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African Journal of Biotechnology

ARTICLES

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academic**Journals**

African Journal of Biotechnology

Full Length Research Paper

Ascorbic acid, β-carotene, total phenolic compound and microbiological quality of organic and conventional citrus and strawberry grown in Egypt

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A two year study at Alexandria University compared ascorbic acid, β-carotene, total phenolic compound, nitrite content and microbiological quality of orange and strawberry fruits grown under organic and conventional management techniques to see if producers concerns are valid. Organically grown oranges and strawberries had lower titratable acidity (TA); whereas, there was no significant difference in total soluble solid (TSS) of oranges and strawberries between two production systems. Higher ascorbic acid and β-carotene content was found in organically grown oranges and strawberries, compared to conventionally grown ones. Total phenol content (TPC) was significantly higher in conventional oranges compared to its organic production. Conversely, TPC was significantly higher in organic strawberries than the conventional ones. Comparative analyses of the microbial counts of organic and conventional oranges and strawberries fruit showed that *Escherichia coli* (*E. coli*) were not detected in any sample. However, conventional oranges and strawberries fruits have greater counts of yeasts and mold than organic ones. Nitrites were detected in all samples. The nitrites levels from organic production varied between 0.13 and 0.16 mg/kg fresh weight (FW), whereas those from conventional production ranged from 0.20 to 0.25 mg/kg FW. Our results show that the ascorbic acid, β-carotene, TPC, TA, nitrite content and biological quality were dependent on the agricultural production system, while for TSS%, this dependency was not pronounced.

Key words: Ascorbic, β-carotene, organic, orange, strawberry, yeasts and molds.

INTRODUCTION

Organic food is tasty, healthy and safe. Sales figures and popularity of organic food have been continuously rising for years. The Egyptian organic agriculture movement was started in the 1990s by SEKEM, a non-governmental organization which applied organic techniques. In Egypt, more than 95% of organic products are exported (IFOAM and FiBL, 2006). Egypt has one of the biggest organic sectors of all African nations and likewise one of the

biggest domestic markets, though it is still on the whole quite small. Egypt grows a total of 24, 548 ha in organic agriculture, 0.72% of its total agricultural land. Main organic crops include fresh vegetables, tropical fruits, dried fruits, cotton, herbs and spices, medicinal plants and cereals (IFOAM and FiBL 2006). A private investor is currently seeking to certify over thirty million hectares of desert to be reclaimed for organic farming (Hashem, 2006).

Orange and strawberry are two from the most important fruits production, consumption and export in Egypt. Egypt is among the top four citrus producers in the Mediterranean Basin. Oranges are the main citrus fruit grown in Egypt, accounting for about 60% of total citrus production. Egypt is the sixth largest orange producer and the second biggest exporter (FAOSTAT, 2009). According to Food and Agriculture Organization (FAO) of the United Nations, in 2010, Egypt production of straw-berries increased more than 3 times from 70,000 to 240,000 tons, and Egypt becomes the seventh largest strawberry producer in the world. Moreover, the iden-tification of oranges and strawberries with high nutritive value represent a useful approach to select those fruits with better health-promoting properties.

Organic oranges and strawberries achieved higher prices than conventional ones, because these products are often linked to sew up the environment, better quality (taste, storage) and most people believe that they are healthier. Moreover, research results on the effects of organic and conventional production on quality some-times are contradictory. In terms of quality, some studies report better taste, higher vitamin C contents and higher levels of other quality related compounds for organically grown products (Mitchell et al., 2007; Caris-Veyrat et al., 2004), whereas, several other studies have found the opposite or no differences in quality characteristics bet-ween organically and conventionally grown fruits and vegetables (Caris-Veyrat et al., 2004). In addition, based on the fact that organically grown fruit and vegetables often rely on manure as fertilizer, these products are perceived to pose a greater risk for foodborne disease (*Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli*) than conventional crops (Johannessen et al., 2004). However, there have been very few scientist reports that have conducted microbiological analysis of organic fruit and vegetables.

People often buy organic fruits and vegetables because they consider organic fruits and vegetables to be more beneficial to both human health and environment, and with better flavor than conventional or integrated counter-parts (Lester et al., 2007). Here we evaluated if there are significant differences in orange and strawberry fruit quality from commercial organic and conventional agro-ecosystems in Egypt. There is a scarcity of data on orange and strawberry fruit quality under organic farming system. The aim of the present study was to compare phytochemical characteristics, nitrite content and microbiological quality of Valencia orange and Festival strawberry produced under organic and conventional farming systems in Egypt in order to be able to address consumer's considerations.

MATERIALS AND METHODS

Sampling

A total of 100 orange and strawberry samples (50 organic and 50 conventional) were purchased in Carrefour hypermarket in the city of Alexandria, located in the south region of Egypt. The samples included: orange (*Citrus Sinensis* L., cv. Washington navel) and strawberry (Fragaria vesca L., var. Festival). Organic oranges and strawberries were produced from organic agriculture company located in Nobaria region. The organic fruits had a certificate issued by Demeter and/or Bio-Suisse certification. All samples were taken to laboratory in sterile plastic bags and they were processed immediately. Some samples were used for analysis of ascorbic acid, total soluble solid (TSS %) and titratable acidity on the same day and were therefore stored at room temperature. The other samples were stored in refrigerator at approximately 4°C until tested on the next day for β-carotene and total phenol content. Three replicates per treatment (each replicates contained 10 fruits of oranges and strawberries) were washed under running water and the non-edible parts (orange peel and leaves of strawberry) were removed. Oranges were hand squeezed and strawberries were cut and homogenized. Orange juice and strawberries homogenate were utilized for the ascorbic acid, TSS and acidity determination.

Determination of total soluble solids (TSS) and titratable acidity (TA)

A portion of fresh orange juice and a small fraction of strawberries homogenate were centrifugated at 4000 g for 5 min and the supernatant was analyzed for TSS and TA. The percentage of total soluble solids (TSS) was measured by a hand refractometer (ATC-1E, Atago, Japan), and acidity as tartaric acid (TA) was determined by titration with 0.1 N NaOH according to AOAC (1995).

Extraction and high performance liquid chromatography (HPLC) analysis of ascorbic acid and β-carotene

Ascorbic acid was extracted according to modified method described by Abdulnabi et al. (1997). Homogenized fresh sample (10 g) of strawberries and another sample of 5 ml of orange juice were extracted with a 5 ml solution of (0.3 M) meta-phosphoric acid and (1.4 M) acetic acid. The mixture was placed in a conical flask (wrapped with aluminum foil) and agitated at 100 rpm with the aid of an orbital shaker for 15 min at room temperature. The mixture was then filtered through a Whatman No. 4 filter paper to obtain a clear extract and then injected directly into Shimadzu HPLC (model CR4A, Japan).

β-carotene was extracted according to the slight modified method of Tee et al. (1996). A sample of strawberries (10 g) and another sample of 5 ml of orange juice were homogenized with 40 ml of 99.8% ethanol and 10 ml of 100% potassium hydroxide for 3 min by a blender. The mixture was heated for 30 min then cooled to room temperature. Exactly 50 ml of n-hexane was added to the texture and shaken strongly for a few seconds and the upper layer (hexan extract) allowed to separate and then removed. The aqueous layer was re-extracted twice with 50 ml of n-hexane in each time. The extract was filtered through anhydrous sodium sulphate to remove any water residue and exposure to reduced pressure at 45°C to

Table 1. Mean concentration of ascorbic acid, β-carotene, total phenolic content, TSS and acidity in orange fruits produced by organic and conventional farming in 2012 and 2013 seasons.

Farming system	Ascorbic acid (mg/100 g)	β-Carotene (IU/100 g)	Total phenolic content (mg/100 g)	TSS (%)	Acidity (%)
2012					
Conventional	55.57 ±0.57[z]b	543.67 ±1.52b	165.28 ±0.34a	9.98 ±0.12a	1.40 ±0.05a
Organic	77.62 ±1.18a	582.77 ±1.45a	126.20 ±0.57b	10.00 ±0.15a	0.90 ±0.12b
2013					
Conventional	55.71 ±0.50[z]b	542.89 ±2.04b	164.65 ±0.68a	10.40±0.12a	1.42±0.03a
Organic	78.43 ±1.12a	587.46 ±1.70a	121.29 ±2.10b	10.46±0.03a	0.90±0.12b

Means in a column followed by a different letter differ significantly at P = 0.05 by L.S.D test. [z]Means ± SE.

Table 2. Mean concentration of ascorbic acid, β-carotene, total phenolic content, TSS and acidity in strawberry fruits produced by organic and conventional farming in 2012and 2013 seasons.

Farming system	Ascorbic acid (mg/100 g)	β-Carotene (IU/100 g)	Total phenolic content (mg/100 g)	TSS (%)	Acidity (%)
2012					
Conventional	31.12 ±0.32[z]b	351.55 ±1.02b	7.65 ±0.18b	6.78 ±0.05a	0.39 ±0.02a
Organic	51.27 ±1.01a	407.21 ±0.29a	16.05 ±0.36a	6.80 ±0.05a	0.23 ±0.01b
2013					
Conventional	33.75 ± 0.61[z]b	351.57 ±2.50b	8.24 ±0.18b	6.78 ±0.05a	0.38 ±0.02a
Organic	51.27 ±1.01a	413.50 ±2.59a	17.04 ±0.12a	6.80 ±0.05a	0.22 ±0.01b

Means in a column followed by a different letter differ significantly at P = 0.05 by L.S.D test. [z]Means ± SE.

remove hexane residue then injected directly into a shimadzu HPLC.

HPLC analysis of ascorbic acid and β-carotene were performed on LC- 6A equipment consisting of LC- 6AD pumps, an in-line degasser, a CTO-6A column oven, a SCL-6A system controller, a SPD 6Avp, a photo diode array detector, a refractive index detector and operated by LC solution software (Shimadzu, Japan).

Determination of total phenol content

Homogenized fresh sample (0.5 g) of strawberries and another sample of 5 ml of orange juice were extracted with a 5 ml 75% (v/v) ethanol under periodical stirring at 45°C (Roussos et al., 2009). After centrifugation (4000 x g for 10 min), a quantity of 0.5 ml Folin-Denis reagent was added to 1 ml of the alcoholic extract and after 5 min, 7 ml saturated sodium carbonate solution was added, shaken and left for 0.5 h. Optical density was measured at 750 nm and total phenols were calculated from a standard curve of tannic acid. These data were expressed as the mg tannic acid equivalents per gram of fresh weight basis according to Slinkard and Singteton (1977).

Determination of nitrite

The nitrite contents in the oranges and strawberries were determined by a spectrophotometric method on foodstuff and water. The nitrite is determined by diazotizing with sulfanilamide and coupling with N-(1- naphthyl)-ethylenediamine dihydrochloride to

form a highly colored azo dye that is measured at 540 nm (Merino, 2009). Three replicates were analyzed; nitrite levels were expressed as mg/kg fresh weight (FW).

Microbiological analysis

Fruit were kept in refrigerator for no longer than 24 h prior to analysis. Each fruit was transferred to an individual, sterile plastic bag using gloves with 30 ml of 0.1% buffered peptone water (BPW). The sterile bags were hand- rubbed for a minute to remove surface microorganisms (Parish et al., 2001). Number of colony (yeasts and molds) was counted by methods (Beuchat and Cousin, 2001; Morton, 2001). *E. coli* were counted by the fast Petrifilm TM method (Kornacki and Johnson, 2001), and the results were reported as colony forming units per gram (CFU/g).

RESULTS AND DISCUSSION

There was no significant difference in TSS content between organically grown oranges and strawberries (Tables 1 and 2). Compared with conventional production system, higher TSS content was reported in oranges, lemons and mandarin grown under organic production system (Duarte et al., 2010). Consistent with our results, no significant differences in TSS percentage were found for citrus and strawberries fruits between organic and

conventional systems (Nunes et al., 2010; Camin et al., 2011 and Roussos, 2011).

Organically grown oranges and strawberries had lower TA than conventionally grown oranges and strawberries (Tables 1 and 2). This is in contrast to what was observed by Koneru (2013) who found that compared with conventional farming, higher TA content was reported in organically grown peaches. In agreement with our results, organic 'Washington Navel' oranges showed lower TA than organic ones (Candir et al., 2013). Based on previous reports, there is a slight difference on the organic acid concentration in the juice according to farming system. This could be attributed to the difference in fertilization system.

The data in Tables 1 and 2 clearly indicates that organically grown oranges and strawberries had higher ascorbic acid content than conventionally grown oranges and strawberries during both seasons. For example, in 2012 season, conventionally and organically grown oranges contained 55.57 and 77.62 mg/100 g, respectively. The corresponding values for strawberries were 31.12 and 51.27 mg/100 g, respectively. Many investigators also reported this increase in ascorbic acid content such as Duarte et al. (2010) on 'Valencia late' and 'Baia' oranges, Lester et al. (2007) on 'Rio Red' grapefruit, Asami et al. (2005) on Northwest Totem strawberry variety and (Jin et al., 2011) on Earliglow and Allstar strawberries. They concluded that a significantly higher ascorbic acid concentration for organically grown versus conventionally grown citrus and strawberries. A review in 2006 reported that organic foods had higher amounts of antioxidant (ascorbic acid) and lower levels of pesticide residues, nitrates and heavy metals contaminations than conventionally grown crops. Through that, organic crops had higher nutritional value and lower risk of causing disease due to contamination (Gyorene et al., 2006). Moreover, Duarte et al. (2012) demonstrated that the higher ascorbic acid content in citrus fruit juice from organic production system depend on species and cultivar.

According to previous studies, the possible interpretation for this finding is that nitrogen fertilizers under high rates seems to decrease the concentration of ascorbic acid content in fruits and vegetables (Lee and Kader, 2000) Besides Lee and Kader (2000) reported that the use of agrochemicals and pesticides may affect the nutritional quality of fruits and vegetables.

A greater β-carotene content in oranges and strawberries from organic compared to conventional farming systems was found (Tables 1 and 2). Roussos (2011) reported that organic management increased carotenoid concentration significantly compared to integrated farming system. The accumulation of carotenoid under organic farming system could be attributed to fertilization strategy. According to Gross (1987), soil fertilization is one of the factors that affects the biosynthesis of carotenoids in fruits.

A common explanation for reported differences in phytochemicals between organic and conventional produce is that organic systems are more stressful than conventional systems due to the limited and restricted use of pesticides in organic systems, thus allowing for greater incidence of biotic stresses (Tarozzi et al., 2006).

Among the TPC detected in the orange juice, significant differences were observed, where the juice of conventional produced fruits exhibited higher values (Tables 1 and 2). In contrast, the results indicated that there were significantly lower concentrations of TP in conventionally grown strawberry than the organically grown one. Jin et al. (2011) indicated that the TPC was significantly higher in organically cultivated strawberries than in conventionally cultivated strawberries. Biosynthesis of phenolic compounds in plants is strongly affected by the cultivator techniques, environmental conditions and the fertilizers used. Häkkinen and Törrönen (2000) reported that, of three strawberry cultivars tested by sampling from organic and conventional farms that increased, phenolic compounds only occurred in one cultivar under organic conditions, possibly due to pathogen attack. It has previously been reported that the phenol concentration is influenced by level of available nitrogen (Brandt and Molgaard, 2001). Increase in phenolic compounds is related to the defense role they play in plants under stressed conditions (Dixon and Paiva, 1995). In the absence of pesticides, plants could contain higher levels of antioxidant components as a result of enhanced synthesis of active phytochemicals produced in defense against biotic and abiotic stress (Tarozzi et al., 2006).

The microbiological quality of organic and conventional oranges and strawberries was determined by analysis of yeasts and molds and *E. coli* (Table 3). *E. coli.* (0.0 MPN/100 g) was not detected in oranges and strawberries fruits under organic and conventional production system.

In oranges, yeasts and molds were present in smaller accounts (1.0×10^2 CFU/g) under organic production system whereas conventional oranges presented higher counts (4.0×10^2 cfu/gm) than organic ones. Conventional samples of strawberries presented higher yeasts and molds counts than organic samples. In strawberries, yeasts and molds count ranged from 11×10 to 14×10 CFU/g under organic production system, whereas the count was from 60×10^4 to 77×10^4 CFU/g under conventional production system. These results contradict those of Maffei et al. (2013) who reported that *E. coli* was found in organic and conventional vegetables and higher microbial count of organic vegetables compared with conventional ones. Although yeasts and molds are associated with food spoilage, the mycotoxins produced by molds may be dangerous to health (Maffei et al., 2013). Mycotoxins caused many diseases, including carcinogen and immunosuppressive effects (Kovacs, 2004). Many other studies would be necessary to confirm these

Table 3. Yeasts and molds and *Escherichia coli* in orange and strawberry fruits produced by organic and conventional farming in 2012 and 2013 seasons.

| Sample | Yeasts and molds (cfu/g) | | | | *Escherichia coli* | | | |
| | Organic | | Conventional | | Organic | | Conventional | |
	2012	2013	2012	2013	2012	2013	2012	2013
Citrus	1.0×10^2	0.0×10	2.0×10^2	4.0×10^2	0.00	0.00	0.00	0.00
Strawberry	11.0×10	14.0×10	60×10^4	77×10^4	0.00	0.00	0.00	0.00

Table 4. Mean concentrations of nitrates in orange and strawberry fruits produced by organic and conventional farming in 2012 and 2013 seasons.

| Sample | Nitrates (mg/l) | | | |
| | Organic | | Conventional | |
	2012	2013	2012	2013
Citrus	0.20 ± 0.01^za	0.20 ± 0.01^za	0.14 ± 0.01b	0.13 ± 0.01b
Strawberry	0.25 ± 0.01a	0.24 ± 0.01a	0.16 ± 0.02b	0.15 ± 0.01b

Means in a column followed by a different letter differ significantly at $P = 0.05$ by L.S.D test.
^{z}Means ± SE.

observations. In addition, handling condition especially in Egypt should be considered, since they may impact the microbial profile of organic and conventional fruits.

To our knowledge, this is the first report that compares the nitrite level in oranges and strawberries fruit in organic and conventional production systems under Egypt conditions. The results obtained for nitrite levels are shown in Table 4. The results show a considerable significant variation in the average levels of nitrite contents between the two production systems. The average levels of nitrites were higher in conventional oranges and strawberries fruits. The nitrites levels from organic production varied between 0.13 and 0.16 mg/kg fresh weight (FW), whereas those from conventional production ranged from 0.20 to 0.25 mg/kg FW. Similar tendency was found by Gonzalez et al. (2010) and Aires et al. (2012); they reported significant differences in the average levels of nitrate contents from organic and conventional produce. The limits detected for nitrite in our samples are within the legal limits (0-1 mg/kg FW for orange and strawberry) recommended by European Union regulations (1995); thus, from the point of view of nitrites, this type of fruits are safe.

From this study, we can conclude that farm management techniques can affect the overall quality of orange and strawberry fruit. Organically grown oranges and strawberries had higher ascorbic acid and β-carotene content and lower titratable acidity than conventionally grown ones. Total soluble solid (TSS) of oranges and strawberries were not affected by the production systems. Total phenol content (TPC) was significantly higher in conventional oranges compared to its organic production. Conversely, TPC was significantly higher in organic strawberries than the conventional ones. Conventional oranges and strawberries fruits have greater counts of yeasts and mold than organic ones. The average levels of nitrites were lower in organic oranges and strawberries fruits. Organic fruits and vegetables seem to become popular because of the concerns over environmental contamination and health benefits. However, it is important to analyze a wide variety of fruit and vegetables to elucidate the possible benefits of the consumption of organic foods as part of a whole diet.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Influence of the reuse of progesterone implants in a fixed-time artificial insemination protocol on the conception rates of lactating cows during two different seasons

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This study aimed to evaluate the influence of reusing progesterone intravaginal implants using a fixed-time artificial insemination (FTAI) protocol on the conception rates of 593 primiparous and multiparous lactating cows during two different seasons. The cows were divided into two categories: multiparous and primiparous. The experiment was conducted during seasons with high and mild temperatures. To compare the conception rates during both seasons, the animals were randomly divided into three groups according to use progesterone implant (1st use, 2nd use and 3rd use) on the first day of the synchronization protocol for FTAI within each category. A pregnancy diagnosis was performed at 30 days following FTAI. The temperature and humidity index was higher (P<0.01) during the season with higher temperatures (74.4 ± 0.26) than during the season with mild temperatures (67.5 ± 0.34). The different categories (multiparous and primiparous) did not influence the conception rate in terms of the seasonal temperatures or the reuse of progesterone implants. Nevertheless, a 3rd use implant administered during the season with high temperatures was 1.98 times less likely to result in a pregnancy compared with a 1st use implant (P=0.01) and 2.83 less likely to result in a pregnancy than the other implants used during the season with mild temperatures (P=0.005). The results indicate that during the season with mild temperatures, the conception rates at 30 days are not influenced by reusing the P_4 implant in any of the categories analyzed. However, during the season with high temperatures, the reuse of the P_4 implant can influence the conception rates of dairy cows.

Key words: Heat stress, Holstein cows, pregnancy.

INTRODUCTION

The increasing demand for animals with higher milk productivity has intensified genetic breeding of herds; however, there has also been a decrease in the reproductive efficiency of these cows (Lucy, 2001). To meet this production requirement, there is a need for a high dry matter intake, which promotes an increase in hepatic blood flow and in the rate of steroid hormone metabolism, thus decreasing blood concentrations of

steroids, which can negatively affect reproductive function (Sangsritavong et al., 2002). The progesterone is involved in oocyte quality, follicular dominance, embryonic growth and maintenance of pregnancy (Leonhardt and Edwards, 2002). Low pre-insemination progesterone levels are responsible for the persistence of dominant follicles, producing a low quality oocyte upon ovulation (Bisinotto et al., 2013). Mann and Lamming (2001) reported that embryonic development is impaired when there are low levels of progesterone exposure following conception due to the low levels of interferon-tau release and, therefore, prostaglandin $F_{2\alpha}$ release. Reproductive challenges increase with heat stress, reproductive diseases, inadequate nutrition, and increased animal productivity. The problem of repro-ductive inefficiency in high producing dairy cows may be minimized with genetic selection and breeding (Banos et al., 2013). All these factors have leveraged the use of reproductive biotechnologies as artificial insemination, multiple ovulation, *in vitro* fertilization and cloning to optimize the production and reproductive performance of the herd in a more rational and economical way (Binelli et al., 2009). Thus, fixed-time artificial insemination (FTAI) has helped in reducing the number of days in lactation until the first insemination and the calving interval in dairy herds (Mapletoft et al., 2009).

Even though others reports have compared the progesterone concentration achieved after insertion of new or reused progesterone implants (Zuluaga and Williams, 2008; Abdallah and El Rahim, 2014). However, there are no previous reports of the association of reusing progesterone implants in different categories of dairy cows and seasons. This study aimed to determine the influence of reusing progesterone implants in FTAI protocols on the conception rate in primiparous and multiparous lactating cows during seasons with high temperatures and mild temperatures.

MATERIALS AND METHODS

Location

The experiment was conducted on a dairy farm in the city of Montividiu in southwestern Goiás State, Brazil (latitude 17°20'5.7" and longitude 51°18'46.7"). During the experiment, lactating Holstein cows were confined in wooded feedlots and supplied with drinking troughs. The animals received a complete diet consisting of quality corn silage and a concentrated diet balanced for milk production (NRC, 2001), which was distributed four times a day using a mixing wagon.

Experimental procedures

The ambient temperature and relative humidity were measured daily throughout the experimental period. During the season with mild temperatures (May to August 2012), the average temperature was 21.1 ± 1.3°C, with a maximum of 23.4 ± 1.2°C and a minimum of 18.9 ± 1.5°C; the average relative humidity was 63 ± 14%. During the season with high temperatures (September 2012 to April 2013), the average temperature was 24.8 ± 0.7°C, with a maximum of 28.3 ± 0.6°C and a minimum of 20.1 ± 0.5°C; the average relative humidity was 78 ± 2%. Heat stress was indicated by the temperature-humidity index (THI) for each period, which was calculated using the following formula used by Mader et al. (2006): THI = T x 0.8 + [(RH (%) ÷ 100 x (T - 14.4)] + 46.4, where T is the temperature in degrees Celsius and RH is the relative humidity of the air. The mean and standard error of the THI was 67.5 ± 0.34 for the season with mild temperatures and 74.4 ± 0.26 for the season with high temperatures.

Animals

The experimental animals (n = 593) were divided into two categories: multiparous (mean of 28.7 ± 3.6 kg milk.day^{-1} [n = 342]) and primiparous (mean of 25 ± 4.3 kg milk.day^{-1} [n = 251]). The selected cows were 58.7 ± 9.3 days postpartum; had a body condition score between 2.5 and 3.5 on a scale from 1 to 5, with 1 being very thin and 5 being very fat (Ferguson et al., 1994); and had a uterus without signs of infection upon clinical examination and ultrasonography (Mindray® DP3300 VET).

Experimental groups

To compare the conception rates of each category during both seasons, the animals were randomly divided into three groups according to use progesterone implant (1st use, 2nd use and 3rd use) on the first day of the fixed-time artificial insemination protocol.

Hormonal treatment

The synchronization of follicular wave started on the first day of the fixed-time artificial insemination protocol (D0), when the animals in each group received a 1st, 2nd, or 3rd use intravaginal progesterone implant (Cronipres®, Biogénesis-Bagó, Garin, province of Buenos Aires, Argentina) and intramuscular application of 2 mg of estradiol benzoate (Bioestrogen ®, Biogenesis-Bagó, Garin, province of Buenos Aires, Argentina). After eight days (D8), the implant was removed, and 0.15 mg of sodium cloprostenol (Bioestrogen®, Biogénesis-Bagó, Garin, province of Buenos Aires, Argentina), 300 IU of equine chorionic gonadotropin (Folligon®, Intervet International B.V., Boxmeer, Holland), and 1 mg of estradiol cypionate were administered (ECP®, Pfizer, Pharmacia and Upjohn Company, NY, USA). Forty-eight hours after implant removal (D10), 0.004 mg of buserelin acetate (Sincroforte®, Ouro Fino, Cravinhos, SP, Brazil) was administered intramuscularly. The artificial insemination was performed with semen from the same bull and single inseminator. A pregnancy diagnosis was performed at 30 days following FTAI using an ultrasound device equipped with a 5.0 MHz linear transducer (Mindray® DP3300 VET).

Statistical analysis

The experimental design was completely randomized using a 2 x 2

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Table 1. Conception rates at 30 days in lactating cows according to the category (multiparous and primiparous), reuse of progesterone implants (1st, 2nd, and 3rd use) in the FTAI protocol, and seasons with mild and high temperatures.

Season	Implant	Conception rate at 30 days	
		Multiparous	**Primiparous**
Mild temperatures	1st use	40.0% (10/25)	54.5% (6/11)
	2nd use	43.9% (25/57)	55.0% (11/20)
	3rd use	50.0% (14/28)	52.6% (10/19)
High temperatures	1st use	39.3% (57/145)	46.3% (50/108)
	2nd use	34.6% (18/52)	41.0% (16/39)
	3rd use	28.6% (10/35)	25.9% (14/54)

Table 2. Analysis of deviance for the conception rate at 30 days in lactating cows in a factorial design for the different seasons, progesterone implants and categories.

Variable	DF	Deviance	ΔDF	Δ Deviance	P value
Null			592	801.17	-
Season	1	4.2373	591	796.93	0.03954
Implant	2	3.6727	589	793.26	0.15940
Category	1	1.9182	588	791.34	0.16606
Season : implant	2	3.6636	586	787.68	0.05012
Implant : category	2	0.8949	583	786.64	0.63924
Season : implant : category	2	0.0154	581	786.63	0.99234

x 3 factorial design to evaluate the effect of two categories (multiparous and primiparous), two seasons (high and mild temperatures), and three treatments (1st, 2nd, and 3rd use implants). The data of conception rate were analyzed using multiple logistic regression with a fixed regression model, considering a significance level of 5%. The model included the effects of treatment, season, implant and THI; because, it is unbalanced data, the variables and interactions between significant variables in the analysis of deviance ($P<0.05$) were considered in the final model. The odds ratios and their respective confidence intervals at 95% probability were calculated for the variables considered in the final model. The program R software (R Core Team, 2014) was used to perform the analysis.

RESULTS

The THI was higher ($P<0.01$) during the season with the high temperatures (74.4 ± 0.26) than during the season with the mild temperatures (67.5 ± 0.34). The conception rates at 30 days for multiparous and primiparous cows according to the progesterone implant used in the FTAI protocol during the seasons of high and mild temperatures are listed in Table 1.

The analysis of deviance for the conception rate at 30 days in lactating cows is presented in Table 2. The season of the year was a significant effect in the concept rate ($P=0.03954$). The different categories (primiparous

and multiparous) and the reuse of progesterone implants did not influence the conception rates.

However, there was a significant interaction of the reuse of progesterone implants and the season of the year with respect to the conception rate (Table 2). The logistic regression model applied to the variables that showed a significant interaction to conception rate of lactating cows in the analysis of deviance is presented in Table 3.

According to the logistic regression model, a 3rd use implant administered during the season with high temperatures was 1.98 times less likely to result in a pregnancy compared with a 1st use implant ($P=0.01$) and 2.83 times less likely to result in a pregnancy than the other implants used during the season with mild temperatures ($P=0.005$).

DISCUSSION

In both categories, there were no statistically significant differences in the conception rate with the reuse of 1st, 2nd, and 3rd use implants during the season with mild temperatures. These results are consistent with those observed by Mendonça et al. (2012), who reported no significant differences in the conception rates of

Table 3. Logistic regression for the conception rate of lactating cows following FTAI with 1st, 2nd, and 3rd use progesterone implants during seasons with high and mild temperatures.

Variable	Factor	Odds ratio	Confidence interval (95%)	P value
Implant - 1st use	-	1.00	-	-
Implant - 2nd use	Mild temperatures	0.91	0.41 - 2.02	0.82
	High temperatures	- 1.23	0.75 - 2.01	0.41
Implant - 3rd use	Mild temperatures	0.77	0.32 - 1.83	0.55
	High temperatures	- 1.98	1.17 - 3.37	0.01
Mild season	-	1.00	-	-
Hot season	Implant - 1st use	1.09	0.54 - 2.21	0.81
	Implant - 2nd use	- 1.47	0.79 - 2.73	0.22
	Implant - 3rd use	- 2.83	1.35 - 5.92	0.005

crossbred dairy cows when implants containing 1.9 g of progesterone were reused in an FTAI protocol. Rocha et al. (2007) also found no difference in the conception rates of Nellore cows upon the reuse of implants containing one gram of progesterone for up to four times. Guido et al. (2008) reported similar conception rates in lactating goats synchronized with new and used progesterone implants (53.3 versus 46.7%). High-producing dairy cows ingest diets that are denser, have an increased hepatic blood flow, and consequently exhibit an increase rate of steroid hormone metabolism that leads to a decrease in blood progesterone concentrations (Sangsritavong et al., 2002). Therefore, the implants should be reused with caution during periods with high temperatures, which can reduce the conception rates because of the metabolic obstacles to which cows are subjected. Climate factors are highly relevant for conception rate (Garcia-Ispierto et al., 2007). The THI during the season with the high temperatures had effect in conception rate of lactating dairy cows. According to Wheelock et al. (2010), values below 65 are within the range of thermal comfort for dairy cows. The action of heat stress on the follicle tends to produce oocytes with a lower fertilization capacity, and if this process occurs, the embryo does not develop normally (Hansen, 2002). In this study, 3rd use implants were unable to maintain similar conception rates in the different categories between the seasons evaluated (Table 3). This finding may be due to heat stress observed during the season with the high temperatures, reducing the concentrations of plasma progesterone necessary for recognizing and maintaining a pregnancy. According to Barbosa et al. (2011), the conception rate during the fall/winter (42.55%) is higher than the conception rate during the spring/summer (25%) when using implants containing 1.9 g of progesterone in an FTAI protocol. However, this difference was not observed when they compared the reuse of implants. Nevertheless, Barbosa et al. (2011) found no difference in the conception rates of crossbred dairy cows in the presence or absence of a corpus luteum at the beginning of the FTAI protocol.

Conclusion

The reuse of progesterone implants in FTAI protocols is an alternative for achieving good reproductive rates in dairy cows. However, the implants should be reused with caution during periods with high temperatures. As a result of non-pregnancy, mismanagement can lead to an increase in the calving-to-conception interval and in the calving interval.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Genetic diversity of Bambara groundnut (*Vigna subterranea* (L.) verdc.) landraces in Kenya using microsatellite markers

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The existence of genetic diversity in germplasm collections is crucial for cultivar development. Genetic relationships among 105 Bambara groundnuts (*Vigna subterranea* (L) Verdc.) accessions from Kenya were evaluated using 12 microsatellite markers. The Bambara landraces were collected from farmers in the western region and the National Genebank of Kenya. A total number of 24 alleles were revealed with a mean of 2 alleles per locus. The polymorphic information content and gene diversity values averaged 0.28 and 0.35, respectively indicating low genetic diversity among the evaluated Bambara groundnut germplasm. Genetic distance based on Jaccard's similarity coefficient from the simple sequence repeat (SSR) marker analysis ranged from 0.08 to 1.16 among the landraces. Cluster analysis distinctly grouped the 105 accessions into three major clusters. The analysis of molecular variance (AMOVA) revealed that 98% of the total genetic variation was within accessions whereas the genetic variation among accessions accounted for 2% of the total genetic variation. The genetic diversity observed in this study provides the basis for selection of appropriate parental genotypes for breeding programmes and mapping populations to further broaden the genetic base of Bambara groundnut germplasm in Kenya.

Key words: *Vigna subterranean*, accessions, Kenya, microsatellite markers, gene diversity, cluster analysis.

