in maize as well. The *X. vasicola* pathovars (*Xvv* and *Xvh)* only caused disease in maize. Maize was included in the study as it is a close relative of sorghum and also the source of isolation for *Xvv* 206 NCPPB (National Collection of Plant Pathogenic Bacteria). However the current proposed names of the *X. vasicola* pathovars were considered invalid according to the pathovar naming standards and were placed as one *X.vasicola* species (Garrity, 2005). The proposal to rename *Xcm* was no longer further supported. This was not helped by the lack of enough insufficient pathogenicity studies of the *X.vasicola* species.

The purpose of this study was to provide the much needed substantial data on pathogenicity of the *X. vasicola* species and *Xcm* on maize, sugarcane, and banana and to establish or support the current pathovar designations of these particular strains. It was assumed that *Xvv* and *Xvh* caused similar symptoms on maize and sugarcane and did not cause disease in banana while *Xcm* only caused disease in maize and banana. The green house trials also included other plant pathogenic *Xanthomonas* pathovars and non-*Xanthomonas* bacteria to bring out the distinct pathogenicity of the *X. vasicola* strains.

MATERIALS AND METHODS

Bacterial strains

28 bacteria strains from NCPPB (National Collection of Plant Pathogenic Bacteria) were used in this study and are listed in Tables 1 and 2. 9 *Xcm*, 4 *Xvv*, 2 *Xvh*, 2 *X.axonopodis* pv. *vasculorum*, one *X. arboricola* pv. *celebensis,* 2 *X. campestris* pv. *perlargonii*, 3 *X. campestris* pv. *campestris* strains and one non *Xanthomonas* strain Paenibacillus larvae. These strains were also tested and evaluated by PCR using the different available BXW primers (Hodgetts et al., 2014). The bacteria were cultured on YDC (yeast dextrose chalk; (Bacto Agar 15 g/l, yeast extract 10 g/l, $CaCO_3$ 20 g/l, D-glucose 20 g/l (dextrose) and distilled/de-ionized water 1000 ml, autoclaved at 121°C for 15 min) media and incubated at 28°C (optimum temperature for *Xanthomonas*) for 48 h. Bacteria culturing was done 48 h before inoculation of the plants. After 48 h, 10 µl loop of the bacteria was then resuspended in sterile water and the concentration adjusted to 10^7 CFU/ml, using the spectrophotometer by addition of water or bacteria. The suspension was then inoculated into the plants using sterile 1 ml syringes (Sinclair and Dhingra, 1995).

Pathogenicity trials

Pilot trials

Pilot trials on banana (Dwarf Tropicana and Dwarf Cavendish) and maize were carried out to determine the level/concentration of bacteria inoculums that would enable pathogenicity and to know what symptoms to expect and when to expect them. Four (4) Tropicana and three of Cavendish dwarf banana were also inoculated with the 200 µl of *Xcm* suspension (NCPPB 2005, 4392) while two Tropicana and three Cavendish dwarf bananas were inoculated with *Xvh* (NCPPB 1060). Two (2) Tropicana and three Cavendish dwarf bananas were left as controls.

Eight (8) maize plants for each pathovar (*Xcm*, *Xvv*, and *Xvh)* were inoculated with 200 ul of bacterial suspension containing 10^7CFU/ml of one of eight bacterial strains: *Xcm* (NCPPB 4344, NCPPB 4378), *Xvv* (NCPPB 702, NCPPB 795) and *Xvh* (NCPPB 1060), *Xanthomonas axonopodis* pv. *vasculorum (Xav,* NCPPB 796, NCPPB 899*.* Four maize plants were controls; two treated with sterile water and two were left untreated.

Eight (8) sorghum seedlings were also inoculated with 200 µl of bacterial suspensions of *Xcm* (NCPPB 4434, NCPPB 4378) and eight with *Xvh* (NCPPB 1060). Four (4) sorghum seedlings were control plants. Control plants were either left untreated or were inoculated with sterile water. Photographic evidence of symptoms was taken. A pilot study on sugarcane was not attempted due to a shortage of sugarcane plants.

Large-scale pathogenicity trials

One hundred and twelve (112) plants of maize (var. *cisko*) and 112 of banana (Dwarf Cavendish) were used in the pathogenicity trial (Table 1 for experiment design). 8 maize plants and 8 banana plants were left untreated and 8 plants of each were inoculated with

Table 1. Experiment design and Bacterial isolates used in the full pathogenicity trial of banana, maize, and sugarcane.

