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Full Length Research Paper

Identification of acetic acid bacteria isolated from Tunisian palm sap

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Date palm sap (P*hoenix dactylifera* L.), called *Lagmi,* is a sugary substrate that readily ferments through the activity of native microflora that consists mainly of yeasts, lactic acid bacteria and acetic acid bacteria (AAB). The aim of this work was to perform a preliminary step in the isolation and identification of AAB species from different samples of palm sap collected in Southern Tunisia, in order to use them as a starter in vinegar production. AAB were isolated on GEY agar from the fresh palm sap (day (D) 0) and following spontaneous fermentation at room temperature after 3 (D3) and 7 days (D7). A preliminary phenotypic identification of 18 isolates was made by means of Gram, catalase, oxidase and ethanol oxidation to acetic acid, and then further to CO_2 and H_2O. Subsequently, genotypic identification was conducted by sequencing the gene coding for 16SrRNA and a phylogenetic analysis was obtained based on 16S rRNA sequences. Isolates were identified as *Acetobacter tropicalis* (11 strains), *Acetobacter pasteurianus* (3 strains), *Acetobacter senegalensis* (1 strain), *Acetobacter indonesiensis* (1 strain), *Acetobacter cerevisiaie* (1 strain), and *Gluconacetobacter liquefacienes* (1 strain). The application of selected strains as pure starter cultures for the production of vinegar from palm sap is under investigation in our laboratory.

Key words: Date palm sap, *Lagmi*, acetic acid bacteria (AAB), starter, vinegar.

INTRODUCTION

The date palm, *Phoenix dactylifera* L., belonging to the *Arecaceae* family, represents an important economic and ecological resource for many countries in North Africa and in the Arabian Gulf. In Southern Tunisia, date palm has a notable role since it constitutes the main source of revenue and economic base for the people living in the Tunisian Sahara (Ben Thabet et al., 2009; Ziadi et al., 2014; Hamza et al., 2015). Date palm sap, called "*Lagmi*" or "*Legmi*", is a clear juice obtained by tapping palm trees using a traditional local method. Sap exudes from the trunk of the date palm tree during the tapping operation. *Lagmi* is a popular juice appreciated for its sweet taste

and its typical flavour. Palm sap is a nutritional and refreshing beverage enjoyed by people in parts of Africa, Asia and South America (Ben Thabet et al., 2009; Lasekan and Abbas, 2010; Salvi and Katewa, 2012; Girhepuje and Mondal, 2015). In Tunisia, the sap is collected throughout the spring over approximately four months (March-June) with a yield of 8 to 10 L per day and per palm (Barreveld, 1993). However *Lagmi* is rapidly fermented by autochthonous microflora due to the availability of sugars (*ca* 92–95% dry matter basis) (Ben Thabet et al., 2009). The endogenous microflora is composed essentially of yeasts, lactic acid bacteria (LAB) and acetic acid bacteria (AAC) (Stringini et al., 2009). The sap derived from other palm trees, like *Arenga pinnata*, *Cocos nucifera* and *Phoenix sylvestris*, is widely used in Asian countries for the production of palm sugar which is used in cakes, desserts and food coating mixed with drinks (Barreveld, 1993; Apriyantono et al., 2009; Ho et al., 2007). Palms such as *Borassus flabellifer, Metroxylan sago*, *Phoenix humilis, Raphia hookeri* and *Elaeis guineensis* are also tapped to collect the sap which is converted into an alcoholic beverage obtained from natural fermentation in Sri Lanka, India, the Philippines, Indonesia and in West Africa (Benin, Ivory Coast and Nigeria) (Atputharajah et al., 1986; Naidu and Misra, 1998; Umerie, 2000). Furthermore, palm wine may undergo further alcoholic fermentation by local distillers, who add sugar to the product and allow it to ferment for a further 4 to 7 days and then distilled to produce a potent gin, known by various names in West Africa, for example *akpeteshie* in Ghana (Amoa-Awua et al., 2007).

So far, to the authors' knowledge, no studies have been undertaken in Tunisia to evaluate date palm sap as a potential starting material for vinegar production. Indeed, the high sugar content of *Lagmi* is ideal for the growth of yeast resulting in alcohol formation. The alcohol can subsequently be converted by selected starter bacteria to a vinegar with a pleasing flavour and aroma. This study focused on the preliminary isolation and identification of acetic acid bacteria to be used as potential starters for making vinegar from palm sap since using defined starter cultures can lead to an efficient and well-controlled fermenting process.

MATERIALS AND METHODS

Sample collection

Palm sap samples were collected from different oases located in four regions of Southern Tunisia (Kebili, Gabès, Medenine and Tataouine). Four different varieties of *Lagmi* were surveyed: Kinta, El Hammouri, Deglet Noor and Alligue. Sample collection was performed in the early morning. The extraction of the sap was carried out according to the practices used by local collectors (Barreveld, 1993). The samples were collected in Stomacher sterile bags and transferred in ice boxes (4°C) for analysis at the Laboratoire d'Ecologie et de Technologie Microbienne, Institut National des Sciences Appliquées et de Technologie (Tunis). A total of 12 palm sap samples of different varieties were collected in

the same month, from different oases. Microorganisms were enumerated on arrival in laboratory (D0), after 3 days (D3), and after 7 days (D7) (Table 1). Counts were carried out in three replicates.

Microbiological analysis

The palm sap sample was shaken by hand in the Stomacher bag and 10 ml was homogenized in 90 ml sterile salt peptone solution containing 0.1% bacteriological peptone and 0.9% NaCl as the 1:10 dilution. After serial dilution, Aerobic mesophilics bacteria (AMB) were enumerated by pour plate on Plate Count Agar (PCA, Oxoid, Basingstoke, UK) incubated aerobically at 32°C for 48 h.

Lactic acid bacteria (LAB) were enumerated on MRS agar (Oxoid) supplemented with cycloheximide 0.005% (Sigma-Aldrich, St. Louis, USA), to inhibit yeast growth. Plates were incubated at 30°C for 3 days under anaerobic conditions. Yeasts (YEs) were counted on Sabouraud agar medium (Oxoid) with chloramphenicol (500 µg/ml) (Sigma- Aldrich) to suppress bacteria growth and incubated at 25°C for 3 days. Acetic acid bacteria (AAB) were isolated by spread plate on GEY agar, composed of 2% D-glucose; 0.8% yeast extract; 0.5% ethanol; 0.5% peptone; 0.3% $CaCO_3$ and 1.5% agar (Yamada et al., 1999). The medium was supplemented with 10 mg ml^{-1} of cycloheximide (Sigma–Aldrich) made up in 50% ethanol and 20 ml l^{-1} of penicillin (Sigma–Aldrich) prepared from a 0.25% stock solution to inhibit the growth of yeasts and lactic acid bacteria, respectively. GEY agar plates were incubated under aerobic conditions at 30°C for up to 1 week. Following incubation, the number of colony forming unit (cfu) was recorded, followed by morphological characterization and counts of each colony type obtained. Isolates originating from GEY agar plates were maintained on agar slants of AG medium as described by Katsura et al. (2001).

Isolation and putative identification of AAB

Representative colonies (65 strains) chosen at random were subcultured from GEY agar by streaking repeatedly on the same substrate until pure cultures were obtained. Only isolates which were able to produce clear halos around the colonies were further characterized. Selected isolates that were Gram negative, catalase positive and oxidase negative were examined by their ability to grow on differential agar including YPM, modified Carr medium, yeast extract-ethanol broth containing bromocresol purple for over-oxidation of ethanol (Carr, 1968; Du Toit and Lambrechts, 2002) and GYPG medium to examine morphological and cultural characteristics of the isolates. The YPM medium contained: g l^{-1} distilled water; 5, yeast extract (Oxoid); 3, peptone (Oxoid); 25, mannitol (Merck), and 12, agar (Oxoid). The modified Carr medium contained: gl^{-1} distilled water; 30, yeast extract (Merck) and 20, ethanol. For over-oxidation of ethanol, the broth medium contained: g l^{-1} distilled water; 30, yeast extract (Oxoid); 20, ethanol and 0.022 g of bromocresol blue with an inverted Durham tube to trap the CO_2 generated by over-oxidation of ethanol. GYPG medium was composed of 1.0% D-glucose, 1.0% glycerol, 0.5% yeast extract, 1.0% peptone and 1.5% agar. The sixty-five (65) acetic acid bacterial isolates were reduced further to 18 isolates, representatives of all sampling zones and times, based on physiological and morphological similarities.

Molecular identification: DNA extraction and PCR

DNA extraction was conducted from a 72 h preculture of each isolated strain, using the Ultraclean Microbial DNA isolation kit (MoBio, Carlsbad, Ca - USA) following the supplier's instruction.

Table 1. Microbial counts of palm sap collected from four different oasis in south of Tunisia (results are mean values from three replicates).

Oasis	D_0[a]				D_3				D_7			
	AMB[b]	LAB[c]	Yes[d]	AAB[e]	AMB	LAB	YEs	AAB	AMB	LAB	YEs	AAB
Kebeli	5.3×10^6	1.9×10^5	1.5×10^6	Nd[f]	7.9×10^8	6.3×10^7	8.6×10^7	3.4×10^4	9.6×10^8	5.1×10^5	3.8×10^5	6.3×10^6
Gabes	2.2×10^7	3.9×10^4	3.9×10^5	Nd	8.1×10^8	3.3×10^7	7.9×10^7	4.5×10^4	8.3×10^8	7.3×10^5	4.9×10^5	7.3×10^6
Medenine	8.8×10^7	5.6×10^5	6.8×10^6	Nd	5.1×10^8	6.6×10^7	8.7×10^7	6.4×10^4	9.1×10^8	5.2×10^5	1.3×10^5	6.5×10^6
Tataouine	9.7×10^7	6.9×10^5	7.7×10^6	Nd	6.3×10^8	7.5×10^7	9.2×10^6	5.3×10^3	8.9×10^8	6.4×10^5	1.5×10^5	9.1×10^6

[a]Counts at arrival of samples in laboratory (D0); after three days (D3) and after seven days (D7); [b]AMB, Aerobic mesophilic bacteria; [c]LAB, Lactic acid bacteria; [d]Yes, Yeasts; [e]AAB, Acetic acid bacteria; [f]Nd, not determined

DNA quality was checked by 1.2% agarose gel electrophoresis in TAE 1X buffer (TAE 1X: 1 mM Na_2EDTA, 40 mM Tris-acetate, pH 7.6), stained with GelRed (Biotium, Hayward, Ca - USA). PCR amplification of the 16S rDNA was carried out with primers BSF8 (5'-AGAG-TTTGATCCTGGCTCAG-3') and BSR 1541 (5'AAGGAGGTGATCCAGCCGCA-3') (Wilmotte et al., 1993). Primers were synthesized by Sigma Proligo (Hamburg, Germany). PCR reaction was conducted by using Taq PCR Core Kit (Qiagen, Milan Italy). Each reaction (final volume of 25 µl) consisted of: 1X buffer, 0.4 µmol l^{-1} dNTP, 0.6 µmol l^{-1} of each primer, 1.25 U of Taq DNA polymerase, 20-40 ng of template DNA and sterile deionised water until final volume. PCR reactions were carried out in a MyCycler thermal cycler (Bio-Rad, Hecules, Ca - USA) according to the following conditions: an initial denaturation cycle at 94°C for 4 min, then 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min and a final extension cycle at 72°C for 5 min. Positive control (DNA from *Lactobacillus plantarum*), negative control (DNA from *Saccharomyces cerevisiae*) and no template control were added in the PCR assay. PCR products were checked on 1.2% agarose gel electrophoresis in TAE buffer (1X) stained with GelRed (Biotium). The molecular weight of the PCR products was estimated by comparing a molecular weight marker (1 KB DNA ladder, Promega, Milan - Italy). The PCR reaction product was purified using the Qia-quick PCR Purification Kit (QIAGEN) and quantified on 1.2% agarose gel.

Molecular identification: 16S rDNA sequencing and phylogenetic analysis

The purified PCR products were sequenced by using ABI PRISM Big Dye Terminator Kit (Applied Biosystems, Foster City, Ca - USA). Sequences were assembled and analyzed aided by Bioedit software (Hall, 1999). Assembled sequences were compared with the entries of the Ribosomal Database Project (RDP Release 10, Update 29; http:// rdp.cme.msu.edu/) and with the sequences available in the Genbank database using the Basic Local Alignment Search Tool version 2.2.27 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic analyses were conducted using MEGA version 6.0 (Tamura et al., 2007). Sequences were deposited into the Genbank nucleotide sequence database under accession numbers from KX424628 to -645.

RESULTS AND DISCUSSION

Microbial counts in palm sap

Table 1 shows the variation in the population of microorganisms found in samples of sap collected from different palm oasis in Southern Tunisia. Aerobic mesophilic bacteria varied from 5.3 x 10^6 to 9.7×10^7 cfu ml^{-1} at Day 0 (when the samples were examined immediately after their arrival at laboratory), from 5.1×10^8 to 8.1×10^8 cfu ml^{-1} at Day 3, and they remained almost at the same concentration (10^8) at Day 7. The microbial counts assessed on MRS medium (LAB) showed a drastic increase from 3.9×10^4 (in sample from Gabès oasis at D0) to 7.5×10^7 cfu ml^{-1} (in sample

from Tataouine at D3). After this increase in numbers, the LAB population dipped slightly at D7. Yeasts ranged from 3.9×10^5 (Gabès, D0) to 4.9×10^5 (Gabès, D7). All these counts, even if slightly lower, are in agreement with those reported by Ziadi et al. (2011). Apart from few reports that show physicochemical characteristics of Tunisian palm sap (Ben Thabet, 2007, 2009) and the microbiological analysis carried out by Ziadi et al. (2011), there are no other information in literature on the microflora of Tunisian date palm sap "*Lagmi*".