INTRODUCTION

Evaluation of available genetic diversity is a pre-requisite for genetic improvement in crop plants, especially in underutilized crops such as Bambara groundnut (Olukolu et al., 2012). Investigation of genetic diversity in both wild and domesticated species is equally important. Wild populations are known to be a potential source of useful genes and traits which could be introduced into the domesticated gene pool (Cattan-Toupance et al., 1998). Wild populations in centers of diversity or domestication constitute the initial gene pool of crops species. Crop

failures and dispersal of germplasm within the centre of origin or limited introduction or isolated locations could lead to reduced genetic diversity in particular breeding populations (Trethowan and Mujeeb-Kazi, 2008).

The genus *Vigna* (Family *Leguminosae*) is an important legume taxon. It comprises about 90 described species of which seven species are cultivated as economic crops in various regions. Several species are cultivated as minor crops and some wildly grown species are harvested for food and feed. Bambara groundnut is the third most important food legume of Africa after peanut and cowpea. The crop is a very important source of dietary protein for poor people in Africa who cannot afford expensive animal protein (Baryeh, 2001). Thus it has high potential for food security in unpredictable drought regions.

Average yield of Bambara groundnut is rather low compared with other cultivated *Vigna* crops; this, is mainly due to the fact that all of Bambara groundnut cultivars grown are landraces. No improved cultivars were developed by a selective breeding program because an efficient hybridization technique has just been developed (Suwanprasert et al., 2006). Before setting up a breeding program for Bambara groundnut, a thorough under-standing on its genetic diversity is necessary. Like many other orphan crops, there are only a few studies on genetic diversity in a large set of Bambara groundnut germplasm. Goli et al. (1997) and Olukolu et al. (2012) studied diversity based on seed patterns in 1,384 and 1,973 accessions, respectively. Olukolu et al. (2012) found that Bambara groundnut from Cameroon/Nigeria region had a higher diversity than those from the other geographical regions. Diversity study in 124 accessions using 28 quantitative traits and in 40 accessions using 554 Diversity Arrays Technique (DArT) markers by the same authors revealed the highest diversity in Cameroon/Nigeria region. The results support the view of Hepper (1963) that center of origin/domestication of Bambara groundnut is in the Cameroon/Nigeria region. In contrast, Somta et al. (2011) studied diversity in a collection of 240 Bambara groundnut accessions using 22 simple sequence repeat (SSR) markers and found highest diversity in West African (excluding Cameroon and Nigeria). This was also reported by Rungnoi et al. (2012). These studies suggest that the center of diversity and origin of Bambara groundnuts is still inconclusive and more evidence is needed to elucidate them. The avai-lable literature reveals a number of studies of genetic diversity in Bambara groundnut in the wild and domes-tication material. They offer a reasonable start to understanding the genetic basis of the domestication event(s) in this crop, potentially enabling parents with a wide genetic base to be identified for developing mapping populations and subsequent QTL analysis.

In this study we evaluated diversity in a collection of 105 Bambara groundnut accessions from several geographical origins in Kenya using simple sequence repeat (SSR) markers. The objective was to provide more evidences on genetic diversity and genetic relationships among different Bambara groundnut accessions in Kenya.

MATERIALS AND METHODS

Plant materials and DNA isolation

A total of 105 Bambara groundnuts accessions (Table 1) from Busia (39), the National Genebank of Kenya (37), Kakamega (6), Bungoma (6) and Vihiga (2) were planted in pots containing 0.00141 m^3 of soil in the greenhouse at the Kenya Agricultural Research Institute (KARI) Njoro, Kenya. To ensure sufficient tissue, young leaf samples (two weeks old) from four plants per accession were collected for DNA isolation and analysis using a modified protocol of the Doyle and Doyle (1990) CTAB protocol. The modifications involved omission of Ammonium acetate stage and longer (12 h) DNA precipitation. DNA Quantification was carried out by 0.8% agarose gel and Nanodrop 200c spectrophotometer (Thermo scientific corp.) and was diluted to 10 ng μl^{-1} for PCR.

Microsatellite marker analysis

Twelve (12) microsatellite primers (Molosiwa, 2012) (Table 2) were used to assess the genetic diversity of the 105 Bambara groundnuts accessions. The PCR amplification was performed in a 10 µl volume mix consisting of 5 U Dreamtaq polymerase enzyme (Thermo scientific corp, Lithuania), x 6 Dreamtaq buffer (Thermo scientific corp, Lithuania), 2.5 mM of each dNTPS (Bioneer corp, Republic of Korea), MgCl$_2$, 5 µM of each primer (Inqaba biotec, S.A) and 30 ng DNA template in an Applied Biosystems 2720 thermocycler (Life Technologies Holdings Pte Ltd,Singapore). The PCR cycles consisted initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54-59.7°C (depending on the primer), extension at 72°C for 1 min followed by one cycle of final extension at 72°C for 10 min. The amplicons were mixed with 6x Orange DNA loading dye (Thermo scientific corp, Lithuania) and separated on a 2% agarose gels (Duchefa, Netherlands) stained with Invitrogen life technologies ethidium bromide (Invitrogen corp, U.S.A) in a 0.5x TBE buffer. The separated amplicons were visualized on an Ebox-VX5 gel visualization system (Vilber Lourmat inc, France). The alleles were scored as absent or present based on the size of the amplified product using a 100 bp O'geneRuler ready to use DNA Ladder (Thermo Scientific Corp, Lithuania).

Data analysis

Molecular data was recorded in binary fashion for SSR marker loci analysed and scoring was based on presence and absence of band for each primer set with one (1) and zero (0) being the respective scores. The summary statistics on major allele frequency, allele number, gene diversity, and PIC values (Botstein et al., 1980) were calculated using Power Marker version 3.25 (Liu and Muse, 2006) while Shannon's information index (*I;* Lewontin, 1972) of each locus was calculated using software popGene32 version 1.32 (Yeh et al., 2000). Analysis of molecular variance (AMOVA) was performed using Arlequin v.3.1 (Excoffier et al., 2005). Genetic dissimilarities between all the accessions was calculated using DARwin version 5.0 (Perrier et al., 2003; Perrier and Jacquemoud-Collet, 2006) using simple matching coefficient. The dissimilarity coefficients were then used to generate an unweighted neighbour-joining tree

Table 1. Names and sources of one hundred and five Bambara groundnut landraces used in the study.

Accession	County	Characteristic features	Accession	County	Characteristic features
KE/BN/1/1	Busia	Black	KE/BN/23/2	Kakamega	Dark Red
KE/BN/1/2	Busia	Dark Red	KE/BN/23/3	Kakamega	Light Red
KE/BN/2/1	Kakamega	Cream entire	KE/BN/24	Vihiga	Dark Red
KE/BN/2/2	Kakamega	Cream spotted	KE/BN/25/1	Kakamega	Black
KE/BN/3/1	Bungoma	Red	KE/BN/25/2	Kakamega	Light Red
KE/BN/4/1	Kakamega	Black	KE/BN/26/1	Busia	Light Red
KE/BN/4/2	Kakamega	Brown	KE/BN/26/2	Busia	Dark Red
KE/BN/4/3	Kakamega	Light Red	KE/BN/27	Busia	Dark Red
KE/BN/5/1	Busia	Cream entire	KE/BN/28	Busia	Brown red black spotted
KE/BN/5/2	Busia	Cream one side spotting	KE/BN/29	Busia	Light Red
KE/BN/8/1	Busia	Lght Red	KE/BN/30/1	Busia	Brown
KE/BN/8/2	Bungoma	Dark Red	KE/BN/30/2	Busia	Red
KE/BN/9	Kakamega	Dark Red	KE/BN/31/1	Busia	Black
KE/BN/10	Vihiga	Brown	KE/BN/31/2	Busia	Dark Red
KE/BN/12/1	Kakamega	Black	KE/BN/32/1	Busia	Black
KE/BN/12/2	Kakamega	Light Red	KE/BN/32/2	Busia	Light Red
KE/BN/12/3	Kakamega	Brown spotted	KE/BN/34/1	Busia	Brown
KE/BN/13/1	Busia	Black	KE/BN/35/1	Busia	Black
KE/BN/13/2	Busia	Light Red	KE/BN/35/2	Busia	Dark Red
KE/BN/13/3	Busia	Dark Red	KE/BN/36	Busia	Cream Red spotted
KE/BN/13/4	Busia	Brown	KE/BN/37/1	Bungoma	Light Red
KE/BN/13/5	Busia	Brown Black spotted	KE/BN/37/2	Bungoma	Dark Red
KE/BN/14/1	Kakamega	Brown entire	KE/BN/38/2	Busia	Light Red
KE/BN/14/2	Kakamega	Brown spotted	KE/BN/39/1	Kakamega	Cream
KE/BN/15/1	Kakamega	Black	GBK/050490	Genebank	Cream entire white eye
KE/BN/16/1	Busia	Dark Red	GBK/050491	Genebank	Cream entire white eye
KE/BN/16/2	Busia	Light Red	GBK/050492	Genebank	Cream spotted white eye
KE/BN/16/3	Busia	Black	GBK/050493	Genebank	Red brown white eye
KE/BN/17/1	Kakamega	Dark Red	GBK/050494	Genebank	Red Brown spotted white eye
KE/BN/17/2	Kakamega	Light Red	GBK/050495	Genebank	Orange brown white eye
KE/BN/18/1	Kakamega	Black	GBK/050496	Genebank	Orange brown white eye
KE/BN/19/1	Busia	Cream entire	GBK/050499	Genebank	Brown white eye
KE/BN/19/2	Busia	Cream spotted	GBK/050501	Genebank	Cream white white eye
KE/BN/20/2	Busia	Light Red	GBK/050502	Genebank	Cream white white eye
KE/BN/21/1	Bungoma	Black	GBK/050649	Genebank	Black white eye
KE/BN/21/2	Bungoma	Light Red	GBK/050650	Genebank	Black white eye/red brown white eyes
KE/BN/22/2	Busia	Dark Red spotted	GBK/050653	Genebank	Dark brown white eye
KE/BN/22/3	Busia	Light Red	GBK/050654	Genebank	Red brown white eye
KE/BN/23/1	Kakamega	Brown	GBK/050655	Genebank	Black white eye
GBK/050656	Genebank	Black white eye	GBK/050671	Genebank	Black white eye
GBK/050657	Genebank	Light red white eye	GBK/050672	Genebank	Black white eye
GBK/050658	Genebank	Black white eye	GBK/050673	Genebank	Black white eye
GBK/050659	Genebank	Black brown white eye	KE/BN/40	Busia	Black white eye
GBK/050660	Genebank	Cream spotted white eye	KE/BN/41	Busia	Black white eye
GBK/050661	Genebank	Black white eye/ Brown spotted white eye	KE/BN/42	Busia	Black white eye
GBK/050663	Genebank	Light red white eye	KE/BN/43	Busia	Black white eye
GBK/050664	Genebank	Black white eye	KE/BN/44	Busia	Black white eye
GBK/050665	Genebank	Black white eye/light red white eye	KE/BN/45	Busia	Black white eye
GBK/050666	Genebank	Black white eye/light red white eye	KE/BN/46	Busia	Black white eye
GBK/050667	Genebank	Light red white eye	KE/BN/47	Busia	Black white eye
GBK/050668	Genebank	Brown white eye/black white eye	KE/BN/48	Busia	Black white eye
GBK/050669	Genebank	Light red white eye	KE/BN/49	Busia	Black white eye
GBK/050670	Genebank	Black white eye			

Table 2. Primer information of twelve SSR markers used for amplification of DNA isolated from 105 accessions of Bambara groundnut germplasm.

Marker	Sequence (5´-3´)	Product size (bp)	Ta (°C)
PRIMER 1F	AGGCAAAAACGTTTCAGTTC		55.3
PRIMER 1R	TTCATGAAGGTTGAGTTTGTCA	273	55.3
PRIMER 2F	AGGAGCAGAAGCTGAAGCAG		55.3
PRIMER 2R	CCAATGCTTTTGAACCAACA	212	55.3
PRIMER 3F	TTCACCTGAACCCCTTAACC		57.6
PRIMER 3R	AGGCTTCACTCACGGGTATG	247	57.6
PRIMER 4F	ACGCTTCTTCCCTCATCAGA		57.6
PRIMER 4R	TATGAATCCAGTGCGTGTGA	197	57.6
PRIMER 5F	TCAGTGCTTCAACCATCAGC		55.3
PRIMER 5R	GACCAAACCATTGCCAAACT	260	55.3
PRIMER 6F	CCGGAACAGAAAACAACAAC		57.6
PRIMER 6R	CGTCGATGACAAAGAGCTTG	189	57.6
PRIMER 7F	TGTGGGCGAAAATACACAAA		59.7
PRIMER 7R	TCGTCGAATACCTGACTCATTG	198	59.7
PRIMER 8F	CAAACTCCACTCCACAAGCA		57.6
PRIMER 8R	CCAACGACTTGTAAGCCTCA	250	57.6
G358B2-D15F	TGACGGAGGCTTAATAGATTTTTC		59.0
G358B2-D15R	GACTAGACACTTCAACAGCCAATG	193	59.0
mBam2co80F	GAGTCCAATAACTGCTCCCGTTTG		59.0
mBam2co80R	ACGGCAAGCCCTAACTCTTCATTT	220	59.0
G180B2-D11F	GAGGAAATAACCAAACAAACC		59.0
G180B2-D11R	CTTACGCTCATTTTAACCAGACCT	198	59.0
G358B3-D15F	TGACGGAGGCTTAATAGATTTTTC		59.0
G358B3-D15R	GACTAGACACTTCAACAGCCAATG	196	59.0

(Saitou and Nei, 1987) with Jaccard's Similarity Coefficient with a bootstrapping value of 1,000 using the same software (DARwin 5.0).

RESULTS

Marker polymorphism, diversity within accessions and genetic distance among accessions

SSR analysis in the 105 Bambara groundnut accessions (Table 3) revealed that the number of reproducible DNA bands per primer ranged from 70 (Primer 1) to 97 (mBam2co80) totalling to 958 with an average of 79.83 bands.

Polymorphic information content (PIC) ranged from 0.13 to 0.35, (marker 10 and 5, respectively) with an average of 0.28 (Table 3). Accessions from Kakamega, Bungoma and Vihiga counties had the greatest gene diversity (H_E = 0.5) and Shannon's diversity index (I =0.6931), followed by those from the National Genebank of Kenya (H_E = 0.49, I =0.6928) and Busia (H_E = 0.47, I =0.6663). The National Genebank of Kenya accessions had the lowest H_E and I with 0.1023 and 0.2103, respectively followed by accessions from Busia county with 0.1420 and 0.2712. Kakamega and Bungoma counties accessions both had genetic diversity of 0.2778

Table 3. Estimate of genetic diversity of Bambara groundnut germplasm collections using 12 SSR markers.

Locus	na*	ne*	h*	I*	Major allele frequency	PIC	No. of amplified bands
Primer 1	2.0000	1.8000	0.4444	0.6365	0.67	0.35	70
Primer 2	2.0000	1.2771	0.2170	0.3744	0.88	0.19	92
Primer 3	2.0000	1.8202	0.4506	0.6429	0.66	0.35	69
Primer 4	2.0000	1.4953	0.3312	0.5133	0.79	0.28	83
Primer 5	2.0000	1.8396	0.4564	0.6489	0.65	0.35	68
Primer 6	2.0000	1.5448	0.3527	0.5375	0.77	0.29	81
Primer 7	2.0000	1.9489	0.4869	0.6800	0.58	0.37	61
Primer 8	2.0000	1.5448	0.3527	0.5375	0.77	0.29	81
G358B2-D15	2.0000	1.5201	0.3421	0.5257	0.78	0.28	82
mBam2co80	2.0000	1.1638	0.1408	0.2694	0.92	0.13	97
G180B2-D11	2.0000	1.4213	0.2964	0.4728	0.82	0.25	86
G358B3-D15	2.0000	1.3725	0.2714	0.4428	0.84	0.23	88
Mean	2.0000	1.5624	0.3452	0.5235	0.76	0.28	79.83

na* = Observed number of alleles; ne* = Effective number of alleles [Kimura and Crow (1964)]; h*= Nei's (1973) gene diversity; I* = Shannon's Information index [Lewontin (1972)].

Table 4. Analysis of molecular variance (AMOVA) for 105 bambara groundnut genotypes.

Source of variation	df	SS	MS	Variance components	Variation
Among Accessions	4	15.275	3.819	0.065	2%
Within Accessions	100	263.772	2.638	2.638	98%
Total	104	279.048		2.703	100%

and Shannon's diversity index of 0.4506.

Analysis of molecular variance (AMOVA) was done on the dataset to partition the total genetic variation among and within the accessions (Table 4). This revealed that the highest proportion of the total variation (98%) was among individuals within accessions. The proportions of variation among the accessions were lower at 2%.

Genetic distance based on Jaccard's similarity coefficient from the SSR marker analysis ranged from 0.08 to 1.17 among the landraces. Accessions from Bungoma county had the least genetic distance (0.41) indicating close genetic relationship while greatest genetic distance was recorded in accessions from Busia county (1.11) indicating genetic distance relatedness. Accessions from the National Genebank of Kenya (1.03) also had a high genetic distance.

UPGMA, Principal coordinate analyses

A dendogram generated by UPGMA cluster analysis failed to illustrate clear pattern of germplasm clusters based on their places of origin (Figure 1). In most cases, accessions from different regions or counties were clustered with one another. However, it demonstrated that accessions from Busia county and the National Genebank of Kenya tended to agglomerate together in cluster III. All the 105 individual genotypes were grouped

into three (I, II, III) main clusters (Figure 1). Except for cluster I all the remaining clusters had sub- clusters. There was a general trend as those accessions from the National Genebank of Kenya and Busia county tended to group together in cluster III while those from Kakamega county tended to cluster together in cluster I. Accessions from Vihiga and Bungoma counties were found in clusters I and III. Two clusters with the highest number of genotypes were cluster II and III with 27 and 58 individual genotypes respectively. Grouping of the genotypes of these landraces into sub- clusters indicated substantial level of intra-landrace polymorphism. Similarly high level of intra-landrace polymorphism can be said of the landraces in cluster II and III all of which had their individual genotypes grouped into more than two sub-cluster units. Cluster I had all the individual genotypes clustered into only one unit suggesting lesser level of intra-landrace polymorphism within the cluster compared to the rest of the landrace clusters.

Principal coordinate analysis (PCoA) (Figures 2 and 3) was performed to reveal genetic relationship among Bambara groundnut accessions. The first three axes accounted for 84.3% of the total variations (Table 5) with each axes explaining 63.58, 12.21 and 8.24% variation in that order. The first three axes accounted for the highest variation (96.81%) for Bungoma county accessions followed by accessions from Busia county (71.4%), Kakamega county (59.59%) and Genebank (59.31%).
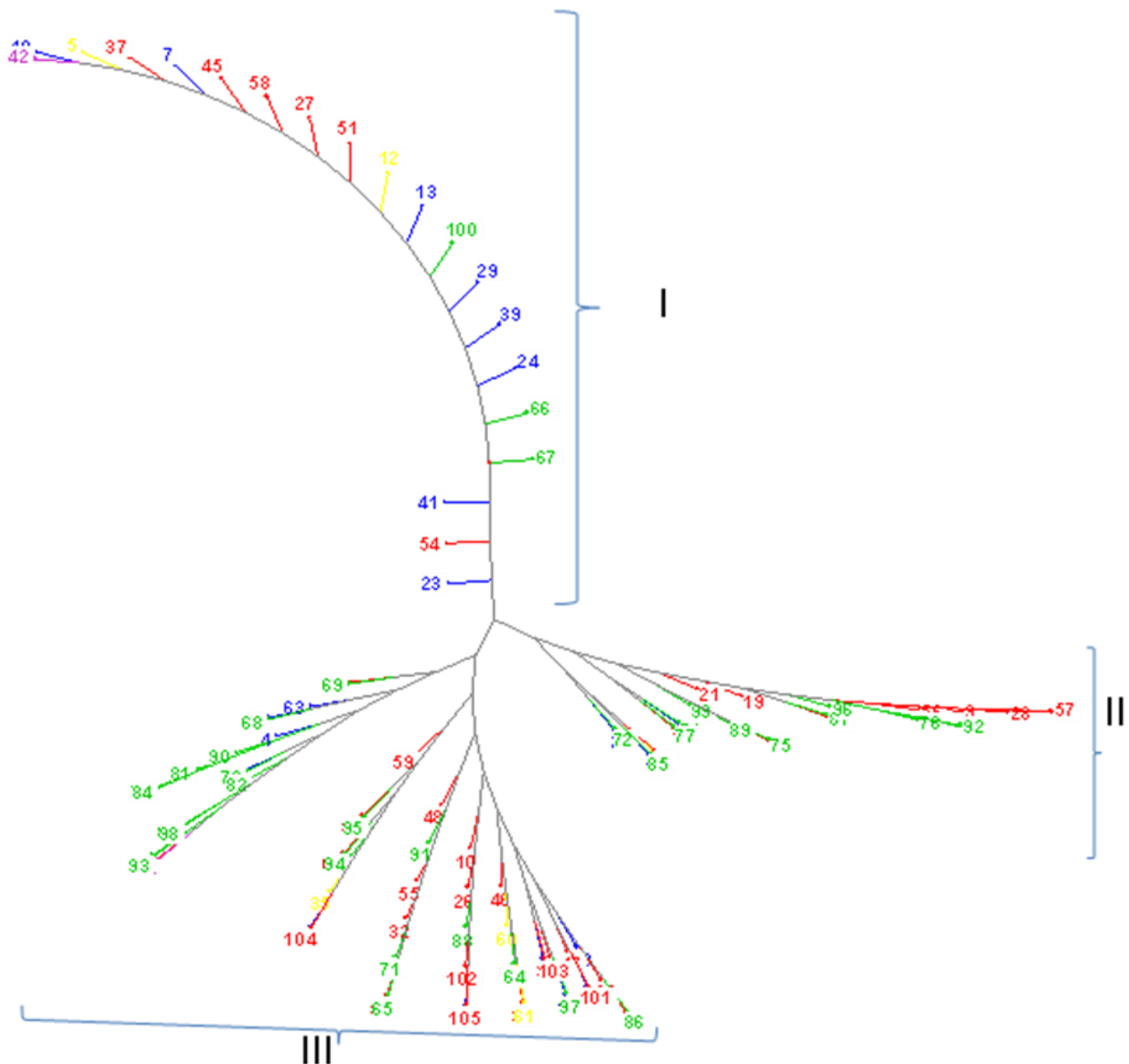
Figure 1. Genetic relationships generated by Jaccard's similarity coefficients among 105 Bambara groundnut accessions. Accessions given in red were from Busia, blue from Kakamega, purple from Vihiga, yellow from Bungoma and green from Genebank.

Principal component analysis failed to differentiate accessions according to their area of origin. Most of the accessions overlapped demonstrating close genetic relationships. This suggests that these accessions could have originated from the same source. From the PCoA plot of the accessions (Figure 3), principal axes 1 and 2 showed that KE/BN/34/1, KE/BN/13/5, KE/BN/16/3 from Busia county and GBK/050668 from the National Genebank of Kenya were the most distinct from the other accessions.

DISCUSSION

Genetic analysis of diversity is very critical as it gives more accurate measure of polymorphism compared to morphological characterizations. In the present study, extent and organization of genetic diversity within 105 accessions of Bambara groundnut from Western Kenya and the National Genebank of Kenya was assessed using 12 polymorphic SSR bands. The 12 SSR markers revealed the availability of polymorphism among the
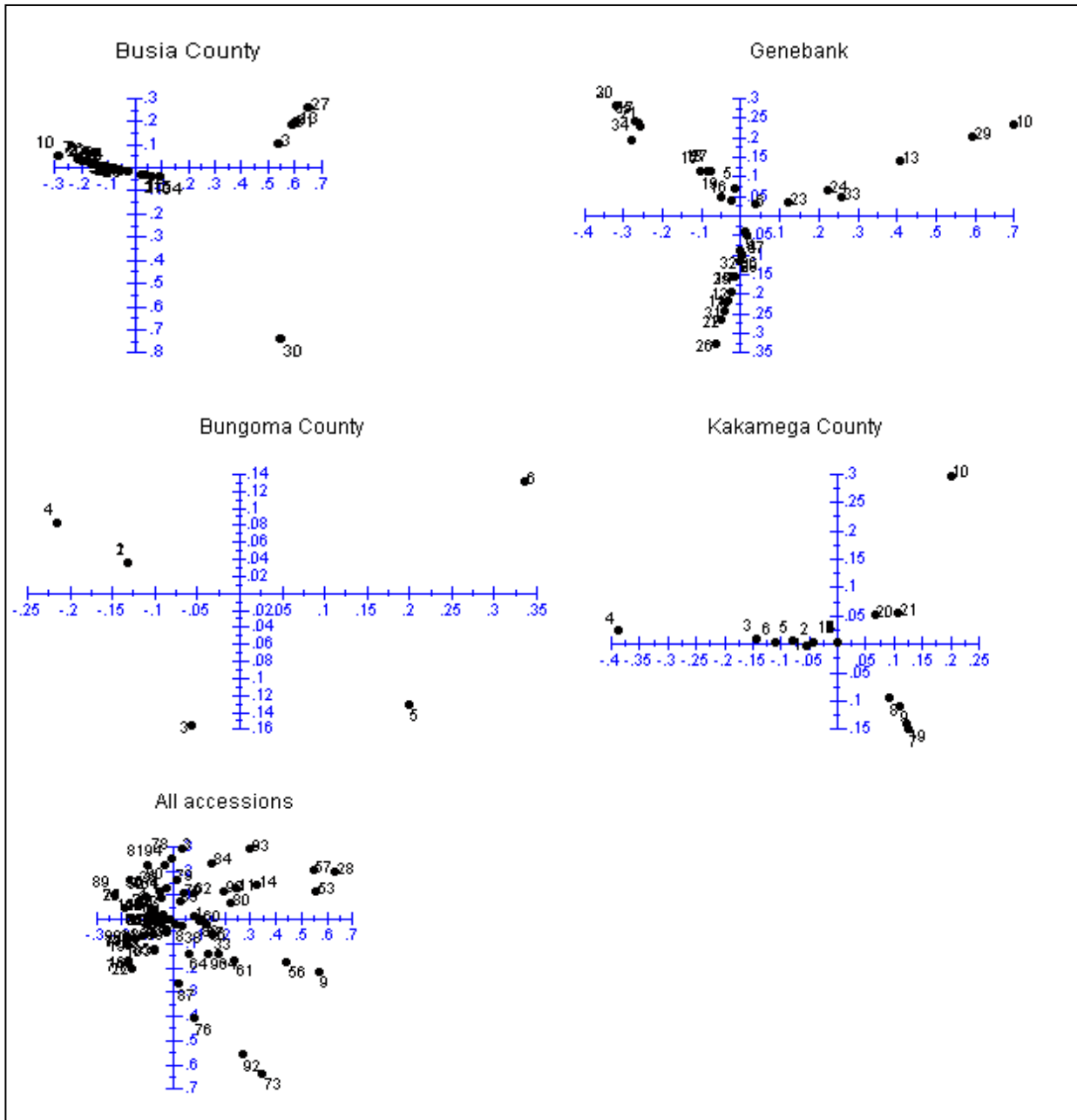
Figure 2. PCoA of axes 1 and 2 based on dissimilarity of 12 SSR markers across 105 Bambara groundnut landraces from different regions.

landraces of bambara groundnuts as evidenced in genetic distances and the cluster analysis (Figure 1). Previous studies by Massawe et al. (2002) based on AFLP molecular marker analysis revealed extensive genetic diversity between 12 African Bambara groundnut landraces from diverse origin. Amadou et al. (2001) also reported considerable genetic diversity among 25 African Bambara groundnut accessions from International Institute for Tropical Agriculture (IITA), Ibadan, Nigeria, using random amplified polymorphic DNA (RAPD)

markers. They demonstrated two main groups of accessions mainly along the lines of their geographic origin. Similarly Somta et al. (2011) reported high genetic diversity among 240 Bambara groundnut accessions from Africa and Southeast Asia using SSR markers as did Olukolu et al. (2012) and Aliyu et al. (2013).

In contrast, based on isozyme analysis, Pasquet et al. (1999) reported that both wild and domesticated bambara groundnuts were characterized by low genetic diversity, indicating that wild Bambara groundnut is the progenitor
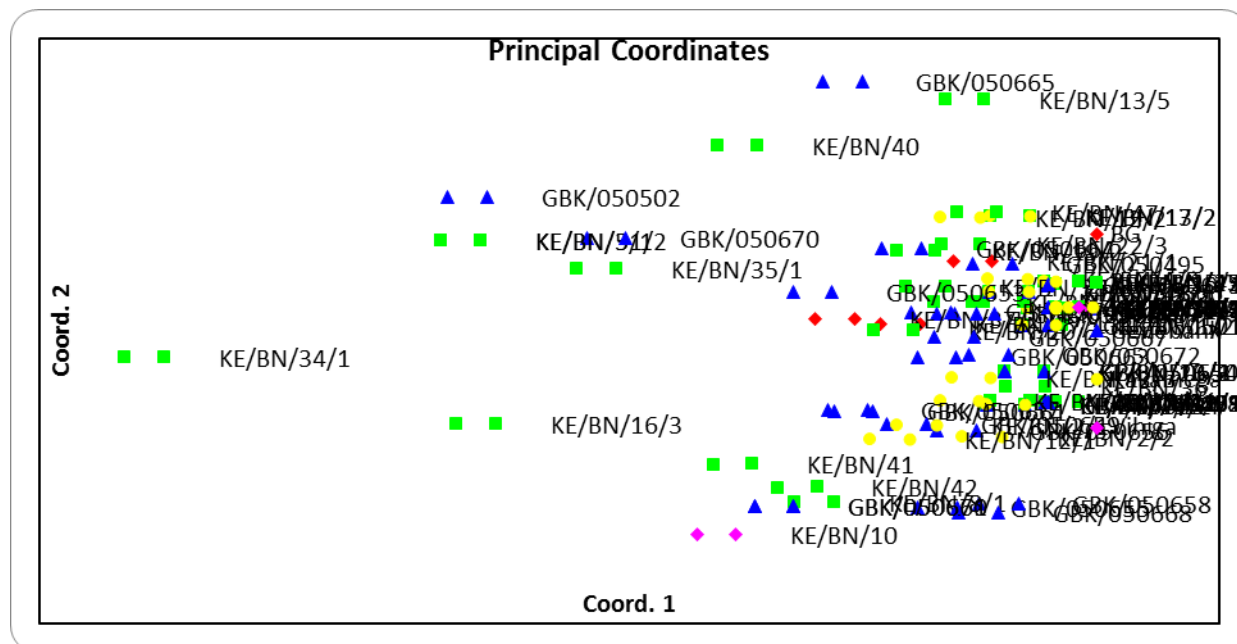
Figure 3. Configuration of bambara groundnut accessions under principal component axis 1 and 2. BG- Bungoma county.

Table 5. Eigen value and percentage of total variation accounted for by the first three component axes.

Axis	Eigen value	Proportion (%)	Cumulative (%)
1	29.21	63.58	63.85
2	5.612	12.21	76.06
3	3.786	8.24	84.30

of the domesticated form. This is expected as isozymes are generally limited by the low levels of polymorphism detectable and may fail to discriminate cultivars differing only slightly in genetic make up.

In this work, genotypes were clustered into three clusters (I, II and III) with clusters II and III forming sub-clusters. There was substantial intra-landrace polymor-phism as two of the three clusters had sub-clusters with distinct genotypes though from different regions. The high level of intra-landrace polymorphism could be attributed to seed exchange between farmers as well as the geographical proximity of the areas. Contrary to the higher intra-polymorphism of most of the landraces, genotypes in cluster I appeared less heterogeneous. Accessions from Kakamega, Busia counties and the National Genebank of Kenya tended to form a clear group (cluster III). This was elucidated further by AMOVA, which partitioned the total genetic variation among and within accessions. This showed that majority of genetic variation observed in the germplasm (98%) was due to the variation among individuals instead of being between specific accession groups. Divergent accessions may have good breeding value, which may

be utilized for direct selection and as parents of crosses with accessions from different clusters. The mixture of accessions in cluster I, II and III mainly from the counties of Busia, Kakamega and National Genebank of Kenya indicated that Bambara groundnut accessions in this group constituted a more heterogenic group, with variable genetic backgrounds. This can also be explained by the high frequency of bambara groundnut seed exchange by farmers over wide geographic-ethnic regions as well as the different informal names given to landraces from one region to another which may give room for genotype duplications as was suggested by Hudu and Saaka (2011).

The low level of genetic diversity revealed in this work could be supported by the fact that small scale farmers in Eastern Africa generally tend to exchange seeds frequently. A farmers field survey (Ntundu, 2002) indicated that at least 44% of farmers in Tanzania obtain their bambara groundnut seeds from others farmers within (39%) and outside (5%) of their regions, annually. In their survey on seed market assessment in Dodoma, Iringa and Morogoro regions in Tanzania, Ashimogo and Rukulantile (2000) reported that 35.4% of farmers obtained maize (*Zea mays* L.) seeds from their fellow farmers, while 60.1% use only their own seeds. Similar practice has been reported to be common among growers of cucurbits (*Cucurbita moschata* Duch) in Zambia where at least 40% of the farmers obtain their seeds from other farmers (Gwanama and Nichterlein, 1995) within their neighbouring growing regions. Further studies showed that sources of seed for planting of Bambara groundnut in Ghana include farmer saved seed,

exchange and market purchase (Berchie et al., 2010).

Principal component analysis (PCA) is a descriptive technique which reveals the pattern of character variation among genotype (Aremu et al., 2007). PCA failed to group accessions according to their areas of origin. This could have lead to a generally low coefficient of variation observed in Bambara groundnut accessions, an indication of a high level of uniformity. This suggested that the source of these accessions could be same due to seed exchange among the farmers.

Conclusion

Our study reported herein shows that Bambara groundnut landraces from Kenya, form a genetically diverse population and SSR markers can be effectively employed to assess genetic diversity and to measure the extent of genetic relationship among accessions. Knowledge of the degree of genetic relationships between bambara groundnut accessions will be of importance for crop improvement and may help to establish a core collection as part of the germplasm collection management to sample a maximum of genetic variation of accessions. This study reveals that Bambara groundnut accessions from Western Kenya and the National Genebank of Kenya constitute three major genetic clusters. The study revealed a low genetic variability among the accessions but a high genetic variability within them.