Plant number	NCPPB no.	Species name	Plant host
1 2 3 4	-	Untreated	
5 6 7 8	-	Dummy inoculated	
9 10 11 12	2985	*Xanthomonas campestris* pv. *perlargonii*	*Pelargonium peltatum*
13 14 15 16	2198	*Xanthomonas arboricola* pv. *celebensis*	Musa spp.
17 18 19 20	796	*Xanthomonas axonopodis* pv. *vasculorum*	*Saccharum officinarum*
21 22 23 24	899	*Xanthomonas axonopodis* pv. *vasculorum*	*Saccharum officinarum*
25 26 27 28	1060	*Xanthomonas vasicola* pv. *holcicola*	*Sorghum vulgare*
29 30 31 32	3129	*Xanthomonas vasicola* pv. *holcicola*	*Sorghum* sp.
33 34 35 36	895	*Xanthomonas vasicola* pv. *vasculorum*	*Saccharum officinarum*
37 38 39 40	702	*Xanthomonas vasicola* pv. *vasculorum*	*Saccharum officinarum*
41 42 43 44	890	*Xanthomonas vasicola* pv. *vasculorum*	*Saccharum officinarum*

Table 1. Contd.

Plant number	NCPPB no.	Species name	Plant host
45 46 47 48	422	*Xanthomonas campestris* pv. *vesicatoria*	*Lycopersicon esculentum*
49 50 51 52	701	*Xanthomonas campestris* pv. *vesicatoria*	*Lycopersicon esculentum*
53 54 55 56	4379	*Xanthomonas campestris* pv. *musacearum*	*Musa* spp.
57 58 59 60	4387	*Xanthomonas campestris* pv. *musacearum*	*Musa* spp.
61 62 63 64	4390	*Xanthomonas campestris* pv. *musacearum*	*Musa* spp.
65 66 67 68	206	*Xanthomonas vasicola* pv. *vasculorum*	*Zea mays*
69 70 71 72	2005	*Xanthomonas campestris* pv. *musacearum*	*Enset*
73 74 75 76	4434	*Xanthomonas campestris* pv. *musacearum*	Musa spp.
77 78 79 80	4433	*Xanthomonas campestris* pv. *musacearum*	Musa spp.
81 82 83 84	529	*Xanthomonas campestris* pv. *campestris*	*Brassica oleracea var. capitata*
85 86 87 88	4031	*Xanthomonas campestris* pv. *perlargonii*	*Pelargonium x hortorum*
89 90 91 92	1131	*Xanthomonas* spp.	*Musa paradisiacal*

Table 1. Contd.

Plant number	NCPPB no.	Species name	Plant host
93 94 95 96	1132	*Xanthomonas* spp.	*Musa canksii var. samoensis*
97 98 99 100	4393	*Xanthomonas* spp.	*Musa* sp.
101 102 103 104	P	Paenibacillus larvae	Cultured
105 106 107 108	-	Untreated	
109 110 111 112	-	Dummy inoculated	

Table 2. Summary of the comparative pathogenicity studies of the *X.vasicola* pathovars and *Xcm*

Strain name	NCPPB No.	Banana	Maize	Sugarcane
Xanthomonas arboricola pv. *celebensis*	1630	+	-	-
Xanthomonas axonopodis pv. *vasculorum*	186	-	++	++
Xanthomonas axonopodis pv. *vasculorum*	899	-	++	++
Xanthomonas axonopodis pv. *vasculorum*	796	-	++	++
Xanthomonas campestris pv. *musacearum*	4378	++	+	++
Xanthomonas campestris pv. *musacearum*	4433	++	+	++
Xanthomonas campestris pv. *musacearum*	4434	++	+	++
Xanthomonas campestris pv. *musacearum*	2005	++	+	++
Xanthomonas campestris pv. *musacearum*	4379	++	+	++
Xanthomonas campestris pv. *musacearum*	4387	++	+	++
Xanthomonas campestris pv. *musacearum*	4390	++	+	++
Xanthomonas campestris pv. *musacearum*	4392	++	+	++
Xanthomonas campestris pv. *musacearum*	4393	++	+	++
Xanthomonas campestris pv. *campestris*	529	-	-	-
Xanthomonas campestris pv. *perlargonii*	4031	-	-	-/+
Xanthomonas campestris pv. *perlargonii*	2985	-	-	-/+
Xanthomonas vasicola pv. *holcicola*	1060	-	++	++
Xanthomonas vasicola pv. *holcicola*	3126	-	++	++
Xanthomonas vasicola pv. *vasculorum*	895	-	++	++
Xanthomonas vasicola pv. *vasculorum*	702	-	++	++
Xanthomonas vasicola pv. *vasculorum*	206	-	++	++
Xanthomonas vasicola pv. *vasculorum*	890	-	++	++
Paenibacillus larvae	3205	-	-	-

**++ Means the pathogen is highly pathogenic on the host while -/+ means the pathogen caused hypersensitive reactions around inoculation sites , + means is pathogenic on the host depending on the different conditions and - means it is not pathogenic on the host.