Counts and phenotypic identification of acetic acid bacteria

All AAB were absent on Day0, but were isolated in all samples on the third day (D3) showing a concentration between 5.3×10^3 (Tataouine oasis) and 6.4×10^4 cfu ml^{-1} (Medenine oasis) (Table 1). The bacteria increased in number of about 2 log orders of magnitude in all oases on D7. Overall, the largest group of microorganisms (not considering aerobic mesophilic bacteria) at D0 and D3 was represented by lactic acid bacteria and yeasts, whereas at D7 acetic acid bacteria became the dominant group. A similar trend was observed by Pereira et al. (2012) in a different

Table 2. Alignments of *16S rDNA* sequences grouped by closest sequence matches via the EMBL database.

Strain	Type strain	Closest relative Acession number of 16SrRNA ref seq.	Similarity (%)
1 Mc	*A. tropicalis*	NR_036881.1	99
1 RSb			99
2 RSb			99
1 HSb			99
2 HSb			99
1DG.b			100
1 DSb			99
1 RCb			99
3 KH.b			99
1 KH.b			99
2 KH.b			99
1 SRc	*A. pasteurianus*	NR_102925.1	100
1 RMc			99
1 HSc			99
1 RCc	*A. senegalensis*	NR_043252.1	100
2 HSc	*A. cerevisiae*	NR025512.1	99
1HTb	*A. indonesiensis*	NR_113847.1	99
3 HSb	*G. liquefaciens*	NR026132.1	99

eco-system (cocoa fermentation) where the simultaneous growth of the yeasts and LAB, was gradually replaced by the AAB. AAB have been ascribed to the phenomenon of viable but non-culturable state (VBNC) (Millet and Lonvaud-Funel, 2000) where the population of AAB is very often underestimated by the inability to culture the population on growth media. This phenomenon, along with the fact that AAB are difficult to isolate and cultivate on artificial medium, despite the great number of growth media proposed (Bartowsky and Henschke, 2008), could explain their absence on D 0 in the present work.

Acetic acid bacteria isolated (*n*=18) from palm sap were all Gram-negative, rod-shaped, catalase-positive, oxidase negative and were able to dissolve the calcium carbonate on the GEY agar plates, due to acid production during growth.

However, isolates which were considered to be *Acetobacter* species redeposited the calcium carbonate after a few days. The colonies of the isolates were circular, raised or convex with entire edges, and white to cream on GYPG agar plates. Growth of these isolates on modified Carr medium with bromocresol blue and Durham tube resulted in a change in colour from purple to yellow, and on further incubation reverted to purple. They also produced gas in the Durham tubes. These results showed that the isolates were able to oxidize ethanol to acetic acid and then further to CO_2 and H_2O showing that they probably belong to *Acetobacter* genus (Du Toit and Lambrechts, 2002; Sengun and Karabiyikli, 2011), *Acetobacter* genus is currently located in the *Acetobacteraceae* family of the *α-proteobacteria* class (Komagata et al., 2014).

Genotypic identification and phylogenetic analysis

Comparison of 16S rRNA gene sequence of the strains with the total nucleotide collection in the EMBL-EBI nucleotide database was used to assign the bacterial name with ≥99% similarity. The AAB species identified in palm sap samples were mainly distributed within two genera, namely *Acetobacter* and *Gluconoacetobacter* (Table 2).

In particular, sequences of 11 strains (61%) were matched with the highest omology (>98%) with *Acetobacter tropicalis*. Three strains (17%) closely matched (>99%) with *Acetobacter pasteurianus* sequences. One strain (5% each) matched with *Acetobacter indonesiensis* (1HTb), *Acetobacter senegalensis* (1RCc), *Acetobacter cerevisiaie* (2HSc), and *Gluconacetobacter* (*Ga*) *liquefaciens* (3HSb). The two predominant groups were thus identified as *A. tropicalis* and *A. pasteurianus*. The phylogenetic tree as illustrated in Figure 1 was constructed from evolutionary distance by using the neighbour joining method.

Based on this analysis, it was observed that phylogenetic tree reflects the results obtained in Table 2. Particularly, 13 of isolated strains (72%) from different oasis and at different fermentation time, belong to the *A. aceti* cluster and 3 (17%) *A. pasteurianus* cluster. The relative low diversity of species isolated could be due to the selective effect of fermentation process, that allowed recovering only the strains that actively take part on the fermentations of palm sap. Thus, isolated strains, selected by the process itself can be all considered a promising representative of AAB that could be used as

Figure 1. Phylogenetic tree based on 16S rDNA gene sequences for Tunisian isolates assigned to the genus *Acetobacter.* The phylogenetic tree was constructed by the neighbor-joining method. The type strain of *Frateuria aurantia* IFO13333 was used as an outgroup.

fermentation starters. Of note is the fact that the 2HS strain is in the same subcluster that regroup *A. orleanensis* and *A. cerevisiaie*. These two later species are very closely related (Cleenwerck and De Vos, 2008). Lisdiyanti et al. (2006) studying the diversity of AAB in Indonesia, Thailand and the Philippines, found *A. pasteurianus* only in fermented foods such as palm vinegar, palm wine, rice wine, pickles, etc., while *A.*

tropicalis was isolated from fermented foods (palm wine and rice wine), fruits (lime, orange, guava, coconut), and coconut juice. In addition, Ndoye et al. (2007) isolated two strains of *A. tropicalis*, and *A.pasteurianus* with ability to grow at temperature around 40°C. These thermotolerant strains were proposed for the industrial production of vinegar in hot and tropical countries, as they considerably reduce the costs associated with the

use of cooling water. Nielsen et al. (2007) also showed that *A. pasteurianus* and *A. tropicalis*, were part of predominant acetic acid bacteria during the fermentation of cocoa from Ghana. Du Toit and Lambrechts (2002) showed that *A. pasteurianus* was the dominant species in the middle (Day 6), and at the end of fermentation of wine (day eleven), due to its high tolerance to ethanol. Similarly, in the present work, it was observed that all strains identified as *A. pasteurianus*, were isolated after 1 week of spontaneous fermentation. Strains identified as *A. tropicalis*, except of 1Mc strain (Tale 2) were isolated also after one week (D 7) of spontaneous fermentation. *A. senegalensis* and *A. indonensiensis* were isolated by Jia Wu et al. (2012) among the non-*A. pasteurianus* strains in the acetic acid fermentation of traditional Shanxi Chinese vinegar. Hidalgo et al. (2013) used an *A. cerevisiae* strain to produce vinegar from blueberry as one method to preserve this seasonal fruit and allow extended consumption. Lastly, *G. liquefaciens,* as in this work, has been isolated from palm, sugar cane and coconut juice by Seearunruangchai et al. (2004). Some species of genus *Gluconacetobacter* have been described mainly at the end of acetification in wine the vinegar process, when acetic acid concentration is high (Seearunruangchai et al., 2004; Vegas et al., 2010) It has been proposed that *A. pasteurianus* pioneers the production of vinegar, and is followed by *Gluconacetobacter* spp. which may be more resistant to acetic acid and less resistant to ethanol (Gullo et al., 2009; Vegas et al, 2010).

Overall, in the present study, AAB isolates belong to *Acetobacter* and *Gluconacetobacter*, 2 separated cluster. *Acetobacter* spp. and *Gluconacetobacter* spp. have been isolated from various sources around the world and they are the primary species used in vinegar fermentation due to their strong ability to oxidize ethanol and to tolerate high acetic acid concentrations (Gullo et al., 2014). They produce acetic acid from ethanol by two sequential oxidation reactions involving alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Adachi et al., 2003; Perumpuli et al., 2014). In our laboratory, further studies are in progress for a complete characterization of the technological potential of the isolated strains (measurement of ethanol and lactic acid oxidizing power, amount of acetic acid produced, and tolerance to acid, heat, acetic acid, and ethanol) to establish their usefulness in industrial application.

Conclusions

To the authors' knowledge, no studies have focused on the use of acetic acid bacteria as starters for making vinegar from fresh palm sap. The results indicate that *Lagmi* collected in Southern Tunisia revealed the presence of AAB from the third day onwards. *Acetobacter* strains were isolated and identified based on the

phenotypic characteristics (at genus level) and molecular aspects (at species level). Strains were identified belonging 11 type strain *A. tropicalis*, 3 type strain *A. pasteurianus* , and 1 type strain each *A. senegalensis, A. cerevisiae, A. indonesiensis* and *Gluconacetobacter liquefaciens.* This data suggest that 16S rRNA gene sequence allows differentiation of species or group/species and represents a tool for a rapid and cost effective preliminary profiling of AAB genera. The molecular technique can also be useful to highlight the phylogenetically closely related species. This first knowledge of the acetic acid bacteria will serve as a guide in selecting starter for the production of vinegar from fresh palm sap.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Evaluation of banana genotype resistant to *Xanthomonas* wilts disease (*Xanthomonas campestris* pv. *musacearum)* in south east of Ethiopia

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Xanthomonas wilt caused by *Xanthomonas campestris* pv.*musacearum* (Xcm) is one of the most important constraints to banana production. The use of resistant banana varieties would be a long-term and cost-effective solution to control any pathogen. Therefore, identifying this pathogen resistant banana genotype is one of the basic requirements for effective management. The current study was therefore initiated to evaluate banana genotypes for resistance to banana *Xanthomonas* wilt. The experiment was conducted at Arsi University greenhouse from 2014 to 2015 GC. Banana and enset disease were collected and bacterial isolates were isolated and characterized based on different characterization tests. Twelve (12) banana genotypes were inoculated with three Xcm isolates (I_1, I_2 and I_3) in a factorial experiment arranged in CRD with six replications. Disease assessment data was conducted and analyzed. Bacterial isolates were isolated and the identity of the isolated strains was confirmed as Xcm. The analysis of variance for incubation period, wilting incidence and disease severity revealed significant variations (p<0.05) among banana genotype and isolates. The results revealed that "Cadaba" genotype was found to have the lowest wilt incidence of 16.67%, severity index 15.07% and longest incubation period 5.28 and 9.33 weeks for initial and complete wilting respectively, moderately resistant to the pathogen and producers preferred for multiplication. Butuza, Grandy nani, Robusta and Willams genotype were determined as moderately susceptible having wilting incidence of 21-30%. However, "Nijuru Genotype" showed the highest wilting incidence of 66.67%, severity index 38.78% followed by "Matooke that could be used as highly susceptible checks in future screening trail. Results also revealed that among Xcm isolates, isolate- I_2 is the most aggressive, while isolate I_1 is the least aggressive. As the current work revealed, the potential variation among banana genotype reaction to Xcm infection, genotype that showed moderately susceptible reaction should be further evaluated against Xcm.

Key words: Banana genotype, incubation period, wilt incidence, *Xanthomonas campestris* pv. *musacearum.*

INTRODUCTION

Banana is the world's fifth most important food crops after maize, rice, wheat and cassava (Tripathi, 2011). In Ethiopia, it is the second major fruit crop after citrus. It is grown in southern and western parts of the country, which are mainly confined to low to mid altitudes where there is adequate rainfall or irrigation (Seifu, 1999). The main banana growing areas are located at Arba Minch in southern Ethiopia and south western Ethiopia (Temesgen

et al., 2004). Bananas are produced mainly in traditional agricultural systems by small-scale farmers throughout the country. Its' production is largely for the local market and home consumption. Banana (*Musa spp.*) is typically a cultivated fruit crop mainly as food and all its cultivars are eaten as dessert in Ethiopia. According to the CSA (2008), the total area under banana production is estimated to be over 29064.03 hectares with the annual production of about 1943331 quintal. The total annual banana production in Ethiopia is 66.86 qt/ha.

Despite banana's importance as a food crop, its production and productivity are threatened by various biotic and abiotic factors (Sharrock et al., 2002). Mainly biotic stresses like disease, insect (mealy bug) and nematodes are a leading cause of banana loss. Based on the distribution and the damage incurred on the banana production, *Xanthomonas* wilt caused by *Xanthomonas campestris* pv. *musacearum* is considered to be the most serious production constraint. It is known to be the most threatening and important problem to banana production (Melese et al., 2014).

This pathogen is very destructive and completely kills the plant at all growth stage and cause heavy total yield loss at many localities in Ethiopia (Dagnachew and Bradbury, 1968, 1974; Dereje, 1985; Gizachew, 2000; Quimio and Mesfin, 1996). It is widely distributed in the high, mid and lower altitude areas of the central, southern and southwestern enset growing regions of Ethiopia with different degree of severity (Dereje, 1985; Spring et al., 1996). More recently, the disease has been reported as more common on banana than enset in western Ethiopia (Temesgen et al., 2004). Today, banana yield losses due to banana xanthomonas wilt have been estimated at 30 to 52% of the annual production (Karamura et al., 2006). In addition, 70 to 80% of disease incidence and 100% yield loss were recorded for many juice bananas in Uganda (Tushemereirwe et al., 2002). Once the pathogen has initiated infection, damage limitation is extremely difficult and the disease is impossible to cure (Eden-Green, 2004).

The *Xanthomonas* wilt disease has been endemic to Ethiopia, significant constraint on enset and banana production in the Ethiopian highlands for over four decades and was first reported and described in the late 1960's (Dagnachew and Bradbury, 1968, 1974). However, in recent years, the epidemics of *Xanthomonas* wilt with significant damage have been reported on banana in Uganda in 2001 (Tushemereirwe et al., 2004). Further outbreak and establishment were also confirmed on banana in eastern Congo, in the Lake Victoria region of Tanzania, Rwanda and Kenya (Aritua et al., 2008; Biruma et al., 2007 and Ndungo et al., 2006). Banana production losses caused by this pathogen threaten the

food security of about 100 million people and the income of millions of farmers in the Great Lakes region of Central and Eastern Africa, who depend on banana fruit for food and export trade (Tripathi et al., 2009) and also threatens food security of over 15 million of Ethiopians' who utilize Enset as a staple or co-staple food (Brandt et al., 1997).