ACKNOWLEDGEMENTS

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Full Length Research Paper

Activity of *Cinnamomum zeylanicum* essential oil and ethanolic extract against extended-spectrum β-lactamase-producing bacteria

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The antibacterial effects of Cinnamon (*Cinnamomum zeylanicum*) essential oil and ethanolic extract against extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Pseudomonas aeruginosa* strains were studied in the present study. The essential oil and ethanolic fraction of *C. zeylanicum* showed significant activities against all tested microorganisms and minimal inhibitory concentration values (MIC) of the essential oil ranged from 0.8 to 20.2 µg/ml. The MIC of ethanolic fraction at 60°C were in the range from 8 to 62.12 µg/ml, although at room temperature showed the highest and lowest activity at 14.5 and 64.11 µg/ml, respectively. The results show by these extracts recommends their potential use against multidrug resistant microorganisms. This study also shows that *C. zeylanicum* could be a potential source of new antimicrobial agents. PCR amplification reaction showed the presence of CTX-M β-lactamases gene in all tested organisms.

Key words: *Cinnamon*, CTX-Mβ-lactam-resistant bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*.

INTRODUCTION

Medicinal plants are natural resources for the preparation of valuable products that can be used in the treatment of various ailments. Plant materials remain an important resource for combating illnesses, including infectious diseases, and many plants have been investigated with the intention to produce novel drugs for the development of new therapeutic agents (Tajkarimi et al., 2010; Sienkiewicz et al., 2013). Thus the emergence of multiple drug resistance of pathogenic organisms has caused an extensive research to find new antimicrobial substances from other sources including plants (Lin et al., 2005; Warnke et al., 2009; Yap et al., 2013). This interest have

been triggered due to the increasing frequency of microorganisms that are resistant to common and generally accepted antibiotics which is on the increase. Antibiotic resistance refers to the ability of a microorganism to withstand the effects of an antibiotic. Resistance rate to these drugs is higher in developing compared to developed countries because of the extensive and indiscriminate use of antibiotics over the last few decades (Akram et al., 2007) and people propensity to self-medicate without a prescription from an expert.

Among the wide array of antibiotics, beta (β)-Lactam

(penicillins, cephamycins etc.) are the most varied and widely used (Bronson and Barrett, 2001). The most common cause of bacterial resistance to β-lactam antibiotics is the production of β-lactamases, enzymes that break the antibiotic structure. The majority of these enzymes have been described in Gram negative bacteria which are responsible for numerous infectious diseases and are generally multidrug resistant. Gram-negative bacteria are more resistant to antibiotics than the Gram-positive bacteria due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Shanab et al., 2004).

Bacterial resistance to β-lactam antibiotics has been attributed to the spread of plasmid-mediated extended spectrum β-lactamases (ESBLs) (Khan et al., 2010; Sienkiewicz et al., 2013). ESBL-producing Enterobacteriaceae and other kinds of bacteria have been reported widely. Infections caused by ESBL-producing bacteria have become a clinical and therapeutic problem because these organisms are resistant not only to β-lactamases but also to many other antimicrobial agents (Velasco et al., 2007). A feasible approach of limiting the transmission of these pathogens is the use of essential oils as alternative or topical agents.

Cinnamon is a plant that belongs to the family of Lauraceae most noted for its bark, which provides the world with the commonly known culinary spice, cinnamon. Cinnamon has medicinal property and has been used to treat gastrointestinal complaints and other ailments (Cao and Anderson, 2011; Varalakshmi et al., 2014) and has been known as a popular remedy. It is reported to exhibit antimicrobial activity (Souza et al., 2007; Mishra et al., 2009; Pritam et al., 2013). Its potential therapeutic roles such as diaphoretic, carminative, antispasmodic, antiseptic, insecticidal and parasiticide properties have also been recognized.

The CTX-M β-lactamases are one of the groups of extended spectrum β-lactamases (ESBLs) identified after the introduction of the broad-spectrum cephalosporins. These enzymes are worldwide spread and 54 different types have already been identified in the last decade. The bla CTX-M genes are located on large plasmids (58-320 kb) that are transformed into *Escherichia coli* and resulted in multiresistant transformants (Soge et al., 2006).

In the present study, essential oil and ethanolic extract of *Cinnamomum zeylanicum* were tested to screen their antimicrobial activity (MIC) against multi-drug resistant *E. coli* (ESBL positive) and *Pseudomonas aeruginosa* strains with verification of CTX-M β-lactamases.

MATERIALS AND METHODS

Plant materials

The essential oil and the powder of *C. zeylanicum* were purchased from the Aroma Trading market (SH pharma), Egypt.

Preparation of plant extract

The bark powder of *C. zeylanicum* (10 g) was refluxed with absolute ethanol (100 ml) for 6 h. The solvents were evaporated at a constant temperature of 60°C until a very concentrated extract was obtained (5 ml). Identification tests for the various chemicals were conducted to test the presence of different chemical constituents.

Preliminary phytochemical analysis

Qualitative phytochemical screening for various chemical constituents including alkaloids, flavonoids, glycosides, phenols, resins, sugars, amino acids, protein, steroids/terpenes and tannins were performed using the crude extract of *C. zeylanicum*. The presence of resins, alkaloids, tannin and proteins was determined according to the methods described by Afaq et al. (1994) and Evans (2002). The presence of amino acids, glycosides, sterols/terpenes, phenols and carbohydrates was demonstrated by the methods of Evans (2002). For the confirmation of the presence of flavonoids, the method of Fornasworth (1966) was used.

Test organisms

The bacterial strains were either reference strain acquired from King Khalid University, Medicine Collage, KSA or clinical isolates from Mansoura University hospital, Egypt. Clinical strains were identified by conventional techniques (Fornasworth, 1966). The isolates studied included Gram-negative bacteria *E. coli* spp. (six strains) and *P. aeruginosa* (two strains). They were tested against their ability to produce β-lactamases enzyme. The isolates studied were resistant to at least one β-lactam antibiotic. Reference bacterial strain was β-lactamase producer *E. coli* ATCC O1577. Antimicrobial susceptibility of these strains was determined by disc diffusion method test. Each strain was routinely sub-cultured, at 37°C, on nutrient agar plate (Difco).

Antibiotic susceptibility testing

Susceptibility testing was performed according to Clinical and Laboratory Standards Institute (NCCLS) recommendations by using microtitre plates containing different dehydrated antibiotics. The initial screening was performed by testing the zone diameter for ceftriaxone, ceftazidime and cefotaxim (Aligiannis et al., 2001) alone and in combination with 4 g/mL clavulanic acid for phenotypic detection of β-lactamases.

Antimicrobial activity test

The antimicrobial activities of the essential oil and the ethanolic plant extract of *C. zeylanicum* were measured *in vitro* against the 9 microbial cultures by using disc diffusion method (Anon, 1997). Filter paper discs (5 mm diameter) were placed on the pre-inoculated agar surface and impregnated with 30 µl of each essential oil or ethanolic fraction. The plates were then incubated at 37°C for 24 h before the diameters of inhibition zones around each disc were measured. All tests were performed twice and the antimicrobial activity was expressed as the mean of inhibition diameters (mm).

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was defined as the lowest concentration that prevents visible growth of the bacteria

(Delaquis et al., 2002). One ml from the dissolved essential oil in ethanol and ethanolic extract (100 µg/ml) was used to determine MIC by serial dilution with nutrient broth (Murray et al., 1995). The bacterial cell number was adjusted at 10^6 bacterial cells/ml (0.1 ml inoculum / tube) for the tested strains. All samples were incubated at 37°C during 18-24 h. As control, ethanol was used.

DNA extraction

Bacterial chromosomal DNA was obtained using Maxwell 16 DNA cell purification Kit with automatic DNA extraction machine according to instruction manual. The chromosomal DNA was checked by electro-phoresis in agarose gels, and the concentrations of the different extracts were standardized by spectrophotometric measurements (Sambrook et al., 1989).

Detection of the CTX-M gene

DNA from different *E. coli* strains and *P. aeruginosa* strains was used as the template in a PCR amplification. Amplification reactions were performed in a final volume of 50 µl. Mg^{2+}-free PCR buffer was purchased as a 10× concentrate consisting of 500 mM KCl, 100 mMTris-HCl (pH 9.0), and 1% Triton X-100 (Perkin-Elmer, Roche Molecular Systems, Inc., Nutley, N.J.) with 200 µM (each) dATP, dCTP, dGTP, and dTTP (Perkin-Elmer, Roche Molecular Systems, Inc.). The Mg^{2+} concentration was 2.5 mM, and the primers were used at 0.5 µM each. The primer pair CTX-M-1 (5′-AACACGGATTGACCGTATTG-3′) and CTX-M-2 (5′-TTACAGCCCTTCGGCGAT-3′) was used to amplify the CTX-M-14 gene in the plasmid DNA. Amplification reactions were carried out in T100 thermal cycler (Bio-Rad, Germany), with an initial denaturation (10 min at 94°C) followed by 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 58°C), and extension (2 min at 72°C), with a single final extension of 10 min at 72°C. Aliquots (15 µl) of each sample were subjected to electrophoresis in 1.0% agarose gels. Amplified products were detected after staining with ethidium bromide (50 µg/ml) and photographed with gel documentation system (BioScience, Taiwan).

RESULTS AND DISCUSSION

With the rise in the emergence of various multidrug resistant microorganisms and the scenario worsening through the indiscriminate use of antibiotics, new and/or alternative antimicrobial compounds must be developed to treat common infections (Shakil et al., 2010). New and safe antimicrobial agents are needed to prevent and overcome severe bacterial infections and the problems of bacterial resistance (Sienkiewicz et al., 2013). Plants essential oils and extracts especially cinnamon have been used for many thousands of years, in pharmaceuticals, alternative medicine, and natural therapies (Pritam et al., 2013). It is necessary to investigate this plant scientifically to improve the quality of healthcare.

The initial screening was carried out to test inhibition zones of ceftriaxone, ceftazidime and cefotaxime, and the corresponding inhibitory diameters were 17, 15 and 14 mm, respectively. Thus, it was possible that this strain might produce ESBLs. Moreover, the confirmation test of ESBL production indicated that inhibition zones of

cefotaxime and ceftazidime with clavulanic acid were 20 and 17 mm, respectively. The increase of 6 mm between the zones of cefotaxime and cefotaxime combined with clavulanic acid was considered to indicate the production of ESBLs. These results were confirmed with the results of Hongbin et al. (2008) who stated that the increased zone diameter for ceftazidime or cefotaxim tested in combination with clavulanate versus the zone when tested alone was considered indicative of ESBL production from *E. coli* isolates obtained from chicken livers.

Results obtained in the present study reveal that the essential oil and the fractions of the plant extract tested possess potential antibacterial activity against the tested organisms. There are differences in the antimicrobial activities of the essential oil and the ethanolic plant extract as each might possess different compounds. This is in agreement with the report of Khan et al. (2010) which stated that the ability of the oil and extract of some medicinal plants to inhibit bacteria suggested the presence of broad spectrum antibiotic compounds. Schmidt et al. (2006) showed that the essential oil of *C. zeylanicum* is rich in eugenol and cinnamaldehyde which are the two major chemical components that are mainly responsible for its antimicrobial properties. Snyder (1997) stated that eugenol, a phenol compound, inhibits mold and adds flavor and aroma to bakery items. It also possesses antiviral properties *in vitro* while, Ali et al. (2005) confirmed that eugenol and cinnamaldehyde inhibit *H. pylori* growth at a low pH, showing their efficacy in eliminating the bacteria present in the human stomach.

It has been published that various plant extracts have been demonstrated to possess antibacterial activity against microbial pathogens. The antimicrobial activity observed could be due to the varied phytochemicals presents (Aduol et al., 2014). Several metabolites from medicinal plants such as sterols, tannins and alkaloids have previously been associated with antimicrobial activity (Leven et al., 1979). It is necessary to identify the phytochemical component of the local medicinal plants to explain some of the biological activity of certain plant extracts observed.

The phytochemical analysis of ethanolic extract of *C. zeylanicum* showed positive tests at room temperature and at 60°C for the presence of many compounds like resins, sterols /terpenes, tannins, glycosides, alkaloids and flavonoids while reducing sugars, amino acids, phenols and proteins were not detected (Table 1).

The antibacterial activity of cinnammon ethanolic extract could be due to the presence of resins, sterols, terpenes, tannins, glycosides, alkaloids and flavonoids. Shuaib et al. (2013) concluded that the resin rich methanolic extracts of *C. myrrha*, *O. turpethum* and *P. Roxburghii* exhibited some degree of antimicrobial activity against Gram-positive (*S. aureus, B. subtilis, M. luteus, E. faecalis*) and Gram-negative bacterial strains (*E. coli, P. aeruginosa, S.*

Table 1. Qualitative test for various phytochemical constituents in ethanolic extract.

Compound	Ethanolic extract (at 60°C)	Ethanolic extract (at room temperature)
Resins	+ve	+ve
Sterols/terpenes	+ve	+ve
Reducing sugers	-ve	-ve
Tannins	+ve	+ve
Glycosides	+ve	+ve
Alkaloid	+ve	+ve
Amino acids	-ve	-ve
Flavonoids	+ve	+ve
Phenols	-ve	-ve
Proteins	-ve	-ve

Table 2. MIC (µg/ml) values of essential oil and ethanolic extract of *C. zeylanicum* studied against multi-drug resistant strains of bacteria.

Strain number	Essential oil	Ethanolic extract (at 60°C)	Ethanolic extract (at room temperature)
P. aeruginosa 1	20.20	64.00	64.11
P. aeruginosa 2	20.00	62.12	64.00
E. coli 1	0.8	8.22	16.20
E. coli 2	12.70	8.00	14.50
E. coli 3	4.88	32.00	38.35
E. coli 4	4.00	24.80	24.00
E. coli 5	4.00	24.12	22.30
E. coli 6	16.33	30.00	32.11
E. coli ATCC O1577	16.00	32.00	60.00

typhi, S. dysenteriae). Dherbomez et al. (1995), stated that a sterol, 7-amino- cholesterol displayed antibiotic activity against *Saccharomyces cerevisiae*, *S. aureus*, *Enterococcus hirae* and *Bacillus cereus*, while, Cantrell et al. (2001), investigated the importance of terpenes and terpenoids for their antimicrobial activity. Haslam (1996) reported that a wide range of anti-infective actions have been assigned to tannins. Iyengaroside-A (2), a glycoside isolated from the methanolic extract of the marine green alga *Codium iyengarii* has been reported to show bactericidal activity (Ali et al., 2002). Bromotyrosine alkaloids have demonstrated high antimicrobial activity against a number of Gram-positive organisms, including Mycobacteria and Staphylococci (Pick et al., 2006). Flavonoids have been found to show *in vitro* antimicrobial activity against a wide range of bacteria (Cowan, 1999).

The ethanolic extract of cinnamon showed significant activity against the investigated bacterial strains, which is promising. It is interesting to note that the extract is not pure compounds and in spite of it, antimicrobial results were obtained, which only suggests the potency of this extract. The potential for developing antimicrobials from plants is rewarding as it will lead to the development of a phytomedicine to act against microbes. Plant based antimicrobials have enormous therapeutic potential as they can serve the purpose without any adverse effects that are often associated with synthetic compounds; hence isolation and purification of phytoconstituents from our plant may yield significant novel antimicrobials.

The essential oil and ethanolic fraction of *C. zeylanicum* showed significant activities against all the microorganisms and the values of MICs for the essential oil ranged from 0.8 (*E.coli* no.1) to 20.2 µg/ml (*P. aeruginosa* no.1) (Table 2). On the other hand, MICs of the ethanolic fraction at 60°C showed highest activity against *E. coli* no.2 (8 µg/ml) while the lowest activity was against *P. aeruginosa* no.1 (64.00 µg/ml). The MICs of ethanolic fraction at room temperature showed the highest activity against *E. coli* no.2 (14.5 µg/ml) and the lowest in *P. aeruginosa* no.1 (64.11 µg/ml). Ethanol was used as a control which did not show any inhibitory activity against each bacterial species.
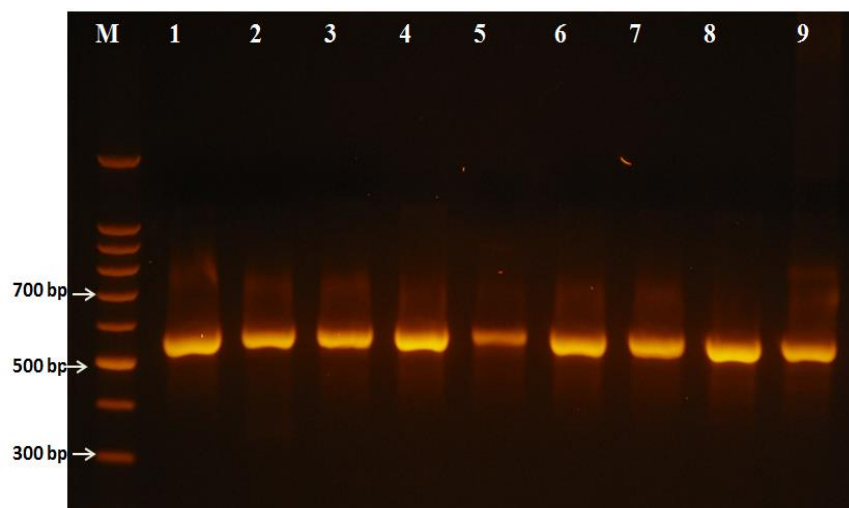
Figure 1. PCR amplification products of CTX-M gene. From left to right; DNA marker: 100bp DNA ladder; lanes 1-6, *E. coli* isolates; lanes 7 and 8 *P. aeruginosa* isolates; lane 9, positive control *E. coli* ATCC O1577 strain containing of CTX-M gene of about 550 bp.

Tests have shown that cinnamon oil have strong antimicrobial effects against the most bacteria and fungi, even more than the cinnamon ethanolic extract. Gupta et al. (2008), showed that cinnamon oil was effective against 10 examined bacteria, including *S. aureus*, *Listeria monocytogenes*, and *E. coli* while cinnamon extract was only effective against most of the food-borne microorganisms. In another study which examined the antibacterial effects of various plant essential oils, cinnamon oil showed maximum activity against the gram positive bacteria *B. subtilis* and *K. pneumoniae* and the gram negative bacteria *P. aeruginosa* and *E. coli* 18 (Ankri and Mirelman, 1999).

There has been a dramatic increase in the number of organisms reported in the literature that produce CTX-M-β-lactamases. This class of β-lactamases has been recognized worldwide as an important mechanism of resistance to oxyimino-cephalosporins used by gram-negative pathogens (Bonnet, 2004) (Figure 1).

To overtake the technical difficulties encountered in molecular detection and characterization of these β-lactamases simultaneous amplification PCR methods have been successfully developed (Colom et al., 2003). The specific fragments at 550 bp was clearly separated and visualized by gel electrophoresis. *E. coli* isolates harboring ESBLs are significantly more frequently found to be resistant to other antibiotics, in particular fluroquinolones. Due to the increased complexity of β-lactam resistance in Gram-negative organisms, the key to effective surveillance is the use of both phenotypic and genotypic analyses (Eisner et al., 2006). This high ESBL frequency may have been caused by the excessive use of broad-spectrum antibiotics in our hospital, together with a lack of attention to laboratory screening of ESBL production by clinical isolates. On the other hand, the high rate of ESBL production could possibly be due to the spread of 1 single clone and/or plasmid within our hospital setting. Owing to a number of limitations, we could not exclude this possibility by determining plasmid profiles and pulsed-field gel electrophoresis patterns of the isolates. As the available treatment options are limited, antibiotic control policies together with the implementation of infection control measures remain of high importance.

Conclusion

The essential oil and the ethanolic extract of *C. zeylanicum* showed varying degrees of antimicrobial activity against the ESBL producing multidrug resistant isolates. The presence of phytochemicals may be responsible for its therapeutic effects. This plant could be a source of new antibiotic compounds which could be more effective against multidrug resistant strains of microorganisms. To test and identify the specific antimicrobial compounds, further work is needed.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Paclobutrazol biodegradation in unsaturated soil in the Semi-Arid Northeast of Brazil

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Paclobutrazol (PBZ) is a plant growth regulator, increasing flowe ring and yield that is widely used in mango cultivation in the semi-arid northeastern Brazil. PBZ remains active in the soil for several years. However, it can severely affect the growth and development of subsequent crops, mainly by reducing vegetative vigor. The aim of this study was to investigate PBZ biodegradation in four samples of soil: P-G (with PBZ application history and with addition of glycerol); NP-G (without PBZ application history and with addition of glycerol); P-NG (with PBZ application history and without addition of glycerol) and NP-NG (without PBZ application history and without addition of glycerol). The biodegradation experiments were carried out in 125 ml flasks containing 10 g soil, at room temperature for 63 days. Mathematical models to analyze the kinetics of degradation of PBZ were applied. PBZ residue was less than 1% in soils with a history, regardless the addition of glycerol. The three models (first-order kinetics, double first-order kinetics and logistic) were well adjusted in these cases (P-G and P-NG). On the other hand, PBZ biodegradation in soil NP-G and NP-NG was 64%, and followed the model of double kinetic. PBZ biodegradation in soil with history was successful, probably because the native microbial had adapted to local environmental conditions.

Key words: Paclobutrazol, biodegradation, mathematical models.

INTRODUCTION

Paclobutrazol (PBZ), a derivative of the triazole group, is now commercially used in many tropical and subtropical fruit crops for regulation of growth, flowering and yield and commonly (Srivastav et al., 2010) used in mango cultivation in the semi-arid northeastern Brazil. However, PBZ remains active in soil for several years and can severely affect the growth and development of

subsequent crops, mainly by reducing vegetative vigor (Jackson et al., 1996). Through biodegradation, chemical compounds may be removed or reduced in the soil, but the process is expensive. In this process, the introduction of additional nutrient sources enhances microbial degradation (China et al., 2004).

Mathematical models can help measure high levels of

toxic substances in the soil, or in the fruit of plants treated with pesticides, and may indicate when such substances should be systematically monitored. Mathematical models are available for various agricultural purposes, among which is the simulation of absorption of organic substances by plants (Fujisawa et al., 2002), as well as the behavior of organic substances in the soil (Gang et al., 2003; Milfont et al., 2008).

The first-order kinetic model has been widely used to describe the kinetics of dissipation of various herbicides found in the soil under field conditions, or in the laboratory (Martins and Mermoud, 1998; López-Galindo et al., 2010). However, for many complex compounds, kinetics first order is not appropriate because the biodegradation of these compounds can occur at different rates throughout the process. In this case, the kinetic model results, using double first-order, have shown to work well, especially when the biodegradation occurs primarily in a fast reaction and then decays at a slow rate (López-Galindo et al., 2010).

Vaz et al. (2012) made a study of PBZ biodegradation in saturated soil without the addition of microorganisms (or only with the participation of microorganisms naturally present in the ground). These authors achieved further adjustments in modeling of PBZ biodegradation using double first-order kinetics.

The aim of the present study was the modeling of biodegradation of PBZ in unsaturated soil in the semi-arid Northeast of Brazil with and without history of application.

MATERIALS AND METHODS

Soil

Soil samples used in this study were collected in January, 2012, from a farm located in Petrolina (PE), Brazil. The soil has a sandy texture and is classified as Quartzipsamments Neosoil. This region had been consecutively treated with PBZ for six years (once a year), with an average dose of 3.57 g of active ingredient per plant. The soil samples were collected 30 days after the last application. An average of 1.5 kg of soil at a depth of 15 and 30 cm was collected from four points around eight plants. These samples were stored in a refrigerator until the beginning of the biodegradation tests. The samples without PBZ application history were also taken from the same farm.

Paclobutrazol

PBZ used for quantification was obtained from Sigma Aldrich (St. Louis, MO, USA). The commercial product (Cultar 25 SC), containing 25% of the active compound, was used in biodegradation experiments.

Experiments of biodegradation

Experiments of biodegradation were conducted with the collected soil samples. To each 10 g of soil with (P-G, P-NG) and without (NP-G, NP-NG) PBZ application history, 30 µg/g PBZ were added from a solution prepared with the commercial product (Cultar 25

SC). The experiments were carried out in 125 ml flasks at room temperature, without stirring for 63 days and in triplicate. Samples were withdrawn after 0, 7, 14, 21, 35, 49 and 63 days for the quantification of native microbial and residual PBZ. In experiments with the addition of glycerol (P-G, NP-G), the concentration of this compound in the soil was 150 µg/g. Micro-organisms were not added to the soil.

Paclobutrazol quantification

To each 10 g of soil, 10 ml of methanol (HPLC grade, Mallinckrodt Baker - Phillipsburg, NJ, USA) was added. This suspension was subjected to vacuum filtration, using a membrane of 0.45 µm. The filtrate was analyzed by high performance liquid chromatography (HPLC) using an Agilent chromatograph (HP 1100). The chromatographic conditions were: methanol/water (80/20), 0.4 mL/min, 30°C, and C-18 column (Kinetex; Phenomenex).

Growth quantification

The native microbial was quantified by the method of counting viable cells. Samples of 1 g of soil were diluted in sterile water, and plated on Petri dishes with the Tryptone Soy Agar (TSA) culture medium, and incubated for 48 h at 30°C. The results were expressed as colony forming unit per milliliter (CFU/ml).

Kinetic modeling

Three mathematical models (Table 1) were applied to kinetic modeling of PBZ biodegradation: first-order kinetic equation, double first-order kinetics equation and logistic equation (Martins and Mermoud, 1998; Varela et al., 2002; Gang et al., 2003). The models were applied using Sigma Plot 11.0. Analysis of variance was performed using Microcal Origin 6.0 (Table 1).

RESULTS AND DISCUSSION

Figure 1 shows the PBZ biodegradation kinetics in soil without and with application history, with and without addition of glycerol. Experiments in P-G and P-NG soil showed a sustained reduction after the 14th day and only around 1% PBZ remained in the 63th day. This ability of the native microbiota to degrade paclobutrazol was probably due to the historical application. After repeated applications of some pesticides, the microorganisms of the soil can degrade these compounds as they become suited for agrochemical use as a source of carbon for energy production and growth. Although there are some other factors affecting the persistence of agrochemicals in the soil, such as temperature, pH of the soil, chemical hydrolysis and the water content of the soil, micro-organisms seem to play an important role in the degradation of these compounds (Abdelrahman, 2004) (Figure 1).

The residue PBZ in soil without application history of PBZ, containing glycerol (NP-G) or not (NP-NG), was approximately 64% with 14 days. The lower biodegradation rate in NP-G and NP-NG was due to microbial not being adapted to PBZ, since this soil had no history of application. This rate remained constant probably

Table 1. Kinetic models for the biodegradation of substrates.

Model		Equation	Analytical solution
First-Order		$-\dfrac{dC}{dt} = k_1 C$	$C(t) = C_0\, e^{-k_1 t}$
Double First-Order	Reaction fast	$\dfrac{dC_R}{dt} = k_f C$	$C(t) = C_0\left\{ f . e^{-k_f t} + (1-f) . e^{-k_s t} \right\}$
	Reaction slow	$-\dfrac{dC_L}{dt} = k_s C$	
Logistic		$\dfrac{dC}{dt} = kC\left(1 - \dfrac{C}{K}\right)$	$C(t) = \dfrac{C_0 K e^{kt}}{K + C_0\left(e^{kt} - 1\right)}$

Nomenclature

$C(t)$	Paclobutrazol concentration at any time t (%)
C_0	Initial paclobutrazol concentration (%)
t	time
k_1	Fist order rate (1/t)
f	Fraction of the paclobutrazol decay attributed to rapid reactions (dimensionless)
k_f	First order rate constant for rapid reactions (1/t)
k_s	First order rate constant for slow reactions (1/t)
K	Final concentration asymptotic, concentration at which the biodegradation of the compound stabilizes and this is no longer degraded
k	First order Pseudo-constant and is therefore related to the degradation rate (1/t).
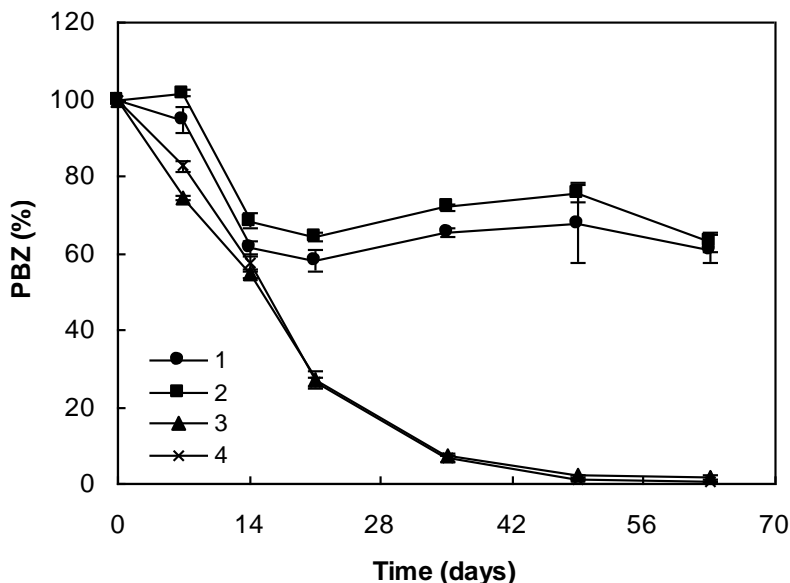
Figure 1. Time course of PBZ. 1, NP-NG; 2, NP-G; 3, P-NG; 4, P-G.

because the native microbiota was not enough to continue the degradation, which was confirmed by lower growth than that obtained in P-G and P-NG (Figure 2).

The biodegradation in NP-G or NP-NG soils were clearly lower than that in P-G or P-NG soils. Biodegradation were not significantly different up to 49 days for experiments in NP-G and NP-NG soils (F = 2.42; α=0.55). Similarly, in soil P-G and P-NG soils, the biodegradation were not significantly different up to 14 days (F = 0.09; α= 0.05). Maximum biodegradation occurred in soil with a PBZ application history with 63 days, regardless of the presence of glycerol. Probably
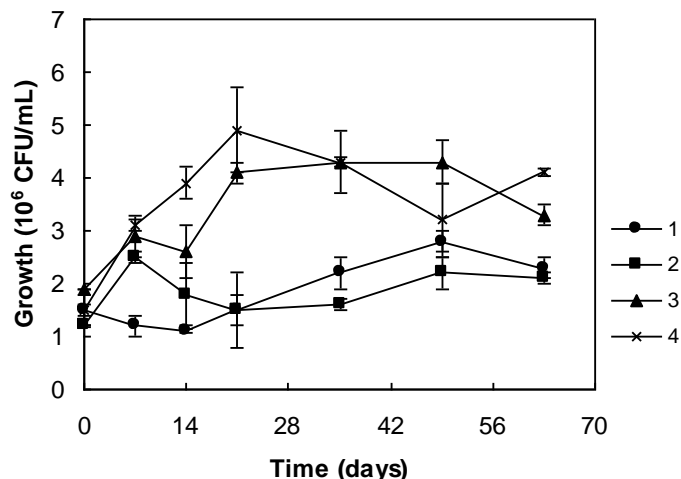
Figure 2. Time course of native microbiota: 1, NP-NG; 2, NP-G; 3, P-NG; 4, P-G.

(a)

(b)

Figure 3. Modeling kinetics of soil NP-NG (a) and NP-G (b).

this was due to the low concentration of glycerol added. Growth was similar; regardless the addition of glycerol, comparing NP-G with NP-NG soils and P-G- with P-NG soils (Figure 2). However, with respect to the soil with and without history, there was higher growth to soil with history (P-G and P-NG).

The PBZ biodegradation kinetics modeling in the experiments without history, and without (NP-NG) and with (NP-G) glycerol are showed in Figure 3a and b, respectively. Constants and kinetic parameters of three models (first-order, double first-order kinetic and logistic equation) are found in Table 2. The highest fit for NP-NG followed a double first-order kinetic (R = 0.89) and logistic equation (R = 0.88). On the other hand, only the model of double first-order kinetics was reasonably fitted to the NP-G (R = 0.81). The PBZ biodegradation kinetics modeling in the experiments with history, and without (P-NG) and with (P-G) glycerol are showed in Figure 4a and b, respectively. The three models were well fitted to P-NG and P-G soils (R >0.93).

Adjustment of the first-order kinetic model indicates that a single microbial system is involved in biodegradation. On the other hand, when the biodegradation first occurs at a fast reaction and then decays at a slow rate, the double first-order kinetic model is more suitable. As both models were well adjusted at P-NG and P-G soils, the constants of first-order models (k_1) were equal to those of the fast reaction of double first-order kinetic model (k_f): k_1 (P-NG) = 0.057; k_1 (P-G) = 0.054; k_f (P-NG) = 0.057; k_f (P-G) = 0.054 (Table 2).
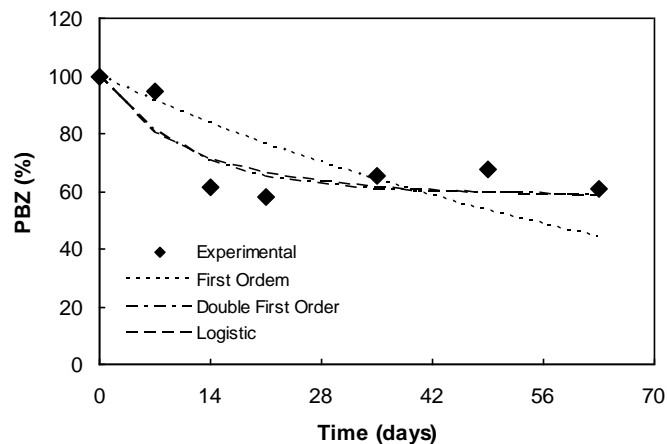
Due to the excellent fit of the first-order kinetic model for P-NG and P-G soils, the constants of the slow reaction were equal to those of the fast reaction of double first-order kinetic model ($k_1 = k_f = k_s$). The first-order kinetic model could not be adjusted for NP-NG or NP-G soils, since that the stabilization of the biodegradation occurred
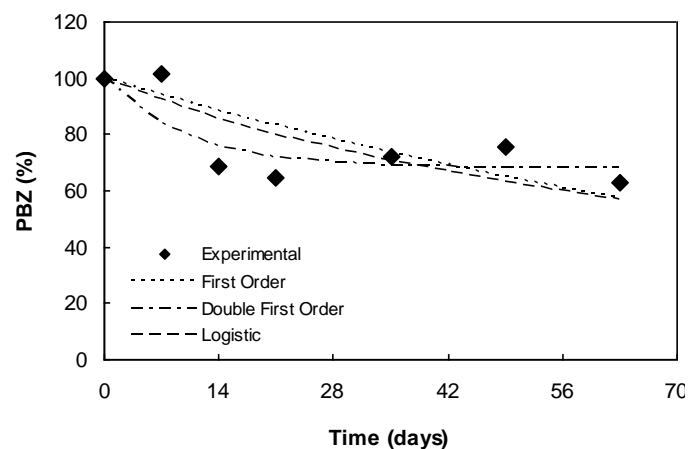
in 64%. On the other hand, logistic equation could not be adjusted for only NP-G, since that K was $5.00.10^{-04}$ and the concentration at which the biodegradation stabilized in this soil was 64%.