sterile water (16 control plants) for each trial. For the sugarcane trial, we were only able to acquire 84 plants and therefore included 12 control plants rather than 16. As in the pilot trial, the plants were inoculated with 200 µl of bacterial suspension adjusted to 10^7 CFU/ml, using sterile 1ml syringes and hypodermic needles. 100 µl of bacterial suspension was also spread on Yeast Dextrose Chalk (YDC) media, plates incubated at 28°C for 48 h to confirm the viability of the inoculums. The greenhouse temperature was set between 28 to 30°C. The inoculated plants were assessed every day for the appearance of symptoms and photographs were taken of any suspected visible symptoms. For the banana pathogenicity trial, symptom severity was scored as follows; 0 - no visible symptoms, 1 - slight wilting/folding of lower leaves, 2 - pronounced wilting/yellowing of most leaves, 3 - pronounced necrosis of the whole plant, 4 - complete death, rotting of the plant. For the maize pathogenicity trial, symptom severity was scored as follows of disease; 0 - no visible symptoms, 1 - water-soaked like streaks, 2 - yellow or brown or white streaks, 3 - brown lesions and 4 - deformation of plant or stunted growth. For sugarcane main trial symptom scores of the leaves were scored according to the type of symptom rather than severity of the disease: 0 - no visible symptoms, 1 - white streaks or lesions, 2 - reddish-brown streaks or lesions, 3 - yellow streaks.

Re-isolation of the pathogens was done from plants inoculated with *Xcm, Xvv and Xvh* irrespective of whether the plants exhibited symptoms or not. One or two leaves or leaf stalks of each treatment were picked, and the part of the leaf bordering between the diseased and healthy was cut up in to pieces (0.1 to 0.3 g). The leaf pieces were then soaked, crushed in 1 ml of phosphate buffered saline solution (PBS) and left to stand for at least 10 min to allow bacteria to ooze out. 100 µl of the crushed leaf-PBS solution was then placed and spread on YDC media, incubated at 28 to 30°C for 48 to 72 h.

Confirmation of the isolates was done by extracting DNA using the QiAamp DNA Mini Kit following the manufacturer's protocol and then testing them with the PCR assays specific for *Xcm* (GspDmFR), (Adriko et al*.,* 2011) and those that are able to detect *Xvv* and *Xvh* strains (BXW1F/3R, Lewis Ivey et al., 2010 and *Xcm*44FR (Adikini et al., 2011). Re-isolation from plants that had been inoculated with other *Xanthomonas* strains was also done and identified visually; colonies that looked like *Xanthomonas* were assumed to be of the same strain that had been inoculated into that particular plant, since no *Xanthomonas* was isolated from control plants.

RESULTS

Banana and maize pilot trial

Two hundred microliters (200 µl) of bacterial inoculum at a concentration of 10^7 CFU/ml was enough to cause virulence in maize and banana. All control plants; untreated and those inoculated with sterile water remained healthy, that is no leaf wilting, streaks, necrosis, or yellowing. The 3 Dwarf Cavendish and 4 Dwarf Tropicana banana plants that had been inoculated with *Xcm* (NCPPB 4392 and NCPPB 2005) developed necrotic leaf symptoms, wilting 3 weeks after inoculation and eventually necrosis of whole plant by the end of trial (5 weeks after inoculation).The 2 Tropicana and 3 Cavendish dwarf banana plants that were inoculated with the *Xanthomonas vasicola* pathovar *Xvh* (NCPPB 1060) had no visible symptoms and remained healthy.

The 4 maize control plants remained healthy, that is the leaves showed no signs of lesions, chlorosis, or streaks and no folding of stems. The 8 maize plants that had been inoculated for each *Xanthomonas vasicola* pathovars *Xvv* (NCPPB 702, NCPPB 795) and *Xvh* (NCPPB 1060) and *Xanthomonas axonopodis* pv. *vasculorum* (NCPPB 796, NCPPB 899) strains exhibited leaf chlorosis, yellow-brown or water soaked streaks or lesions on the leaves, usually beginning in the center of the leaf, 1 week after inoculation. The most severe symptoms included deformation of the plant and retarded growth by *Xvv* and *Xvh* (NCPPB1060) 5 weeks after inoculation respectively*.* Out of the 8 maize plants inoculated with *Xcm* (NCPPB 4434*,* NCPPB 4378)*,* only one plant (inoculated with *Xcm* NCPPB 4378) showed identical symptoms as those seen on plants inoculated with *Xvv*; the leaves only had yellow-brown streaks. All the maize controls remained healthy and did exhibit any leaf lesions or wilting. The pilot trial for sorghum was unsuccessful as all the plants including those that had been inoculated with *Xcm* and *Xvh* did not show any signs of disease. Unfortunately, fresh sorghum seedlings were still not available in time for the main large scale trial.

Banana large scale pathogenicity trial

The 16 banana control plants remained healthy. Out of the 24 plants that had been inoculated with *Xcm,* 20 showed severe typical symptoms of *Xanthomonas* wilt of bananas (Figure 1 a-c) and refer to Table 3). The 4 plants that were inoculated with *Xanthomonas arboricola* pv. *celebensis (Xac)* that causes Banana blood disease remained healthy and re-isolation of the bacteria was unsuccessful. This strain may have lost its ability to cause disease in banana as it had been got from -80°C storage. The 24 plants that had been inoculated with the *Xanthomonas vasicola* pathovars (16 with *Xvv* and 8 with *Xvh*) also remained healthy and re-isolation of these strains was successful. The 8 plants that had also been inoculated with *Xanthomonas* species NCPPB 1131 and 1132 originally isolated from banana plants also remained healthy. All 112 banana plants had their older leaves yellowing with scorched appearance throughout the trial most likely due to natural ageing rather than disease infection.