According to Ssekiwoko et al. (2006a), there is no effective control measure against the *Xanthomonas* wilt, except the use of different cultural strategies. Currently, the control of *Xanthomonas* wilt depends on the use of cultural practices that include the use of disease-free planting materials, early detection and distraction of the diseased plants, cleaning and disinfecting of farming equipment, and rotation of infected sites with non-host crops and restriction of introduction of foreign plant materials into gardens (Brandt et al., 1997). Adoption of de-budding, meaning removal of the male bud by forked stick had been able to prevent insect vector spread to cultural practices (Blomme et al., 2005). Despite this cultural work, *Xanthomonas* wilt epidemics are increasingly more difficult to control because recommended cultural practices are labor exhaustive and not timeliness being implemented by producers.

Host resistance is the most cost-effective and simplest method of controlling any disease caused by plant pathogens (Young and Danesh, 1994). Based on a screening trial of local and exotic banana genotypes for reaction to Xcm, no genotype was found to be 'immune' to infection (Temsgen et al., 2006; Awasa Agriculture Research Center Progress Report, 2000). However, Musa balbisiana, a wild type of banana, was identified as the most resistant to *Xanthomonas* wilt in Uganda (Ssekiwoko et al., 2006a). Therefore, further exploring resistant banana genotype or identifying the most resistant banana genotype is a base for developing cultivars with resistant to *Xanthomonas* wilt through conventional breeding or biotechnology which would be a long-term and cost-effective solution.

Since its discovery in the 1960s in Ethiopia and recently in East Africa, some studies have been conducted to control *Xanthomonas* wilt in the country. According to Tripathi et al. (2008) and Getachew et al. (2006), even though no natural banana cultivars and genome groups have complete genetic resistance to Xw, they differ in degree of susceptibility. However, research on *Xanthomonas* wilt that involves searching for resistant banana cultivars which generated under tissue culture protocol has been given very little due attention. Therefore, exploring and identifying the most pathogen resistant banana genotype which are developed through tissue culture is one of the basic requirements for effective and sustained implementation of integrated disease management program. There is limited knowledge on the

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Table 1. Description of the *Xanthomonas campestris* pv. *musacearum* isolates used for the pathogenicity tests.

Isolate code	Location	Altitude (mas)	Plant species sampled
I$_1$	West/Dire Inchini/Bola	2560	Enset Sabbara clone
I$_2$	Southern /Sidama/Yirglem/Dale	2749	Banana Pisawak genotype
I$_3$	Southwest/Wonchi/	2671	Enset clone Hiniba

pathogenicity and aggressiveness of *X. campestris* pv. *musacearum* (Xcm) strains to various banana genotypes. Thus, for effective control of this pathogen, the current study was designed to evaluate different banana genotypes for resistance to banana Xanthomonas wilt under artificial inoculation conditions.

MATERIALS AND METHODS

Collection of diseased banana and enset samples, plant species and pathogen characterization

Diseased banana and enset samples were collected from the major banana and enset growing districts Southern region, west and south-west of Ethiopia, viz., Southern region/Sidama, West/Dire Inchini and Southwest/Wonchi districts. Diseased pseudostem samples were collected from one kebele in each district in random sample. The samples were labeled properly and brought into Arsi University College of agriculture and environmental science for further studies. After isolation and detection it was labeled as I$_1$ from enset in Dire inchini, I$_2$ from banana in Sidama Yirglem and I$_3$ from enset in Wonchi (Table 1). Identity of the isolated bacteria was confirmed following colony growth on semi selective medium (sucrose peptone agar medium: 20 g sucrose, 5 g peptone, 0.5 g K$_2$H$_3$PO$_4$, 0.25 g MgSO$_4$ and 15 g agar in 1 L sterilized distilled water) (Mwangi et al., 2007) and Gram staining reaction tests (Schaad, 1988). In addition, physiological tests, that is, gelatin liquefaction and starch hydrolysis testes as well as catalase reaction were carried out (Dickey and Kelman, 1988). For pathogenicity test, different banana genotypes were collected as described in Table 2.

Preparation of inoculum

Pathogenicity tests were carried out on the different banana genotypes using the three different Xcm isolates. The Xcm isolates were isolated from the sample of naturally infected cultivated enset and banana, and used for this purpose as mentioned above. Before inoculation of test plants, the isolates were grown on YDA and incubated at 28°C for 2 days. The concentration of each bacterial suspension was adjusted to 0.3 OD at 460 nm, which is equivalent to 10^8 cfu/ml bacteria cells, using spectrophotometer (Gizachew, 2000).

Pathogenicity testing on different banana genotypes

A factorial experiment with three Xcm isolates (I$_1$, I$_2$ and I$_3$) as sub-factors, and 12 banana genotype as main-factors were carried out to determine the pathogenicity of the isolates on banana genotype. The experiment was laid in completely randomized design (CRD) with six replications. For this purpose, twenty four suckers of each

Table 2. Plant materials used for pathogenicity test and their sources.

Tested plant type Banana genotype	Source
Willams	MARC
Giant Cavandish	MARC
Dwarf Cavandish	MARC
Nijuru	MARC
Robusta	MARC
Cardaba	MARC
Grandy nani	MARC
Poyo	MARC
Butuza	MARC
Kitawire	MARC
Ducasse hybrid	MARC
Matoke	MARC

MARC-Melkassa Agriculture Research Center.

genotype was used and planted into plastic bag (100 cm in diameter and 100 cm height), which were filled with sun-dried mixture of top soil : sand : manure at a ratio of 3:2:1 (Quimio, 1992) and allowed to establish for three months (Figure 1). Three months after planting, at four to seven leaf stages, each genotype was inoculated with 3 ml of a virulent *Xcm* isolate suspension whose cell concentration was adjusted to 1x10^8 cfu/ml at lower base of the newly expanded central leaf petiole using 10 ml sterile hypodermic syringe needle (Dereje, 1985; Gizachew et al., 2008a). One plant per each banana genotype was inoculated with each three Xcm isolates (I1, I$_2$ and I$_3$) and one plant per banana genotype but the control plant was inoculated with the same volume of sterile. Then, inoculated plants were covered with a wet plastic bag for 48 h. Each treatment was replicated six times (Figure 2).

Disease assessment

Disease assessment for the pot experiments were started one week after inoculation and proceeded at a week interval for four consecutive months after inoculation. Wilt incidence, incubation period for the first wilting symptom and time for complete wilting was recorded at time of disease assessment. In addition, disease severity was assessed using standard disease scales of 0-5 (Winstead and Kelman, 1952) where 0: no symptom; 1: only the inoculated leaf wilted; 2: 2-3 leaves wilted; 3: four leaves wilted; 4: all leaves wilted and 5: plant dead). The severity grades were converted into percentage of severity index for analysis (Cooke, 2006). The area under percent severity index progress curve

Figure 1. Established banana genotype for artificial inoculation of Xanthomonous wilt under pot culture condition for three months.

Figure 2. Artificial inoculation of banana genotype by *Xanthomonous* wilt pathogen.

(AUPSiPC) for each treatment was computed using the formula adopted from Jerger and Vijanen-Rollinson (2001). The severity grades were converted into percentage of severity index for analysis (Cooke, 2006).

$$PSI = \frac{SNR \times 100}{NPR \times MSS}$$

Where PSI is percent severity index; SNR is the sum of the numerical rating; NPR is number of plant rated, MSS is the maximum score of the scale. Means of severity from each scoring date was used in the data analysis. Disease incidence/wilt incidence was calculated according to the following formula:

$$WI = \frac{NPCW \times 100}{NPPT}$$

Where: WI– Wilt incidence, NPSWS– number plants completely wilted, NPPT- number of plants per treatment. The area under percent severity index progress curve (AUPSiPC) for each treatment was computed using the formula adopted from Jerger and Vijanen-Rollinson (2001).

$$AUPSiPC = \sum_{i-1}^{n-i} 1/2 \, (x_i + x_{i+1}) \, (t_{i+1} - t_i)$$

Where n is total number of assessment times, ti is the time of the ith assessment in weeks from the first assessment date, xi is the percentage of the disease severity or disease incidence at i^{th} assessment. AUPSIC-area under percent severity index progress curve was expressed in percent-weeks because severity (x) was expressed in percent and time (t) in weeks.

Data analysis

Analysis of variance was performed for data of disease parameters (wilt incidence, percent of severity index and Incubation period) using General Linear Model of SAS computer package (SAS, Institute Inc. 2003). Means were separated with LSD at 5% probability level using the t-test. The degree of resistant to Xw was determined on the basis of WI (%) as: highly resistant (HR): 0% wilting; resistant (R): 1-10% wilting; moderately resistant (MR): 11-20% wilting; moderately susceptible (MS): 21-30% wilting; susceptible (S): 31-50% wilting; highly susceptible: > 50% wilting (Bayoumi and El-Bramawy, 2007).

RESULTS AND DISCUSSION

Isolation, identification and morphological, biochemical and physiologically of the bacterial isolates

Out of the collected symptomatic banana and enset samples, three had detectable Xcm isolates. Spreading the bacteria ooze obtained from the symptomatic banana and enset pseudostem on the cellobiose cephalexin agar (CCA) media resulted in the growth of typical bacterial isolates with smooth mucoid colonies having light to deep yellow and creamy color after three days of incubation period at 28°C. And also after leaf and pseudostem pieces were plated, the mucoid growth of bacterial isolates was observed on the media. These observations were consistent with the finding of Kidist (2003), who indicated the Xcm colonies from banana and cultivated enset as being light to yellow and creamy.

Pink to reddish colored cells were observed after Gram staining, and isolates were classified under Gram negative bacteria. All tested bacteria isolates did not dissolve in 3% KOH solution rather caused the KOH solution to become a thin strand of slime in appearance, when the mixed bacteria culture in the solution was lifted with the inoculating loops, further confirming their identity as Gram negative. All isolates have formed gas bubbles, when dissolved with three drops of $3\%H_2O_2$, and hence were catalase positive. The reaction of the three isolates to Gram staining, catalase reaction and KOH reaction were found to be consistent with the description given by Kidist (2003) and Gizachew (2000) and are also similar to general characteristic of *X. campestris* described by Bradbury (1984).

Further, each isolate was tested for gelatin liquefaction after 3 to 7 days of incubation. When inoculated gelatin tubes were observed without tilting, there was circle formed by growth of bacteria, floating at the upper part of the inoculated test tubes as compared to the non-inoculated control. When inoculated tubes were tilted, gelatin liquefaction was observed in all inoculated test tubes and was taken as positive and the non-inoculated control remained solid. Hence, the bacterial isolates were capable of hydrolyzing gelatine. This result is in agreement with the description given by Dagnachew and Bradbury (1968), who stated ability of some Xcm isolates to liquefy gelatin.

With regard to starch hydrolysis reaction, each isolate showed 1 to 2 cm clear zone around their growth, which is an indication of starch hydrolysis but un-inoculated plate remain unchanged. The result of morphological, physiological and biochemical tests indicated that all isolates from symptomatic samples, fit the characteristics of *X. campestris* pv.*musacearum*. These results are in line with description of cultural characteristics of the Xanthomonas wilt pathogen by Kidist (2003) and Dereje (1985).

Evaluation of banana genotype for resistance to Xcm pathogen

Incubation period for symptom expression and wilt incidence, disease severity and area under disease progress curve

Disease assessment started a week after inoculation, and the earliest external typical disease symptoms were observed 3 to 5 weeks post inoculation on the infected banana genotype. These include collapse of plantlets and folding down of the leaf blade along the midribs followed by scalding and dull green appearance of the central inoculated leaf. This was followed by yellowing starting at the apex, stepwise wilting of leaves, drying and wilting of the whole plant and finally death and rotting (Figure 3). Yellowish bacterial ooze was observed, when pseudostem and leaf petiole were cut. Such typical symptoms were described under field and experiment condition by Gizachew (2000), Dereje (1985) and Alemayehu et al. (2016).

The analysis of variance for incubation period in relation to the appearance of both initially wilting and complete wilting of plants, wilt incidence and disease severity index revealed significant differences (p<0.05) among genotypes and isolates. However, the interaction effect was not significant.

The mean of incubation period ranged from 3 to 5 weeks for initial wilting, while the period varied between 6 and 11 weeks for complete wilting (Table 3). The result showed that banana cultivars vary with regards to earliness and intensity of symptom expression. In this experiment, among the tested genotypes, Matooke was found to have the shortest incubation period (3 weeks) followed by Nijuru, (3.11 weeks), Gaint Cavendish (3.11) and Dwarf cavendish (3.39 weeks), while Butuza and Carduba had the longest incubation period (5.11 and 5.28 weeks respectively) for appearance of initial wilting. Matoke, Nijuru, Dwarf cavendish and Gaint cavendish were significantly differently from other genotypes for early showing disease symptom (Table 3). So the current finding showed that Xcm can survive in banana tissue for periods of over three weeks without showing any external

Wilted Robusta   Wilted Butuza

Control Butuza   Giant cavendish

Grandy nani

Figure 3. The appearance of disease symptoms three weeks after inoculation on cultivated different banana genotype.

symptoms or latently found in the side of banana tissue. Ibrahim (2013) found that the period of symptom expression for Xcm isolate inoculated banana leaves was within five weeks. Mwangi et al. (2006) also found that banana cultivars vary with regards to earliness and intensity of symptom expression.

The number of weeks required to complete wilting varied between six and 10 weeks among banana genotypes and six to nine among isolates (Table 3). This result was in conformity with Temesgen et al. (2006), who reported complete wilting between six and 16 weeks after inoculation. Among banana genotypes, Cardaba took

longer (9.33 weeks) to show complete wilting as compared to other tested banana genotype, while Nijur had the shortest incubation period (5.8 weeks) for complete wilting. There was significant difference between Cardaba and Nijuru for incubation period for complete wilting, while there were no statistically significant differences among Robusta, Kitawire, Poyo, Willams, Ducasse hybrid, Grandy nani and Butuza and also among Matoke, Gaint cavandish and Dwarf cavandish (Table 3). Such kind of variation in symptom expression for either initial or final completely wilting of entire plant could indicate the degree of susceptibility or tolerance

Table 3. Incubation period for initial (IP) and complete wilting (CWP) (weeks), percent of wilt incidence (%DI) and percent severity index progress (PSI) on 12 banana genotype inoculated with three *Xcm* isolates under greenhouse condition.