PBZ has been shown to be efficient in treating mango trees in semi-arid conditions (Mouco and Albuquerque, 2005). Because it needs to be applied into the soil, it is inconvenient since it remains and effects future planting. Further, it is difficult to determine the dosage for each future use when only empirical methods are used, as there remains residue from the previous cycle of application (Rademacher et al., 2006). No quantification is done nor is it always taken into consideration when deciding the dose. Thus, the applied amount of PBZ in the soil is not always appropriate and the risks of using doses above the recommended are great.

The inflorescences on trees treated with high doses have been very compact (Mouco and Albuquerque, 2005), creating suitable conditions for the incidence of diseases and pests (Winston, 1992), whose control is also hampered by the format of the panicles. Besides the phytosanitary problems, excessive doses of PBZ can

Table 2. Constants and kinetic parameters of three models applied at biodegradation.

Soils	C_0 (%)	First order		Double first order				Logistics		
		K	R	f	k_f	k_s	R	K	k	R
NP-NG	100	0.013	0.62	0.42	0.089	$7.12.10^{-11}$	0.89	57.6	$5.84.10^{-02}$	0.88
NP-G	100	0.009	0.51	0.32	0.103	$2.69.10^{-11}$	0.81	$5.00.10^{-04}$	$6.09.10^{-08}$	0.60
P-NG	100	0.057	0.99	1.60	0.057	0.057	0.99	$9.00.10^{-04}$	$9.44.10^{-07}$	0.95
P-G	100	0.054	0.98	1.00	0.054	0.054	0.98	$3.77.10^{-05}$	$3.72.10^{-08}$	0.93

(a)
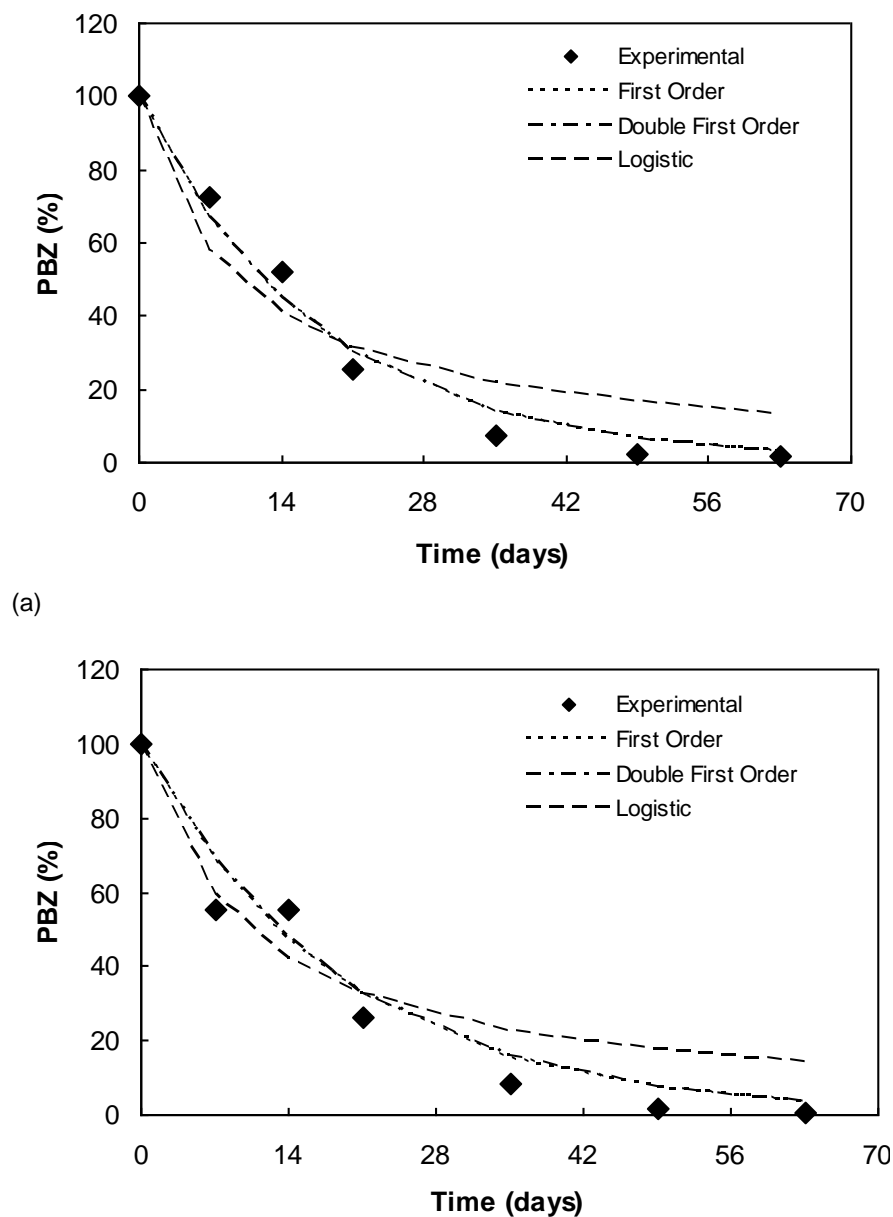
Figure 4. Modeling kinetics of soil P-NG (a) and P-G (b).

inhibit vegetative and floral sprouting longer than desirable, requiring nitrate sprays to stimulate flowering. Thus, in addition to increasing the cost of crop production, for all the reasons that have been mentioned, there is accumulation of chemicals in the ground without knowing the long-term consequences for the production system.

Conclusion

Biodegradation of PBZ in unsaturated soils was more efficient when soil samples with a history of application of PBZ were used. We concluded that this soil bacterium is better adapted for the degradation of the compound. Mathematical models can help to identify high levels of toxic substances in soil treated with pesticides and indicate that such substances should be systematically monitored.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Polymorphism of *Pfatpase6* in Côte d'Ivoire: Detection of a four new point mutations

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Over the past decade, the number of malaria cases has dropped by more than half in many malaria-endemic countries. However, recent parasite resistance to artemisinin undermines that progress. Artemisinin-based combination therapy (ACTs) is recommended for the treatment of *Plasmodium falciparum* malaria. Among the potential genes that are associated to resistance of *P. falciparum* to artemisinin include *PfATPase6* gene that encodes the protein SERCA: the specific target of drugs in the parasite. *PfATPase6* was the subject of many studies across the world to highlight its' involvement in the resistance of *P. falciprum* to artemisinin. It was found in this work that this gene has a polymorphism but its' involvement in the resistance of the parasite has not been demonstrated. The objective of this study was to describe the basic polymorphism of clinical isolates of *P. falciparum* in Côte d'Ivoire during the period when the country national anti- malaria program introduced ACTs in the treatment of malaria. Thus, 82 DNA fragments from 41 clinical isolates divided into regions A and B were analyzed using automatic sequencing method. The results show more points mutation of DNA fragments of PfATP6 but the most significant are D734Y (29.2%), Q254H (9.7%), N669Y (14.6%) and S670C (12.2%). Other mutations emerged in marginal proportions. We therefore recommend strict monitoring of gene polymorphism in *PfATPase6* in as much as the effectiveness of artemisinin derivatives is concerned; but the fact remains that their involvement in the resistance of *P falciparum* to artemisinin is still very low.

Key words: Côte d'Ivoire, detection, mutations, *PfATPase6*, polymorphism.

INTRODUCTION

Artemisinin (ART) and its derivatives play an indispensable role in the malaria elimination campaigns currently being unfolded in many regions where malaria is endemic. To reduce the chance of resistance development and prolong the life span of this group of drugs, the World Health Organization (WHO) has endorsed ART-based combination therapies (ACTs) as the first-line treatment for *Plasmodium falciparum* malaria

(Nosten and White, 2007). Since the adoption of the ACT policy in many regions where *P. falciparum* malaria is endemic (Bosman and Mendis, 2007), a trend of steady reduction in global malaria incidence has been observed (WHO, 2011). However, the recent detection of emerging low-grade resistance to ARTs in Western Cambodia, which manifested as delayed parasite clearance, has raised a major concern (Noedl et al., 2008; Dondorp et al., 2009). As recommended by WHO Global Plan for Artemisinin Resistance Containment (GPARC), research aimed to decipher the underlying mechanisms of ART resistance has become a priority. ARTs contain an endoperoxide bridge that is essential for the parasite-killing activities (White, 2008).

Although the structure of ART was solved over three decades ago, the mode of action of this group of drugs has not been unequivocally determined (Cui and Su, 2009; O'Neill et al., 2010; Ding et al., 2011). The most-studied model suggests that heme-mediated activation of ARTs results in C-centered free radicals that alkylate biomolecules in the parasite, leading to parasite death (Meshnick, 1994; Meshnick, 2002; Krishna et al., 2004). Evidence supporting the involvement of heme in the action of ARTs includes antagonistic actions of iron chelators and the requirement of hemoglobin digestion for the activity of ART (Meshnick et al., 1993; Klonis et al., 2011). This also correlates with the tolerance pheno-menon of ring-stage parasites to ARTs, when hemoglobin digestion activity is low. The reduced metabolic activity at the ring stage is reflected further in ART induced temporary arrest of growth (dormancy) at this stage (Chavchich et al., 2010; Witkowski et al., 2010). Whereas, this may partially explain the prolonged parasite clearance observed in clinical studies (Nosten, 2010), the possibility of host factors that may play a crucial role in determining prolonged parasite clearance times observed *in vivo* has not been investigated (Vattanaviboon et al., 1998; Charoenteeraboon et al., 2000). In addition, it has been proposed that ARTs may interfere with the mitochondrial function of the parasite (Li et al., 2005; Wang et al., 2010). Other postulated cellular targets of ARTs include the multidrug resistance 1 (*mdr1*) gene, ABC transporter genes *G7* and *G49* (Anderson et al., 2005), translationally controlled tumor protein (Bhisutthibhan et al., 1998), and the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) ortholog PfATP6 (Eshetu et al., 2010). Moreover, none of these candidate genes appears to be responsible for the observed ART resistance in areas of endemicity (Dondorp et al., 2009; Imwong et al., 2010). The proposal of PfATP6 as the primary target of ARTs in malaria parasites was initially based on the structural resemblance of ARTs to thapsigargin, a specific inhibitor of mammalian SERCAs. Since PfATP6 is the only SERCA-type Ca^{2+}-ATPase in the malaria parasite's genome, it was evaluated as the target of ARTs. When expressed in *Xenopus laevis* oocytes, PfATP6ase can be

specifically inhibited by ART as well as thapsigargin (Eckstein-Ludwig et al., 2003). Modeling of PfATP6 and docking simulations suggest that ARTs bind to PfATP6 through hydrophobic interactions (Jung et al., 2005; Naik et al., 2011). Variations at a single residue, 263, located in the predicted ART-binding pocket of PfATP6ase, tremendously affect the sensitivity of the enzyme to ARTs (Uhlemann et al., 2005). When assayed in *X. laevis* oocytes, the introduction of a single substitution, L263A or L263S (residues in *Plasmodium vivax* and *Plasmodium berghei* SERCAs, respectively) resulted in an approximately 3-fold increase or decrease of sensitivity to ARTs, respectively. Furthermore, the L263E replacement led to complete abolishment of inhibition by ART (Uhlemann et al., 2005). However, this observation was not extended to *P. falciparum*, where introduction of the L263E mutation through transgenics resulted in borderline non-significant changes in the 50% inhibitory concentrations (IC_{50}s) for ART and its derivatives (Valderramos et al., 2010).

P. falciparum resistance to drugs remains a major public issue in Côte d'Ivoire. The therapeutic failure index for chloroquine and sulphadoxine-pyrimethamine are respectively 62.2 and 35.4% (Ouattara et al., 2010). Since the year 2005, Côte d'Ivoire switched as its first-line treatment policy for uncomplicated cases of malaria to the use of artemisinin-based combination therapy (ACT). Having started in 2006, the implementation has now reached the whole country. The main goal of the study, performed at a time when the national imple-mentation of the ACT policy had just begun, was to describe the genetic polymorphism of the *PfATPase6* gene. This should provide useful baseline data for the Ivorian Malaria Control Program.

MATERIALS AND METHODS

Blood collection and DNA extraction

This study was carried out in two health centers of Abobo Township, El Rapha and Anokoua Kouté in the Northern of Abidjan district at 15 km from the center of the capital city. The study took place from February to December, 2006. During the study, only infected patients with *P. falciparum* diagnosis was carried out by microscopic examination of Giemsa-stained thick blood films as recommended by the Ivorian Malaria Control Program. Patients were aged between 2 to 45 years. Being given that it is a molecular study, only finger-pricked capillary blood was imbibed onto filter paper (Isocode Stix®, Schleichler and Schuell, Ecquevilly, France) obtained from infected patients with haemoglobin rate > 6 g/dL. However, all patients were treated after blood was taken free of charge with artemether-lumefantrine (AL) recommended by the Ivoirian Malaria Control Program. Imbibed blood filter paper samples were dried, and conserved until molecular analysis at Paris-Sud XI University, UMR 8080, Orsay (France). DNA was extracted from filter papers by the boiling method as follows. After rinsing two times with 500 µL of distilled water, the filter papers were immersed in 75 µL of distilled water in a 0.5 mL micro-tube and incubated at 99°C for 30 min. For each PCR, 12.5 µL of the supernatant was used. Informed consent was obtained from

Table 1. Oligonucleotide primers used for PCR amplification of *PfATPase6* gene.

Primer pair	Sequence 5' ⟶ 3'	Target region (pb)	Size (pb)
Sense PFATP-1	ATPGAAGAAGAGGTTATTAAGAATGCTCATACA	1-871 (A)	871
Antisense PFATP-2R	ATTCATGGTTCATTTTTATATGGTTGTTTA		
Sense PFATP-4	GATTCTTTAACAGAATACCAACTATGTCAA	1138-2431 (B)	1294
Antisense PFATP-5R	TGCCATATGGCTGGTATACGTGTATTTATG		
Sense PFATP-3	GGTTTGAATGAATTAGAAGTAGAAAAGAAG	121-871 (A)	751
Antisense PFATP-2R	ATTCATGGTTCATTTTTATATGGTTGTTTA		
Sense PFATP-6	ACAGAATACCAACTATGTCAAAAAGGGGAT	1147-2431 (B)	2431
Antisense PFATP-5R	TGCCATATGGCTGGTATACGTGTATTTATG		

Table 2. Single nucleotide polymorphisms (SNPs) and their corresponding amino acid point mutations in *PfATPase6* gene.

Wide/mutant type	SNPs	Frequency (%)
TAT- T**TT**[§]	Y148 F	1 (2.4)
GAT- GA**A**	D153E	1 (2.4)
AGT- A**A**T	S158N	1 (2.4)
GGT- GG**A**	G160G*	1 (2.4)
GGT- GG**A**	G223 G*	1 (2.4)
CAA- CA**C**	**Q254H**	**4 (9.7)**
CAT- C**C**T	H638P	1 (2.4)
TCA- TC**C**	S658S*	1 (2.4)
AAT- **U**AT	N663Y	1 (2.4)
AAT- **T**AT	N668Y	1 (2.4)
AAT- **T**AT	**N669**Y	**6 (14.6)**
AGT- **T**GT	**S670**C	**5 (12.2)**
TGG- **G**GG	W681G	1 (2.4)
GAT-**T**AT	**D734Y**	**12 (29.2)**

*Synonymous polymorphisms do not lead to any amino acid change. [§]Transferred nucleotides are in greasiness. N = 41. **Bold:** Sites where mutation have occurred

patients or guardians accompanying the sick children. The study was approved by the National Ethics Committee of Côte d'Ivoire.

PCRs amplication and sequencing

The following mixture was prepared in 50 µL final volume: genomic DNA extracted as described above, specific primers [SIGMA® Aldrich] (10 pmol), buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTPs and one unit of Taq DNA polymerase. Given the large size of the gene (3000 bp) and the points mutations described above, *PfATPase6* was divided into two regions A and B. For the primary PCR, the primers pairs used were PFATP-1/PFATP-2R (region A) and PFATP-4/PFATP-5R (region B). The PTC-100 thermal cycler (MJ Research, Watertown, MA) was programmed to carry out 30 cycles of 94°C for 2 min (first cycle) or 1 min (rest of the cycle), 50°C x 1 min, and 72°C x 1 min, followed by 72°C x 12 min at the end of 30 cycles. Due to the small quantity of DNA imbibed onto filter paper, nested PCR was necessary to obtain sufficient amount of amplified products for direct PCR sequencing or PCR-RFLP. The primary amplification was performed as described above, using 12.5 µL of DNA suspension. The nested-PCRs were performed with primers pairs PFATP-3/PFATP-2R for region A (900 bp fragment) and PFATP-6/PFATP-5R for region B (1359 bp fragment). All the primers used are presented in the Table 1. A known DNA of *P. falciparum* was used as positive control during the performed PCRs. Amplification products were sequenced by an automated sequencer (MWG Biotech.) and electropherograms were analyzed by using 4 peaks, DNA Strider and Ape software.

Analysis

Data were included in a data file with EPI-INFO v. 6.0 and bivariate relations were analysed by the kappa test of Cohen. The degree of agreement (k) was scored as follows: 0-0.20, slight agreement; 0.21-0.4, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, good agreement; and > 0.81, very good agreement.

RESULTS

Samples characteristics

Samples from 41 patients with acute *P. falciparum* malaria ranging from 2 to 45 years old (48% of which were males) were studied. Four samples (8.8%) of the 45 collected isolates had parasites densities lower than 4,000 ring/µL and had been excluded. Sequencing was successful in the all 82 DNA fragments (41 fragments for each region). The size of region A was 900 bp while the region B was 1359 bp. The mean age was 12 years old and the H/F sex ratio was 1.07. The mean parasitemia was 54,831 ring/µL.

Genetic polymorphism and global prevalence of mutation

Genetic polymorphism was found at 14 point mutations. All the mutations were rare except four codons of the *PfATPase6* gene (Table 2). In our analysis, we have considered only four major mutations as follow: Q254H,
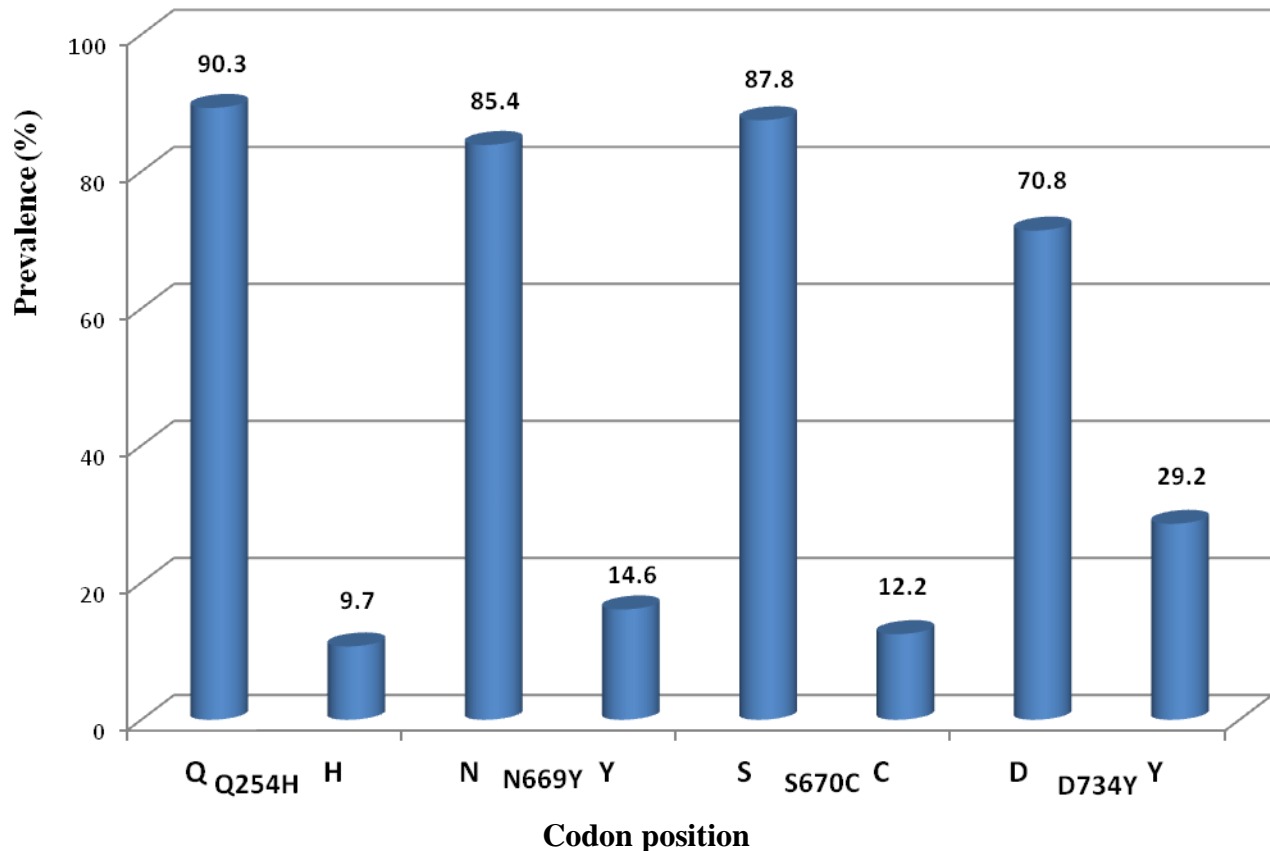
Figure 1. Prevalence of genetic polymorphism in *PfATPase* 6 gene.

N669H, S670C and D734Y. The mutation at codon 254 lead to the amino acid change glutamine in histidine. While the second and third mutations at codons 669 and 670 lead to the amino acid change asparagine in histidine and serine in cysteine, respectively. The fourth mutation at 734 lead to the amino acid change of aspartic acid in tyrosine. The prevalence of each mutation was: 9.7% for Q254H, 14.6% for N669H, 12.2% for S670C and 29.2% for D734Y (Figure 1). The G2306A mutation (encoding S769N) was absent in all the samples.

Bivariate relation of *Pfatpase* mutation according to parasitemia, age and blood collection area

Neither the parasitemia, nor the sex, nor the age, nor the blood collection area was related to the *Pfatpase* mutations (k<0.1).

DISCUSSION

The study on the evaluation of polymorphism in *PfATPase6* gene is the first of its kind to be undertaken in Côte d'Ivoire. This explains why local data is rarely available; but, this gene was tipped to be the genetic support of *P. falciparum* resistance to artemisinin (Imwong et al., 2010). In Côte d'Ivoire, several genetic markers of *P. falciparum* resistance to antimalarial drugs have regularly been the subject of study (Djaman et al., 2010). Those studies have shown, for example, the link between PfcrtK76T mutation and resistance to chloroquine, thus justifying the withdrawal of this molecule as first-line anti-malarial drug. Following example of molecular studies carried out in other countries, we wanted to evaluate the polymorphism in *PfATPase6* gene in Côte d'Ivoire in order to make local data available. This work took place just after the introduction of ACTs as first-line anti-malaria treatment in Côte d'Ivoire in 2005. The previous data indicated that *PfATPase6* gene is polymorphic at codons 89, 243, 263, 401, 431 568, 623, 630, 642, 769 and 898, and that these mutations would be responsible for the decreased in sensitivity of *P. falciparum* to artemisinin derivatives (Ulhemann et al., 2005; Jambou et al., 2005, Mugittu et al., 2006).

Grouping the gene into regions A and B has highlighted these haplotypes described above. Thus, all codons implicated in the resistance to artemisinin were explored in this present study. Apart from these haplotypes, sequencing was used to analyze more than 2400 base pairs (bp). Our results reveal 14 mutations at various

points; meanwhile, four codons had mutated in relatively high proportions. They are 254, 669, 670 and 734. None of the previously described haplotypes has mutated. These point mutations are new compared to the data available on the polymorphism in *PfATPase6* gene. Similar studies were conducted in Sao Tome and Principe and showed a silent mutation of T2694A (Ferreira et al., 2007). Ferreira et al. (2008) also published a similar paper with Brazilian samples (Ferreira et al., 2008). They described the analysis of four SNPs in isolates from Pará in nucleotide positions: 110, 1916, 2306 and 2694. The same previous study was checked for five *PfATPase*6 gene SNPs (538, 574, 623, 683 and 769) by DNA-microarrays (Crameri et al., 2007). By DNA-microarrays, the *PfATPaseA623E* mutation was found in 4.7% of the Niger samples, but sequencing did not confirm this. These abundant and often conflicting data on the polymorphism of *PfATPase6* gene clearly show the non-involvement of this gene in the resistance of *P. falciparum* to artemisinin. Recently, S769N mutation tipped to be the key mutation was found not associated with resistance of *P. falciparum* to artemether (Cui et al., 2012); meanwhile, the efficacy of artemisinin the current first molecule in malaria treatment is in sharp decline (Witkowski et al., 2013). Therefore, we must look for other molecular markers associated with *P. falciparum* resistance to artemisinin to better understand the mechanisms of resistance and thus improve malaria treatment (Ariey et al., 2014; Kamau et al., 2014).

Conclusion

The present work did not show the haplotypes; presently candidates as molecular markers for artemisinin resistance. However, the molecular diversity of *PfATP6* seems more pronounced than previously demonstrated as 14 mutations were detected with four relatively frequent. Whereas these mutations may be spontaneous and as such without any particular role in the parasite response to artemisinin, continual monitoring of *P. falciparum* susceptibility to artemisinin is highly welcome. In addition, molecular epidemiology should be part of routine surveillance to produce complementary information to assess the appropriateness of the current national anti-malarial drug policy.

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Full Length Research Paper

Amylolytic strains of *Lactobacillus plantarum* isolated from barley

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Two α-amylase-producing strains of *Lactobacillus plantarum* were isolated from South African barley. The extracellular α-amylase activity produced by strain A.S1.2 coincided with cell growth, while strain B.S1.6 produced α-amylase mainly during stationary growth. Cell wall α-amylases in both strains were approximately five times higher than recorded for extracellular α-amylases. Both strains demonstrated highest extracellular α-amylase activity in 2% (w/v) maltose, followed by 2% (w/v) malt extract and 2% (w/v) starch, respectively. The α-amylase produced by the two strains functioned optimally at 50°C and under alkaline conditions. The two strains of *L. plantarum* fermented carbohydrates naturally present in barley, and produced cell-bound and cell-free α-amylase at alkaline conditions. The two strains may be developed into starter cultures to facilitate the germination of barley and produce malt with a higher fermentable sugar content.

Key words: Lactobacillus plantarum, starch hydrolysis, barley, malting.

INTRODUCTION

Lactic acid bacteria (LAB) are fastidious and usually only grow in environments rich in monosaccharides, disaccharides, amino acids, peptides, nucleotide bases, vitamins, minerals and fatty acids (Hammes and Hertel, 2009). Complex carbohydrates are seldom fermented, especially in environments rich in glucose or disaccharides such as sucrose (Hammes and Hertel, 2009; Reddy et al., 2008). However, a few strains degrade starch in the presence of easier fermentable carbohydrates. Amylolytic (starch hydrolysing) strains of *Lactobacillus plantarum* have been isolated from fermented cassava, maize and sorghum (Guyot, 2010; Haydersah et al., 2012; Nwankwo et al., 1989; Sanni et

al., 2002; Songré-Ouattara et al., 2008), from burong isda prepared from fermented fish and rice (Olympia et al., 1995) and fermented rice noodles (Kanpiengjai et al., 2014). Amylolytic strains of *L. fermentum* were isolated from ogi and mawè, a Benin maize sourdough product (Agati et al., 1998; Tchekessi et al., 2014), from traditionally fermented Nigerian foods (Sanni et al., 2002) and a starchy soil waste in the Cameroon (Fossi and Tavea, 2013). Amylolytic strains of *L. manihotivorans* were isolated from fermented cassava (Morlon-Guyot et al., 1998) and sorghum silage (Chahrour et al., 2013), and *L. amylolyticus* from beer malt (Bohak et al., 1998) and swine waste (Nakamura and Crowell, 1979). A

starch-hydrolysing strain of *Streptococcus macedonicus* was isolated from pozol, a Mexican fermented maize beverage (Díaz-Ruiz et al., 2003).

Amylolytic LAB (ALAB) have also been isolated from raw starch, corn and potato and play an important role in cereal-based fermented foods such as European sour rye bread, Asian salt bread, sour porridges, dumplings and non-alcoholic beverage production (Chatterjee et al., 1997; Nakamura, 1981; Rodriguez-Sanoja et al., 2000).

Knowledge regarding ALAB in barley is poor. However, lactic acid bacteria proliferate extensively on barley when fermentable sugars become available during malting (Booysen et al., 2002; O`Sullivan et al., 1999; Petters et al., 1988). In the brewing industry, malting is essential to supply yeast with fermentable substrates, and the optimization of this process demands sufficient starch degradation (Laitila et al., 2007). We are the first to report on *L. plantarum*, isolated from barley, with extracellular and cell wall α-amylases. The strains may be exploited as starter cultures during malting to contribute to barley germination, and hence malt with a higher fermentable sugar content.

MATERIALS AND METHODS

Screening for amylolytic lactic acid bacteria and differentiation of isolates

Barley (10 g) from eight different samples, collected in the Western Cape, South Africa, was suspended in 100 ml sterile distilled water and incubated at 30°C for 48 h. The suspensions were serially diluted in sterile distilled water, plated onto modified MRS agar and incubated at 30°C for 48 h. The composition of the medium was as described by De Man et al. (1960), except that glucose was replaced with 2% (w/v) soluble starch (Merck) (MRS-starch medium) and 200 µg/ml Delvocid (Gist Brocades NV, Delft, The Netherlands) was added to prevent the growth of yeasts and molds. Plates were flooded with a potassium iodide solution (0.6%, w/v, K and 0.3%, w/v, I crystals, dissolved in distilled water). Colonies surrounded by the largest clear zones were selected and streaked onto MRS-starch plates to obtain pure cultures. Starch hydrolysis was confirmed by inoculating the isolates (10%, v/v) into MRS-starch broth. After 48 h of incubation at 30°C, the starch hydrolysed was calculated from the residual, thus unfermented starch and expressed as a percentage of the original concentration. The spectrophotometric method of Nakamura (1981) was used.

The isolates were Gram-stained and visualised with a phase contrast microscope. Catalase activity was determined by covering colonies on MRS agar plates with 5% (v/v) hydrogen peroxide. Phenotypic and genetic relatedness of the isolates were determined by carbohydrate sugar fermentation reactions and RAPD-PCR, respectively. The API 50 CHL system (BioMerieux, Maray 1'Etoile, France) was used, according to instructions of the manufacturer. DNA was extracted from each isolate using the ZR Fungal/Bacterial DNA kit (Zymo Research Corporation, Orange, California, USA). The PCR method described by Van Reenen and Dicks (1993) was used, but with a few modifications. Each reaction of 25 µl contained 40 ng genomic DNA, 1.25 mmol/l of each dNTP, 1 µmol/l of a single 10 base primer and 2.5 units Taq Polymerase. Three single primers [5'-CCAGCAGCTT-3' (OPL-03), 5'-GGGCGGTACT-3' (OPL-12), 5'-AGGTTGCAGG-3' (OPL-16)] were used. The cycling program included an initial cycle of 94°C for 4 min, followed by 45 cycles of

94°C (1 min), 36°C (1 min), and 72°C (1 min). Final incubation was at 72°C for 5 min and ended by cooling to 4°C. PCR were performed in triplicate. DNA banding patterns were visualized by electrophoresis in 1.4% (w/v) agarose gels. Lambda DNA, digested with *Hind*III and *Eco*R1 (Boehringer Mannheim, Darmstadt, Germany) was used as molecular marker.

Identification of strains

Strains with unique phenotypic and genetic characteristics were identified to species level by sequencing the 16S rRNA and *rec*A genes. The 16S rDNA from each isolate was amplified using primers 8F (5'-CACGGATCCAGACTTTGATYMTGGCTCAG-3') and 1512R (5'-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3'), as described by Felske et al. (1997). The *rec*A gene was amplified using primers AmpF (5'-GCCCTAAAAAARATYGAAAAGAAHTTYGGTAAAGG-3') and AmpR (5'-AATGGT GGCGCYACYTTGTTTTTHACAACTTT-3'), according to the method of Endo and Okada (2008). PCR products of the 16S rRNA gene and *rec*A were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, California, USA). The DNA fragments were cloned into pGEM-T Easy vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into *E. coli* DH5α. Competent *E. coli* DH5α cells were prepared as described by Neveling et al. (2012). Plasmids were isolated using the Qiagen plasmid miniprep kit (Qiagen). The 16S rRNA and *rec*A genes were sequenced using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, California) as prescribed by the manufacturer. Blast analysis was conducted on the 16S rDNA and *rec*A sequences and aligned with ClustalW. Phylogenetic trees were constructed with sequences of approximately 1500 bp 16S rDNA and 600 bp *rec*A. Phylogenetic analysis was done using the neighbour-joining method, maximum-likelihood (Cavalli-Sforza and Edwards, 1967) and maximum parsimony algorithms (Kluge and Farris, 1969). Bootstrapping was performed as described by Felsenstein (1985).