The 20 plants inoculated with *Xcm* showed severe typical symptoms of BXW 3 weeks after inoculation. The disease affected the younger leaves first, beginning with dull green coloring of the lamina, folding of the two halves of the midrib touching each other, yellowing of the leaves, reddish brown streaks on the leaf, eventually all the leaves wilted and entire plant rotted away. Most of the *Xcm* inoculated plants were dead by the 7^{th} week of the trial. Re-isolation of the *Xcm* from most *Xcm*-inoculated

Figure 1a. BXW symptom score 1; pale colouring of the lamina, folding of the leaves along the mid-rib with the two halves touching; 5-weeks after inoculation.

Figure 1b. BXW symptom score 2; yellowing of the leaves and appearance of reddish-brown streaks on the leaves; 5-weeks after inoculation.

plants was successful but rather difficult from plants that had already died as it was apparent that other microbes had already invaded the plants.

Other pathovars such as *X. axonopodis* pv. *vasculorum*, *X. campestris* pv. *pelagonii*, *X. campestris* pv. *campestris* and *X.campestris* pv. *vesicatoria* did not cause disease in banana but were successfully-re-isolated. The non-*Xanthomonas* strain *Paenibacillus* larvae were not successfully re-isolated (Table 4).

Figure 1c. BXW symptom score 3; wilting of most leaves and entire necrosis of the plant; 5-weeks after inoculation.

Maize large scale pathogenicity trial

The 16 control plants; eight inoculated with sterile water and eight left untreated remained healthy throughout the trial. Out of the 24 plants inoculated with *Xanthomonas vasicola* pathovars (*X.vasicola* pv. *vasculorum* and *X.vasicola* pv. *holcicola*), 4 maize plants inoculated with *Xvh* NCPPB 3129 remained healthy (Table 5). The other 20 plants and the eight inoculated with *Xanthomonas axonopodis* pv. *vasculorum* displayed symptoms consistent with the known pathogen profiles of *Xvv, Xvh* and *Xav* in the pilot studies. Symptoms appeared 6 days after inoculation, these included; yellow, brown, white or water soaked streaks as well as brown lesions on the leaves, usually beginning in the center of the leaf. The most severe symptoms were deformation of the plant and retarded growth by *X. vasicola* pv. *vasculorum* and *X. vasicola* pv. *holcicola* respectively (Figure 2a-b). These symptoms separated *Xvv* and *Xvh* The 24 plants that had been inoculated with *X. campestris* pv. *musacearum* did not exhibit any of the symptoms throughout the trial, suggesting that *Xcm* did not affect maize under these conditions and re-isolation from a few of these plants was successful even five weeks after inoculation. Plants

Table 3. Symptom scores for *Xcm*-inoculated individual banana plants of the main pathogen trial.

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 4	Week 5	Week 7
Xcm NCPPB 2005	1	0	0	0	0	2	2
	2	0	0	1	1	3	4
	3	0	0	1	1	2	3
	4	0	0	0	0	2	2
Xcm NCPPB 4379	1	0	0	0	0	0	0
	2	0	0	0	0	0	0
	3	0	1	2	3	4	4
	4	0	0	0	2	2	3
Xcm NCPPB 4387	1	0	0	2	2	4	4
	2	0	0	2	2	3	4
	3	0	0	2	2	3	4
	4	0	0	1	2	3	4
Xcm NCPPB 4390	1	0	0	0	0	0	0
	2	0	0	0	1	1	1
	3	0	0	0	1	1	1
	4	0	0	1	3	4	4
Xcm NCPPB 4433	1	0	0	0	2	3	3
	2	0	0	0	0	0	0
	3	0	0	2	4	4	4
	4	0	0	0	0	0	1
Xcm NCPPB 4434	1	0	0	0	1	1	3
	2	0	0	1	2	3	4
	3	0	0	2	2	4	4
	4	0	0	2	2	3	4

0 - No visible symptoms, 1 - slight wilting/folding of younger leaves, 2 - pronounced wilting/yellowing of most leaves, 3 - pronounced necrosis of the whole plant, 4 - complete death, rotting of the whole plant.

Table 4. Symptom scores of the banana plants inoculated with other *Xanthomonas* pathovars and non- *Xanthomonas* strain.

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 4	Week 5
Xcp 2985	1,2,3,4	0	0	0	0	0
Xcp 4031	1,2,3,4	0	0	0	0	0
Xav 796	1,2,3,4	0	0	0	0	0
Xav 899	1,2,3,4	0	0	0	0	0
Xvh 1060	1,2,3,4	0	0	0	0	0
Xvh 3129	1,2,3,4	0	0	0	0	0
Xvv 895	1,2,3,4	0	0	0	0	0
Xvv 702	1,2,3,4	0	0	0	0	0
Xvv 890	1,2,3,4	0	0	0	0	0
Xcv 422	1,2,3,4	0	0	0	0	0
Xcv 701	1,2,3,4	0	0	0	0	0
Xcc 529	1,2,3,4	0	0	0	0	0

*0 - No visible symptoms, 1 - slight wilting/folding of younger leaves, 2 - pronounced wilting/yellowing of most leaves, 3 - pronounced necrosis of the whole plant, 4 - complete death, rotting of the whole plant. Xcp - *X.campestris* pv. pelargonii, *Xvv* - X.*vasicola* pv. vasculorum, *Xvh* - X.*vasicola* pv. holicicola, Xcc - *X.campestris* pv. *campestris*, Xcv - *X.campestris* pv. *vesicatoria*, X.spp - Xanthomonas strain,Xac - X.arboricola pv. celebensis, *Xav* - X.axonopodis pv. Vasculorum.