Banana genotype	IP (week)	CWP (week)	%DI	%PSI
Matooke	3.06[b]	6.4[fg]	66.67[a]	38.78[a]
Nijuru	3.11[b]	5.8[g]	55.56[ab]	30.05[ab]
Giant cavandish	3.11[b]	6.75defg	44.44[abc]	28.45[ab]
Dwarf cavandish	3.39[b]	6.5[efg]	38.89[bcd]	25.71[ab]
Robusta	3.89[ab]	6.8[bcdef]	38.89[bcd]	28.45[ab]
Kitawire	3.78[ab]	7.43[bcdef]	33.33[bcd]	21.11[ab]
Poyo	3.5[b]	7.5[bcde]	33.33[bcd]	18.71[ab]
Willams	3.94[ab]	7.86[bc]	27.78[cd]	23.60[ab]
Ducasse hybrid	4.33[ab]	7.83[bc]	27.78[cd]	24.49[ab]
Grandy nani	4.06[ab]	8.0[b]	22.78[cd]	21.61[ab]
Butuza	5.11[a]	7.75[bcd]	22.22[cd]	29.74[ab]
Cardaba	5.28[a]	9.33[a]	16.67[d]	15.87[b]
LSD	1.97	1.128	21.58	14.32
Isolates				
I$_1$	4.49[a]	8.83[a]	83.33[a]	41.22[a]
I$_2$	3.39[b]	7.08[b]	41.11[b]	19.60[b]
I$_3$	3.76[b]	6.88[b]	28.33[b]	16.15[b]
LSD	0.72	0.75	10.79	5.839
%CV	23.803	10.94	23.01	21.20

Means followed by the same letter in the column are not significantly different at 5% level of significance, I$_1$- Isolate from infected enset West Showa, I$_2$- Isolatefrom infected banana southern region, I$_3$- isolate from infected Enset South western region, data represent mean of six replications.

of the genotypes. Thus, Carduba delaying of initial and complete wilting symptom expression showed tolerance to Xcm infection, while Matooke showed the earliest initial and complete wilting found susceptible for this pathogen.

Comparisons were made across the isolates for initial and complete wilting, isolate I$_2$ and I$_1$ caused initial wilting the earliest (3.39 and 3.76 weeks after inoculation, respectively), while Isolates I$_3$ took about 4.46 weeks for first wilting of the host. Most plantlets inoculated with Isolates I$_2$ and I$_3$ completely wilted but most of the banana genotype plantlet inoculated with isolate I$_1$ was not completely wilted. Among the three isolates of Xcm used in this study, Xcm isolate I$_2$ and I$_1$ had shorter incubation periods (7.08 and 8.83 weeks, respectively) for causing the complete wilting as compared to isolates I$_3$ (9.21 weeks). As a result, among the three isolates of *Xcm* used in this study, I$_2$ isolated from banana genotype from highland area resulted in the earliest causing initial and complete wilting of most tested banana genotype. This suggested the most virulence and aggressive nature of isolates I$_2$. However, isolate I$_1$ was found to be a weaker pathogen as compared to other isolates. The result of the present study is in accordance with the finding of Alemayehu et al. (2016), who reported that

significance differences among isolates were recorded to induce initial wilting three to four weeks after inoculation under greenhouse condition. Similarly, Ibrahim (2013) reported that there was difference among Xcm isolates for inducing disease symptom on inoculated genotype.

Disease severity was high for most banana genotypes after inoculation with isolates I$_2$ and I$_3$. 80-100% of disease severity indexes were recorded for banana genotype Nijuru, Giant cavandish, Matoke, Dwarf cavandish and Ducasse hybrid at 8 to 9 weeks after being inoculated with isolate I$_2$. Isolate I$_3$ caused 30to 70% severity at 9 to 12 weeks after inoculation, while isolate I$_1$ resulted in 6.67 to 53.33% severity at 8 to 12 weeks after inoculation (Figure 4). Averagely, 83.33% of disease incidence and 41.22% of disease severity index were caused by isolate I$_2$. Isolate I$_3$ caused average wilt incidence of 43.11%, and 19.6% disease severity index. Isolate I$_1$ had significantly lower disease incidence (28.33%) and severity index (16.15%) (Table 3). This further confirmed the most aggressive nature of isolate I$_2$ as compared to the remaining two isolates.

Complete disease severity index (100%) on Giant cavandish, Dwarf cavandish and Ducasse hybrid, 90% on Grande nani and Robusta, and 86.7% on Matooke were
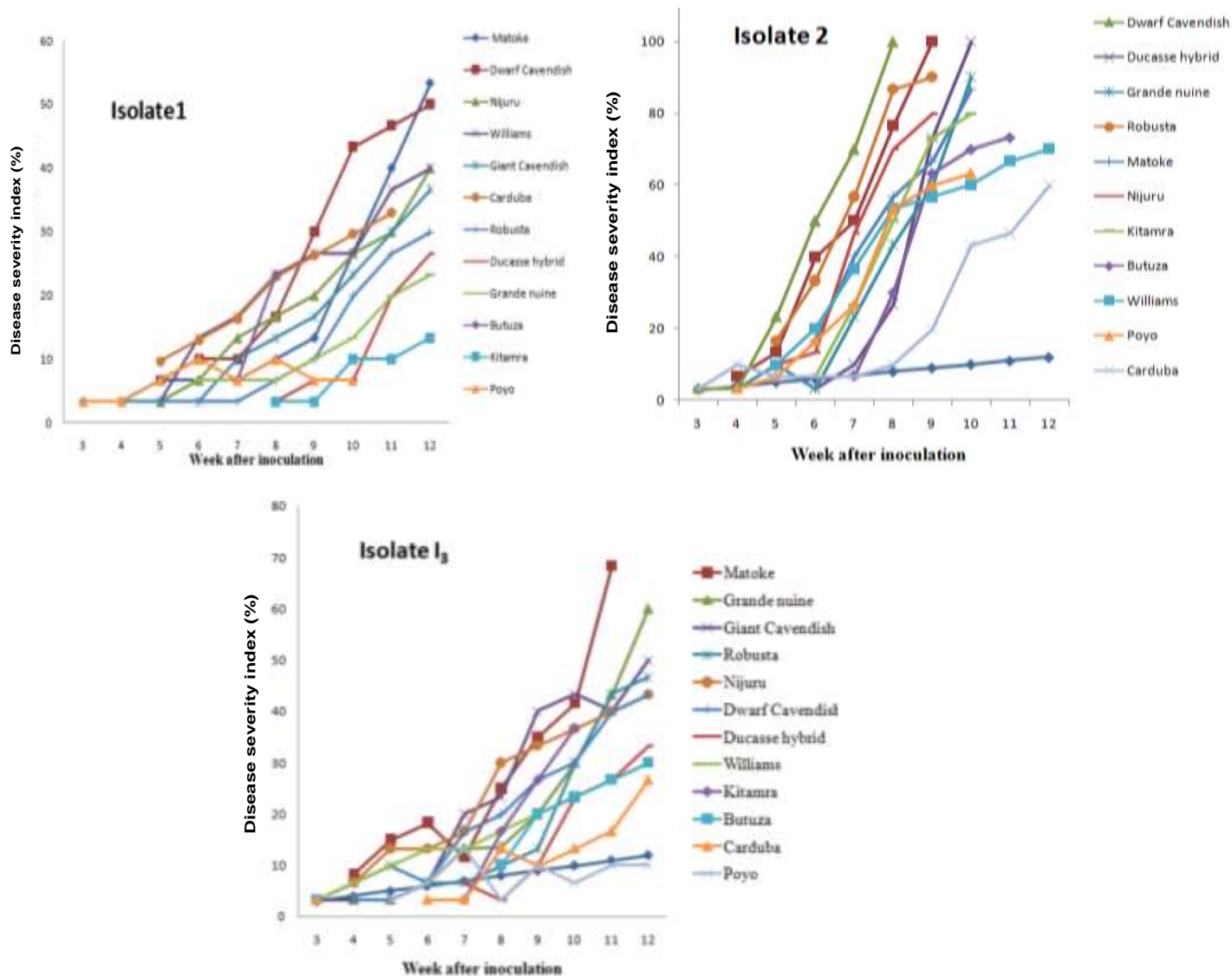
Figure 4. *Xanthomonas* wilt disease development expressed as percent of severity index (%) on twelve banana genotype under greenhouse condition over time after inoculation (week) Note: Xcm Isolates I$_1$ and I$_3$ from enset and Isolate I$_2$ from banana.

recorded at 8 to 9 weeks after inoculated with isolate I$_2$. Similarly, 68.33% of disease severity on Matooke and 60% severity index on Grande nani were recorded at 11 and 12 weeks, respectively after inoculation with isolates I$_3$. Less than 50% of disease severity index were recorded on the other banana genotype being inoculated with Isolates I$_1$ (Figure 4).

Based on the evaluation of their reaction, none of the twelve banana genotype types had complete resistance to Xcm isolates used in this study. Among banana genotypes type tested in the current experiment, Cardaba was significantly different from the others having lowest wilting incidence (16.67%) and disease severity (15.07%) and categorized as moderately resistant. Other banana genotypes include Butuza, Grandy nani, Robusta and

Willams were determined as moderately susceptible having wilting incidence of 21 to 30%. On the other hand, Nijuru and Matooke were relatively highly susceptible with 66.67 and 55.6% average wilting incidence, 38.78 and 32.05% of severity index, respectively. 31 to 50% wilting incidence were recorded on Ducasse hybrid, Poyo, Gaint Cavandish, Dwarf cavandish and Kitawire were determined as susceptible (Table 3). The results suggest higher susceptibility of Nijuru and Matoke, and moderately resistance of Cardaba to Xcm infection. Hence, this banana genotype showed moderate resistance to *Xanthomonas* wilt making pathogen to be multiplied and used by producers and help as one disease management option in addition to cultural practices.

Conclusion

Banana is one of the major food crops in the low to mid lands of the East of Africa. However, its production is threatened by a number of aboitic and biotic factors. Among biotic factor, Xanthomonas wilt caused by *X. campestris* pv. *musacearum* is one of the most important constraints to banana production. The only recommended control measures for Xanthomonas wilt are cultural practices. So, in addition to cultural practices, the use of resistant banana varieties would be a long-term and cost-effective solution. Therefore, exploring tolerance banana genotype is one of the basic requirements for control of this pathogen. Thus, the current study was designed with the objective to evaluate different banana genotypes for resistance to banana Xanthomonas wilt under artificial inoculation conditions.

Diseased samples of banana and enset were collected and bacterial isolates were isolated from collected symptomatic samples and characterized based on pathogenicity, morphological, physiological and biochemical tests. All the tests confirmed the identity of the isolated strains as *X. campestris* pv. *musacearum.* 12 banana genotypes were inoculated with three isolates in a factorial experiment arranged in CRD with six replications. Disease assessment was carried out every week for four months and all the disease parameters data were collected, measured and analyzed.

Pathogenicity tests involving inoculation of different banana genotype with three isolates of *Xcm* revealed significant variations ($p < 0.05$) among the isolates and genotype in terms of incubation period, wilting incidence and disease severity. Among *Xcm* isolate, I_2 was found to be the most aggressive, while I_1 was the least aggressive. In the study of evaluation of 12 banana genotype tolerance to *Xcm*, among all, "Cardaba" genotype exhibited moderately tolerant against Xanthomonas wilt. Therefore, "this banana genotype could be considered as tolerant genotype to the pathogen and it can be used as a Xanthomonas wilt management component. Butuza, Grandy nani, Robusta and Willams genotype were determined as moderately susceptible having wilting incidence of 21 to 30%. On the other hand, genotype "Nijuru" showed the highest wilting incidence, severity index and shortest incubation period followed by "Matoke" and both genotype could be used as highly susceptible checks in future screening trail. The results suggest the higher susceptibility of Nijuru and Matooke and moderate resistance of Cardaba to *Xcm* infection.

In general, the current study showed that banana genotype varies in reaction to Xanthomonas wilt pathogen. In these regards, use of tolerant genotype along with cultural practices and sanitary control measure is viewed to be the most feasible of the Xanthomans wilt management. In the future, producers or farmers should prefer to multiply Cardaba genotype due to its moderate resistance to Xw. Additionally, the banana genotype that showed a moderately susceptible reaction to the wilt pathogen should be further evaluated against *Xcm* isolate under field conditions. More research is needed considering the various banana genotype from the different banana growing regions and research center to explore resistant gene in banana genotype.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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Full Length Research Paper

Comparative study for growth and sporulation of some mycotoxigenic fungi in relation to water activity effects

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This study examined the effect of water activity (0.85-0.995 a_w) on growth rate and asexual spore production for four mycotoxigenic strains (*Aspergillus flavus*, *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Penicillium verrucosum*) on Malt Extract Agar (MEA). The water activity levels of MEA media were modified ionically (NaCl) and non-ionically (glycerol). Results showed that the optimum a_w for growth was at 0.98-0.995 for all species using both solutes. However, when water stress was inflicted, there was a slower growth for all species. The limit for growth of the strains was at 0.85-0.9 a_w, there was no growth at 0.9 a_w for *A. carbonarius* using NaCl solute and 0.85 a_w for *A. carbonarius* and *A. flavus* using glycerol solute. *A. ochraceus* and *P. verrucosum* had a higher tolerance to lower water activity than *A. carbonarius* and *A. flavus* when modified with NaCl. There were significant differences in sporulation between species on glycerol and NaCl-amended media. The optimum conditions for production of asexual spores is often very different from that for growth. Little amount of conidial spore occurred at 0.93-0.95 a_w modified with NaCl in cultures of *A. carbonarius* and *P. verrucosum* but high amounts were produced by *A. ochraceus* and *A. flavus*. Optimum water activity for spore production was 0.995 a_w for *A. carbonarius*, 0.98 a_w for *P. verrucosum*, 0.95 a_w for *A. flavus* and 0.85 a_w for *A. ochraceus* on modified media with glycerol. This is the first detailed study to examine the similarities and differences in growth and sporulation in response to the change of water activity level of important mycotoxigenic species. This study can help in understanding why these species are varied in mycotoxin production, so the results obtained in this study may be useful for application in systems of food safety management.