Determination of α-amylase activity

The isolates were grown in MRS broth (Biolab) to mid-exponential phase, inoculated (4%, v/v) into 200 ml MRS-starch broth (pH 7.0) and incubated at 30°C for 40 h, without pH control, on an orbital shaker (120 rpm). Samples of 1 ml were collected every 2 h from the 200 ml culture. The cells were harvested (10 000 *g*, 15 min) and the cell-free supernatant tested for extracellular α-amylase activity, using the Cerelpha method (Megazyme International, Ireland, Ltd, County Wicklow, Ireland). Results were expressed in Cerelpha Units (CU)/ml, with one unit defined as the amount of enzyme required to release one micromole p-nitrophenol from a blocked p-nitrophenyl maltoheptaoside oligosaccharide substrate (BPNPG7) per min. Cell wall α-amylase activity was measured after 24 h of incubation at 30°C. The cells were harvested as before, washed twice in sterile saline (0.85 %, w/v) and resuspended in extraction buffer (1 mol/l Na-malate, 1 mol/l NaCl, 40 mmol/l CaCl, pH 5.4). Malt flour was used as positive control and an isolate of *L. plantarum* with no α-amylase activity on plate assays, was used as negative control. All experiments were performed in triplicate.

Effect of carbon source, temperature and pH on α-amylase activity

Production of α-amylase from different carbon sources was determined by inoculating active-growing cells (4%, v/v) of each isolate into MRS broth and MRS broth with glucose replaced by 2%
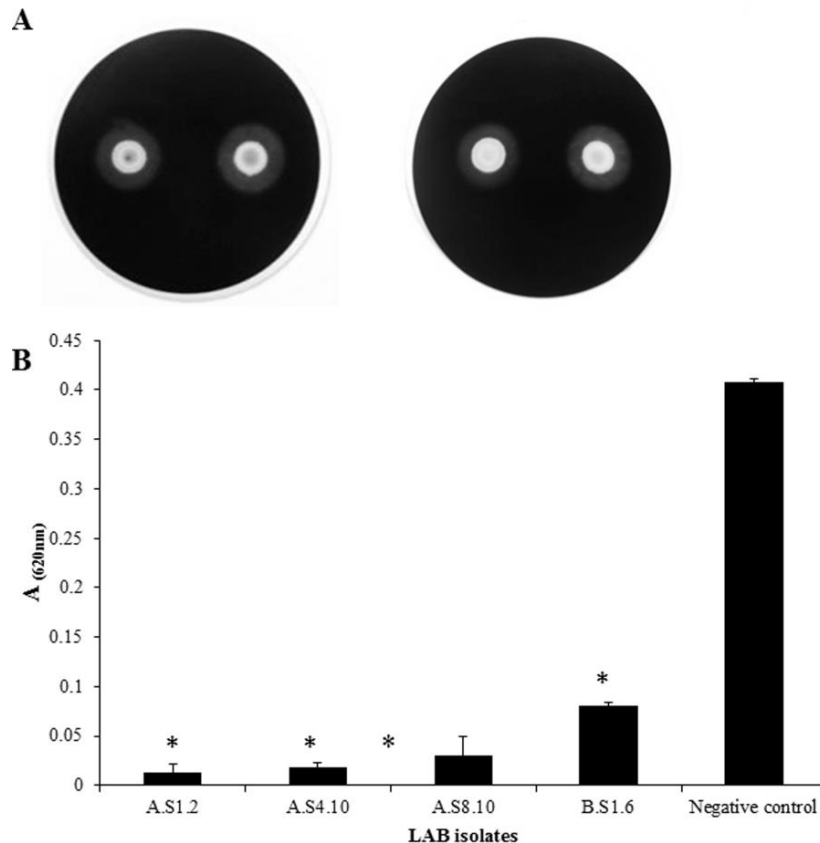
Figure 1. A, Starch hydrolysis on MRS-starch agar plates, showing clear zones. Staining was with KI. From left to right: Isolates A.S1.2, A.S4.10, A.S8.10 and B.S1.6. B, Residual starch content after fermentation in MRS-starch broth, as determined using the method of Nakamura (1981). Significant levels in the paired t-test: *$p < 0.05$.

(w/v) starch (Merck, Darmstadt, Germany), 2% (w/v) maltose (Sigma-Aldrich, St. Louis, Missouri), 2% (w/v) cellobiose (Sigma), and 2, 4 and 6% (w/v) malt extract (Muntons, Suffolk, England), respectively. In another experiment, 2% (w/v) maltose was added to MRS broth. An additional experiment was performed by inoculating cells into MRS broth supplemented with a combination of 2% (w/v) malt extract and 2% (w/v) maltose. All cultures were incubated at 30°C for 24 h. Viable cell numbers were determined by plating onto MRS agar. Extracellular α-amylase activity was measured in the cell-free supernatants, as described before. All experiments were performed in triplicate.

The stability of extracellular α-amylase at different temperatures and pH conditions was determined by using cell-free supernatants collected from 24 h-old cultures grown in MRS-starch broth at 30°C. Temperature effects were determined by incubating cell-free supernatants at 20 to 70°C (with 10°C intervals) for 10 min. The effect of pH was determined by incubating cell-free supernatants at pH 3-9, with increases of one pH unit, for 10 min. Citrate phosphate buffer (0.1 mol/l) was used for pH 3-6, phosphate buffer (0.1 mol/l) for pH 7 and 8 and Tris buffer (0.1 mol/l) for pH 9. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis was done with Statistica (v. 10, StatSoft, Inc.). The students'-test was performed at 95 % confidence levels.

RESULTS

Selection of amylolytic strains

From the 185 colonies on the MRS-starch plates, 90 were surrounded by clearing zones, suggesting that they hydrolysed starch. Four of the 90 isolates (A.S1.2, A.S4.10, A.S8.10 and B.S1.6) produced the largest clearing zones and hydrolysed at least 75% of the starch in MRS-starch medium (Figure 1). All four isolates were Gram-positive and catalase negative. Isolates A.S1.2, A.S4.10 and A.S8.10 hydrolysed approximately the same amount of starch within 48 h (Figure 1), had an identical sugar fermentation profile (not shown) and displayed similar RAPD-PCR banding patterns (Figure 2). Isolate B.S1.6, on the other hand, degraded less starch (Figure 1) and had a different DNA profile (Figure 2). Furthermore, strain B.S1.6 differed from the other three strains by fermenting L-arabinose, rhamnose and lactose, but not α-methyl-D-mannoside and inuline. Strains A.S1.2 and B.S1.6 formed a tight cluster with other strains of *L. plantarum* when 16S rRNA (Figure 3) and *rec*A sequences (not shown) were compared. All four strains
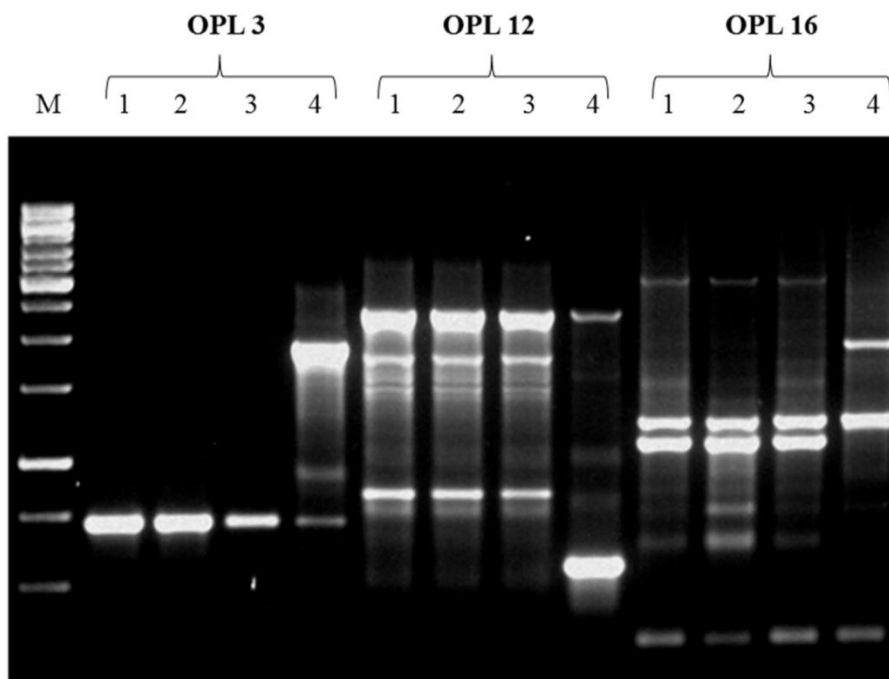
Figure 2. DNA banding patterns obtained with RAPD-PCR. Three primer sets were used (OPL 3, OPL 12 and OPL 16). Order of isolates per primer set: Lane 1 = A.S1.2, lane 2 = A.S4.10, lane 3 = A.S8.10 and lane 4 = B.S1.6. M =lambda DNA digested with *Hind*III and *Eco*RI.

were classified as *L. plantarum* based on RAPD-PCR profiles (Figure 2) and comparison of 16S rRNA sequences (Figure 3). Strain A.S1.2 was selected as the representative of three strains with the same RAPD-PCR profile (Figure 2). The RAPD-PCR profile of strain B.S1.6 was, however, different (Figure 2). Strains A.S1.2 and B.S1.6 were thus selected for further studies.

α-Amylase activity

Extracellular α-amylase production of strain A.S1.2 coincided with cell growth (Figure 4A). Highest cell numbers of strain A.S1.2 (4×10^8 CFU/ml) were recorded after 22 h of inoculation. Starch hydrolysis started 8 h after inoculation, approximately 2 h before the first extracellular α-amylase activity was recorded. α-Amylase activity increased to ~ 0.1 CU/ml after 18 h of inoculation and increased rapidly to 0.7 CU/ml over the next 4 h. After 22 h of fermentation, α-amylase activity decreased to 0.3 CU/ml within 8 h and remained close to this level for the duration of the 40-h fermentation. The pH decreased from an initial 7.0 to approximately 3.8 towards the end of fermentation.

Extracellular α-amylase production of strain B.S1.6 did not coincide with cell growth (Figure 4B). Cell numbers increased to 2×10^8 CFU/ml after 14 h of inoculation and remained close to this level throughout the experiment.

As recorded for strain A.S1.2, starch hydrolysis started 8 h after inoculation, and approximately 10 h before the first extracellular α-amylase activity was recorded. α-Amylase activity increased to ~ 0.7 CU/ml after 28 h of incubation and slowly increased to 0.75 CU/ml towards the end of the 40-h fermentation. In contrast to strain A.S1.2, α-amylase activity increased as the culture pH decreased. No starch was degraded by the negative control, a non-amylolytic strain of *L. plantarum,* despite some limited growth (Figure 4C).

Cell-bound α-amylase activities recorded for strains A.S1.2 and B.S1.6 after 24 h of fermentation were 3.2 and 1.7 CU/ml, respectively (not shown), which is approximately five times higher than the extracellular α-amylase activities recorded (Figure 4A and B).

Effect of carbon source, temperature and pH on α-amylase activity

For both strains A.S1.2 and B.S1.6 highest α-amylase activity was recorded in the presence of 2% (w/v) maltose (Figure 5A and 5B, respectively). Activity of α-amylase recorded from growth in the presence of other carbohydrates is expressed as activity relative to that recorded for growth in the presence of maltose. In the case of both strains, highest α-amylase activity was recorded in the presence of 2% (w/v) maltose, followed
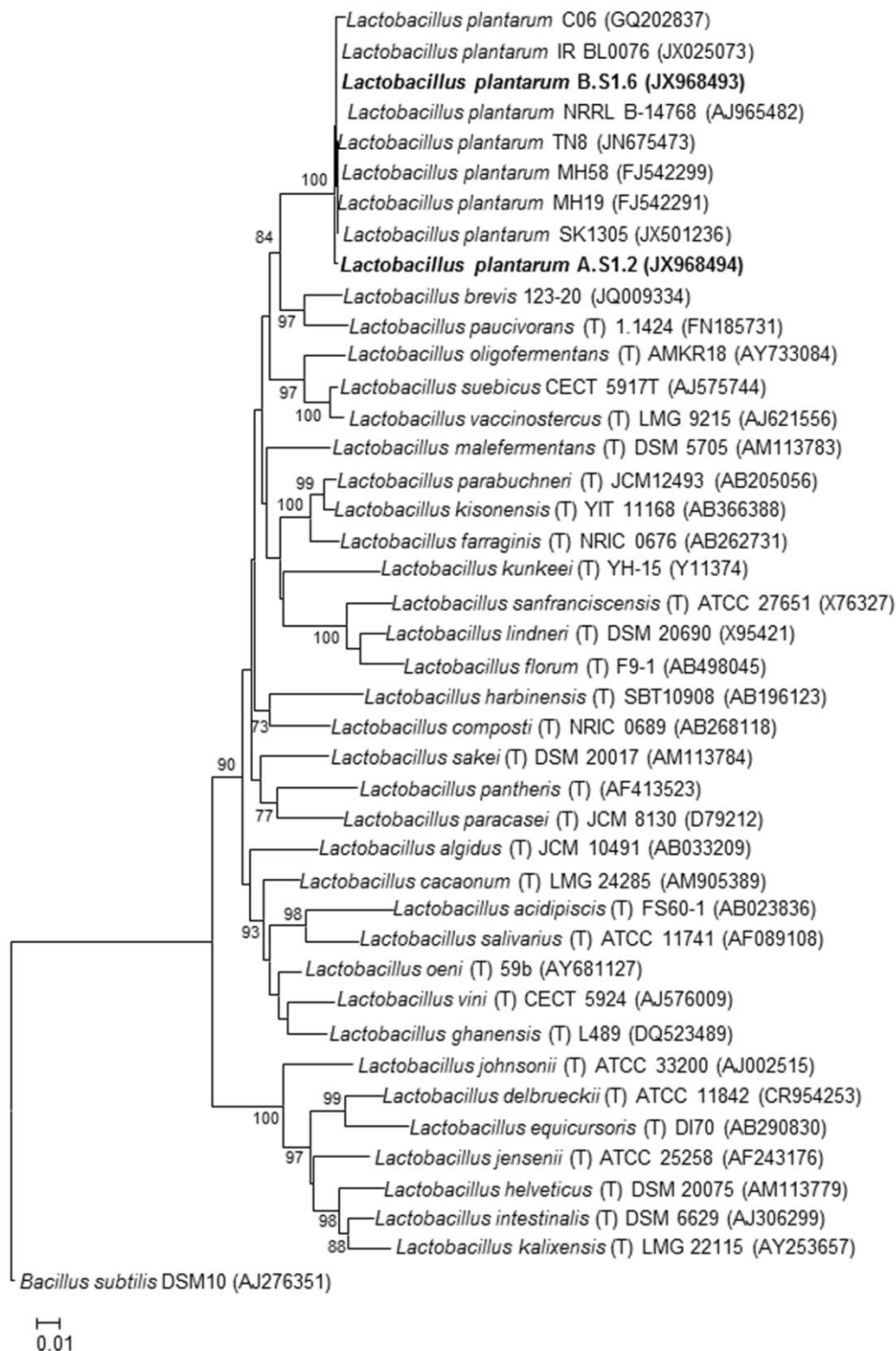
Figure 3. Phylogenetic relatedness of strains A.S1.2 and B.S1.6 to *L. plantarum*, based on partial 16S rRNA sequences. The maximum-likelihood method was used to construct the phylogenetic tree. *Bacillus subtilis* was used as an out-group.

by growth in the presence of 2% (w/v) malt extract and 4% (w/v) malt extract. Growth in the presence of a combination of 2% (w/v) maltose and 2% (w/v) malt extract yielded the same level of relative α-amylase activity as when cells were grown in the presence of 2% (w/v) starch. Growth in the presence of 6% (w/v) malt extract yielded less relative α-amylase activity compared to growth in the presence of 2% (w/v) maltose with 2%
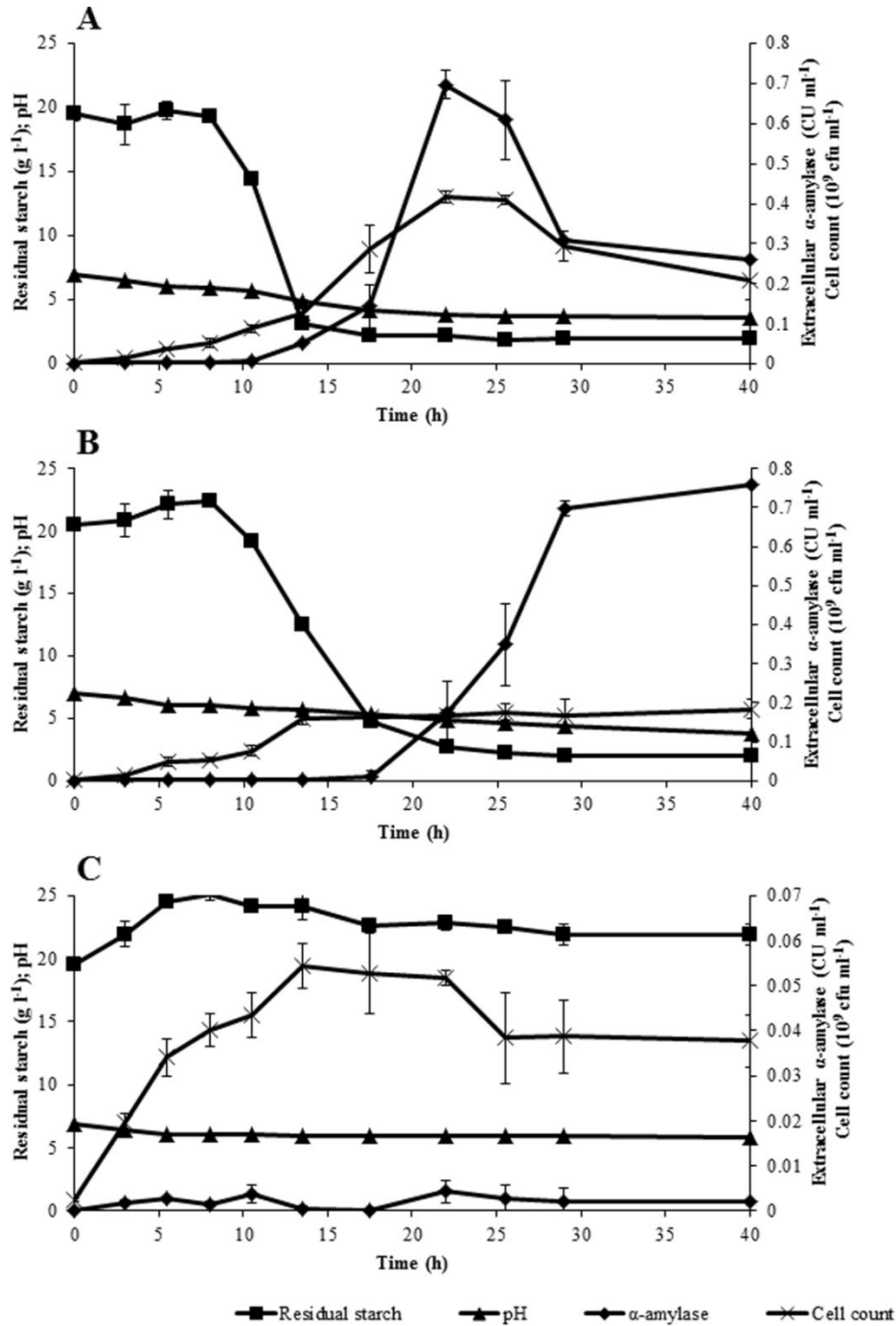
Figure 4. Growth and starch hydrolysis of *L. plantarum* strains A.S1.2 (A), B.S1.6 (B) and a non-amylolytic strain of *L. plantarum* (C) in MRS-starch broth. Incubation was at 30°C, without pH control.

(w/v) malt extract, or 2% starch. Low relative α-amylase activity was recorded when the strains were grown in the presence of 2% (w/v) glucose, 2% (w/v) cellobiose, and a combination of 2% (w/v) glucose with 2% (w/v) maltose. An inverse correlation was observed between cell numbers and α-amylase activity, that is highest activity

was observed in media with the lowest viable cell numbers (not shown).

The temperature profiles of α-amylases from strains A.S1.2 and B.S1.6 were similar, with maximum activity recorded at 50°C (Figure 6A and B) and in alkaline conditions (Figures 6C and D).
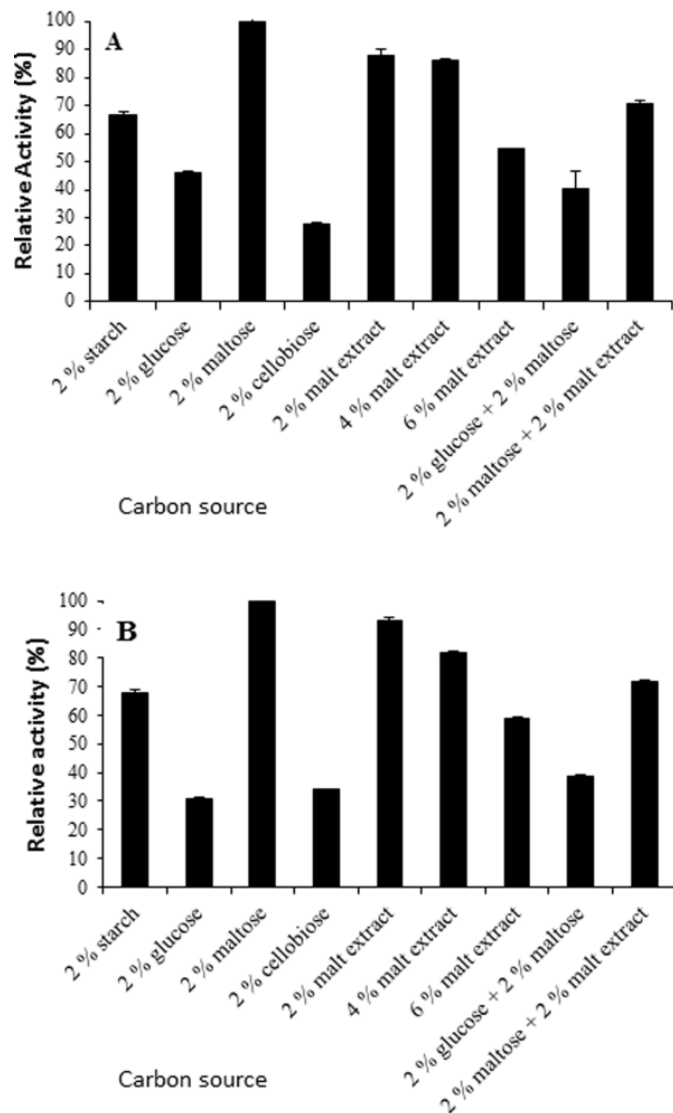
Figure 5. Extracellular α-amylase activity of *L. plantarum* A.S1.2 (A) and *L. plantarum* B.S1.6 (B). Results are expressed as relative values compared to that obtained in MRS with maltose as carbon source.

DISCUSSION

Strains A.S1.2 and B.S1.6 are phenotypically and genetically different. However, based on biochemical reactions, 16S rRNA (Figure 3) and *rec*A sequencing, both strains belong to the species *L. plantarum.* A number of studies have reported the presence of ALAB, including *L. plantarum*, in fermented cereals (Agati et al., 1998; Giraud et al., 1991; Morlon-Guyot et al., 1998; Sanni et al., 2002). However, little is known about the presence of these bacteria in barley. Due to their fastidious nature, LAB will only grow in the presence of fermentable nutrients, present during malting (Booysen et al., 2002; O`Sullivan et al., 1999; Petters et al., 1988).

For the majority of ALAB, starch fermentations are characterised by rapid starch hydrolysis, growth-linked amylase production and a reduction in the pH of growth media (Calderon et al., 2001; Fossi et al., 2011). The decrease in α-amylase activity of strain A.S1.2 after 22 h of growth (Figure 4A) may be ascribed to proteolytic enzymes released from lysed cells, or sensitivity of the enzyme at low pH (3.8).

Production of α-amylase by stationary phase cells has been reported for *L. plantarum* (Giraud et al., 1994). Strain B.S1.6 seems to follow the same pattern of α-amylase production. Strain B.S1.6 degraded starch for 18 h in the absence of detectable levels of α-amylase (Figure 4B). This suggests that the α-amylase remained attached to the cells for at least 18 h. Limited information is available for *L. plantarum* on cell wall α-amylase activity. Most studies focused on extracellular α-amylase activity (Giraud et al., 1991; Olympia et al., 1995; Sanni et al., 2002). The cell-bound α-amylase activity recorded for strain B.S1.6 (1.7 CU/ml) was much higher than the 0.1 to 0.4 C U/ml reported for amylolytic strains of *L. plantarum* (Songré-Ouattara et al., 2008). The strains studied by these authors did not produce α-amylases in cell-free supernatants. The increase in activity recorded for α-amylase produced by strain B.S1.6 towards the end of growth (Figure 4B) may suggest that the enzyme is released from the cells at approximately pH 3.8 and that it is not degraded by proteolytic enzymes. Since no decline in B.S1.6 cell numbers were recorded towards the end of growth (Figure 4B), cell lysis is expected to be minimal, as also the release of intracellular proteolytic enzymes. Production of α-amylase by cells in stationary phase, especially at low pH and in nutrient deprived medium, is unlikely. The hypothesis that the α-amylase of strain B.S1.6 is cell wall bound needs to be verified.

Malt contains glucose, maltose, starch and cellobiose. *L. plantarum* A.S1.2 and B.S1.6 produced highest extracellular α-amylase activity in 2% (w/v) maltose, followed by 2% (w/v) malt extract and 2% (w/v) starch. This correlates with previous studies showing highest α-amylase production in the presence of maltose (Calderon et al., 2001; Fossi et al., 2011; Guyot and Morlon-Guyot, 2001). Strains A.S1.2 and B.S1.6 produced α-amylase in malt extract that contains mono- and disaccharides, suggesting that they may be used as starter cultures in malting.

An inverse correlation was observed between extracellular α-amylase activity and growth in both strains (not shown). The reason for this is not known. Low cell numbers produce less lactic acid, which in turn is less stringent on α-amylase production or activity. Experiments of extracellular α-amylase activity at different pH values (Figures 6C and D) supports the hypothesis, since the extracellular α-amylases produced by both isolates were more active at higher pH values. However, the α-amylase produced by isolate B.S1.6 (Figure 6D) was more resistant to low pH compared to
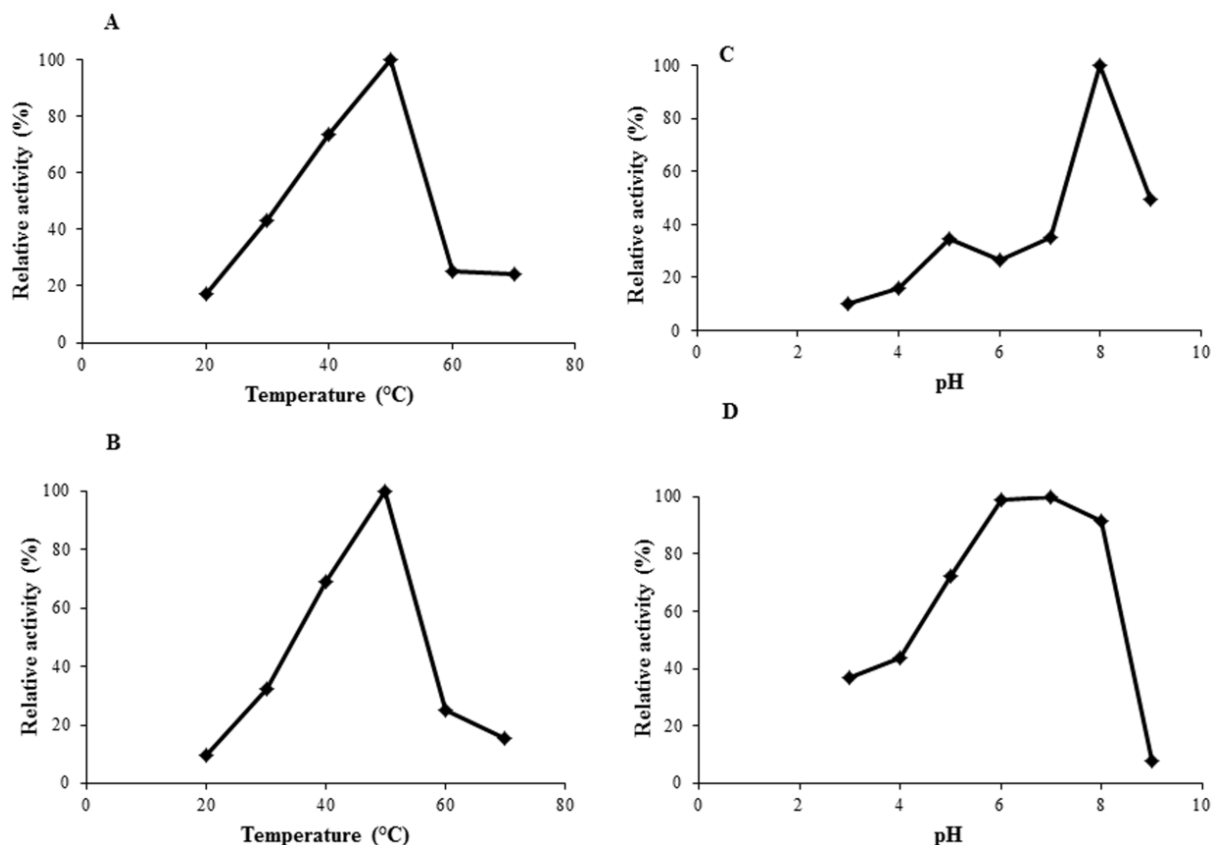
Figure 6. Effect of temperature on the activity of extracellular α-amylase produced by (A) *L. plantarum* A.S1.2 and (B) *L. plantarum* B.S1.6. The effect of pH on the activity of extracellular α-amylase produced by strains A.S1.2 and B.S1.6 is shown in C and D, respectively.

the α-amylase produced by isolate A.S1.2 (Figure 6C). These findings correlated with higher α-amylase activity recorded during stationary growth of isolate B.S1.6 (Figure 4B) and during late exponential growth of isolate A.S1.2 (Figure 4A).

The majority of ALAB, including *L. plantarum*, has an optimum pH of 4 to 5 (Agati et al., 1998; Aguilar et al., 2000; Champ et al., 1983; Giraud et al., 1991; Olympia et al., 1995; Ogunremi and Sanni, 2011). Amylolytic LAB that functions at alkaline environments have been identified and isolates from this study closely resembles *S. equines* and *L. dextranicum* with optimum pH ranges of 6.5 to 9.0 and 5.0 to 8.0, respectively (Dunican and Seeley, 1962; Lindgren and Refai, 1984).

The extracellular α-amylases from both strains function optimally at 50°C, which is in accordance with other ALAB (Ogunremi and Sanni, 2011). Malting is usually conducted at 20°C to activate amylases (Ullrich, 2011). However, optimal extracellular amylase activities at 50°C were reported for a strain of *L. fermentum* (Ogunremi and Sanni, 2011). Lactic acid bacteria with amylolytic activity at higher temperatures allows for optimization of fermentation processes and microbiological safety of fermented foods, and are thus highly desirable as starter

cultures. The amylases produced by *L. plantarum* A.S1.2 and B.S1.6 may contribute to the enzymatic pool during mashing when added as starter cultures in malting. Studies on the expression of the genes encoding α-amylase production at these pH values should provide more information.

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Biodegradation of low density polyethylene (LDPE) by a new biosurfactant-producing thermophilic *Streptomyces coelicoflavus* NBRC 15399[T]

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In the present study, we tested 83 actinomycete isolates for low density polyethylene (LDPE) degradation in the laboratory, and these isolates were previously isolated for biosurfactant production from the oil contaminated soil sample near Naval Dockyard in Visakhapatnam. Among them, 20 were positive using 2,3,5-triphenyl tetrazolium chloride (TTC) as a viability indicator and able to grow in a mineral salts medium containing LDPE as a sole source of carbon. Of these, NDYS-4 isolate showed prominent result with redox probe 2,3,5-triphenyltetrazolium chloride (TTC) as a viability indicator, which forms pink colour insoluble triphenylformazan (TPF) on mineral salts media containing emulsified polyethylene as a carbon source within five to seven days, and it was also grown prominently on mineral salt agar plates containing LDPE in comparison to other isolates, and it was selected for detailed analysis. The weight loss of polyethylene films was approximately 30% after four weeks of incubation with selected isolate. The viability and metabolic activity of this isolate growing on the polyethylene surface was confirmed using a TTC reduction test. The metabolic activity was also correlated with a concomitant increase in the protein density of the biomass on the surface of the polyethylene. The microbial degradation of LDPE was also analyzed by the change in pH of the culture media and microscopic analysis. Based on the results, this degradation may be mediated by enzymatic activity and may also involve cell-surface hydrophobicity. It was also able to grow on other polymers such as polyvinyl acetate (PVA), polycaprolactone (PCL), polyethylene oxide (PEO) and polyethylene glycol (PEG) using TTC as a viability indicator and also showed prominent growth on mineral salts agar plates containing polyethylene as a source of carbon. Based on the results of phenotypic characteristics, phylogenetic analysis, biochemical characteristics and 16S rRNA gene sequence homology, the isolate NDYS-4 was identified as *Streptomyces coelicoflavus* NBRC 15399[T]. It could be concluded that the PE degrading actinomycete selected in this study showed diverse and varying capacities to degrade polyethylene and other polymers and can be exploited for cleaning up polyethylene contaminated sites.