Table 4. Contd.

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 4	Week 5
X.spp 1131	1,2,3,4	0	0	0	0	0
X.spp 1132	1,2,3,4	0	0	0	0	0
X.spp 4393	1,2,3,4	0	0	0	0	0
P	1,2,3,4	0	0	0	0	0
Untreated	1,2,3,4,5,6,7,8	0	0	0	0	0
Dummy	1,2,3,4,5,6,7,8	0	0	0	0	0

Table 5. Symptom scores for individual maize plants that had been inoculated with *Xav, Xvh, Xvv* and *Xcm* in the main maize pathogen trial;1-week after inoculation: 0 - no visible symptoms, 1 - Water-soaked streaks, 2 - Yellow brown or white streaks, 3 - Brown lesions, 4 - Deformation of plant or stunted growth.

Treatment	Plant no.	Week 1	Week 2	Week 6
Xav NCPPB 796	1	1,2	2,3	0
	2	1,2	3	0
	3	1,2	2,3	2
	4	1,2	2	2
Xav NCPPB 899	1	1	3	0
	2	0	3	2
	3	0	3	2
	4	0	2	2
Xvh NCPPB1060	1	2	4	2
	2	1	3	2,3
	3	1	1,2	2
	4	1,2	2,3	2,3
Xvh NCPPB 3162	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xvv NCPPB 895	1	1	2	0
	2	1	1,2	2,3
	3	1,2	2,3	2,3
	4	1	2,3	2,3
Xvv NCPPB 702	1	1,2,3	2,3	0
	2	1,2	2,3	0
	3	1,2	3	1,2
	4	2	3	0
Xvv NCPPB 890	1	1	1,2	0
	2	1,2	2	0
	3	1,2	3	0
	4	1,2	2,3	0
Xvv NCPPB 206	1	2,4	4	4
	2	1,3	2,3	3
	3	2,3	2,3	2
	4	1	2,4	4

Table 5. Contd.

Treatment	Plant no.	Week 1	Week 2	Week 6
Xcm NCPPB 4379	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4387	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4390	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 2005	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4434	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
Xcm NCPPB 4433	1	0	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0

Xcm - *X. campestris* pv. musacearum, *Xvv* - *X*. *vasicola* pv. vasculorum, *Xvh* - *X. vasicola* pv. holcicola, *Xav* - *X*. axonopodis pv. vasculorum.

Figure 2a. Stunted growth symptom (score 4) of maize; 3-weeks after inoculation with *Xvh* (NCPPB 1060).

Figure 2b. Deformation of plant symptom (score 4) of maize; 3-weeks after inoculation with *Xvv* (NCPPB 895, NCPPB 702, NCPPB 206, NCPPB 890).

Table 6. Symptom scores of the maize plants inoculated with other *Xanthomonas* pathovars and non- *Xanthomonas* strain in the main maize trial 1-week after inoculation: 0 – no visible symptoms, 1 – Water-soaked streaks, 2 – Yellow brown or white streaks, 3 – Brown lesions, 4 – Deformation of plant or stunted growth**.**

Treatment	Plant no.	Week 1	Week 2	Week 6
Xcp 2985	1,2,3,4	0	0	0
Xcp 4031	1,2,3,4	0	0	0
Xcv 422	1,2,3,4	0	0	0
Xcv 701	1,2,3,4	0	0	0
Xcc 529	1,2,3,4	0	0	0
X.spp 1131	1,2,3,4	0	0	0
X.spp 1132	1,2,3,4	0	0	0
X.spp 4393	1,2,3,4	0	0	0
P	1,2,3,4	0	0	0
Untreated	1,2,3,4,5,6,7,8	0	0	0
Dummy	1,2,3,4,5,6,7,8	0	0	0

* Xcp – *X.campestris* pv. pelargonii, Xcc – *X.campestris* pv. *campestris*, Xcv – *X.campestris* pv. *vesicatoria*, X.spp – Xanthomonas species, Xac – X.arboricola pv. celebensis.

inoculated with other common plant pathovars; *Xanthomonas campestris* pv. *campestris, Xanthomonas campestris* pv. *vesicatoria* and *X. campestris* pv. *perlargonii* did not show any signs of disease (Table 6).