Key words: Food fungi, osmotic, abiotic parameters, conidiospores

INTRODUCTION

Food and animal feed can be contaminated with mycotoxigenic filamentous fungi by producing one or more potential mycotoxins. Globally, 25 to 50% of crops that are produced for food and feed are vulnerable for

contamination with mycotoxins. The percentage could be more in tropical areas and it is documented that up to 80% of the crops are reported to contain major amounts of mycotoxins (Konietzny and Greiner, 2003). Food and feed can be easily contaminated with aflatoxigenic and ochratoxigenic fungi, especially in warm climates. *Aspergillus ochraceus* and black aspergilli, such as *Aspergillus niger* and *Aspergillus carbonarius*, are ochratoxin producing species most frequently found in warm and tropical regions of the world, while in temperate climates ochratoxin (OTA) is principally produced by *Penicillium verrucosum* (Pitt and Hocking, 1997; Pardo et al., 2005). Exposure to OTA has been linked to the progressive kidney disease known as Balkan endemic nephropathy (BEN) and to the development of urinary tract tumours in humans (Plestina, 1996). *Aspergillus flavus* is the main producer of aflatoxins (AFs), which include the most potent natural carcinogen known (JECFA, 1997). Some isolates of this species are able to produce other mycotoxins, particularly cyclopiazonic acid (CPA), which is toxic to a variety of animals and has been implicated in human poisoning (Dorner et al*.,* 1983; Rao and Husain, 1985).

Spolilage of agricultural products occurs as a result of infection by mycotoxigenic fungi under suitable environmental conditions in the field and may occur at various stages of food chain, for example, pre-harvesting, harvesting, drying, and storage (Sinha, 1995). Depending on the environmental conditions and the specific water activity (a_w), the specific fungal species will grow and produce toxins (Sanchis and Magan, 2004). The role of water activity in determining growth, evolution and adhesion degree of moulds on nutrient substrates is well recognized (Bouras et al., 2009). When the fungal growth is not restricted by environmental factors such as pH or temperature, chemical reactions, enzymatic changes, and microbial growth may occur readily in foods with high water contents. Fungal growth and mycotoxin production in foods and feeds depend on the effect of biotic and abiotic factors including water activity and temperature (Astoreca et al., 2012). Water activity is more useful parameter than water content as it reflects the availability of water for metabolic processes (Sanchis and Magan, 2004).

It has previously been shown that a_w is one of the important criteria for understanding the ecology of spoilage fungi, especially mycotoxigenic species. A significant amount of information is now available on the growth and mycotoxin profiles for many of these species including ochratoxigenic ones and aflatoxigenic onces (Garcia et al., 2011; Astoreca et al., 2012). It has been examined the impact that a_w x temperature factors on growth and mycotoxin production by a wide range of mycotoxigenic fungi (Sanchis and Magan, 2004; Magan and Aldred, 2007). They reported that the range of a_w x temperature for mycotoxin production is generally more

restrictive than those for growth. In contrast to *Penicillium verrucosum* which can grow and produce ochratoxin A under a very similar range of a_w x temperature conditions (Cairns-Fuller et al., 2005). Generally, most moulds do not grow at a water activity (a_w) of 0.8. However, practically no information is available on the similarities and differences between these species, which can produce ochratoxins or aflatoxins. Thus, the aim of this study was to compare the *in vitro* effect of the key ecological parameter of a_w modified by glycerol and NaCl on growth rate, and a sexual spore production of four mycotoxigenic strains.

MATERIALS AND METHODS

Fungal strain

In this study, four mycotoxigenic fungal strains (*A. flavus* 2092, *A. ochraceus* 14027, *A. carbonarius* IT 1102 and *P. verrucosum* 593) were tested. The strains were kindly supplied by Prof Naresh Magan (Applied Mycology Group, Cranfield Health, Cranfield University, Bedford, UK). The strains were sub-cultured on Malt Extract Agar (20 g malt extract, 2 g peptone, 15 g agar per liter) for 7 days at 25°C in the dark.

Adjustment of water activity of media for comparative studies

The media used in this study was Malt Extract Agar (MEA). Water activity of media was modified to five water activity levels (0.98, 0.95, 0.93, 0.90 and 0.85 a_w) by the addition of ionic solute, sodium chloride (NaCl) or the non-ionic solute, glycerol (Dallyn and Fox, 1980) and a control was unmodified media with freely available water (0.995a_w). All the media were sterilized by autoclaving at 121°C, for 20 min. The a_w levels were kept constant by keeping the same a_w treatments in polyethylene bags and checked using a AQUALAB ® 3TE, USA.

Inoculation of fungi and incubation conditions

The spores of the four strains were gently dislodged from the colony surface with a sterile spatula suspended in 10 ml of sterile distilled water containing 0.05% Tween-20 in 25 ml bottles. The spore suspensions prepared could be stored frozen for 5 days or more without loss of viability. The fungal spore concentration was determined using a haemocytometer and adjusted to 10^6 spores ml^{-1}. Petri plates with NaCl and glycerol- modified MEA were inoculated with 5 µl of the spore suspension and incubated at 25°C. Three replicates pre-treatment were used along with all experiments to ensure repetability. Those at the same water activity level were placed in sealed polyethylene bags (Marin et al*.,* 1995).

Measurement of fungal growth

The diameters of fungal colony of three replicate plates were measured in two directions at right angles to each other. The growth measurements were recorded daily until the Petri plates were completely covered by the fungal grwoth (Aldred et al*.,* 1999) and the growth rate was calculated by plotting radial mycelium growth vs. time and radial growth rates (mm day^{-1}) were calculated from the slope by linear regression (Patriarca et al., 2001).
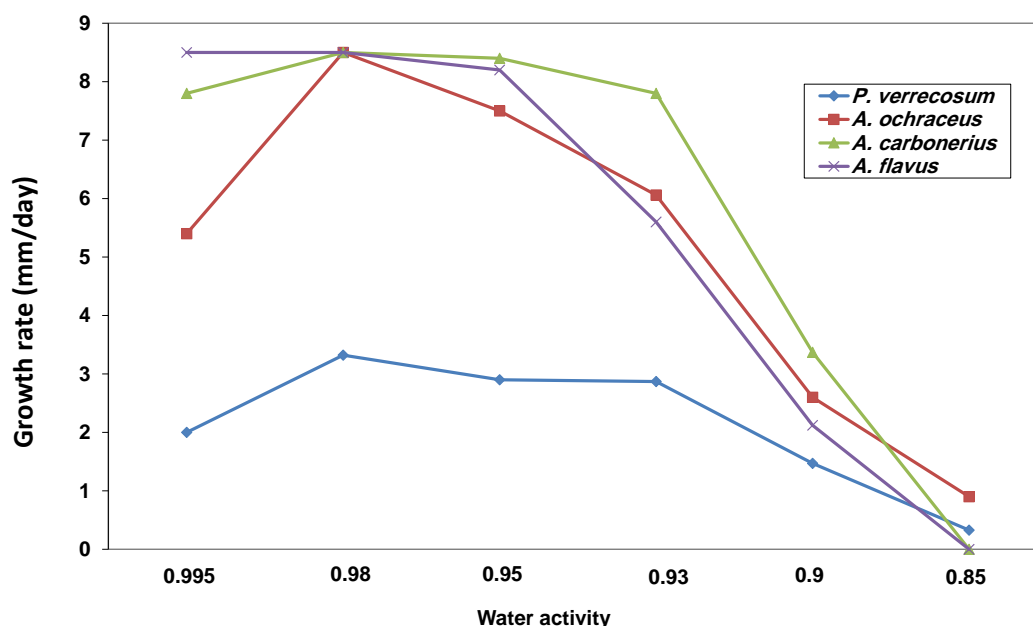
Figure 1. Effect of water activity modified with glycerol on growth of four toxigenic fungi.

Measurement of sporulation

The strains were inoculated on MEA medium overlaid with cellophane; this aided the entire mycelial growth to be removed. The colony was suspended in 10ml of sterile water containing a wetting agent (Tween 80, 0.1%) to wet the spores (Ramos et al., 1999). Fungal spores were filtered and collected through sterile glass wool, and the filtrate was centrifuged to obtain a spore pellet. The number of spores was determined per centimetre (cm) of colony at the end of incubation period using a haemocytometer and a microscope (Ramos et al., 1999).

RESULTS AND DISCUSSION

Effects of water activity on growth rate

Water activity is the most important factor determining growth, evolution and adhesion degree of moulds on nutrient substrates (Bouras et al., 2009). Figures 1 and 2 compares the growth rate of *A. flavus*, *A. ochraceus*, *A. carbonarius* and *P. verrucosum* at different a_w levels modified by glycerol and NaCl respectively. The maximum growth rate was achieved at 0.98 a_w for all species using both solutes except for *A. flavus*, it was at 0.995 a_w in comparison to NaCl modified media. However, when water stess was applied, there was a slower growth for all species until 0.9 a_w, there was no growth for *A. carbonarius* using NaCl solute and 0.85 a_w for *A. carbonarius* and *A. flavus* using glycerol solute. *A. ochraceus* and *P. verrucosum* had a higher tolerance to lower water activity than *A. carbonarius* and *A. flavus* when modified with the NaCl solute. In all cases the a_w range of 0.98-0.93 resulted in a faster growth rate when

compared to the control (0.995 a_w) except *A. flavus* using glycerol solute. The use of glycerol to modify media water availability produced a higher growth rate than with NaCl, probably because it can be utilized as a carbon and energy source and can act directly as a compatible solute (Parra et al., 2004). In contrast, high concentrations of NaCl can be toxic and this may explain the differential growth patterns observed. Belli et al. (2004) who reported optimal growth of isolates of *Aspergillus* section Nigri on synthetic grape juice medium between 0.95 and 0.995 a_w found similar results. They also observed a similar tendency in growth rates of the isolates, which increased with a_w, reaching the optimum at 0.98 a_w and decreasing slightly at 0.995 a_w. Previously, it was reported that the minimum value of a_w required for fungal growth is in range of 0.65 - 0.77. Mitchell et al. (2005) reported that the optimum value of a_w for growth of *A. carbonarius* is in the range of 0.93 - 0.9 while for *A. niger* a_w is 0.97 (Esteban et al., 2006). The value of a_w for fungi growth varies depending on temperature conditions, type of substrate and the region, in which fungi were detected. Recently, Lahouar et al. (2016) reported that maximum diameter growth rates were observed at 0.99 a_w at 37°C for two toxinogenic *A. flavus* isolates. The minimum a_w needed for mycelial growth was 0.91 at 25 and 37°C.

Effects of water activity on asexual sporulation

There were significant differences in sporulation between species on glycerol and NaCl-amended media. The
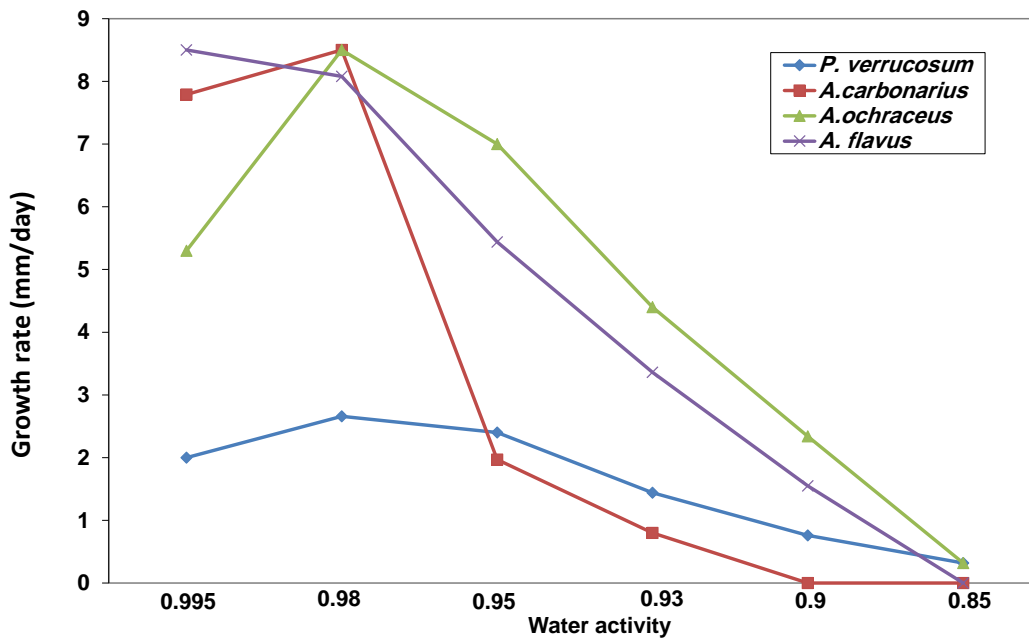
Figure 2. Effect of water activity modified with NaCl on growth of four toxigenic fungi.
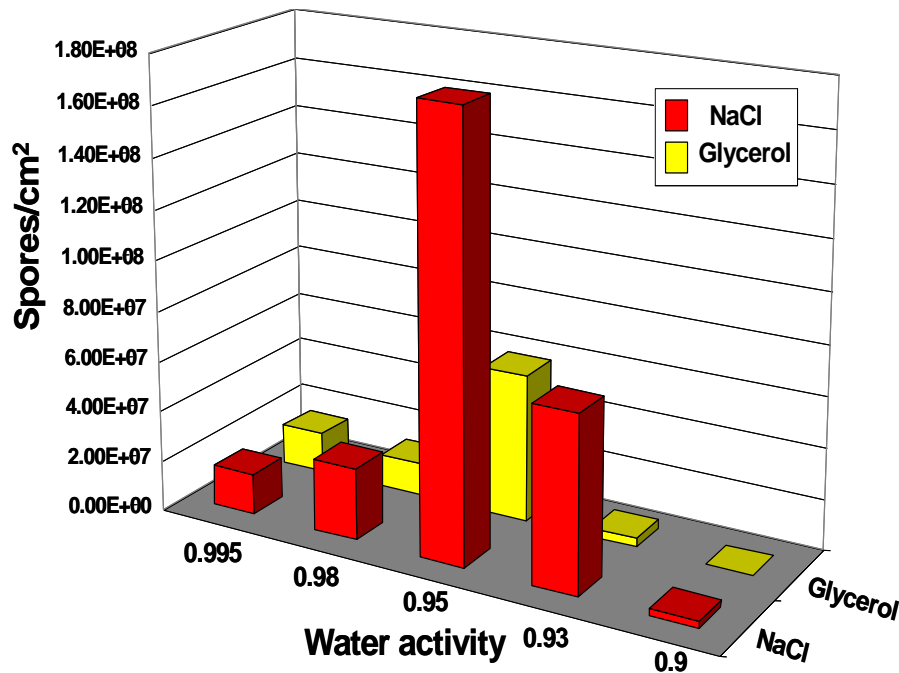
Figure 3. Water activity effects on spore production of *A. flavus* on MEA medium.