Key words: Biosurfactant, low density polyethylene, 2,3,5-triphenyl tetrazolium chloride, mineral salts media, hydrophobicity, sequence homology, phylogenetic analysis.

INTRODUCTION

Biosurfactants are heterogeneous group of secondary metabolites with surface active properties, and are synthesized by a variety of microorganisms (Chandrasekaran et al., 1978). Biological surfactants possess a number of potential advantages over their chemically manufactured counterparts, including lower toxicity, biodegradability (Zajic et al., 1983) and effectiveness at extreme temperatures, pH and salinity (Kretschmer et al., 1982).

The use of synthetic plastic by any means has changed the nature of waste in last three to four decades (Sheavly, 2005). Over this period, it has replaced natural material in various aspects of human life and is non-degradable thereby causing serious environmental and human health problems. These plastics are used extensively because of their availability, durability and light weight and low cost. According to the Central Pollution Control Board (CPCB), New Delhi, India, 8 million tons of plastic products are consumed every year in India. A study on plastic waste generation in 60 major cities in India revealed that approximately 15,340 tons per day of plastic waste is generated in the country (CPCB, New Delhi, India, 2013). The UV irradiation (photo-oxidation) (Esmaeili et al., 2013), thermal and chemical oxidation of PE prior to its exposure to a biotic environment enhances biodegradation (Volke-Sepuleveda et al., 2002). These pre-treatments increase surface hydrophilicity of the polymer by forming polarisable groups such as carbonyl groups that can be utilized by microorganisms (Albertsson, 1978; Albertsson, 1980; Cornell et al., 1984). Among these plastics, low density polyethylene (LDPE) is used mainly as carrier bags, in agricultural and other industrial applications constitutes the major portion of waste problem. LDPE is chemically inert and microbial attack resistance as it possess hydrophobic nature and absence of carbonyl and hydroxyl groups.

The generation of biodegradable polyethylene requires modifying the properties that are responsible for the PE resistance to degradation. A standard test to determine the biodegradation of plastic materials when exposed to soil was developed by the ASTM D 5988 (2003). Moreover, plastics pollute beaches and oceans, and kill marine fauna. These environmental issues raise concerns regarding the use of plastics. Since, the plastics have become an integral part of modern life; it is not possible to discontinue the use of these waste materials. Therefore, there is an emergency requirement of biotechnological approaches to solve these environmental challenges related to plastics. Disintegration of LDPE and subsequent degradation by microorganisms using only polymers as sole source of carbon is the main strategy (Roy et al., 2008).

Furthermore, actinomycetes also play an important role in polyester degradation (Tokiwa and Pranamuda, 2001). Most of the studies about high temperature of PES-degradation were focused on bacteria. A thermophilic bacterium, for example, is able to degrade PES at 50°C (Tansengco and Tokiwa, 1998). Pranamuda et al. (1997) had isolated a PLA-degradable actinomycete, *Amycolatopsis* strain HT-32, and Jarerat and Tokiwa (2003) had isolated the *Saccharothrix wayanrdensis* and *Kibdelosporangium aridum*. Sanchez et al. (2000) had found the polycaprolactone (PCL)-degradable fungi, *Aspergillus* sp. However, the studies about the PES degradation by actinomycetes on high temperature are still rare in the literature. The use of thermophilic actinomycetes for biodegradation of polymers such as polyethylene succinate, polyhydroxybutyrate and polycaprolactone at different temperatures, have gained notable importance because the inefficiency of the physical and chemical methods for disposal of these materials in the environment (Kim-Chi et al., 2007). Biodegradability of plastic wastes under natural conditions is required in the management of these solid wastes (Orhan and Buyukgungor, 2000). Therefore, the present study focused mainly on the biodegradation of LDPE by actinomycetes and its cultural and morphological characteristics.

MATERIALS AND METHODS

Low density polyethylene

The low density polyethylene granules (size range between 35-65 mm) were provided by Sai Polymers, Auto nagar, Visakhapatnam (India). The granules were dissolved in xylene (Fisher Scientific, 97% pure) by heating for 15 min; then, the residue was crushed while it was warm followed by filtration. The powder so obtained was washed with ethanol to remove residual xylene and allowed to evaporate to remove ethanol. The powder was dried in hot air oven at 60°C overnight to remove residual solvent and obtain dry fine powder.

Collection of soil

According to Kalyani et al. (2014), the mixture of soil with petrochemicals produces actinomycetes, with biosurfactant production, which requires further screening of actinomycetes for degradation of polyethylene and other polymers.

Screening of actinomycetes for polyethylene degradation

The biosurfactant producing actinomycetes from oil contaminated

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soil were isolated on humic acid vitamin agar (Hayakawa and Nonomura, 1987) (1.0 g humic acid, 1.0 g yeast extract, 1.7 g KCl, 0.5 g Na_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.01 g $FeSO_4.7H_2O$, 0.02 g $CaCO_3$, 50 mg nalidixic acid, 50 mg cyclohexamide, 0.25 mg biotin, 0.5 mg each of thiamine-HCl, riboflavin, niacin, pyridoxin-HCl, inositol, Ca-pantothenate, p-aminobenzoic acid and agar 18.0 g, distilled water 1.0 L, pH 7.2) plates using dilution technique and incubated at room temperature for one week, then observed in long working distance objective lens. The powdery colonies withbranched hyphae were picked and streaked on Bennett's agar [1.0 g beef extract, 1.0 g yeast extract, 2.0 g N-Z amine A (enzymatic digest of casein), 10 g glucose, 15.0 g agar, distilled water 1.0 L, pH 7.3] plates, then maintained as glycerol suspensions (20% v/v) at -80°C.

These isolates were then assayed for their ability to utilize polyethylene as the sole source of carbon and energy. They were grown in basal medium containing (per liter): yeast extract, 0.1 g; $FeSO_4.7H_2O$, 10 mg; $MgSO_4.7H_2O$, 0.2 g; $(NH_4)_2SO_4$, 1 g; $CaCl_2.2H_2O$, 20 mg; NaCl, 0.1 g; $Na_2MoO_4.2H_2O$, 0.5 mg; $NaWO_4.2H_2O$, 0.5 mg; $MnSO_4.H_2O$, 0.6 mg with a pH 7.2. Then, 20 µl of 1% TTC solution was added to 10 mL of medium as an indicator of viability (Alef and Nannipieri, 1999).

Furthermore, the screening actinomycetes were performed by comparing their growth ability in solid media containing 2% liquid oligomer as the sole source of carbon. The actinomycetes with the ability to grow in the presence of 5% ethylene oligomer were transferred to synthetic mineral medium containing 0.1% LDPE powder as the sole source of carbon for the final screening step (Atefeh et al., 2013).

Morphological and cultural studies

The colour of aerial mycelium, substrate mycelium and soluble pigment when grown on different media were observed. The macro and micro morphological features of the colonies and the colour determination of the aerial mycelium, substrate and soluble pigment were examined every 24 h for 7 to 14 days of incubation. Macro morphology was observed by the naked eye and also by using 10x, 40x magnifying lens.

Micro morphology (spore morphology)

Study of the aerial mycelium and its sporulation characteristics was carried out by inclined cover slip method (Williams and Davies, 1967). Sterile cover slips were placed at an angel 45° into solidifying agar medium in Petri plate such that a part of the cover slip was in the medium. Inoculum was spread along the line where the upper surface of the cover slip meets the medium. After full sporulation, the cover slips were removed and examined directly under the microscope (Labo America Inc., LABOMED USA CXR3 9122100).

Identification of actinomycetes

The molecular identification and characterization of the actinomycete was carried out by 16S rRNA gene sequencing which was performed at Institute of Microbial Technology (IMTECH), Chandigarh (India). The similarity search was conducted in-silico using the basic local alignment search tool (BLAST) database of NCBI. The scanning electron microscope of the actinomycete was done by RUSKA Laboratory's College of Veterinary Sciences, SVVU, Rajendra nagar, Hyderabad (India).

Physiological and biochemical characterization

The ability to grow at various temperatures (12-42°C), pH (5.0-10.0) and different concentrations of NaCl (2-10% w/v) on medium was also tested. The organism was also tested for its ability to utilize carbon sources such as D-glucose, galactose, L-arabinose, D-fructose, raffinose, Meso-inositol, D-mannitol, sucrose, salicin and rhamnose in modified Bennett's broth. Cultural characteristics of this strain were determined following incubation for 10-15 days at 28-42°C on various media. After incubation, the growth and colour of spore mass and diffusible pigment production were observed.

Chemotaxonomy

Isomers of diaminopimelic acid (DAP) and sugars present in hydrolysates of whole cell actinomycete were determined by thin-layer chromatography following the standard methods of Waiksman and Henrici (1943) and Boone and Pine (1968).

Polyethylene film biodegradation assay

The biodegradation tests were performed on samples of low-density polyethylene film (that is, 2×1 cm pieces of polyethylene bags) that had been dried overnight at 60°C, weighed, disinfected (autoclaved at 105°C for 1 h) and added to each flask (approximately 50.0 mg of polyethylene film per flask) containing 50 mL of basal medium. The flasks were inoculated with 3 mL of a mid-exponential phase culture that were maintained in Bennett's agar medium. Before inoculation, the culture was washed with basal medium to remove medium and cellular soluble debris. The cell densities of the inoculums were adjusted to $1.5×10^6$ colony forming units (CFU) per mL. The flasks containing non-inoculated basal medium supplemented with polyethylene film served as the control.

Weight loss measurements

To facilitate accurate measurement of the residual polyethylene weight, the bacterial film colonizing the polyethylene surface was removed by supplementing the cultures with a 2% (v/v) aqueous sodium dodecyl sulfate (SDS) solution. The flasks were then incubated for 4 h at 50°C and further washed with warm distilled water (Sivan et al., 2006). The polyethylene samples were collected on filter paper, rinsed with distilled water and then dried overnight at 60°C before they were finally weighed. The initial weights of the pre-incubated polyethylene samples were measured following the same procedure mentioned above.

Viability and metabolic activity of the isolates

The viability and metabolic activity of the surface-attached actinomycete was measured with the redox probe 2,3,5-triphenyltetrazolium chloride (TTC), which facilitates direct monitoring of actively respiring actinomycete. The colorless TTC is readily reduced by the microbial electron transport system (ETS) to pink-colored insoluble triphenylformazan (TPF). The respiration was monitored by measuring the level of TPF (Alef and Nannipieri, 1999). Surface adhered cells were monitored for their viability. Cell pellets were washed twice with 50 mM phosphate buffer at a pH of 7.6 and centrifuged at 5000 rpm for 10 min. Then, the pellets were re-suspended in 4.5 mL of buffer solution, and 0.5 mL of TTC (0.1 g/L) was added. The mixture was incubated at 37°C for 15 min in a water bath. Then, 5 mL of 96% cold methanol was added to stop the reaction and to begin the extraction of TPF. The enzyme activity was quantified at the absorption spectra of 480 nm in the spectro-

photometer. Ice-cold methanol and a phosphate buffer solution at a 1:1 ratio served as a blank.

Estimation of protein content of the surface attached actinomycetes

The total protein content of the surface-attached biomass was determined after alkaline hydrolysis, as follows. The polyethylene pieces were sampled from flasks containing mineral salt cultures of actinomycete isolates, washed gently with water to remove medium debris and boiled for 30 min in 4.0 mL of 0.5 M NaOH. The extracts were centrifuged to precipitate cell-debris fragments. The super-natants were collected, and the pellets were subjected to the same procedure to minimize the experimental error. The collected supernatants were combined, and the protein content in the extract was determined spectrophotometrically at 280 nm.

Determination of pH change

Study of pH change was adopted to make sure any metabolic activity of the NDYS-4 in supplemented medium, as metabolism shown by microbial cells may greatly support the evidence of degradation. The pH of the medium inoculated with actinomycete was measured daily during the study. The pH probe was inserted in the medium to measure the pH. Initial pH of the medium was ensured to be 7.2 ± 0.3. The medium uninoculated with microbe served as negative control.

Evaluation of cell surface hydrophobicity

The method used to determine bacterial cell-surface hydrophobicity: the bacterial adhesion to hydrocarbon (BATH) test (Rosenberg et al., 1980) was carried out. The BATH assay for bacterial hydrophobicity is based on the affinity of bacterial cells for an organic hydrocarbon such as hexadecane. The more hydrophobic the bacterial cells, the greater their affinity for the hydrocarbon, resulting in a transfer of cells from the aqueous suspension to the organic phase and a consequent reduction in the turbidity of the culture. For this assay, bacteria were cultured in starch casein medium until the mid-logarithmic phase, centrifuged and washed (twice) with PUM buffer containing (per liter): 17 g K_2HPO_4, 7.26 g KH_2PO_4, 1.8 g urea and 0.2 g $MgSO_4 \cdot 7H_2O$. The washed cells were resuspended in PUM buffer to an optical density at 400 nm (OD_{400}) value of 1.0 to 1.2. Aliquots (1.2 mL each) of this suspension were transferred to a set of test tubes, to which were added increasing volumes (range 0 to 0.2 ml) of hexadecane. The test tubes were shaken for 10 min and allowed to stand for 2 min to facilitate phase separation. The OD_{400} of the aqueous suspension was measured. Cell-free buffer served as the blank.

Microscopic observation of the polyethylene

The untreated and treated samples after two weeks days of duration were subjected to microscopic analysis (after washing thrice with 2% (w/v) aqueous sodium dodecyl sulphate and warm distilled water repeatedly through mild shaking for few minutes and additionally flushed with 70% ethanol with the objective of removal of cells so as to get maximum surface to be exposed for visualization, air dried overnight and then, the degradation was observed with a trinocular microscope (America Inc., LABOMED USA CXR3 9122100).

Table 1. Screening of polyethylene degrading actinomycetes using TTC as a viability indicator and their growth on mineral salts agar plates with polyethylene as a carbon source.

Strain	Viability and growth
NDYS-4	+++
NDYS-6	++
NDYS-8	+
NDYS-17	+

+ Indicates intensity of pink colour and growth.

RESULTS

Isolation and screening of PE degrading actinomycetes

A total of 83 strains of biosurfactant producing actinomycetes were tested for biodegradation; only 20 isolates shown positive result with TTC as a viability indicator. Of these, four samples displayed intense pink colour (Supplementary Figure 1), which indicates that the occurrence of degradation microorganisms in the polyethylene. It is the simplest method for investigating microbial degradation of aliphatic polymer since the formation of pink colour in the mineral salts medium indicates solubilisation of the polymer by the enzyme secreted from the microbes (Nishida and Tokiwa, 1993; Pranamuda et al., 1995). The pink colour was formed when strains secrete extracellular enzymes in order to breakdown compounds into soluble materials (Fields et al., 1974). Out of 20 strains, only four isolates of NDYS had shown intense pink colour (Table 1 and Supplementary Figure 1) and of these, NDYS-4 had shown PE degradation efficiently by forming intense pink colour.

In addition, the screening of these 20 isolates was performed by comparing their growth ability in solid media containing synthetic mineral salts medium supple-mented with 2% ethylene oligomer, resulting in the selection of four isolates. Of these isolates, one actinomycete isolate was selected as the final strain by comparing their growth ability in mineral salts agar medium containing LDPE powder as the sole source of carbon (Table 1). This result agrees with the TTC reduction test, which was used to test the viable actinomycetes on mineral salts media.

Identification and characterization of the isolate NDYS-4

Morphology

Outer surface of colonies were perfectly round initially, but later developed thin wavy mycelium. The colour of the
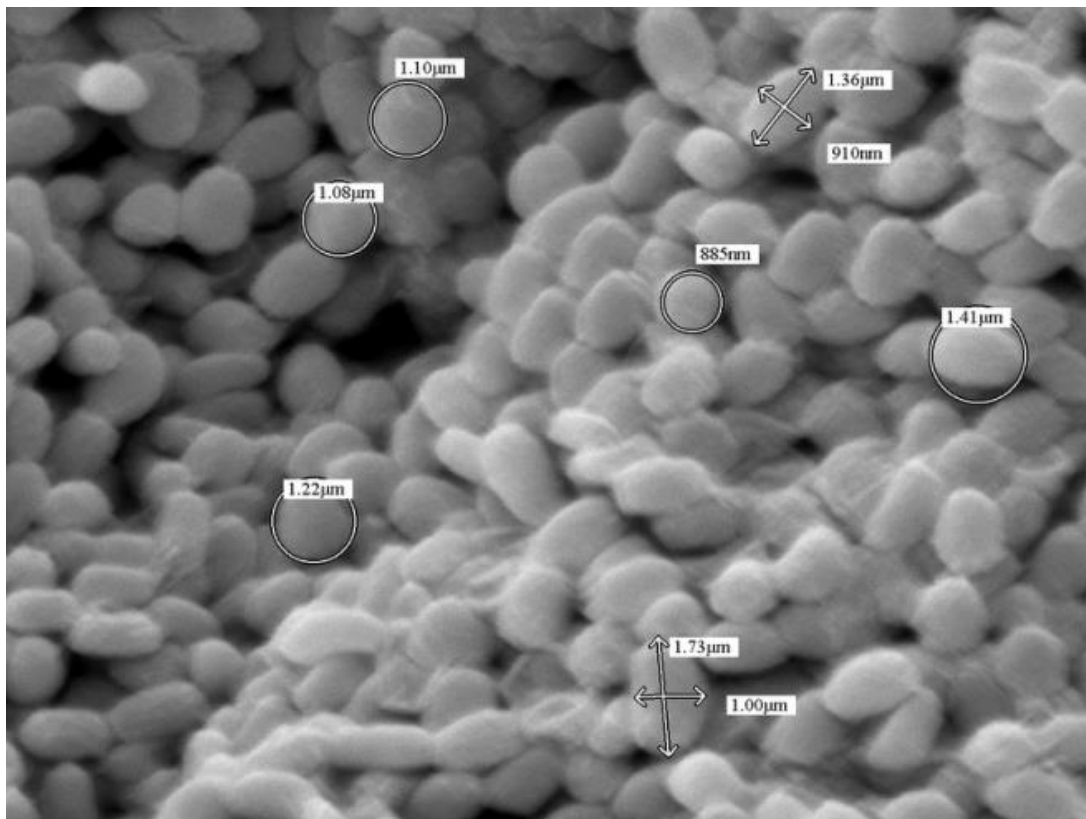
Figure 1. Scanning electron microscopy image of strain NDYS-4.

aerial mycelium observed was white and colour of the substrate mycelium was pink by studying the morphology (Supplementary Figure 2), SEM (Figure 1), 16s rRNA gene sequencing (Supplementary Figure 3), homology (Supplementary Table 1) and phylogenetic tree (Figure 2); the isolated strain was found to be *Streptomyces coelicoflavus.*

Growth on different media

The cultural characteristics of strain NDYS-4 are shown in Supplementary Table 2. The growth was prominent on most of the media and the substrate mycelia on most media tested were light pink. Aerial mycelia were white powdery in color. Diffusable pigments were adsent in all of the media.

Physiological and biochemical characteristics

Citrate utilization, methyl red test, catalase reduction and urea reaction were positive. Starch hydrolysis, casein hydrolysis, gelatin liquefaction, voges-prauskouer test, nitrate reduction, indole production, hydrogen sulphide production, tyrosine reaction and oxidase utilization were not observed. Only LL-diaminopimelic acid and glycine

were detected in whole cell hydrolysates, no diagnostic sugars were present, indicating NDYS-4 has a chemo type I cell wall and can be grouped under the genus *Streptomyces* (Supplementary Figure 3 and Table 3).

The chemical and physiological characteristics of strain NDYS-4

Utilization of carbon source was observed in mannitol, rhamnose and fructose. However, arabinose, glucose, galactose, raffinose, salicin, sucrose and meso-inositol were not utilized. Moderate to good growth was observed with L-histidine, sodium nitrate, yeast extract and L-arginine but no growth was observed with valine. Sodium chloride tolerance range was between 0-10% and optimum concentration was between 2 and 7% (Supplementary Table 4a, b). The temperature and pH tolerance range were 12-42°C and 5.0 to 10.0, respectively. The optimum temperature was 28 ± 2°C.

Determination of weight loss

The selected actinomycete was allowed to degrade the polyethylene under shaking condition for four weeks to observe the percent weight loss of polyethylene films
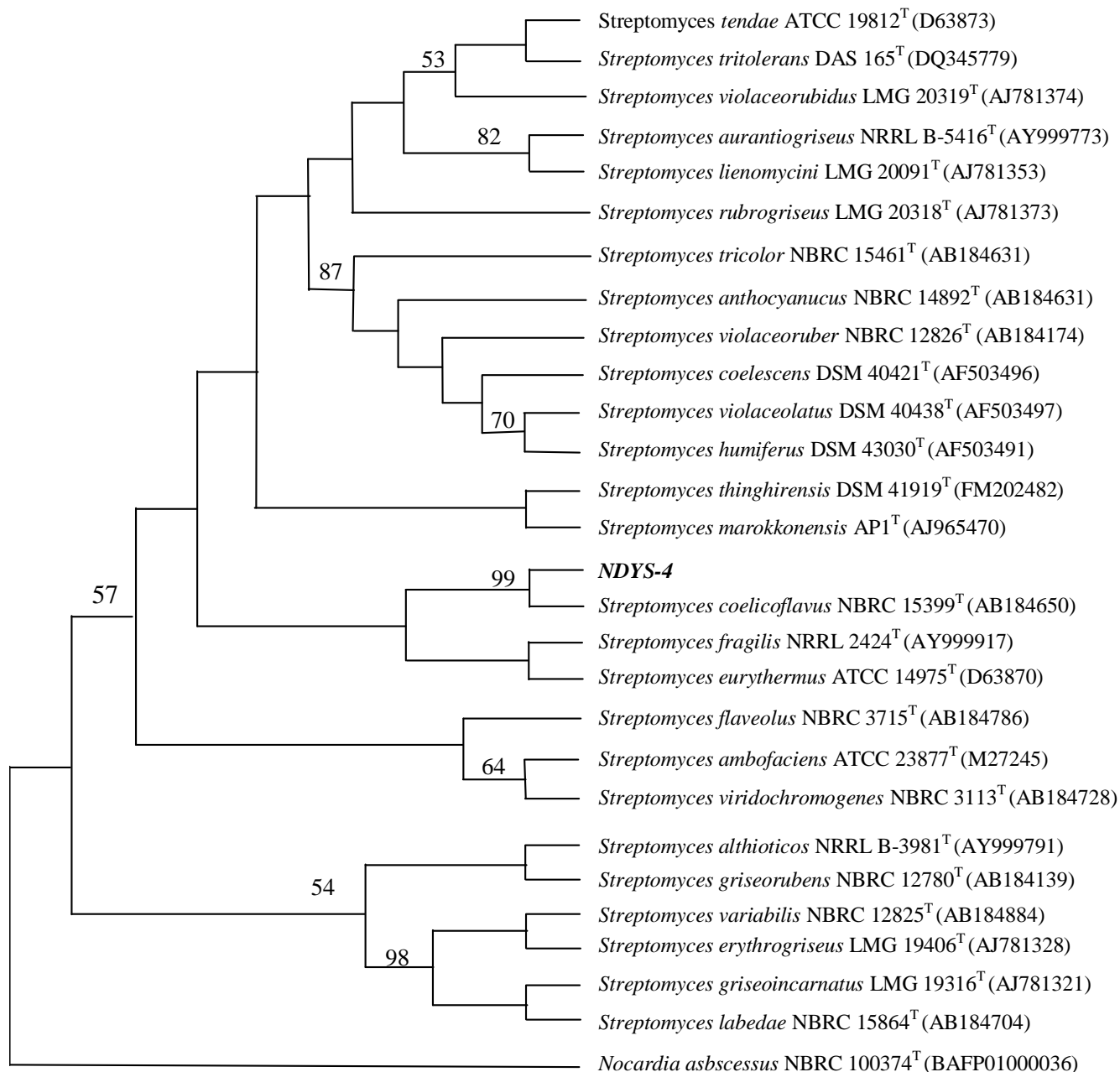
Figure 2. A phylogenetic dendrogram showing the relationship between the 16S rRNA gene sequences. The evolutionary relationship was inferred using the Neighbor-Joining method.

weekly because the complete sporulation of actinomycetes usually takes one week. The percentage of weight reduction was 3, 10, 19 and 30% after incubation for 1, 2, 3 and 4 weeks, respectively with S. coelicoflavus. The low percent weight loss after one week may be due to the complete maturation of actinomycetes which takes six to seven days. The weight loss of the polythene films can be attributed to the breakdown of carbon backbone due to enzymatic degradation by this actinomycete.

Viability and metabolic activity of the actinomycete

The respiration of the surface-attached actinomycete on the polyethylene films was monitored regularly for 30 days. The reduction of TTC to TPF, driven by the ETS, was used to determine the respiration level of the surface-attached bacteria. The formation of TPF increased sharply on the seventh day of incubation, followed by a constant increase until the 6th week (Figure 3). This observation indicates a regular increase in the
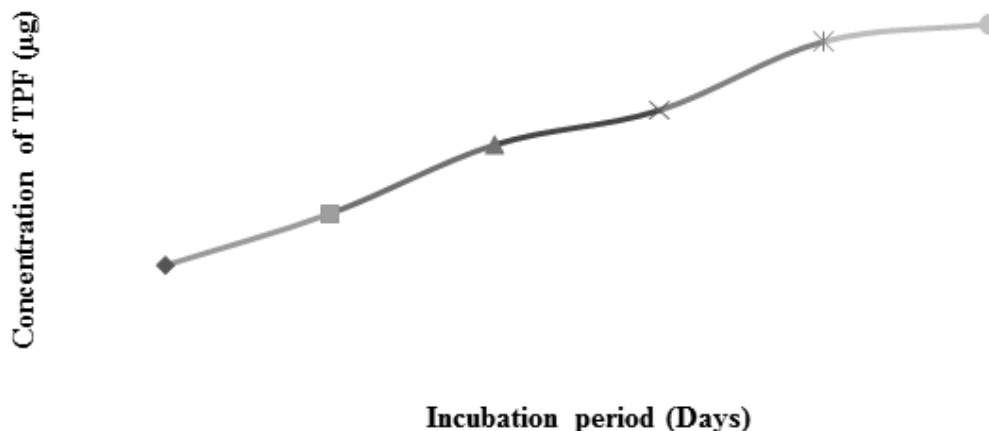
Figure 3. The respiration of surface attached actinomycete culture *Streptomyces coelicoflavus* NBRC 15399[T] in basal medium supplemented with polyethylene as a sole source of carbon. The respiration was measured by the intracellular reduction of TTC by cytochrome of the electron transport system to TPF.
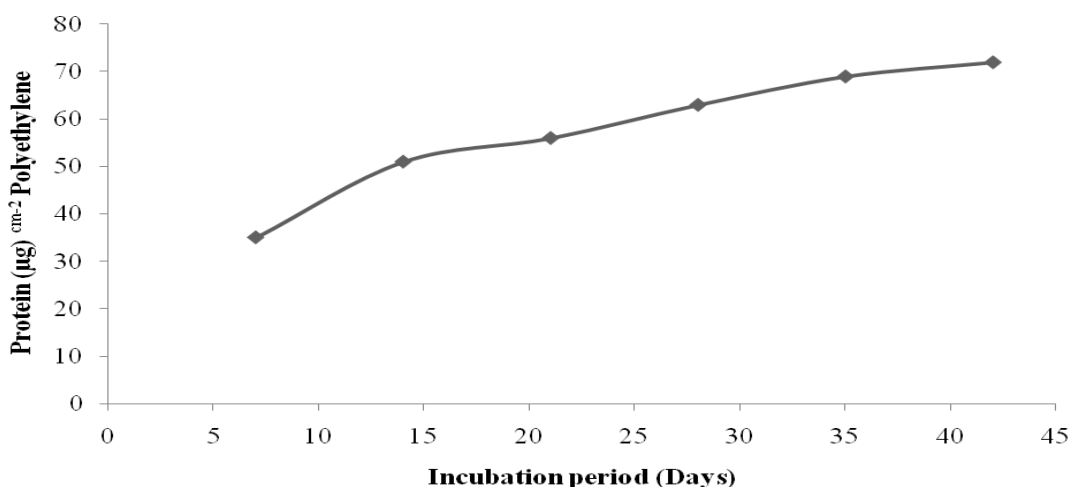
Figure 4. The protein content of the surface attached actinomycete culture *Streptomyces coelicoflavus* in mineral salts medium supplemented with polyethylene as a sole source of carbon.

respiration rate and suggests isolate viability and growth indicating it utilizes the polyethylene as a carbon source.

Growth kinetics of surface attached actinomycete

The growth kinetics of actinomycete on the polyethylene film surface was monitored by quantifying the total protein extracted from the surface of the film. The data depicted in Figure 4 shows increase in protein content over six weeks of incubation and is reflected by an increase in the surface-attached biomass. The continuous increase in extractable protein suggests a growth of isolates on the polyethylene. This data may also suggest that the biomass on the polyethylene is proliferating. This result

agrees with the TTC reduction analysis, which was used to measure the metabolic activity of the actinomycete.

pH change

The pH value is a key factor for the survival and activity of microorganisms. Generally, the pH should be between 6 and 8 (ASTM D 5988, 2003). The daily measurements of the medium pH are presented in Figure 5. The change in pH of the medium indicates the metabolic activity of the actinomycete in the mineral salts medium containing polyethylene films as a sole source of carbon. The pH of the medium starts decreasing on 3rd day of incubation (Figure 5).
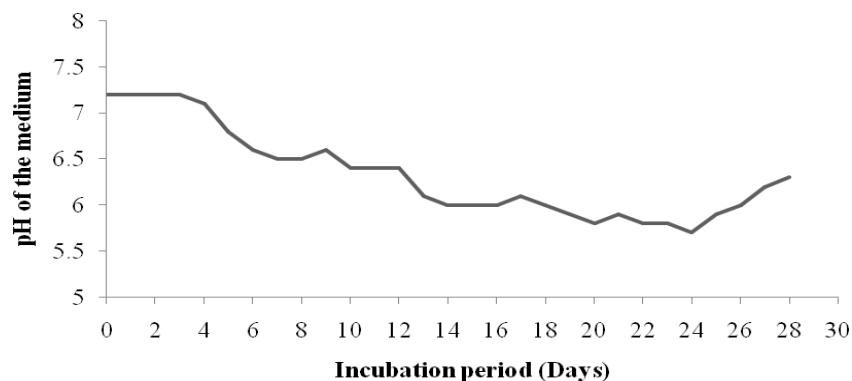
Figure 5. Variation in pH level during biodegradation due to microbial activity.
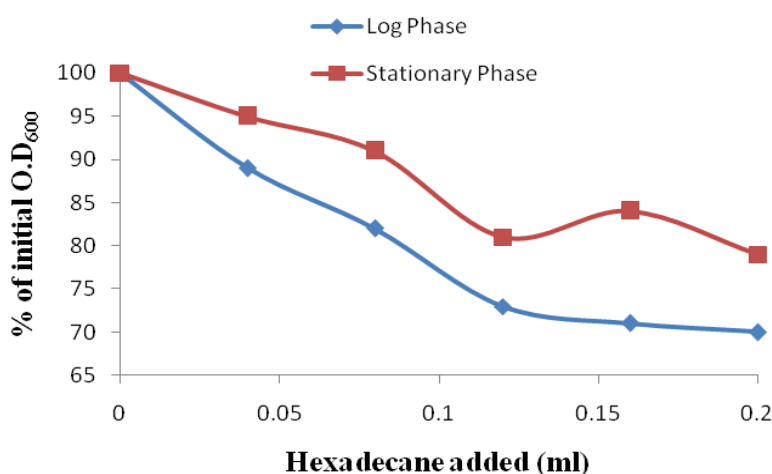
Figure 6. Hydrophobicity of NDYS-4 was determined by the bacterial adhesion to hydrocarbon test. Aliquots of logarithmic and stationary cell suspensions were supplemented with increasing concentrations of hexadecane. The transfer of hydrophobic cells from the aqueous phase to the hexadecane is reflected as a decrease in the turbidity (optical density at 600 nm, $O.D._{600}$) of the bacterial suspension.

Bacterial hydrophobicity

To determine the bacterial interaction with polyethylene, the bacterial cell surface hydrophobicities of the positive isolates, in both log and stationary phases, were assayed using a BATH test and the results are shown in Figure 6. The isolate showed only an 11% reduction in turbidity at the lowest concentration and an approximately 30% reduction at the maximum concentration. The logarithmic cells were more hydrophobic than those of the stationary phase. The greater affinity to hydrocarbon (hydropho-bicity) suggests a higher colonization interaction of the isolate with the polyethylene surface.

Microscopic analysis of polyethylene films

Polyethylene films were incubated in liquid cultures as a

substrate to examine their degradability. The films would conform to degrade at day five and the quick degradation was observed after eight days. The surfaces of PE films became rough and small cracks were formed in the inoculated culture after 15 days (Figure 7b). On the contrary, the film surfaces were smooth in the system without cell inoculation cultures (Figure 7a).

Degradation of other polymers

The appearance of pink colour in basal medium indicated that the polymer could be hydrolyzed by the enzyme into water soluble products (Nishida and Tokiwa, 1993). The pink colour formed not only on PE-emulsified broth but also on PCL, PVA, PEG and PEO indicating that the actinomycete *Streptomyces coelicoflavus* utilize these polymers for its growth. The results are shown in Table 2.

Figure 7. Surface structures of polyethylene films on microscope. (A) Without cell inoculation; (B) With cell inoculation NDYS-4.

Table 2. Viability of the isolate NDYS-4 on other polymers using TTC as a viability indicator.

Polymer	Viability (intensity of pink colour)
Polyethylene	+++
Polycaprolactone	+
Polyvinyl acetate	+
Polyethylene glycol	++
Polyethylene oxide	++

+ Indicates the intensity of pink colour

DISCUSSION

Degradation of LDPE by microorganism had been known for several years and there is no report on biodegradation of LDPE by *S. coelicoflavus* so far. This is the first experimental report on low density polythene (LDPE) utilization as a carbon source under laboratory conditions by showing effective ability of *S. coelicoflavus*.