However by the 5th week of the trial, most of the diseased plants seemed healthier than they had been 1 week after inoculation; the affected leaves were fewer or either drying or falling off. The leaf symptoms such as the streaks and lesions were not exhibited on leaves that had inoculation sites thereby confirming the symptoms were not as a result of HR. Some of the plants inoculated with *Xvv* and *Xav* that had earlier had their leaves with lesions and streaks (one to two weeks after inoculation) later became healthy, showing no more symptoms on any other leaves or on leaves that had the inoculation sites or any other parts of the plant by the 5th week. Four out of the eight plants inoculated with *Xvh (*these were inoculated with *Xvh* NCPPB 3126) remained healthy and throughout the trial (asymptomatic) and did not exhibit any visible symptoms even one week after inoculation, and re-isolation of the *Xvh* bacteria from these plants was successful.

Sugarcane large scale pathogenicity trial

All control plants (inoculated with sterile water and those left untreated) remained healthy throughout the trial. *Xvv*, *Xvh*, *Xcm,* and *Xav* caused foliar symptoms such as reddish-brown streaks and white streak spots (Figure 3a-d, Table 7). *Xcm* also caused white and yellow streaks on the leaves). *Xav* also caused white streaks on the leaves. These symptoms appeared 1 week after inoculation. Out of the 6 plants inoculated with *Xvh*, 3 of them (these had been inoculated with *Xvh* NCPPB 3126) remained healthy. Out of the six plants inoculated with *Xav,* 2 of them remained health. Out of the 12 plants inoculated with *Xvv,* 2 of them remained health. Out of 21 plants inoculated with *Xcm*, 7 remained healthy.

Common plant pathogenic *Xanthomonas* pathovars; *X. campestris* pv. p*elargonii*, *X.campestris pv. campestris*, *X. campestris* pv. *vesicatoria* and the *Xanthomonas* species (NCPPB 1131) originally isolated from banana caused white lesions and patches around the inoculation site on the leaves suspected to be more of a hypersensitive reaction (HR) to the pathogens in sugarcane, one week after inoculation (Figure 3e and Table 8). The other common plant *Xanthomonas* pathovar; *X.arboricola* pv. *celebensis* as well as the non *Xanthomonas* strain *Paenibacillus* larvae did not affect sugarcane.

Viability of the bacterial inoculum

The 100 µl of bacteria inoculum that was plated before each inoculation of a plant did grow within 48 h at 28°C incubation, confirming that the inoculum was viable.

DISCUSSION

This study has been able to provide substantial data on the comparative pathogenicity of the *Xanthomonas vasicola* pathovars and *Xcm* (Table 2). This study also

Table 7. Symptom scores of individual plants inoculated with *Xanthomonas vasicola pathovars, Xanthomonas axonopodis* pv. *vasculorum* and *Xanthomonas ccampestris* pv. *musacearum* in the main sugarcane pathogen trial.

Treatment	Plant no.	Week 1	Week 2	Week 3	Week 6
Xav NCPPB 796	1	0	2	2++	2
	2	0	0	0	0
	3	0	0	0	0
Xav NCPPB 899	1	2	2++	2,3++	2,3
	2	2	2	2,3++	2,3
	3	0	0	1++	1
Xvh NCPPB 1060	1	2	2,3++	2,3++	2,3
	2	2,3	2,3++	2,3++	2,3
	3	2	2+	2+	2
Xvh NCPPB 3162	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
Xvv NCPPB 895	1	2	2,3	2,3+++	2,3
	2	0	0	0	0
	3	2	2	2	2
Xvv NCPPB 702	1	0	2	2	2
	2	2	2	2,3	2,3
	3	0	2	2	2
Xvv NCPPB 890	1	0	2	2	2
	2	0	0	0	0
	3	3	2,3	2,3	2,3
Xvv NCPPB 206	1	2	2	2,3+++	3
	2	2	2	2,3+++	2,3
	3	2	2	2++	2
Xcm NCPPB 4379	1	0	0	0	0
	2	0	0	0	0
	3	1	1	0	0
Xcm NCPPB 4387	1	0	0	0	0
	2	0	0	0	0
	3	1	1,2++	1,2++	1,2
Xcm NCPPB 4390	1	0	0	0	0
	2	0	1	1	1
	3	0	0	0	1
Xcm NCPPB 2005	1	0	0	0	0
	2	0	0	2+	2
	3	2	1,2	1,2++	1,2++
Xcm NCPPB 4434	1	2	2+	2+	2
	2	0	2+	2+	2
	3	2	2+	2+	2
Xcm NCPPB 4433	1	1	1+	1	1
	2	1	1+	1	1

0 - No visible symptoms, 1- white streaks or lesions, 2 - reddish-brown streaks or lesions, 3 - yellow streaks. + means severe, ++ means very severe, *Xcm* - *X. campestris* pv. musacearum, *Xvv* - X.*vasicola* pv. vasculorum, *Xvh* - X. *vasicola* pv. holcicola, *Xav- X. axonopodis* pv. vasculorum.