optimum conditions for production of asexual spores is often very different from that for growth. With the exception of *A. carbonarius* and *P. verrucosum*, often-maximum spores were produced at sub-optimal water availability conditions, regardless of solute used. *A. flavus* produced high concentrations of spores at 0.95 a_w on modified media with glycerol and NaCl (Figure 3), while *A. ochraceus* had markedly high sporulation capacities at 0.93 a_w (Figure 4). There was a dramatically decrease in spores production by *P. verrucosum* when water stress
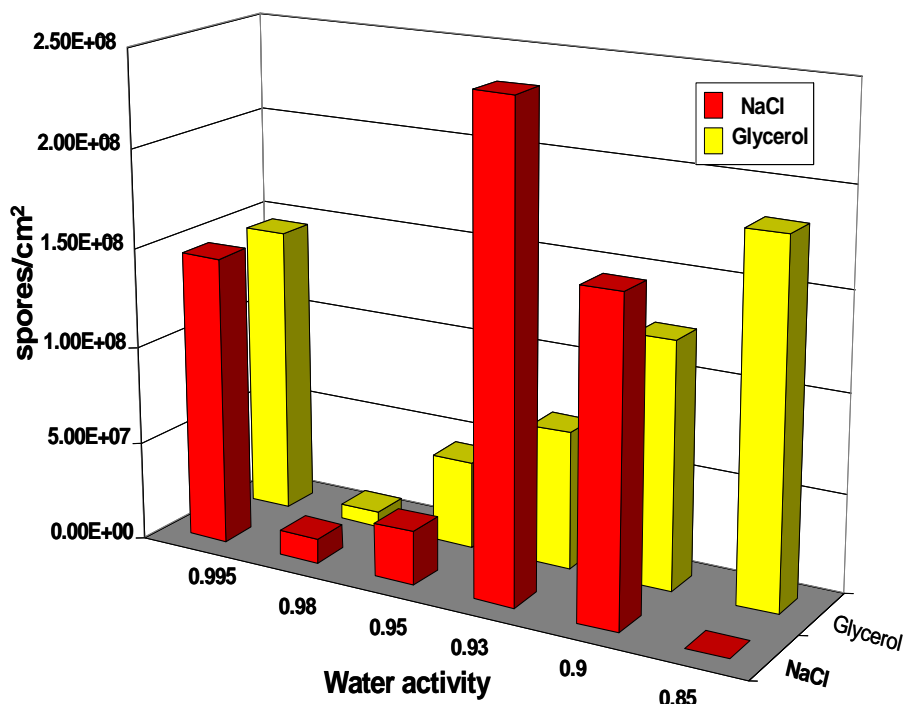
Figure 4. Water activity effects on spore production of *A. ochraceus* on MEA medium.
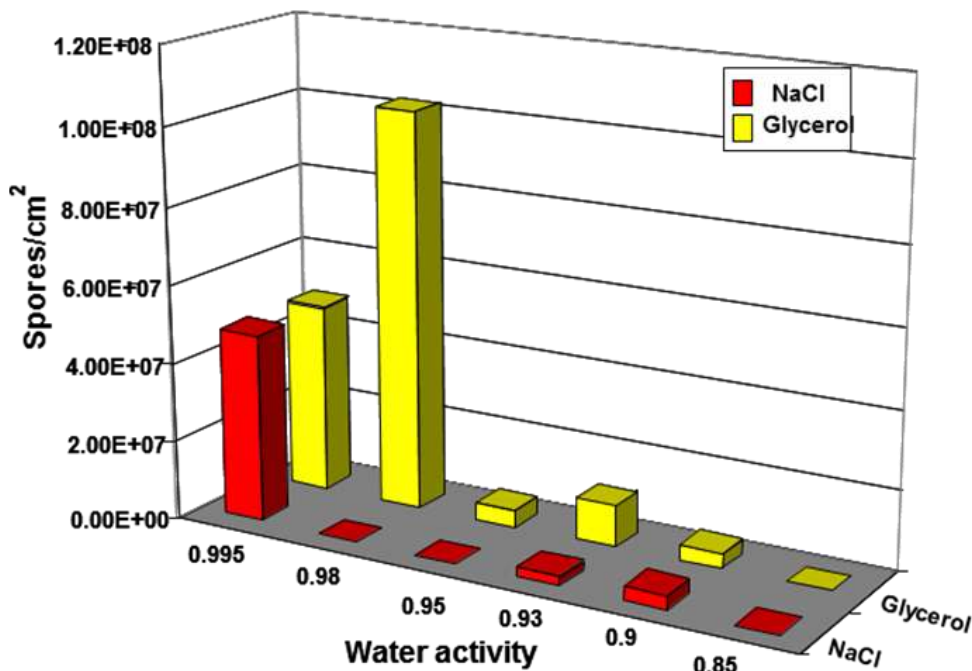
Figure 5. Effect of water activity on sporulation of *P. verrucosum* on MEA medium.

imposed 0.98-0.85a_w with NaCl amended media and 0.95-0.85 with glycerol media (Figure 5). In case of *A.*

carbonarius, the highest amount of spores was produced at control media (0.995 a_w) and no spore production at
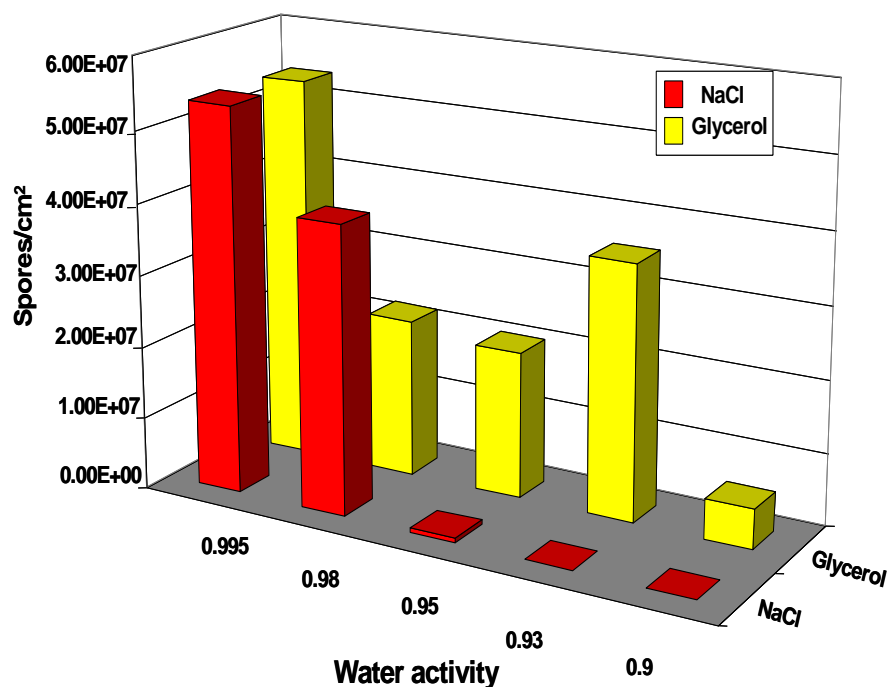
Figure 6. Effect of water activity on sporulation of *A. carbonarius* on MEA medium.

0.93-0.9 a_w with NaCl media (Figure 6). This may be due to osmoregulation which is an energy-requiring process and may affect sporulation process. Our results showed that there was a good relationship between growth and sporulation in case of *A. carbonarius* and *P. verrucosum* with glycerol media where the optimum a_w for growth and sporulation was 0.98-0.99. While, there was no relation in case *A. ochraceus* and *A. flavus*. A study done by Gervais and Molin (2003) with *P. roquefortii* strains from cheese grown optimally at 0.97- 0.98 a_w, while maximum spore production was at 0.96 a_w. Parra et al. (2004) showed that the highest amount of spores produced by a genetically engineered *A. niger* strain was at 0.95 a_w when this was modified with glycerol at 35°C, and by a wild-type strain of *A. niger* at 0.97 a_w and 35°C. Giorni et al. (2008) found that maximum number of spores from strains of *A. flavus* (from Italy) was produced at 0.96 a_w. Recently, Thomas and Ogunkanmi (2014) showed that the highest amount of conidia produced by *A. carbonarius* was at 0.95 a_w followed by 0.99 a_w and 0.98 a_w at all temperatures examined. The relationship between sporulation and mycotoxin production was documented by Mostafa et al. (2005) who determined that most of the mycotoxins produced after the completion of initial fungal growth followed by developmental stage, represented by sporulation, and sclerotial formation. Another study by Atoui et al. (2007) on *A. carbonarius* strains suggests that a significant percentage of the mycotoxin ochratoxin A, was

channelled into the conidia, and this varied with environmental stress.

Conclusion

From this study the growth rate and sporulation of four mycotoxigenic fungi (*A. flavus*, *A. ochraceus*, *A. carbonarius* and *P. verrucosum*), several parameters important for maximizing the growth rate and sporulation have been identified. Water activity had the greatest effect on growth rate and sporulation. *A. flavus* grew significantly better than the other strains examined, while *A. ochraceus* produced higher amount of asexual spores than other strains. For developing control systems on prevention of food contamination by mycotoxigenic fungi and their toxins, it is necessary to study factors influencing on fungal growth and spore production. Controlling water activity will help objectively forecast the contamination level of dried product by fungi and mycotoxins.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

Screening of *Lactobacillus* spp. from raw goat milk showing probiotic activities against pathogenic bacteria

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This research work was executed to isolate potential *Lactobacillus* spp. from local raw goat milk samples for their antimicrobial activities against different human intestinal pathogens. Isolation of such bacteria was carried out by Man Rogosa Sharpe (MRS) agar media. Identification of the isolated strain was conducted according to the morphological and biochemical tests. Furthermore, growth optimization and their antimicrobial activity also studied against the pathogenic bacteria (*Escherichia coli* ATCC 25921, *Pseudomonas aeruginosa* ATCC 9027, *Vibrio cholera* ATCC 14035 and *Salmonella enteric* ATCC 14028). A total of 23 isolates were obtained from the raw goat milk samples, among them four isolates were selected on the basis of their antimicrobial activities against pathogenic bacteria. The *Lactobacillus* spp. showed better growth in 6.5 to 7.5 pH range and temperature range was 35 to 40°C. Thus it can be said that the goat milk provides a natural dwelling place for *Lactobacillus* spp., which are beneficial to human health.

Key words: Antimicrobial activity, growth optimization, intestinal pathogens, *Lactobacillus* spp., Man Rogosa Sharpe (MRS) agar media, pH, raw goat milk.

INTRODUCTION

Lactobacillus is one of the most important genera of lactic acid bacteria (LAB) (Coeuret et al., 2003). They are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary purposes (Fuller, 1989). Probiotics, as defined in the FAO/WHO (2002) report, are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Probiotics are beneficial bacteria in that they favorably alter the intestinal microflora balance, inhibit the growth of harmful bacteria, promote good digestion, boost immune function and increase resistance to infection (Helland et

al., 2004). Other physiological benefits of probiotics include removal of carcinogens, lowering of cholesterol, immunostimulating and allergy lowering effect, synthesis and enhancing the bioavailability of nutrients, alleviation of lactose intolerance (Parvez et al., 2006).

Study on lactic acid bacteria specially *Lactobacillus* spp*.* are drawing a worldwide interests significantly in worldwide due to its applied beneficiary effects in the prevention, control and treatment of diseases and health maintenance (Osuntoki et al., 2008). Now, *Lactobacillus* spp. are widely used as probiotics which play a key role in enhancing resistance to colonization by exogenous, potentially, pathogenic organisms in the intestinal tract. They produce a variety of substances *e.g.*, bacteriocin, nisin, lacticin etc. that are effective against different types of enteric pathogens like *Escherichia coli*, *Salmonella spp.*, *Shigella* spp., *Vibrio* spp., *Bacillus* spp., *Klebsiella* spp., *Staphylococcus* spp., *Pseudomonas* spp., *Proteus* spp. etc. These effects can be described as the improvement of lactose digestion and the treatment of diarrheal disorders (Abdullah and Osman, 2010).

Lactobacillus spp*.* is widely distributed in nature and found as indigenous microflora in raw milk (Rodriguez et al., 2000) and fermented milk with spontaneous fermentation. LAB mostly found in milk of human (Martin et al., 2003) and other animals (Fujisawa and Mitsuoka, 1996). The present study was designed to characterize some *Lactobacillus* spp. from various goat milk samples collected from different regions of Chittagong district to investigate their optimum growth conditions, and some probiotic properties like sensitivity to antibiotics and antagonistic activity to pathogenic bacteria.

MATERIALS AND METHODS

Sample collection

Five different raw goat milk samples were collected from different local area of Noakhali zone under the Chittagong division in Bangladesh in sterile bottles and packed in sterile polythene bags. After packing the bags, the milk samples were carried to microbiology laboratory of globe pharmaceuticals LTD, Bangladesh in an ice box contained ice which maintained the temperature at 4°C within four hours, and used for further studies (isolation, identification and antimicrobial activity). The remaining milk samples were stored at 0°C for further use.