The selected isolate NDYS-4 showed 100% homology with *Streptomyces graminearus*, 99% homology with *Streptomyces fradiae* and 99% homology with *Streptomyces violaceoruber.* The basic local alignment search tool (BLAST) result and phylogenetic analysis confirmed that their similarity to the respective species. The viability was found on basal medium containing LDPE powder as a sole source of carbon within five to seven days of incubation in all the four strains of NDYS; however the growth development was rapid in the case of NDYS-4. In the present study, the colour of the viable cells increased steadily during the first 7 days to 42 days of incubation in basal media. The formation of pink colored insoluble TPF increased sharply on the seventh day of incubation, followed by a constant increase until the 6th week. This indicates a regular increase in the respiration rate and suggests viability and growth of

actinomycete but in case of bacteria the formation of TPF increased sharply on the first day of incubation, followed by a constant increase until the fourteenth day (Kumar and Bhavanth, 2013). Due to their actively increasing metabolism after 42 days of incubation supporting the additional use of this actinomycete for biodegradation of low density polyethylene; dehydrogenases, a group of intracellular enzymes, are involved in microbial oxidoreductases metabolism. These enzymes have been frequently used as an index of microbial activity in soil (Alef and Nannipieri, 1999). The activities of these enzymes are linked to respiratory and energy processes in the cell, which depend on the metabolic state of microorganisms. To determine dehydrogenase activity, tetrazolium salts such as TTC were used as artificial electron acceptors. TTC replaces oxygen as the final H^+/e acceptor and is reduced by the aerobic cytochrome system to water-insoluble, red-colored formazans by microbial activities (Alef and Nannipieri, 1999; Schimmel and Morrison, 1989). From this, it is clearly understood that the viability and growth are occurring conventionally in the presence of LDPE as a carbon source in this study. These results are also supported by the growth kinetics of actinomycete on mineral salts medium containing polyethylene.

The application of biosurfactants in bioremediation process is currently an ambiguous topic. The ability of biosurfactant producing actinomycete for degradation of low density polyethylene in the present study indicates that these two processes are closely related. Several studies confirm that biosurfactants exhibit higher biodegradability compared to surfactants of synthetic origin (Lima et al., 2011a, b). Albertsson et al. (1993) showed that Tween 80 increased the adhesion and biodegradation of polyethylene by *Pseudomonas aeruginosa*, and Yamada-Onodera et al. (2001) reported that the nonionic surfactant Triton X-100 improved the growth of *Penicllium simplicissimum* in a medium contain-

ing polyethylene without being utilized by the fungus.

A simple and quick way to measure the biodegradation of polymers is by determining the weight loss. Microorganisms that grow within the polymer lead to an increase in weight due to accumulation, whereas a loss of polymer integrity leads to weight loss. Weight loss is proportional to the surface area since biodegradation usually is initiated at the surface of the polymer. The isolate NDYS-4 shows approximately 30% reduction in weight loss of polymer after 4th week of incubation. Kumar and Bhavanth (2013) had also reported the loss was 1 ± 0.033%, 1.5 ± 0.038% and 1.75 ± .06% for *K. palustris* M16, *B. pumilus* M27 and *B. subtilis* H1584, respectively after 30 days of incubation.

Figure 5 shows the variation in pH of the medium during this biodegradation study. Microorganisms secrete a variety of enzymes into the soil water, which begin the breakdown of the polymers. Two types of enzymes are involved in the process, namely intracellular and extracellular depolymerases.

Exoenzymes from the microorganisms first breakdown the complex polymers giving short chains or monomers that are small enough to permeate through the cell walls to be utilized as carbon and energy sources by a process of depolymerization (Dey et al., 2012). The similar results were also reported on *Bacillus amyloliquefaciens* (Merina and Santosh, 2014). The isolate NDYS-4 showed the production of some enzymes and metabolites with the indication of pH change supporting the metabolic activity of this isolate on the LDPE substrate and also its degradation.

The ability of a microorganism to utilize any substrate depends on its growth and adherence to that substrate. Bacterial adhesion to either a hydrophilic or hydrophobic substrate is governed by many factors, including the forces by which the bacterium attaches to the surface and the properties of the substrate and microorganism. Generally, a hydrophobic bacterium prefers a hydrophobic surface for attachment, whereas the opposite is true for bacteria with hydrophilic properties.

As the polyethylene surface is hydrophobic in nature, it has been suggested that the more hydrophobic the bacterial cell surface, the higher the interaction with polyethylene. A 20% maximum reduction in turbidity has been reported for *Rhodococcus ruber* (Gilan et al., 2004), and increase (32%) in the hydrophobicity of two marine bacteria, *K. palustris* and *B. subtilis* (Kumar and Bhavanth, 2013). In the present study, we report a significant increase (30%) in the hydrophobicity of *S. coelicoflavus*.

While the growth, viability, weight reduction and pH change and cell hydrophobicity provide solid evidence of polymer biodegradation, whereas the changes of surface of LDPE films were elucidated by microscope. However, our study supports the occurrence of enzymatic activity on polyethylene as microscope photographs of the polyethylene film showed some localized degradation

(Figure 7a, b). There are examples in the literature confirming the ability of the genus *Streptomyces* to degrade PE. The *Streptomyces* shows the degradation of disposable polyethylene containing 6% starch (Hanaa et al., 1998) and *Microbispora* shows the disappearance of PES film within six days in liquid cultures at 50°C (Kim-Chi et al., 2007).

Polyethylene, which has a wide range of applications, is accumulating in the environment. Its inert properties that resist deterioration and degradation are creating a serious environmental concern. This *in vitro* biodegradation study suggests the suitability of *S. coelicoflavus* for the degradation of environmental hazardious materials such as polyethylene. Based on the viability, growth results on the polyethylene surface, hydrophobicity, metabolic activity and we were able to determine that *S. coelicoflavus* is more efficient than the other actinomycetes. Hence, further study on microbial enzymes from actinomycetes in degradation of the LDPE and plastic will pave way for finding technology for degrading these environmentally hazardous plastic materials.

Furthermore, there is a need for the relationship between the biosurfactant production and biodegradation, and investigate the mechanism of polymer degradation and screen for other reported genes to establish the mechanism of biodegradation. There is also need to determine the optimum growth requirements of the isolate, bioaugmentation studies in the field and compare the *S. coelicoflavus* with well characterized actinomycetes and other microbes.

Conflict of interests

The author(s) have not declared any conflict of interests.

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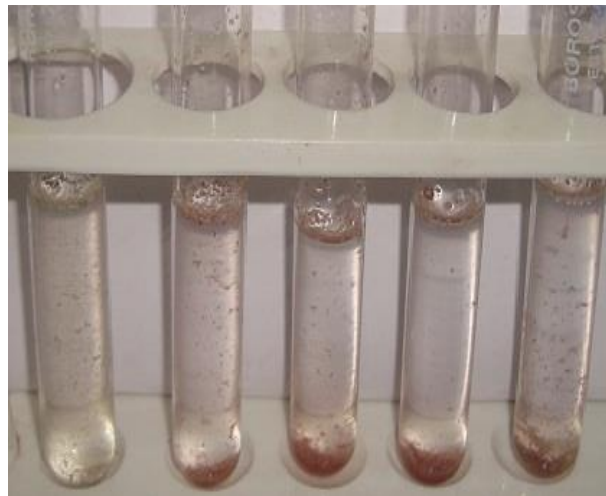
Negative control NDYS-6   NDYS-4   NDYS-8   NDYS-17

Supplementary Figure 1. Growth of actinomycetes in basal media containing TTC as redox indicator and polyethylene as a sole source of carbon.
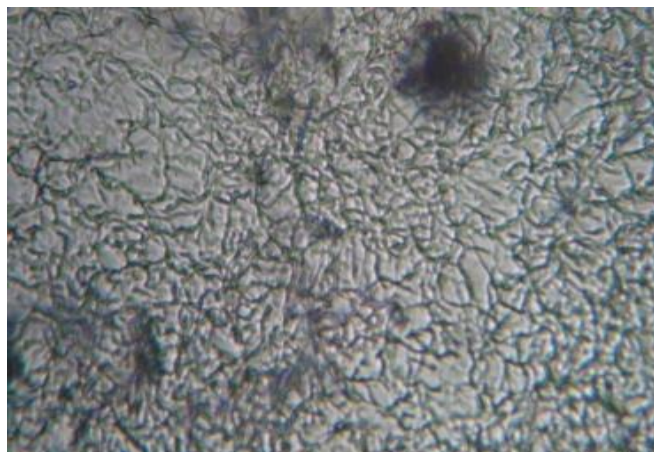
Supplementary Figure 2. Microscopic morphology of the strain NDYS-4.

CATGCAAGTCGAACGATGAACCACCTTCGGGTGGGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCT
GGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGACCTGCCAAGGCATCTTGGCGGGTCGAAAGCTCCGGCGGTGAAGG
ATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGC
GACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAA
GCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGA
GATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTAGGTGTGGGC
AACATTCCACGTTGTCCGTGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGG
AAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCAAGCCCTTCGGGGTGTTGGGGACTCACGGGAGACCGCCGGGGT
CAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAAT
GAGCTGCGATACCGCAAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCG
GAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTC
GGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGACGAAGTC

Supplementary Figure 3. 16S rRNA gene sequence of strain NDYS-4.

Supplementary Table 1. BLAST result showing significant homology with other sequences.

Accession	Description	Max score	Total score	Query coverage (100) (%)	E value	Max identy (%)
AB548687.1	*Streptomyces coelicoflavus* gene for 16S rRNA, partial sequence, strain: USF-6280	2641	2641	100	0.0	100
GQ395243.1	*Streptomyces* sp. XAS589 16S ribosomal RNA gene, partial sequence	2641	2641	100	0.0	100
EF371437.1	*Streptomyces graminearus* strain 14318 16S ribosomal RNA gene, partial sequence	2641	2641	100	0.0	100
NR_041175.1	*Streptomyces coelicoflavus* strain NBRC 15399 16S ribosomal RNA gene, partial sequence	2641	2641	100	0.0	100
JN969016.1	*Streptomyces* sp. CPE291 16S ribosomal RNA gene, partial sequence	2639	2639	99	0.0	100
FJ261963.1	*Streptomyces* sp. 210408 16S ribosomal RNA gene, partial sequence	2636	2636	100	0.0	99
EU201137.1	*Streptomyces* sp. ZG0656 16S ribosomal RNA gene, partial sequence	2595	2595	100	0.0	99
EU257234.1	*Streptomyces* sp. A309Ydz-QZ 16S ribosomal RNA gene, partial sequence	2595	2595	100	0.0	99
FJ267616.1	*Streptomyces* sp. 216701 16S ribosomal RNA gene, partial sequence	2590	2590	100	0.0	99
JF682780.1	*Streptomyces fradiae* strain GOS1 16S ribosomal RNA gene, partial sequence	2586	2586	99	0.0	99
AB184369.1	*Streptomyces violaceoruber* gene for 16S rRNA, partial sequence, strain: NBRC 13385	2586	2586	100	0.0	99
AJ781365.1	*Streptomyces violaceolatus* 16S rRNA gene, type strain LMG 20293	2584	2584	100	0.0	99
AF503495.1	*Streptomyces caesius* 16S ribosomal RNA, partial sequence	2584	2584	100	0.0	99
NR_027222.1	*Streptomyces coelescens* strain AS 4.1594 16S ribosomal RNA gene, partial sequence	2584	2584	100	0.0	99

Supplementary Table 2. Cultural characteristics of the isolate NDYS-4 on various media.

Medium	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigment
Yeast extract malt extract (ISP-2)	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Oat meal agar (ISP-3)	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Inorganic salt starch agar (ISP-4)	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Glycerol asparagine agar (ISP-5	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Nutrient agar	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Starch casein agar	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None
Bennett's agar	Abundant, spreading, powdery	Light pink	Abundant, powdery, white	None

Supplementary Table 3. Physiological and biochemical characteristics of the isolate NDYS-4.

Character	Result
Melanin production:	
ISP-1	-
ISP-6	-
ISP-7	-
Nitrate reduction	-
Starch hydrolysis	-
Casein hydrolysis	-
Gelatin liquefaction	-
Hydrogen sulphide (H_2S) production	-
Tyrosine production	-
Catalase reduction	+
Citrate utilization	+
MR	+
VP	-
Indole production	-
Oxidase utilization	-
Urea	+
Cell wall composition	+
Gram staining	G+
Spore staining	-

+, Yes; -, No

Supplementary Table 4a. Carbon source utilization of the isolate NDYS-4.

Carbon source utilization (1% w/v)	Result
D-Glucose	-
Galactose	-
L-Arabinose	-
D-Fructose	+
Raffinose	-
Meso-Inositol	-
D-Mannitol	+
Sucrose	-
Salicin	-
Rhamnose	+

Supplementary Table 4b. Nitrogen utilization, NaCl, temperature and pH tolerance of the isolate NDYS-4.

Nitrogen Source (0.1% w/v)	Result	NaCl tolerance (w/v) (%)	Result	Temperature tolerance (°C)	Result	pH tolerance
L-Histidine	+	2	+	12	+	5.0
Sodium nitrate	+	5	+	25	+	8.0
Yeast extract	+	7	+	37	+	9.0
L-Valine	-	10	+	42	+	10.0
L-Arginine	+					

+, Present; -, absent.

Full Length Research Paper

Effects of drying temperature on viability of macaw palm (*Acrocomia aculeata*) zygotic embryos

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In response to a growing interest in improving seedling production of oilseed species (like macaw palm), a fruit drying protocol for facilitating seed extraction was proposed. This enabled the production of macaw palm seedlings, but the temperature most suitable for seed extraction without losing its physiological quality is unknown. The goal of this study was to evaluate the effects of different drying temperatures on the physiological quality of macaw palm zygotic embryos to improve previously published drying methods. Fruits were dried in a forced-air drying oven at 57 or 37°C at different time periods (zero, two, four, six and eight days). Following each drying period, the fruits were removed from the drying oven, and the water content of the fruits and seeds were measured in addition to embryo viability and *in vitro* germination. Seed water content could be estimated based on fruit water content at both drying temperatures, eliminating the need to remove the seeds from the fruit. Drying at 57°C decreased the drying time by 50% compared to drying at 37°C; however, it was detrimental to embryo viability and germination. Therefore, drying of fruit at 37°C is recommended. Embryos dried at this temperature were still able to germinate after 16-day drying period, which corresponded to a decrease of 24.8% in the initial fruit water content.

Key words: Arecaceae, biodiesel, dehydration, physiological quality, propagation.

INTRODUCTION

Macaw palm [*Acrocomia aculeata* (Jacq.) Lood. Ex Mart.] is locally known as *macaúba, bocaiúva, coco-de-espinha, macaúva, marcová* and *mucajá*. This palm can reach 10 to 15 m in height and 3 to 4 m in crown diameter. The pinnate leaves are clustered at the stipe apex and are up to 5 m long. The yellow fruits are spherical or slightly flattened, globose and drupe shaped, with a diameter of 2 to 5 cm; have a cartaceous epicarp and a thin and fibrous

mesocarp. The bony endocarp is blackened and strongly attached to the mesocarp. There are generally between one and three seeds, which are oleaginous and edible. They are covered with a thin tegument layer, which, together with the endocarp, determines the high degree of seed dormancy in this species (Almeida et al., 1998; Silva et al., 2001; Lorenzi et al., 2004; Ramos et al., 2008).

Macaw palm is used for feed, ornaments, cosmetic oil and biofuels. Despite having many practical uses, macaw palm, like *tucumã* (*Astrocaryum aculeatum* Meyer) and wild areca palm [*Areca triandra* (Roxb.) ex Buch-Ham], is rarely cultivated. This is mostly due to its poor germination rates, as well as lack of studies that provide information about its germination. Palms are generally propagated through seeds, and palm seed germination is known to be slow and uneven because the seed's protective layer (endocarp) restricts water absorption and oxygen diffusion and exerts mechanical resistance to seedling emergence, resulting in a high degree of physical seed dormancy (Meerow, 1991; Ferreira and Gentil, 2006; Yang et al., 2007).

Due to the environmental and socio- economic importance of macaw palm and the difficulty in propagating it by conventional methods, a seedling production protocol was recently proposed to enable efficient seedling production. With this, it can be used for family farming and will lead to increased profit. The protocol includes drying the fruits at room temperature to ease endocarp removal and seed extraction, followed by mechanical scarification of the tegument, immersion of the seeds in gibberellic acid and sowing of the seeds in a sterilised substrate (Motoike et al., 2007). This protocol can be time consuming, because it necessitates drying for five to seven days. An exploration of alternative protocols is warranted, given that seedling production of this species is still in its initial stage. Mechanical scarification of seeds followed by immersion in gibberellic acid was reported to increase seedling emergence by 50% after four weeks (Ribeiro et al., 2011).

To accelerate seed extraction and ensure the physical quality of the seeds, the water content of the seeds must be decreased to the point that they become detached from the endocarp; thus preventing damage to the seeds during extraction. However, the critical water content for seed survival is relatively high in this family; that is, there can be total loss of seed viability even at high water contents. For açaí palm (*Euterpe oleraceae* Mart.) fruits dehydrated in a drying oven at 30°C, a progressive decrease in seedling germination was observed in seeds with a water content below 15% (Nascimento et al., 2007).

Water content, drying method and storage time can interfere with seed germination in species of the family Arecaceae. This is because the drying process decreases seed moisture and consequently affects metabolic processes (Chien and Chen, 2008; Ribeiro et al.,

2012). For macaw palm, it was observed that drying of fruits for seven days at 37°C allowed for the extraction of physically intact seeds without a loss in their physiological quality. To reduce drying time is necessary for evaluating new drying temperatures. (Neto et al., 2012).

As the drying temperature increases, this can decrease the drying time in oven. The goal of this study is to test the effects of drying temperature and water content on the physiological quality of macaw palm embryos to improve the existing drying protocol.

MATERIALS AND METHODS

The study was performed at the Seed and Plant Tissue Culture Laboratory of the Federal Institute of Goiás (Instituto Federal Goiano), Rio Verde Campus, Goiás - Brazil, using ripe macaw palm fruits collected in January 2011 at the Gameleira farm in the municipality of Montes Claros de Goiás, GO (16° 07' S, 51° 18' W, 592 m altitude).

Fruit drying with varying drying time

Due to their high heterogeneity in size, healthy fruits were divided into three classes according to their total mass. The number of fruits per class corresponded to the size distribution observed in the field; that is, four small fruits (≤ 40.0 g), 14 medium fruits (40.1-55.0 g) and eight large fruits (≥ 55.1 g), making a total of 26 fruits. This was done to homogenize the replicates. The influence of size on seed germination is not known. A total of 40 embryos were removed from these fruits.

The fruits were dried whole in a forced-air drying oven (Tecnal TE 394) at 57 ± 2 or 37 ± 2°C for zero, two, four, six and eight days to test the effect of drying temperature. 57°C was selected as the temperature in order to minimize drying time based on previous studies.

Fruit drying with varying water contents

The effect of drying temperature on fruits with varying water content was tested. At the end of each drying period, the water content of fruits that were partially dried at 57°C was determined using the drying oven method. For this purpose, fruit fresh weight was measured, and the fruits were placed in a 105 ± 2°C drying oven until they reached a constant mass (Brasil, 2009). Subsequently, a second fruit lot dried at 37°C was removed from the oven when the fruits reached the same moisture content, as they dried at 57°C (Figure 1).

In vitro germination

For each drying time and water content tested, a separate fruit lot was removed from the drying oven. The fruits were opened using a 1.5 kg hammer and a concrete block; and the physiological quality of the embryos was evaluated according to Neto et al. (2012). The embryos were inoculated with half-strength MS culture medium (Murashige and Skoog, 1962) and kept in growth chambers at 25 ± 3°C in the dark for 15 days. Following this period, the embryos were subjected to a 16 h light/8 h dark photoperiod at 25 ± 3°C and 40-60 $\mu mol.m^{-2}.s^{-1}$ photosynthetically active radiation supplied by fluorescent bulbs. A daily count of germinated seeds was
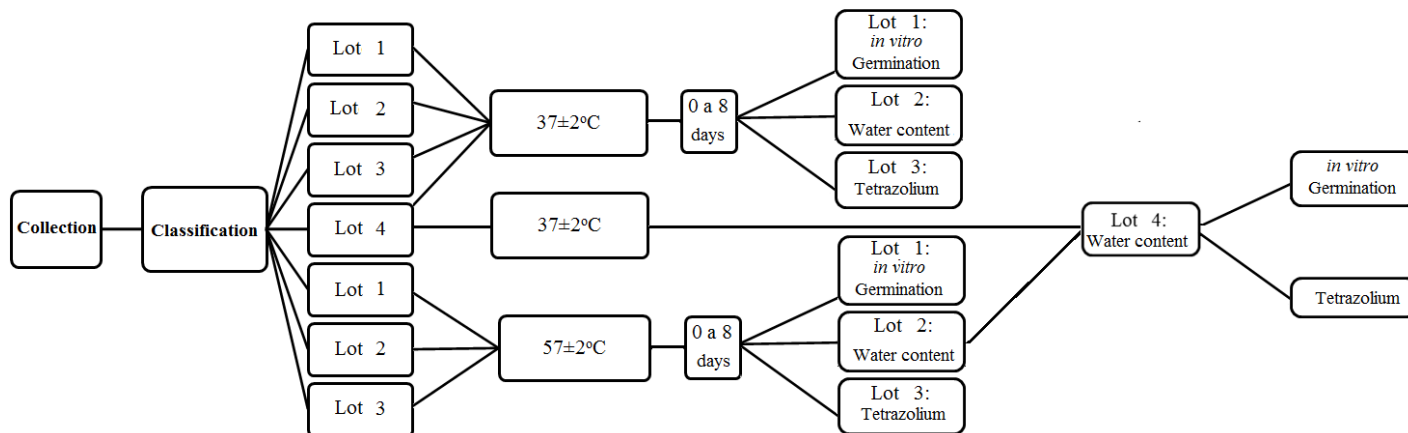
Figure 1. Diagram of procedures used for drying macaw palm fruits and testing i*n vitro* germination and zygotic embryo vigour.

37°C: Y = 43.4009 * $e^{(-0.0593x)}$; r² = 0.8157*
57°C: Y = 40.9654 * $e^{(-0.0631x)}$; r² = 0.6644*

37°C: Y = 25.180 - 0.725 x; r² = 0.8565*
57°C: Y = 25.271 - 0.936 x - 0.1661 x²; r² = 0.9762*
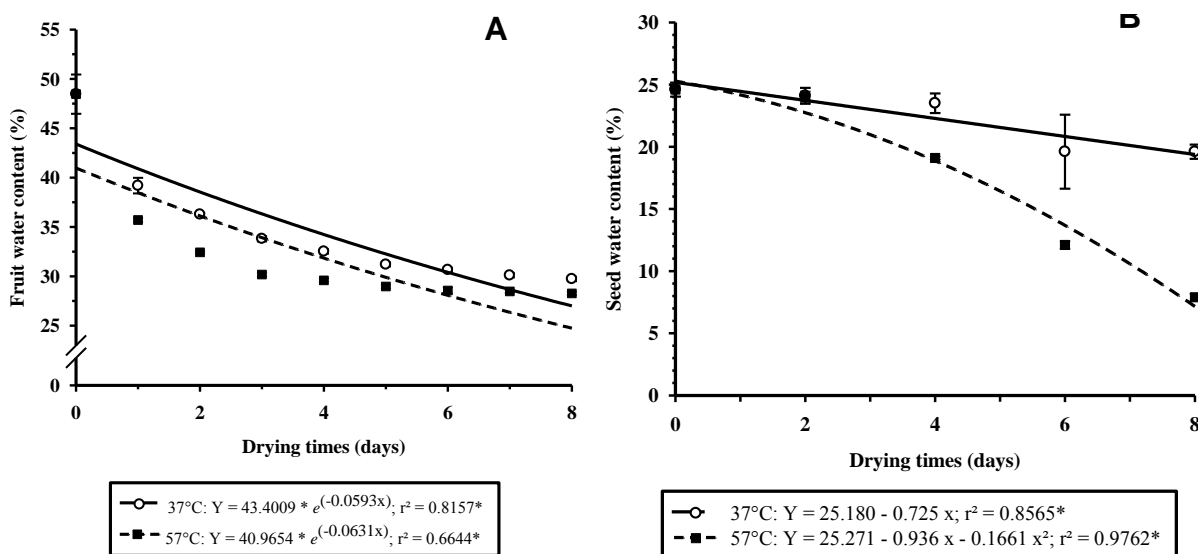
Figure 2. Water content of macaw palm fruits (A) and seeds (B) dried in a forced-air drying oven over varying drying times at two drying temperatures (37 and 57°C). *Significant at $p<0.05$.

performed, and the germination rate index (GRI) (Maguire, 1962) and germination percentage were calculated. The height of the germinated seedlings at 60, 90 and 120 days was also measured.

Tetrazolium test and electrical conductivity

Electrical conductivity and embryo viability were measured using a second fruit lot that was removed from the drying oven at the same time with the fruit lot used for the *in vitro* germination test. Embryos were soaked in deionised water (50 mL) for 24 h at 25°C for the measurement of electrical conductivity. The tetrazolium test was performed according to Ribeiro et al. (2010).

A completely randomised experimental design was used, with a five (drying time and/or water content) x two (drying temperature) factorial scheme with four repetitions of 15 embryos each. An analysis of variance and a regression analysis were performed.

RESULTS AND DISCUSSION

Fruit drying with varying drying time

The results of the regression analysis showed that water loss occurred exponentially at both fruit drying temperatures. The models that fitted the dehydration data were significant and showed a greater decrease in water content at 57°C (Figure 2).

The fruit water content at harvest time was above 40%. Following eight days of drying, fruit water contents of 27 and 24.7% were observed for fruits that were dried at 37 and 57°C, respectively. Water loss was more pronounced in fruits dried at 57°C (Figure 2A), which is consistent with Neto et al. (2012).
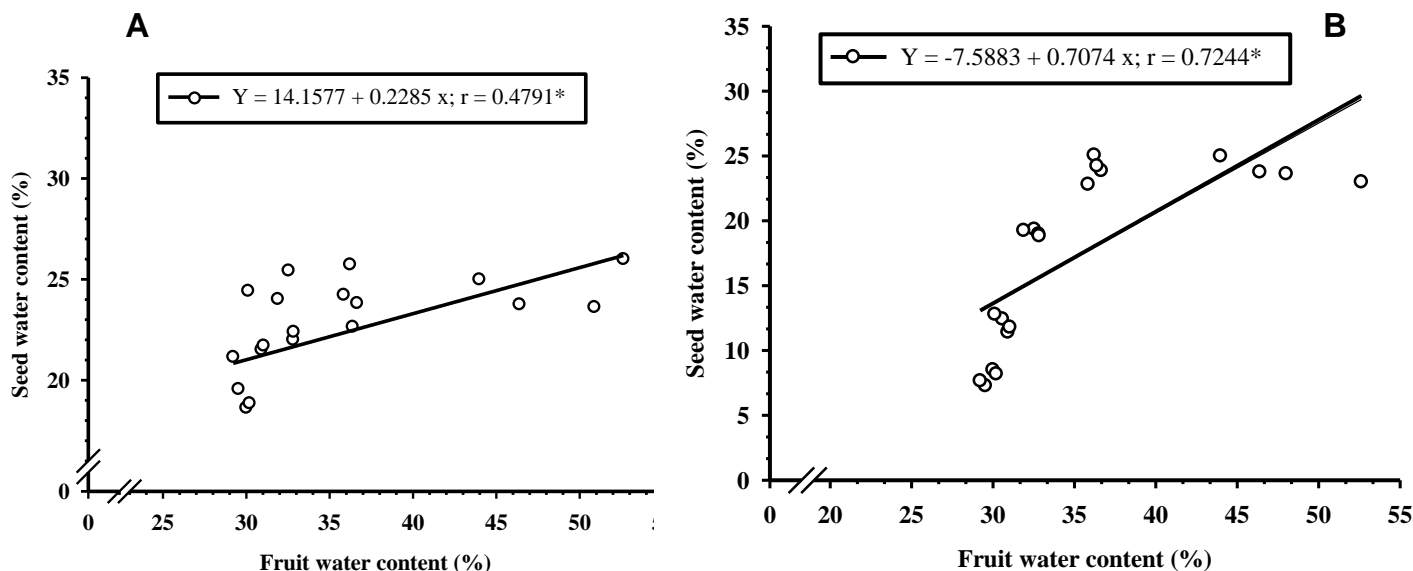
Figure 3. Relationship between water content of macaw palm (*A. aculeata*) fruits and seeds dehydrated in a drying oven for eight days at 37°C (A) and 57°C (B).* Significant at *p*<0.05.
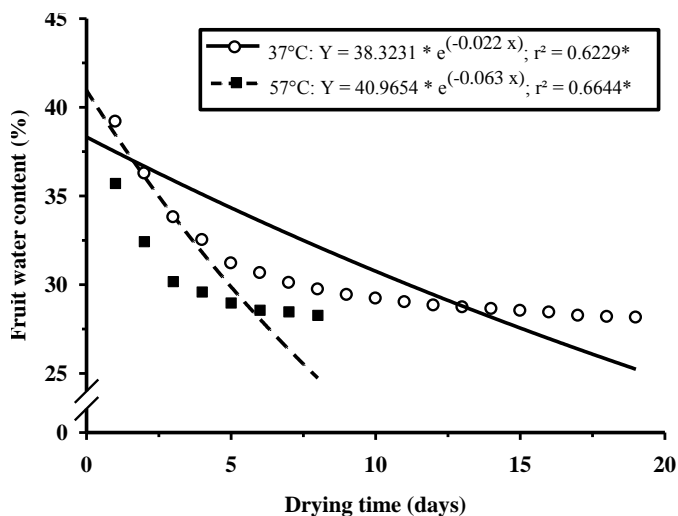
Figure 4. Water content of macaw palm fruits and seeds dried over varying drying times at two temperatures. *Significant at *p*<0.05.

The best explanatory models for seed water loss were different for the different drying temperatures. Drying at 37°C was best described by a linear model, and drying at 57°C was best described by a quadratic model. The same result was observed for fruit drying. Significant models were found for fruit drying, and there was greater water loss in fruits dried at 57°C. Seeds had an initial water content of 25%. Following eight days of drying, seed water contents of 19.4 and 7.15% were observed for fruits that were dried at 37 and 57°C, respectively (Figure 2).

Fruit and seed water content were significantly and positively correlated (r=0.4791* and r=0.7244* for drying temperatures of 37 and 57°C, respectively). The ratio of water loss in fruits and seeds dehydrated at 37°C was 1:0.23% (Figure 3A), and the ratio in fruits and seeds dried at 57°C was 1:0.71% (Figure 3B). Therefore, drying occurred approximately three times faster at 57°C than at 37°C.

Seed water loss resulting from the drying of macaw palm fruits was previously described by Neto et al. (2012). These authors observed a drying ratio that was very similar to the ratio observed in this study (1:0.20%) when fruits were dehydrated at 37°C. A seed water loss ratio of 1:0.87% after drying at 37°C and a decrease in germination percentage after drying at 57°C were observed in babassu (*Orbignya phalerata* Mart.) seeds (Silva et al., 2012). These findings show the importance of determining the effects of fruit drying temperature and drying time on seed water content for each species. With this, the optimal seed water content can be estimated from fruit water content, thereby eliminating the need to remove the seeds and risk destroying them in the process.

Fruit drying with varying water contents

According to the fitted models, the same water content was reached after twice as much drying time at 37°C compared to 57°C. Therefore, the water content obtained after eight days of drying at 57°C (24.8%) was only reached after 16 days of drying at 37±2°C (Figure 4).

According to Rubio Neto et al. (2012), drying macaw palm fruit at 37°C for up to eight days makes the extraction of intact seeds easier and does not result in a loss of embryo vigour or seed viability. In this study, the
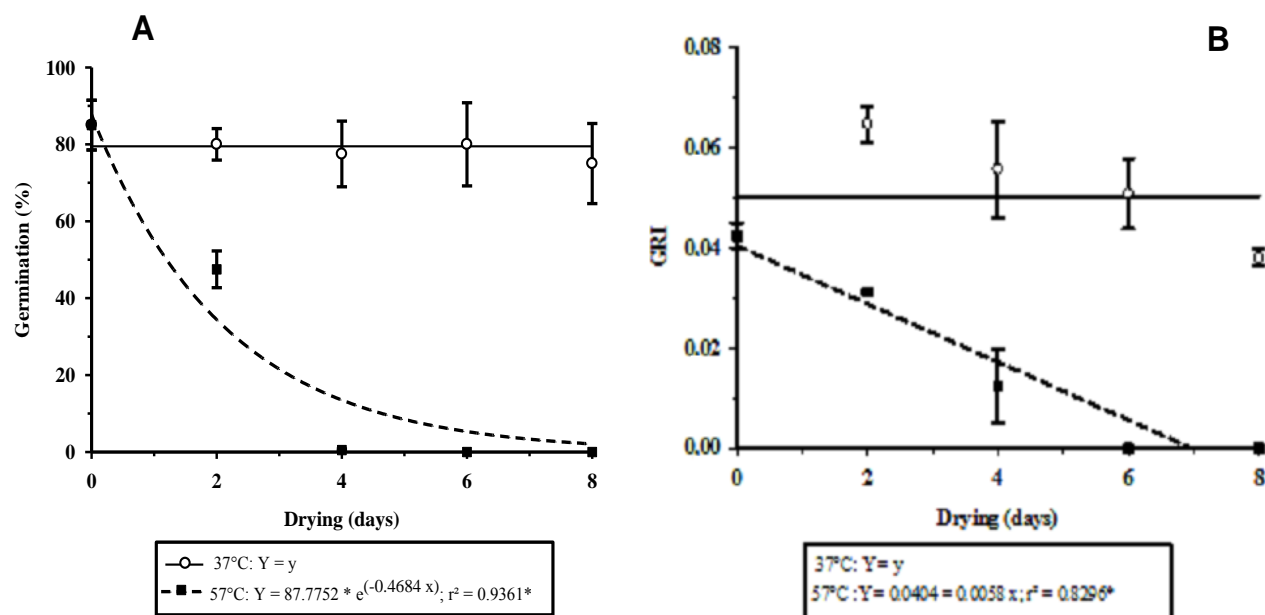
Figure 5. Germination percentage **(A)** and germination rate index (GRI) **(B)** for macaw palm zygotic embryos extracted from fruits dehydrated over varying drying times at two different temperatures (37 and 57°C).

germination of embryos decreased with increasing of temperature, although drying at 37°C did not affect the germination.