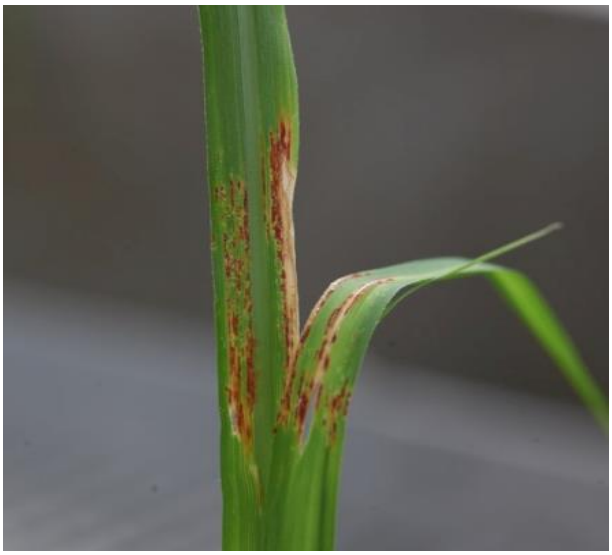

Figure 3a. Reddish/brown leaf symptoms caused by Xvv (NCPPB 895, NCPPB 206); 1-week after inoculation in the main sugarcane pathogenicity trial.

Figure 3c. Reddish/brown leaf symptoms (score 2) caused by Xcm; 1-week after inoculation in the main sugarcane pathogenicity trial.

Figure 3b. Left, reddish/brown leaf symptoms (score 2) caused by Xvh NCPPB 1060; 1-week after inoculation in the main sugarcane pathogenicity trial.

Figure 3d. White streak leaf symptoms (score 1) caused by *Xav* (NCPPB 796); 1-week after inoculation in the main sugarcane pathogenicity trial.

provides for establishment of proper pathovar designations of *X. vasicola* species and further supports the proposal to rename *Xcm* to *X. vasicola* pv. *musacearum*. *X. vasicola* pv. *vasculorum (Xvv)* and *X. vasicola* pv. *holcicola (Xvh)* strains were able to cause disease in maize and sugarcane through artificial inoculation.

Pathogenicity of Xvv, Xvh, and Xcm on maize

Xvv naturally affects sugarcane (Dookun et al., 2000) while *Xvh* naturally affects sorghum (Navi et al., 2002). Even though the two pathogens caused similar symptoms such as lesions or streaks on leaves of maize and sugarcane, they caused distinct symptoms in maize: *Xvv* causes deformation of the plant while *Xvh* causes stunted growth. This then separates them as two different pathovars or warrants different pathovar designations

Figure 3e. Left, white hypersensitive response patches seen about points of inoculation on the leaves of sugarcane inoculated with X.campestris pv pelargonii (NCPPB 2985); 1-week after inoculation in the main sugarcane pathogenicity trial.

based on the differences in symptomology on the same host (Young et al., 2001). Differences in the draft genomes of *Xvv* isolates by Wasukira et al. (2014) may also attempt to explain the possible cause of the difference in symptomology by *Xvv* and *Xvh*. Wasukira's study revealed that an *Xvv* isolate from maize had lost the virulence factor *xopAF* which was present in five other *Xvv* isolates from sugarcane. As *Xvv* and *Xvh* are also closely related, *Xvh* may be lacking this virulence factor *xopAF.*

Unfortunately we were not able to conduct pathogenicity tests on sorghum, to further confirm this symptom difference between *Xvv* and *Xvh*. The pilot trial for sorghum was unsuccessful and this could probably be attributed to either the age of the seedlings or it could be that artificial inoculation of sorghum is usually unsuccessful (Navi et al*.,* 2002). However, some of the maize plants that had been affected one week after inoculation later recovered 5 weeks after inoculation and showed no more signs of disease suggesting that maize may be less susceptible to both pathogens.

A previous study has shown *Xanthomonas campestris* pv. *musacearum (Xcm)* to be able to cause disease in maize (Aritua et al., 2008), however this has been weakly supported in our study. Possible reasons as to why maize plants in the main trial that had been inoculated with *X. campestris* pv. *musacearum* remained healthy; the maize sub-species used in the two studies may have been different, the growth stage of the maize seedlings (in both studies), the greenhouse conditions of the two trials were different. However, based on the three possible reasons, these conditions may have caused a latency period for *Xcm* in maize. Latency period can be defined as period before the pathogen induces symptoms (Verhoeff et al., 1974) or when the symptoms appear due to the changes in the environmental and nutritional stage of maturity in the host or pathogen (Agrios, 1988).This suggests that most likely the conditions of the green house or the maize varieties used in both studies were critical factors.

The successful re-isolation of *Xcm* from the healthy maize plants also further suggests that maize can be a reservoir for *Xcm* strains. This is highly significant and should contribute to the control methods currently used for *Xanthomonas* wilt of bananas especially since maize are among the crops that usually intercropped with bananas in Uganda.

Pathogenicity of Xvv, Xvh, and Xcm on sugarcane

Symptoms caused by both *X. vasicola* pathovars and *Xcm* on sugarcane were similar, that is reddish-brown streaks or lesions on the leaves which may be explained by the genetic closeness the three strains share (Parkinson et al., 2009). Though one strain of *Xvh* NCPPB 3126 used in all the main pathogenicity trials was successfully re-isolated, it did not cause any symptoms in both maize and sugarcane. This strain may have lost its ability to cause disease after being kept in -80˚C and may just have been surviving as an asymptomatic endophyte.