Isolation of *Lactobacillus* spp. from goat milk

Samples were cultured by pour plat method on the Man Rogosa Sharpe (MRS) agar media after serial dilution and incubated at 37°C for 24 to 48 h. The selected strains on the bases of their activity in MRS media were then subcultured onto MRS agar slants which were incubated at 37°C for 24 h and preserved in 20% glycerol (Oxoid, Canada) at -20°C until further used.

Identification of isolated *Lactobacillus* spp*.*

The selected isolates were examined for their morphological properties, such as size, shape, cell arrangement and gram-staining properties. Cultural properties including form, colour, elevation, margin, surface of colonies on MRS agar plate and slant were also recorded. Physiological and biochemical characteristics of the isolates were evaluated by Voges-proskauer, methyl red, indole, and citrate utilization (IMViC) catalase and oxidase tests. The ability of the isolates to ferment a number of sugars including glucose, xylose, arabinose, lactose, glycerol, starch, and manitol were also tested. The isolates were identified up to species based on comparative analysis of the observed characteristics with the standard description of bacterial strains in Bergey's Manual of Determinative Bacteriology.

Optimization of growth parameters

Optimum temperature and pH were determined. Optimum growth temperature was determined by growing the selected isolates in MRS broth and incubating at different temperatures (20 to 45°C) for 24 h. MRS broth of different pH (pH 4.5 to 9) were inoculated and incubated for 24 h to determine the optimum pH. The optimum parameters for highest growth of the identified *Lactobacillus* spp*.* were determined by measuring and comparing the optical density (OD) at 600 nm (OD600) (Barua et al., 2015).

Anti-microbial activity of isolated *Lactobacillus* spp.

Cross-streak method was used to detect the anti-microbial activity of *Lactobacillus* spp. (Lertcanawanichakul and Sawangnop, 2008)*.* against selected gram negative pathogenic bacteria such as *Salmonella* spp*., Vibrio cholerae, Pseudomonas aeruginosa* and Enterotoxigenic *E. coli* (ETEC). Each pure isolated *Lactobacillus* spp. culture from previous MRS agar slant was individually streaked in half portion of different MRS agar plates with a smear line. Then, all plates were incubated at 37°C for 3 days. After 2 days incubation period, the isolates secreted anti-metabolites into the medium and then, test pathogens were cross-streaked along the line of fully grown isolates following Cross-streak method. Each streaking was started from near the edge of the plates and was streaked toward the growth line of the isolated *Lactobacillus* spp. After streaking into the medium, the plates were incubated at 37°C for 24 h. After overnight incubation, a clear zone of inhibition along the growth line of the isolates ware observed which indicated growth inhibition of pathogenic test bacteria due to anti-microbial activity of isolated *Lactobacillus* spp. The microbial interactions were analyzed by the observation of the size of the inhibition zone (Madigan et al., 1997).

Assay of antibiotic sensitivity pattern

To assess the antibiotic sensitivity pattern, disk diffusion method was followed (Ivanova et al., 2000). Culture inoculums of the isolates grown in MRS broth was taken as amount of 0·5 ml, and was mixed in 5 ml of the same medium containing 0·5% agar, and aseptically poured into glass Petri dishes containing MRS agar medium. The antibiotic disks (Ampicillin, Amoxicilline, Tetracycline, Erythromycin and Gentamicin) were placed on the surface of the plate at equidistance. The plates were then kept at 4°C for 1 h for proper diffusion of antibiotics. The plates were incubated at 37°C for 24 h. The antibiotic sensitivity or resistance was determined by observing the presence of zone of inhibition. The zone of inhibition was measured by a millimeter scale.

RESULTS

Isolation

After primary screening of five goat milk samples by MRS

Table 1. Microscopic and biochemical tests of isolates with carbohydrate utilization ability.

Tests	LB$_a$	LB$_b$	LB$_c$	LB$_d$
Gram Staining	+	+	+	+
Form	Rod	Rod	Rod	Rod
Catalase	-	-	-	-
Oxidase	-	-	-	-
IMViC				
Indole	-	-	-	-
MR	-	-	-	-
VP	-	-	-	-
Citrate	-	-	-	-
Carbohydrate fermentation				
D (+) Xylose	+	+	+	+
Lactose	+	+	+	+
Glycerol	+	+	+	+
Starch	+	+	+	+
Manitol	+	+	+	+
Glucose	+	+	+	+

+, Positive result; -, Negative result.

agar media, 23 isolates were found, all the isolates produced small, irregular and round shape with shiny whitish cream or brownish colored which were morphologically similar to *Lactobacillus spp.* and among these isolets only 4 isolates were selected for further study according to their growth inhibition properties against four references pathogenic microorganisms *Salmonella* spp*., Vibrio cholerae, Pseudomonas aeruginosa* and Enterotoxigenic *E. coli* (ETEC).

Identification

All isolates were examined under microscope to observe their microscopic properties. These isolates were found gram positive, short and medium rod shaped non-spore forming bacteria. Furthermore, some biochemical tests such as catalase test, oxidase test, IMViC tests (Indole test, Methyl Red (MR) test, Voges Proskauer (VP) test, citrate utilization test) and carbohydrate fermentation patterns were performed as depictured by Bergey's manual systematic bacteriology (Hensyl, 1994). The isolates were found catalase and oxidase negative and in indole, methyl-red, voges proskauar, citrate utilization (IMViC) tests all isolates were also found negative. In this study, all the four isolates were able to ferment 6 different carbohydrates, that is, Xylose, Lactose, Glycerol, Starch, Mannitol and Glucose indicating that they are able to grow in variety of habitats utilizing different type of carbohydrates. The summarized results of all bacteriological and biochemical tests are presented in Table 1.

Optimum growth parameters for the selected *Lactobacillus* spp.

Growth of selected isolates (four isolates) studied on different growth parameters like pH and temperature to reveal their optimum growth parameter. Most of the isolates showed the best growth at neutral pH and the less growth observed when pH increased and decreased. Furthermore, it also found that the best bacterial growing temperature was between 35 to 40°C. Cellular growth was indicated by turbidity after incubated for 24 h on broth media. The results of morphological and physiological tests over four isolates were shown in the Table 2.

Assay of anti-bacterial activity by cross-streak method

Zone of inhibition produced by the isolates against target pathogens were indicated their antimicrobial properties and identified isolates of *Lactobacillus* spp*.* showed that inhibitory properties against *Salmonella* spp*., Vibrio cholerae, Pseudomonas aeruginosa* and Enterotoxigenic *E. coli* (ETEC) (Table 3).

Susceptibility and resistance to antibiotics

Four potential isolates (LB$_a$, LB$_b$, LB$_c$ and LB$_d$) were subjected to the antibiotic resistance study, where we use five antibiotics (Ampicillin, Amoxicilline, Tetracycline, Erythromycin and Gentamicin). The result showed that all

Table 2. Growth ability of selected isolates in different pH and temperature.

Growth in different pH range				
4.5	+---	+---	+---	+---
5.0	+---	+---	+---	+---
5.5	+---	+---	+---	+---
6.0	++--	++--	+---	++--
6.5	+++-	++--	+++-	++++
7.0	++++	++++	+++-	++++
7.5	++++	+++-	++++	++--
8.0	++--	++--	++++	++--
8.5	+---	+---	++--	+---
9.0	+---	+---	+---	+---
Growth in different temperature (in ºC) range				
20	+---	+---	+---	+---
25	+---	+---	+---	+---
30	++--	++--	+---	++--
35	++++	++++	++++	++++
40	++++	++++	++++	++++
45	++--	++--	++--	++--

+++, Optimum growth; +---, poor growth.

Table 3. Antimicrobial activity test of *Lactobacillus* spp. against pathogenic bacteria.

Isolate	Zone of inhibition (in mm)			
	Salmonella spp.	*V. cholerae*	*P. aeruginosa*	*E. coli*
LB$_a$	6.5	4.5	5.5	7.0
LB$_b$	7.0	6.0	6.0	7.6
LB$_c$	8.0	5.5	6.4	5.4
LB$_d$	7.7	6.8	8.0	6.0

isolates were susceptible to Erythromycin, Gentamicin and Ampicillin. Whereas most of these isolates were resistant against remaining antibiotics (Figure 1).

DISCUSSION

Milk and milk products are usually associated with probiotics bacteria, which provide supplements in maintaining beneficial intestinal balance (Tambekar and Bhutada, 2010). This study was designed to isolate and identify *Lactobacillus* spp. using MRS media and from bacterial morphological and biochemical characteristics. Moreover to determine probiotic activities of the isolates against various pathogens like *Salmonella* spp*., Vibrio cholerae, Pseudomonas aeruginosa* and Enterotoxigenic *E. coli* (ETEC). Man Rogosa Sharpe (MRS) agar media is most commonly used for cultivation *of Lactobacillus spp.* (El-Moez et al., 2001). This is why, after isolation of 23 *Lacobacillus spp.* from five raw goat milk samples using

MRS media, only four isolates showed better activities against pathogenic bacteria mentioned above. The *Lactobacillus spp.* can produce some metabolites such as Bacteriocins like Acidophilin, Acidolin, Lactocidin, Bulgarican, Lactolin, Lactobacillin and Lactobrevin which are antagonistic to various degrees against diarrheagenic intestinal pathogens (Tambekar and Bhutada, 2010). Four potential isolates (out of 23) were characterized during the isolation steps, according to their colony morphology (color, size, shape, appearance, gram staining) on MRS media, which showed better antibacterial activity against four reference strains. All the potential isolates showed moderate or strong inhibitory activity and zone of inhibition was measured. But the entire growth inhibition (in ml) was not same. Some isolates showed positive result against all reference strains but some isolates could not.

Selected isolates (LB$_a$, LB$_b$, LB$_c$ and LB$_d$) found to be gram positive, short and medium rod shaped non-spore forming bacteria which indicated they are members of
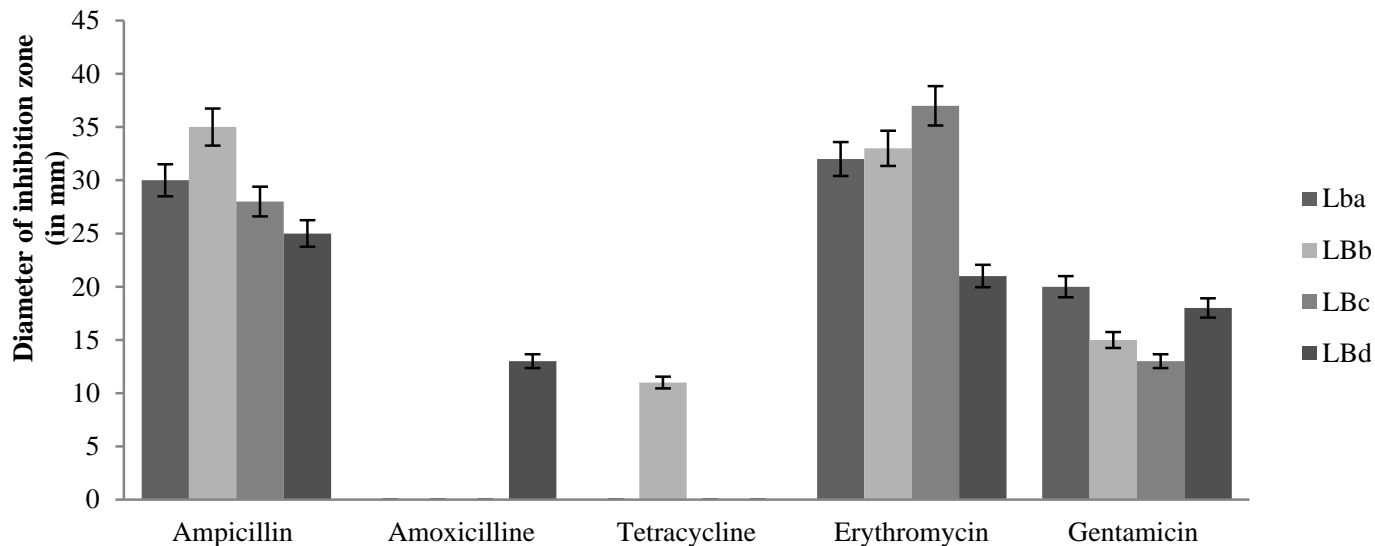
Figure 1. Susceptibility and resistance of four isolates (LB$_a$, LB$_b$, LB$_c$ and LB$_d$) of *Lactobacillus* spp. against antibiotics (Ampicillin, Amoxicillin, Tetracycline, Erythromycin and Gentamicin).

Lactobacillus spp. (Thamaraj and Shah, 2003). The isolates were showed catalase and oxidase negative and in IMViC tests all isolates were also found negative, thereby these might confirm the isolates were Lactobacillus spp. (Dhanasekaran et al., 2010). All these results are found relevant to the findings of Chowdhury et al. (2012).

These four isolates were also subjected to examine their optimum growth condition by allowing them to grow in different pH and temperature for 24 h in broth medium. They showed better result in 6.5 to 7.5 pH range and temperature range was 35 to 40°C. Findings of present study showed more or less similarities with the previous study where optimum pH and temperature for the growth of *Lactobacillus spp.* was neutral (7) and 37°C respectively (Barua et al., 2015). Antibiotic susceptibility and resistance test also observed of these four bacterial isolates, the result showed that all isolates are susceptible to Erythromycin, Gentamicin and Ampicillin. But most of these strains were resistant against remaining antibiotics. Study of Barua et al. (2015) and Anas et al., (2014) also support the present research.

It is clear that these isolates may produce some metabolites that play role on growth inhibition of pathogens. So, isolated *Lactobacillus* spp. might have a further research value.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Association of N₂-fixing cyanobacteria with wheat (*Triticum vulgare* L.) roots

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Two locally prevailing hetyerocystous cyanobacterial isolates, collected from alluvial clay soil and identified as *Nostoc muscorum* and *Anabaena variabilis,* were tested (either singly or mixed) for their potency to colonize the roots of wheat seedlings. *N. muscorum* formed close association with root hairs, whereas *A. variabilis* showed loose binding. The mixture of both cyanobacterial strains formed weak association with the roots of wheat seedlings. The tight colonization of *N. muscorum* led to accumulation of Chl "a" in the roots, as well as showed a beneficial influence on stimulating nitrogenase activity. Such tight association by *N. muscorum* significantly increased the growth parameters of wheat seedlings. Dry weight of the inoculated plant seedlings roots augmented by more than 3-fold of the control, wherever the shoots were promoted by more than 36% as compared to the control has no inoculation. However, the loose attachment shown by *A. variabilis* contributed to the weak association of the mixture of both cyanobacterial agents.

Key words: *Nostoc muscorum, Anabaena variabilis, Triticum variabilis,* cyanobacterial isolates.

INTRODUCTION

It is known that the extensive application of mineral fertilizer is very costly and environmentally hazardous. So, application of biofertilizer is considered one of the most alternative solutions used to reduce soil pollution and agricultural expenses. In the recent years, several attentions have been drawn to use cyanobacterium that exists in the different soils as a biofertilizer for its capability for N₂-fixation and organic compounds assimilation (Prasanna et al., 2004). Efficiency of cyanobacteria to form such successful beneficial collaboration by invading the plant roots is of a considerable agronomic importance, particularly for cereal

production (Singh et al., 2011; Prasanna et al., 2013b). Likewise, some of cyanobacteria showed an effective role in stimulating soil fertility by producing plant growth enhancing substances, consequently increasing nutrients availability and plant uptake (Sergeeva et al., 2002; Prasanna and Kaushik (2006).

Aref and EL-Kassas (2006) pointed out that inoculation of the soil by genera *Nostoc* and/or *Anabaena* of cyanobacteria significantly augmented maize yield. A significant increase in chlorophyll content, dry weight, many enzymes activities as a result of cyanobacterial inoculation of wheat roots were reported by (Babu et al.,

2015).

A significant promotion in dry weight, total nitrogen, chlorophyll a, chlorophyll b, carotenoids and pigments occurred in wheat plants treated with cyanobacterial biofertilizer (Dhar et al., 2015). Cyanobacteria also play an effective role in carbon and nitrogen cycling, wherever it enhance the production of ammonia and indolic compounds. They also reported that analysis of harvest stage of rice elucidated a remarkable augmentation in soil biomass carbon, nitrogen availability, grain yield and 1000 grain weight.

Smith and Crews (2014) showed that cyanobacteria may have a beneficial effect on inhibition plant diseases by reducing the herbivore or pathogen host population. Therefore, the present work aimed to estimate the potential of two locally isolated cyanobacterial strains in Egypt, namely *Nostoc muscorum* and *Anabaena variabilis* for developing association with wheat roots, as well as their effect on plant growth and nitrogenase activity for possibilities of utilizing it as a wide biofertilizer application.

MATERIALS AND METHODS

Isolation and cultivation of Cyanobacteria

Cyanobacterial isolates were collected from the surface layer (0 to 20 cm) of an alluvial clay soil (pH 7.5, EC 2.5 dSm^{-1} and OM 1.07%). The isolated cyanobacteria were purified and identified to be *N. muscorum* and *A. variabilis,* following the technique described by Holt et al. (1994).

The axenic cultures were maintained in BG-11 medium (Allen and Stainer, 1968), and kept under constant conditions with continuous light (3000 lux) provided by four 18 W fluorescent tubes, and at a temperature of 26±2°C.

Root colonization assay of cyanobacterial isolates

Wheat seeds (*Triticum vulgare* L.) were germinated for three days and seedlings were placed in glass jars, each containing 50 ml of BG-11 medium containing 0.5% agar. To evaluate the root colonization ability of each cyanobacterial isolate in wheat seedlings, the prepared glass jars were divided into four groups as follows:

Applied treatments:

1. Control without inoculation *"C"*.
2. Inoculation with *N. muscorum* **"N"**.
3. Inoculation with *A. variabilis* **"A"**.
4. Inoculation with a mixture of **"N"** + **"A"**.

The experimental jars were inoculated with 4 ml of 15 day-old cyanobacterial culture of each strain. Each treatment was performed in three replicates and incubated in the presence of continuous light (at intensity of 3000 lux) and at temperature of 26±2°C. Medium in the jars was maintained at a constant volume via compensation wherever needed. Colonization was tested, after 15 days, by having transverse root sectors made by the microtome technique. The roots transverse sectors were examined under the light microscope by magnification power of 100x.

Nitrogenase activity and chlorophyll "a" content determination

Acetylene reduction technique was used to assay nitrogenase activity (Hardy et al., 1973) for cyanobacterial association with the roots of wheat seedlings. Whole plants (15 day-old) were placed, with their roots immersed in 5 ml medium, in sealed glass vessels each containing 17 ml of 10% (v/v) acetylene in air and incubated on an orbital shaker at a light intensity rate of 3000 lux for 60 min.

Wheat roots were extracted using methanol in the dark at 4°C, and content of chlorophyll **"a"** (Chl **"a"**) was determined spectrophotometrically at 663 nm, according to MacKinney (1941). Moreover, growth of wheat seedlings were estimated in terms of shoot and root dry weights, which were then statistically analyzed, according to Gomez and Gomez (1984).

Statistical analysis

Complete randomize design was applied on the experiment, the LSD of 0.05% was calculated through analysis of variance (ANOVA) to make a comparison among the means of the obtained data of the tested treatments.

RESULTS AND DISCUSSION

Colonization of cyanobacteria with the roots of 15 days old wheat seedlings

Figures 1a, b, 2a and b show that, *N. muscorum* and *A. variabilis* had ability to colonize the roots of wheat seedlings. In the case of the mixture of both cyanobacterial strains, little colonization was observed, due certainly to contribution of *N. muscorum* strain. Such observations were detected in comparison with the control treatment without cyanobacterial inoculation, which showed no colonization occurred (Figure 1a). The attachment of cyanobacteria was characterized by long filaments loosely packed together, growing between root hairs. In the case of *Anabaena*, individual filaments were observed on the surface and around the roots in freshly cut transverse sections (Figure 2b) as they were not intimately associated with root epidermis. On the other hand, *Nostoc* formed tight associations, where packed filaments were observed on wheat roots. The cyanobacterial packages made an intimate contact with the root surface. In contrast, the strain forming a loose attachment (*Anabaena*) was not characterized by abundant sticky mucilage production.

Fadl-Allah et al. (2011) explained the process of colonization for the cyanobacteria forming a tight association with wheat roots, by the first stage of colonization of *N. muscorum* which might occur via migration of hormogonia. The hormogonia developed into long filamentous, and the long filaments were developed at the seriate stage, which consisted of filaments tightly packed in a mucilaginous sheath. They added that, in addition to forming a tight association, *N. muscorum* also appeared to penetrate some root cells as cyanobacterial mass.
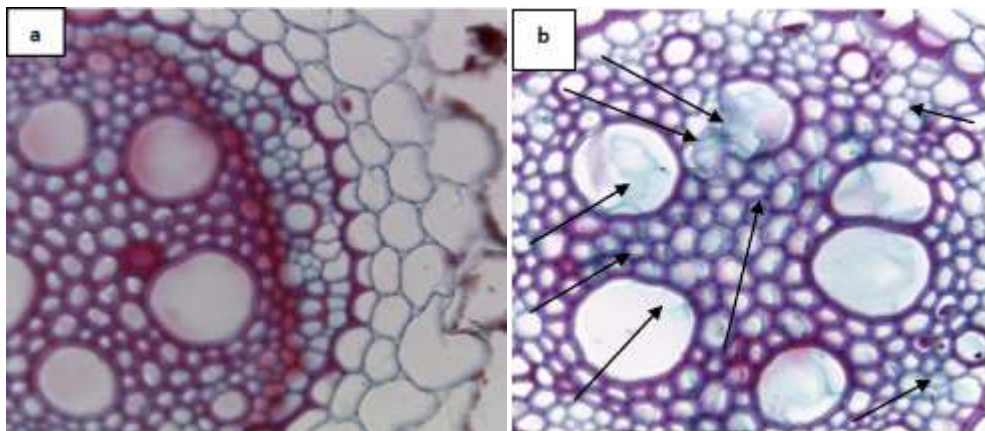
Figure 1. a) Control: wheat root without cyanobacterial inoculation; b) wheat root colonized with *N. muscorum* (as indicated by arrows).
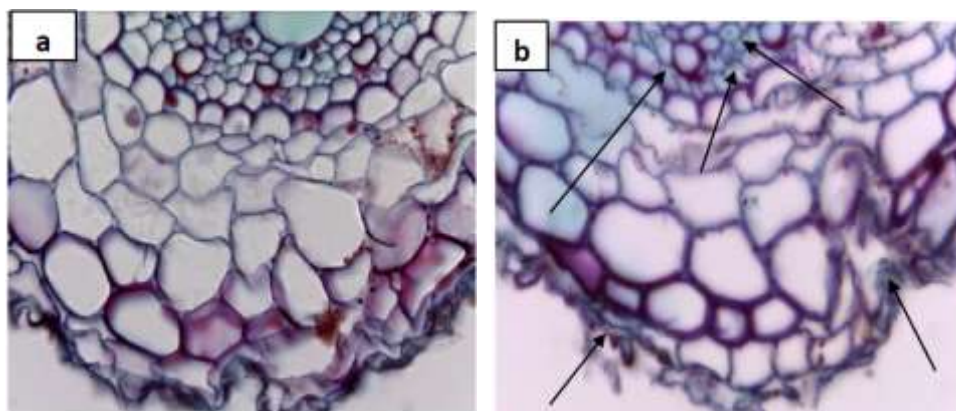

Figure 2. a) Root of wheat seedlings inoculated with *A. variabilis*; b) root of wheat seedlings inoculated with *N. muscorum* and *A. variabilis.* Little colonization is shown by *N. muscorum* (as indicated by arrows).

Table 1. Effect of cyanobacterial inoculation on some growth parameters of wheat seedlings (15 days old).

Treatment	Growth parameters of wheat seedlings							
	Roots					Shoots		
	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)	Chl." a" (µg g^{-1} dwt.)	*N-ase activity	Length (cm)	Fresh wt. (mg)	Dry wt. (mg)
Control	6.7	29.2	2.1	000	0.0	16.5	109.8	19.4
N. muscorum "N"	8.5	88.6	6.8	450	1.60	29.6	158.8	26.5
A. variabilis "A"	7.4	60.5	5.7	000	0.0	24.0	143.5	23.8
"A" + "N"	6.5	58.5	4.9	302	1.28	18.8	140.4	21.2
L. S. D. at 0.05%	1.15	27.0	2.2	–	–	1.7	32.1	1.4

*N-ase: Nitrogenase activity nmol C_2H_4 h^{-1}.

Cyanobacterial inoculation affecting growth of wheat seedlings

Data listed in the Table 1 reveal that, inoculation with cyanobacterial isolates significantly increased the measures of both roots and shoots of wheat seedlings, as compared to the un-inoculated seedlings. However, inoculation with *N. muscorum* was superior to *A. variabilis* and/or to their mixture in promoting the tested growth criteria of the plants, that is, length and fresh and dry

weights.

Results of the current study denoted that, inoculation of wheat plant roots with *N. muscorum* improved wheat seedlings growth, this agreed with previous findings reported by Gantar (2000) and Babu et al. (2015). In addition to its ability to fix N_2 and supply such essential nutrient to plants, cyanobacteria have been shown to produce compounds that stimulate the growth of plants.

In an earlier study by Prasanna et al. (2008, 2013a), it was proven that cyanobacteria had the ability to produce phytohormone "IAA". Nanjappan et al. (2007) evaluated the potential of plant growth promoting cyanobacteria as a biofertilizer for wheat. Gantar (2000) and Prasanna et al. (2012) showed that, the N_2 fixed by *Nostoc* sp. strain in association with wheat was taken up by the plants and supported their growth.

Effect of cyanobacterial inoculation on chlorophyll "a" content and nitrogeanase activity in roots of wheat seedlings

Chlorophyll "a" was not detected in roots grown either in absence of cyanobacteria, generally, or in the presence of *A. variabilis,* that was unable to form tight associations. The tight associations appeared by *N. muscorum* had a high Chl "a" concentration (450 µg Chl "a" g^{-1} root dry wt.), as compared to *A. variabilis* which had a loose association with wheat plant roots.

Nitrogenase activity was not also detected in the roots grown either in the absence of cyanobacteria, or presence of *A. variabilis*. The tight associations of *N. muscorum* occurred a high nitrogenase activity (1.6 nmol C_2H_4 h^{-1}), as compared to *A. variabilis* which had a loose association with wheat roots.

Earlier studies have shown that the heterocystous cyanobacterium *Nostoc* sp. strain 2S9B was unusual among characterized cyanobacteria in its ability to form tight associations with wheat roots and to penetrate both root epidermis and cortical intracellular and intercellular spaces. *Nostoc* sp. strain 2S9B could easily co-exist within wheat tissue, making it a promising organism for achieving a biological association between a N_2-fixing bacterium and wheat. Gantar and Elhai (1999) and Prasanna et al. (2013b) tested four heterocystous cyanobacteria, belonging to the genera, *Anabaena* and *Nostoc* for their ability to form associations with the roots of wheat seedlings under using hydroponics. The cyanobacterial strains formed close associations with wheat plants, and were able to enter through root hairs and penetrate the epidermal layer of wheat roots. Moreover, there was a significant higher indole acetic acid production and chlorophyll accumulation observed in the colonized roots.

Conclusion

The present study showed that the ability of cyanobacterium *N. muscorum* strain to form tight association and colonization with wheat roots, enhanced nitrogenase activity consequently, plant growth through its potential role as a nitrogen fixer bacteria. So, it may be utilized as a biofertilizer and contribute in promoting the amount of nitrogen fertilizer needed by wheat crop. However, further studies must be undertaken to confirm the effect of this strain (*N. muscorum*) on other plants under Egyptian Conditions.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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