Pathogenicity of Xvv, Xvh, and Xcm on banana

The study has also shown the X. *vasicola* pathovars to be non-pathogenic on banana but the strains still able to survive within the plant asymptomatically. This suggests banana can be a host to other *Xanthomonas* strains (apart from *Xcm*) including *Xvv* and *Xvh* strains. *X. axonopodis* pv. *vasculorum (Xav)* is a pathovar that though does not fall within the *X. vasicola* species, causes similar symptoms apart from deformation and stunted growth. This separates *Xav* from the *X. vasicola* pathovars. The study demonstrates that *Xav* is pathogenic on maize and sugarcane but not banana. This suggests that *Xav* may share a few genetic similarities with the *X. vasicola* pathovars in terms of its ability to cause disease both in maize and sugarcane.

Xcm was also clearly pathogenic on sugarcane and the symptoms on sugarcane were also very similar to those caused by the *X. vasicola* pathovars. Only *Xcm* was distinctly pathogenic on banana while the *X. vasicola* pathovars did not affect banana. According to Studholme et al*.,* 2010, the draft genomes of *Xcm* and *Xvv* are significantly similar; however differences in Type III secretion system (T3SS) effectors may explain their differences in host adaptations. *Xcm* encodes two predicted YopJ-like C55 cysteine proteases that are absent from *Xvv.* Previous studies (Aritua et al., 2008, Parkinson et al*.,* 2009) have shown *Xcm* to fall within the

Table 8. Symptom scores of plants inoculated with other *Xanthomonas* pathovars and non- *Xanthomonas* strain in the main sugarcane pathogen trial.

Treatment	Plant no.	Week 1	Week 2	Week 4	Week 6
Xcp 2985	1,2,3	HR	0	0	0
Xcp 4031	1,2,3	HR	0	0	0
Xcv 422	1,2,3	HR	0	0	0
Xcv 701	1,2,3	HR	0	0	0
Xcc 529	1,2,3	HR	0	0	0
X.spp 1131	1,2,3	HR	0	0	0
X.spp 1132	1,2,3	0	0	0	0
X.spp 4393	1,2,3	0	0	0	0
P	1	0	0	0	0
	2,3			HR	
Untreated	1,2,3,4,5,6	0	0	0	0
Dummy	1,2,3,5	0	0	0	0
	4,6			HR	

*0 - No visible symptoms, 1- white streaks or lesions, 2 - reddish-brown streaks or lesions, 3 - yellow streaks. HR – Hypersensitive reaction around inoculation site, Xcp - *X. campestris* pv. pelargonii, Xcc – *X. campestris* pv. *campestris*, Xcv - *X. campestris* pv. *vesicatoria*, X.spp - Xanthomonas species, Xac - *X*. arboricola pv. celebensis.

X. vasicola species and the pathogenicity trials have revealed that there are clearly interspecific pathovar differences within the species. It is still unclear as why some *Xcm*-inoculated banana plants remained healthy; however such a phenomenon is not new. It has been shown that latency infection of BXW does normally occur in bananas (Ocimati et al., 2015) and is the cause of recurring BXW incidences.

Other Xanthomonas strains on banana, maize, and sugarcane

Re-isolation of other *Xanthomonas* bacteria from banana was successful but not from maize or sugarcane, suggesting that banana can be reservoir to common *Xanthomonas* plant pathogens; *X. campestris* pv. *campestris*, *X.campestris* pv. *perlargonii*, *X.campestris* pv. *vesicatoria* and the *X. vasicola* pathovars. *Xanthomonas* species NCPPB 1131 and 1132 originally isolated from bananas (Studholme et al., 2011) were shown to be non-pathogenic on banana.

Conclusion

The pathogenicity trials have shown that the current *X. vasicola* species (*Xvv* and *Xvh*) can cause disease in similar hosts, and there are differences in symptomatology. The genome studies of *Xvh* may explain the differences in symptomology caused by *Xvv* and *Xvh. Xcm* causes disease in the similar hosts (maize and sugarcane), causing similar foliar symptoms which further supports the genetic closeness *Xvv*, *Xvh* and *Xcm* share. Only *Xcm* causes disease in banana. This data also provides additional information on the pathogenicity of *the X. vasicola* pathovars and should also further support the proposal to rename *Xcm* to *Xanthomonas vasicola* pv. *musacearum*.

Conflict of interest

The authors have not declared any conflict of interest.

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Abbreviations

BXW, Banana *Xanthomonas* Wilt; **NCPPB,** National Collection of Plant Pathogenic Bacteria; **pv.,** pathovar; ***Xcm*,** *Xanthomonas campestris* pv. *Musacearum;* ***Xvh*,** *Xanthomonas vasicola* pv. *Holcicola*; ***Xvv*,** *Xanthomonas*

vasicola pv. *Vasculorum.*

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