Another factor to be considered about drying process is the quality of seed extraction because in this study, it was easy to remove physically intact seeds, with *in vitro* germination being maintained across varying drying times (Neto et al., 2012). Similarly, drying at 37°C did not affect germination percentage for embryos of babassu, which had a mean germination percentage of 93.3% following 12 days of drying (Silva et al., 2012).

In vitro germination

The embryo germination rate index decreased linearly with increasing drying time at a drying temperature of 57°C. At a drying temperature of 37°C, both the germination percentage and the germination rate remained stable over time, reaching 0.05 and 81.2%, respectively (Figure 5B). These germination results are considered satisfactory.

Drying at 57°C was more effective for fruit water loss and it drastically decreased embryo physiological quality, like fruit content. This is because germination percentage decreased linearly and germination ceased completely when fruit water content fell below 30%. Fruit drying at 37°C resulted in more superficial drying. A mathematical model that explained the behaviour of the data for drying at 37°C could not be identified. However, drying at 37°C resulted in an overall mean germination percentage of 70.6% (Figure 6A and B).

Tetrazolium test and electrical conductivity

Drying at 57°C, a high temperature, resulted in the absence of vigorous and viable embryos, in addition to a high mortality rate, for all drying times. These results show the deleterious effects of drying at this temperature. At 37°C, the percentage of viable embryos decreased with increased drying time (Figure 7).

An interaction between drying time and temperature was observed. The two factors are discussed here. The tetrazolium method proposed by Ribeiro et al. (2010) was used to classify the embryos by colour, vigor, viable, unviable or dead.

A linear decrease in the percentage of vigorous embryos was observed with increased fruit drying time at 37°C (Figure 8A). A mathematical model could not fit the data from the fruits dried at 57°C, and therefore, the mean values from these fruits were used to fit a model. The percentage of vigorous embryos was low for fruits dried at 57°C, regardless of drying time. Eight days of drying corresponded to an average of 18% vigorous embryos. The linear decrease in the percentage of vigorous embryos is not a concern, so long as the initially vigorous embryos remain viable. Viable embryos represent a class of vigour in which the capacity for *in vitro* germination is retained (Figure 7B). When fruits were dehydrated at 37°C, 39.2% of the embryos were viable.

The highest percentages of unviable and dead embryos, which do not germinate *in vitro*, were observed for drying at 57°C. No model was found that explained the behaviour of the data. However, the deleterious effect
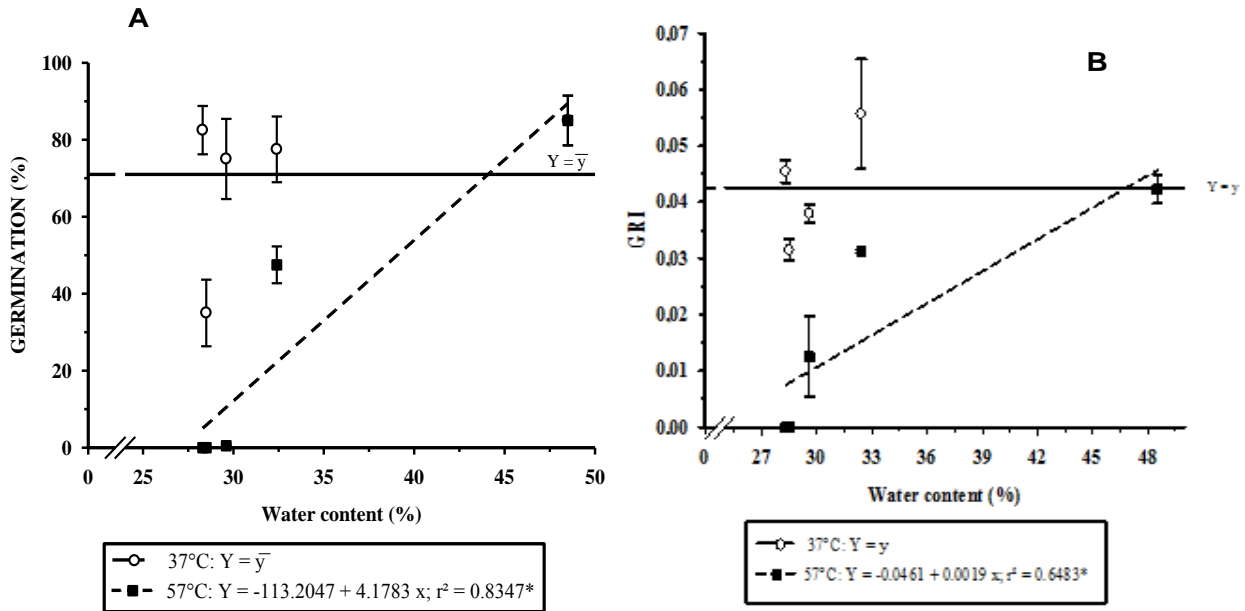
Figure 6. Germination percentage (A) and germination rate index (GRI) (B) for macaw palm embryos from seeds with different water contents.

Drying time (days)
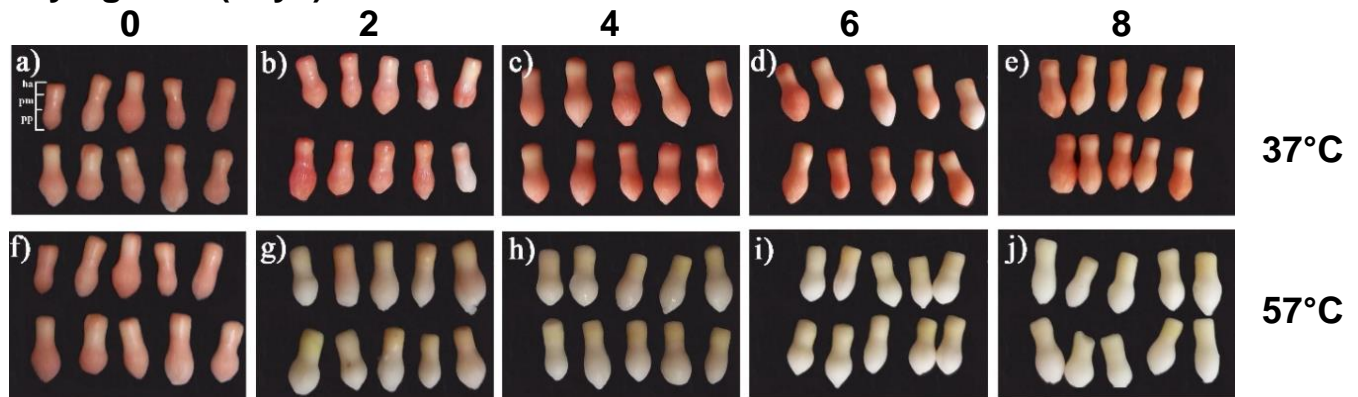
Figure 7. Macaw palm zygotic embryos subjected to the tetrazolium test (bar = 4 mm). The haustorial (ha), medial (pm) and proximal petiole (pp) regions of the embryo can be observed. a) and f) embryos obtained from fresh fruits; b) to e) embryos obtained from fruits dehydrated at 37°C for two, four, six and eight days; g) to j) embryos obtained from fruits dehydrated at 57°C.

of drying on the embryo viability was evident, reaching a mean value of 26%, independent of the drying time (Figure 8C).

Drying at 37°C minimised embryos' mortality, with a mean value of 0.3%. In contrast, drying at 57°C resulted in much faster drying but significantly increased embryos' mortality, with a mean value of 80%. These results show that drying at 37°C is preferable, as it allows for drying without the loss of embryos' physiological quality (Figure 8D).

An exponential decrease in germination percentage

with increased drying time was observed in seeds from fruits dried at 57°C, with germination reaching 2.85% after eight days. For seeds from fruits dried at 37°C, germination remained stable, with an overall mean germination percentage of 80% (Figure 6A). Drying at 57°C resulted in a more pronounced decrease in germination percentage and germination rate, showing that drying at a high temperature compromised the physiological quality of the embryos (Figure 8B).

For seeds dried at both temperatures, ions released from the embryo due to fissures in the embryonic membrane
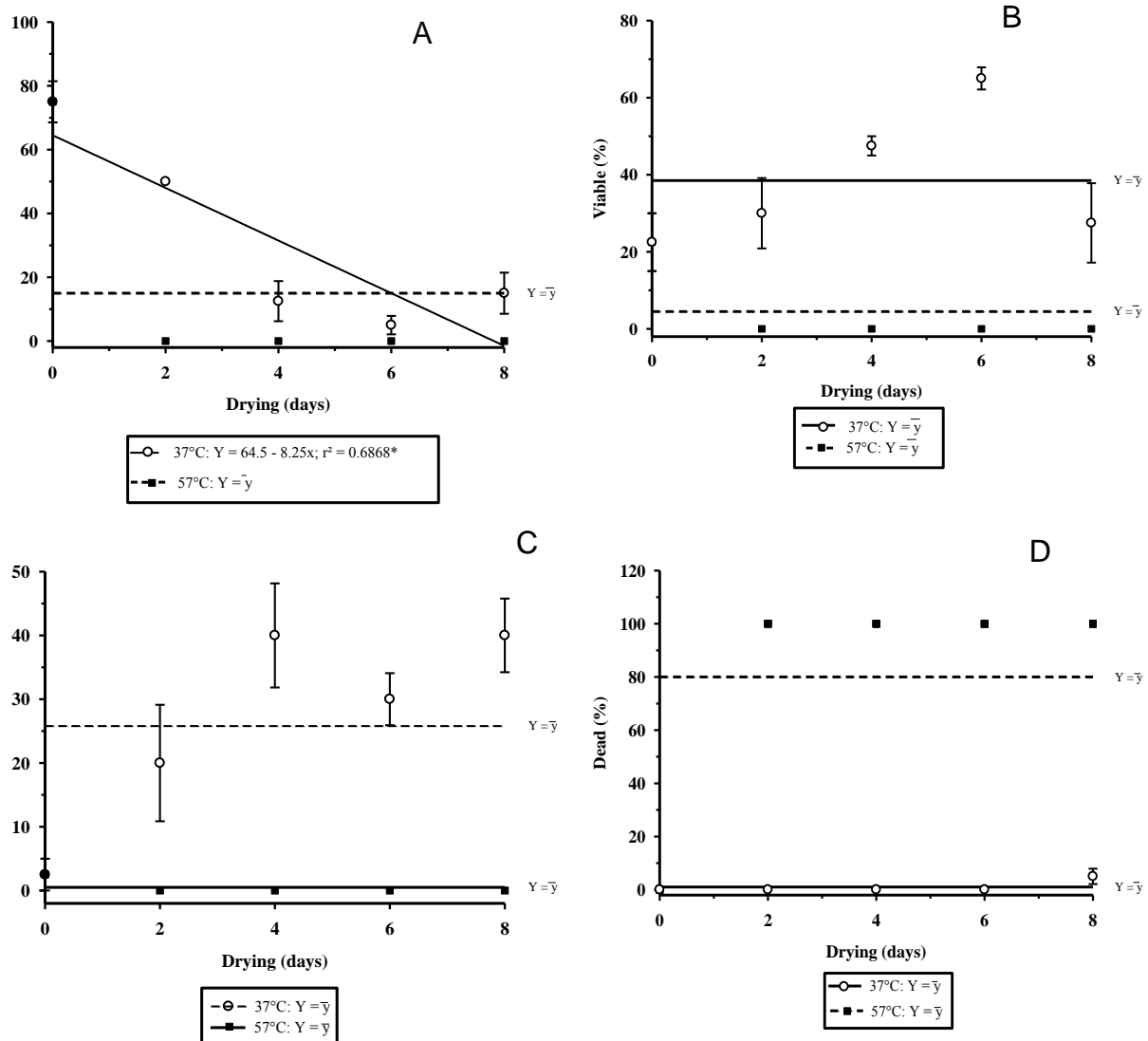
Figure 8. Percentage of vigorous (A), viable (B), unviable (C) and dead (D) macaw palm (*A. aculeata*) zygotic embryos extracted from fruits dehydrated over varying drying times at two different temperatures (37°C and 57°C).

caused by the drying process resulted in an exponential increase in electrical conductivity on the second day of drying. There was no interaction between drying time and temperature for electrical conductivity, and there were no differences in electrical conductivity for different drying times or temperatures. Beginning on the second day of drying, electrical conductivity generally remained stable (Figure 9). This result suggests that further studies are required to improve the methodology for determining electrical conductivity. It is likely that the membranes of dehydrated embryos are damaged when they are subjected to soaking in distilled and deionised water for 24 h (Brasil, 2009). Therefore, soaking the embryos for a shorter time period than the time period suggested in the methodology proposed by Brasil (2009) may be more appropriate.

The tetrazolium test applied according to Ribeiro et al. (2010) resulted in staining of the embryos that was sufficient for the quantification of embryos' vigour. This test has helped researchers to know quickly and accurately the viability of embryos; thus, it was found that increasing the temperature result in decreased viability.

The present study demonstrated that it is possible to estimate the water content of macaw palm seeds based on fruit water content for both drying temperatures tested (37 and 57°C). In contrast to drying at 37°C, drying at 57°C resulted in faster drying of the fruits but hindered embryos' viability and germination. It shows that if the goal is to extract seed to produce seedlings, 37°C is recommended, but if the goal is to extract oil, drying of fruits at 57°C is recommended. This is one of the major findings of this study.

$$37°C: Y = 202.324 (1 - e^{(-1.2149\,x)}); r^2 = 0.9865*$$
$$57°C: Y = 205.237 (1 - e^{(-1.4062\,x)}); r^2 = 0.9769*$$
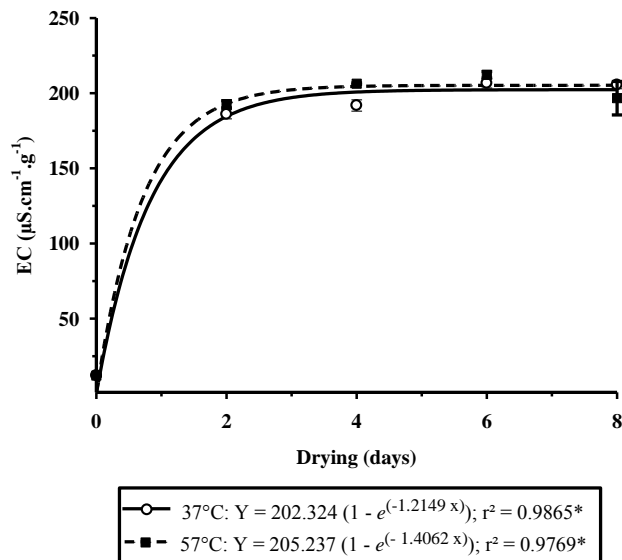
Figure 9. Electrical conductivity (EC) of macaw palm zygotic embryos extracted from fruits dehydrated over varying drying times at two different temperatures (37 and 57°C).

Conflict of interests

The author(s) have not declared any conflict of interests.

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Full Length Research Paper

Genomic composition factors affect codon usage in porcine genome

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The objective of the study was to determine the codon usage bias in the porcine genome and decipher its determinants. To investigate the underlying mechanisms of codon bias, the coding sequence (CDS) from the swine reference sequence (ssc10.2) was extracted using Biomart. An in house built Perl script was used to derive various genomic traits and codon indices. Analysis was done using R statistical package, and correlations and multivariate regressions were performed. We report the existence of codon usage bias that might suggest existence of weak translational selection. The codon bias is feebly related to nucleotide composition (GC%, GC3, CDS length). This study can be explored for designing degenerate primers, necessitate selecting appropriate hosts expression systems to manipulate the expression of target genes *in vivo* or *in vitro* and improve the accuracy of gene prediction from genomic sequences thus maximizing the effectiveness of genetic manipulations in synthetic biology.

Key words: Coding sequence, synomynous codons, selection, translational mutation, pig genome.

INTRODUCTION

The availability of nearly complete genome sequences from different taxa has enabled tremendous advances in evolutionary biology, providing insight to the actions of natural selection on genomes (Whittle et al., 2012). These biological breakthroughs revealed the importance of studying the degeneracy of genetic code, which enables most amino acids to be coded by more than one o called 'synonymous' codon (Wright, 1990). Synonymous codons usage (SCU) bias has been documented both within and between genomes, with huge interspecific and even intragenomic variation (Jia et al., 2009).

Several biological factors such as tRNA abundance (Kanaya et al., 2001), strand specific mutational bias, replicational, transcriptional and translational selection (Hershberg and Petrov, 2008), secondary structure of proteins, mRNA structure, GC composition (Knight et al., 2001) and environmental factors (Basak and Ghosh,

2005; Behura et al., 2013a) have been reported to influence the synonymous codon usage in various organisms. The afore mentioned factors led to two hypotheses on the evolution of codon bias; mutation bias and natural selection for translation accuracy and efficiency (Sharp et al., 2005).

The mutational bias hypothesis predicted that genes in the GC-rich regions of the genome preferentially use G- and C-ending codons, while those in the AT-rich regions use A- and T-ending codons (Zhang et al., 2009) as observed in mammals.

In *E. coli*, Stoletzki and Eyre-Walker (2007) found strong support for the selection for translational accuracy hypothesis; they reported that highly conserved sites and genes have higher codon bias than less conserved sites and genes. In their report, codon bias was positively correlated to gene length and production costs, both indicating selection against missense and nonsense mutations. This was further corroborated in plants such as *Arabidopsis thaliana*, *Oryza sativa* and *Zea mays* where codon usage bias was correlated to the base composition of genes, gene expression level and CDS length (Morton and Wright, 2007).

In higher animals like humans there are reported codon bias which are thought to maximize the speed of elongation, minimize the costs of proofreading thus maximizing the accuracy of translation (Bulmer, 1991). Several studies have failed to disentangle between translation accuracy and efficiency, as both are believed to be intertwined.

However, there are reports correlating the translational efficiency with expression levels and use of codons that match common tRNAs. For instance, in eukaryotes, codon bias have been associated with translation efficiency (Qian et al., 2012), so that the most abundant tRNA can be recognized easily in highly expressed genes. In the study, Qian et al. (2012) hypothesized that different synonymous codons were translated at different speeds due to disparities in codon selection time, and that faster translation were important because it minimize ribosome sequestering and so help alleviate ribosome shortage.

On the contrary, some reports dismiss translation efficiency and accuracy from synonymous codon bias usage in mammals (Reis et al., 2004). Instead splice related biases are evident (Parmley and Hurst, 2007) and selection for the preservation of exonic splicing enhancers (ESEs) where there are high density of regulatory elements, explains low SNP density, low protein evolutionary rates, and low synonymous substitution near intron- exon boundaries (Parmley and Hurst, 2007).

Despite reports on codon usage in mammals, there is no such literature in porcine genome. The study of codon usage would shade light to the known disparity in gene expression levels and quantitative trait loci that may related to genome architectures. In this study, we tested these mutation and translation hypotheses using genomic traits at our disposal. Our results confirm evidence of codon usage bias that was affected by CDS length, GC content and GC3s in the CDS. The analysis of codon usage pattern in pigs might give insight for understanding the mechanism of biased usage of synonymous codons in silent sites.

This could necessitate selecting appropriate hosts expression systems to improve or decrease the expression of target genes *in vivo* or *in vitro*. The codon usage profiles can be explored for designing degenerate primers and improve the accuracy of gene prediction from genomic sequences and protein functional classi-fication thus maximizing the effectiveness of genetic manipulations in synthetic biology (Qian et al., 2012). The study of gene expression traits in the porcine genome is relevant to many fundamental biological processes including species and breed diversity, gene expression and evolution, and adaptation to micro environment. The objective of the study was to determine determinants of codon usage bias in the pig genome to further decipher plasticity of genes.

MATERIALS AND METHODS

Sequence data

Two complete genome sequences were used for analysis. A total of 23,269 coding sequences was extracted from the female Duroc pig breed as the reference genome, (Sus scrofa build 10.2) using BioMart (Ensembl v 68). Only 21,550 coding sequence (CDS) that were more than 50 amino acids (150 bp) were included for analysis. The short CDS were excluded due to large estimation errors for codon usage which are associated with short sequence length. The majority of the excluded genes were microRNAs which are averagely 22 bp in length. The second data was extracted from gene coordinates and Ssc 10.2 reference genome.

Codon indices

Relative synonymous codon usage (RSCU)

Relative synonymous codon usage (RSCU) is the proportion of the observed codon divided by its expected frequency at equilibrium. An RSCU value close to 1 indicates lack of bias, RSCU >1 indicates a codon used more frequently than expected, and RSCU < 1 indicates a codon used less frequently than expected (Sharp et al., 2005).

RSCU values are largely independent of amino acid composition and are particularly useful in comparing codon usage among genes, or sets of genes that differ in their size and amino acid composition. In this study we developed an in house Perl script to calculate RSCU as;

$$RSCU = j \, x \, \frac{C_i}{\sum_{i=1}^{j} C_i}$$

Where, C denotes actual codon counts, j denotes number of synonyms and i denote the codon counts within the synonyms.

Table 1. Comparison of GC and length of both CDS and gene. The GC content of CDS is higher than the content of genes.

Parameter	CDS and gene	Minimum	1st Quad	Mean	3rd Quad	Maximum
GC content	gene	0.0818	0.4065	0.4789	0.47890	0.9800
	CDS	0.2852	0.4587	0.5312	0.6013	0.8123
Length bp	gene	150	1422	27338	1722	830146
	CDS	152	639	1415	1722	22503

We finally derived mean RSCU as;

$$RSCUmean = \frac{\sum_{I=1}^{I} RSCU}{N}$$

Where, *RSCU* is the values derived per gene, *N* is the total number of codon counts per gene. The value of RSCU_Mean ranged from one to infinity depending on the biasness of the gene. It was assumed to be a sister index to genomic Codon Adaptation Index (gCAI) that is explained below.
Another parameter genomic RSCU (RSCU') was calculated as;

$$RSCU' = \frac{RSCU}{n}$$

Where, n is the number of synonymous codons of an amino acid. This parameter measures and compares the usage of all 61 sense codons and is the proportion of use of a codon in all genes.

Genomic codon adaptation index (gCAI)

Classical Codon adaptation index (CAI) was first used to measure gene expression. This measure is species dependent and is the empirical measure for gene in studies investigating mutational and selectional components of codon usage (Goetz and Fuglsang, 2005). A CAI value is always between 0 and 1, and a higher value means a stronger codon usage bias and higher expression level and / or translation efficiency. In most research CAI of a coding sequence (CDS) is computed from the two parameters; the codon frequencies of the CDS and the codon frequencies of a set of known highly expressed genes (often referred to as the reference set). This computation leads to CAI which is used as a proxy for gene expression. In this case CAI values are normalized using codon frequencies in highly expressed gene sets. According to Xia (2007), CAI computation involves first derivation of a column of W values;

$$Wif = \frac{Fij.ref}{MAXfi.ref}$$

Where, Fij.ref is the frequency of codon j in synonymous codon family i, and MAXfi.ref is the maximum codon frequency in synonymous codon family i from a set of highly expressed genes.

The codon adaptation index for a given gene is then given by:

$$CAI = \prod_{i=1}^{L} Wif^{1/L}$$

Where, L is the number of codons from synonymous families in the gene.

In our study, genomic codon adaptation index (gCAI) is calculated as the geometric mean RSCU divided by the highest possible geometric mean of RSCU given the same Amino Acid sequence.

$$gCAI = \frac{\sqrt[n]{\prod_{i=1}^{n} rscu}}{\sqrt[m]{\prod_{i=1}^{m} RSCU}}$$

This value (gCAI) is a proxy for codon bias but not gene expression. This is because the gCAI values are normalized using codon frequencies at equilibrium, thus there is no assumption of gene expression bias.

Analysis tools

An in house Perl script was used to derive codon indices, gene length, GC and GC3 (the frequency of G+C at the third position) for all the CDS. Statistical analysis was conducted using R (V 2.15.0). We used a Sperman's rank correlation to relate codon indices (gCAI, RSCU') with different nucleotide composition variables (that is, GC, GC3 and CDS length). Multivariate regression model was used to predict the biasness and determine contribution of genomic factors to the biasness.

RESULTS

Variation in the CDS length, GC content and GC3s in the Coding sequence

The coding sequence GC content ranged from 0.285 to 0.812 with a mean of 0.531. For the CDS length, the shortest and the longest gene were 151 and 45618 bp respectively, with a mean of 1415 bp. The comparison of GC content between genes (intron and exons) and CDS were on average 47 and 53%, respectively. The mean CDS and gene lengths were 1415 and 27338 bp (Table 1). This confirms that the CDS are generally GC richer than the genes.

Codon usage bias analyses

The observed relative synonymous codon usage (RSCU) clearly indicated that there was a nonrandom usage of

synonymous codons for individual genes (Table 2). To investigate if the observed biasness, favoring specific codons, were beyond specific genes, we performed an overall genome wise analysis by concatenating all the genes into one large sequence string. The rationale was to exclude factors specific for individual genes. Preference of certain synonymous codons was observed in Figure 1. Table 2 shows the variation in RSCU values across codons coding for the same amino acid. This table highlights the biasness for a representative gene. For example valine a four degenerate amino acid has more preference for GTG = 1.5688 and GTC = 1.0139 than GTT = 0.8546 and GTA = 0.565. For Aspartate, GAC = 1.0598 was more preferred than GAT = 0.9401. Figure 1 depicts the codon usage of serine (RSCU) and may act as a representative of all synonyms. In this figure, the codon AGC, TCC and TCA were the most preferred in that order. To compare the usage of the 61 codons, RSCU' was used. In this analysis, the codons from two degenerate amino acids had higher values (for example, TAC = 5.50 x 10^{-6} and TAT = 4.96 x 10^{-6}) as compared to three fold degenerate amino acids (for example, ATC = 3.09 x 10^{-6}, ATT = 3.09 x 10^{-6}, ATA = 2.08 x 10^{-6}). Generally it was noticed that the usage of the codons reduced with the increase of the degeneracy with the six fold degenerate having the lowest usage codons or observed bias.

Correlation between codon indices and nucleotide composition

We found a significant correlation between nucleotide content and codon bias indices. The genomic RSCU (RSCU') correlated positively with the GC content (r^2 = 0.796, p = 2.0e-16) and GC3 (r^2 = 0.162, p = 2.0e-16) but negatively with the CDS length (r^2 = 0.84 p = 2.0e-16). Contrary to our expectation the genomic codon adaptation index (gCAI) significantly correlated negatively with the GC content (r^2 = -0.355, p = 2e-16), GC3 (r^2 = -0.321) and the CDS length (r^2 = -0.773, p = 2e-16) (Table 3). It is worth noting the two indices gCAI and genomic RSCU only differs in mathematical calculation with former using geometric means while the later using arithmetic mean.

We further fitted a model to predict the codon usage bias (CUB) using nucleotide composition factors as independent variables. The fitted model;

[y = β_o + β_1 GC% + β_2 CDS Length + β_3GC3 + error] was used.

Where, y is either gCAI or genomic RSCU.

In this model all the factors/variables were highly significant. The model explained 29% of the observed

gCAI (Table 4). Almost similar results were realized with genomic RSCU except the change in the sign of the coefficients. As can be seen in Table 5, there was negative association between genomic RSCU with GC content, CDS length and the intercept. However, this parameter was positively associated with GC3 and GC frequencies. It is worth noting that coefficients had minimal effect. This showed that nucleotide composition factors play minor but significant roles in shaping codon bias.

DISCUSSION

Previous analyses of codon usage in different taxa have suggested that there exists a huge interspecific variation and clear intragenomic variability (Gagnaire et al., 2012) and this study is no exception. Several biological factors such as tRNA abundance, strand-specific mutational bias, gene expression level, gene length, amino acid composition, protein structure, mRNA structure, nucleotide composition, intron splicing, recombination, gene conversion, DNA packaging, intron number (Qin et al., 2013) and selection for increased translational efficiency or accuracy have been demonstrated to relate to codon usage bias (CUB) (Ingvarsson, 2007). Despite abundance of these reports very few studies have focused on mammalian genomes. It is commonly accepted that both natural selection and genetic drift shape CUB across taxa (Zhao et al., 2007). Selection of codon bias is generally viewed as being weak; therefore, it is expected that selective forces, such as purifying selection against unfavored codons, should be more prominent in organisms with large effective population size (Ne) such as prokaryotes and unicellular eukaryotes or even fruit flies.

Species with low Ne are expected to be more prone to genetic drift and therefore, should show relaxed selective pressure on codon usage. In order to examine synonymous codon usage in the pig genome we first deciphered the genomic composition of the genes (intron and exons) and the CDS as well, followed by analysis of the CUB. We hereby present evidence suggesting that the pattern of synonymous codon choices in the Sus scrofa is as a result of a complex equilibrium between different forces, namely the natural selection at the translational level, nucleotide compositional, mutation bias and the length of each gene.

We report conclusive evidence for codon usage bias in the pig genome. The CUB is evident in the nonrandom usage of synonymous codons as shown by the codon indices. This finding is consistent with other studies involving prokaryotes (Karlin et al., 1997) and eukaryotes (Waldman et al., 2011). The observed preference of some codons could be suggestive of a weak selection force acting on codon pool in the pig genome. The observed

Table 2. Codon usage table of a representative gene (ENSSSCG0000000015) showing biased synonyms of 20 amino acids. The numbers in bold are the subtotal synonyms per amino acid.

Amino acid	Codon	Codon count	RSCU
S (Serine)	TCC	16	1.1707
S	AGC	18	1.3170
S	TCA	12	0.8780
S	TCG	7	0.5121
S	TCT	18	1.3170
S	AGT	11	0.8048
Subtotal		82	
F (Phenylalamine)	TTT	20	1.4814
F	TTC	7	0.5185
Subtotal		27	
T (Threonine)	ACT	8	0.6956
T	ACC	19	1.6521
T	ACA	12	1.0434
T	ACG	7	0.6086
Subtotal		46	
N (Asparagine)	AAC	20	1.0810
N	AAT	17	0.9189
Subtotal		37	
Y (Tyrosine)	TAC	10	1.6666
Y	TAT	2	0.3333
Subtotal		12	
E (Glutamate)	GAA	22	0.9361
E	GAG	25	1.0638
Subtotal		47	
V (Valine)	GTT	10	0.9090
V	GTC	13	1.1818
V	GTG	17	1.5454
V	GTA	4	0.3636
Subtotal		44	
Q (Qlutamine)	CAG	16	1.6000
Q	CAA	4	0.4000
Subtotal		20	
M (Methionine)	ATG	8	1.0000
K (Lysine)	AAA	25	1.0000
K	AAG	25	1.0000
Subtotal		50	
C (Cysteine)	TGC	8	1.6000
C	TGT	2	0.4000
Subtotal		10	
L (Leucine)	TTG	11	1.1379
L	CTT	2	0.2068
L	CTA	2	0.2068
L	CTG	22	2.2758
L	TTA	10	1.0344
L	CTC	11	1.1379
Subtotal		58	

Table 2. Contd.

|			
A (Alaline)	GCG	5	0.3508
A	GCT	10	0.7017
A	GCC	29	2.0350
A	GCA	13	0.9122
Subtotal		57	
W (Tryptophan)	TGG	4	1.0000
P (Proline)	CCA	6	0.5714
P	CCC	19	1.8095
P	CCT	7	0.6666
P	CCG	10	0.9523
Subtotal		42	
H (Histidine)	CAT	5	1.1111
H	CAC	4	0.8888
Subtotal		9	
D (Aspartate)	GAT	14	0.8750
D	GAC	8	1.1250
Subtotal		22	
I (Isoleucine)	ATA	3	0.4090
I	ATT	5	0.6818
I	ATC	14	1.9090
Subtotal		22	
R (Arginine)	AGA	5	1.3636
R	CGG	6	1.6363
R	CGA	1	0.2727
R	AGG	7	1.9090
R	CGT	1	0.2727
R	CGC	2	0.5454
Subtotal		22	
G (Glycine)	GGC	25	1.6949
G	GGG	11	0.7457
G	GGA	12	0.8135
G	GGT	11	0.7457
Subtotal		59	

CUB is further proved to be influenced significantly by nucleotide composition.

However, in contrast to other papers (Rao et al., 2011), we report negative correlation between genomic codon adaptation index (gCAI) or CUB and the CDS length. The GC content and GC3s were consistent with their findings. In humans, the GC content and mutational biases were reported as major factors that influence codon usage. In plants several factors like nucleotide composition of genes, the levels of gene expression and length of the coding sequence contributed to the observed codon usage bias.

In *B. pseudomallei* genome, highly expressed genes had the highest GC content and it tended to use G or C at the third position of the codon (Hershberg and Petrov,

2010). The highly expressed genes in *B. pseudomallei* also had high GC content positively correlated with CAI value and GC3s. Their result purport that the highly expressed genes tend to use 'C' or 'G' at synonymous positions compared with lowly expressed genes. In this study our results points to preferred usage of both C or G and A or T at the synonyms sites as shown in Table 2, with the C or G ending codons being the majority.

However a negative correlation between gCAI and GC content or GC3s is unique. This might be due to the difference in the genome isochore structure, ambiguity (vary with space and time) of the gene expression in mammals, or due to difference in methodology of calculating CAI variants The negative correlation found between gCAI and gene length is consistent with other
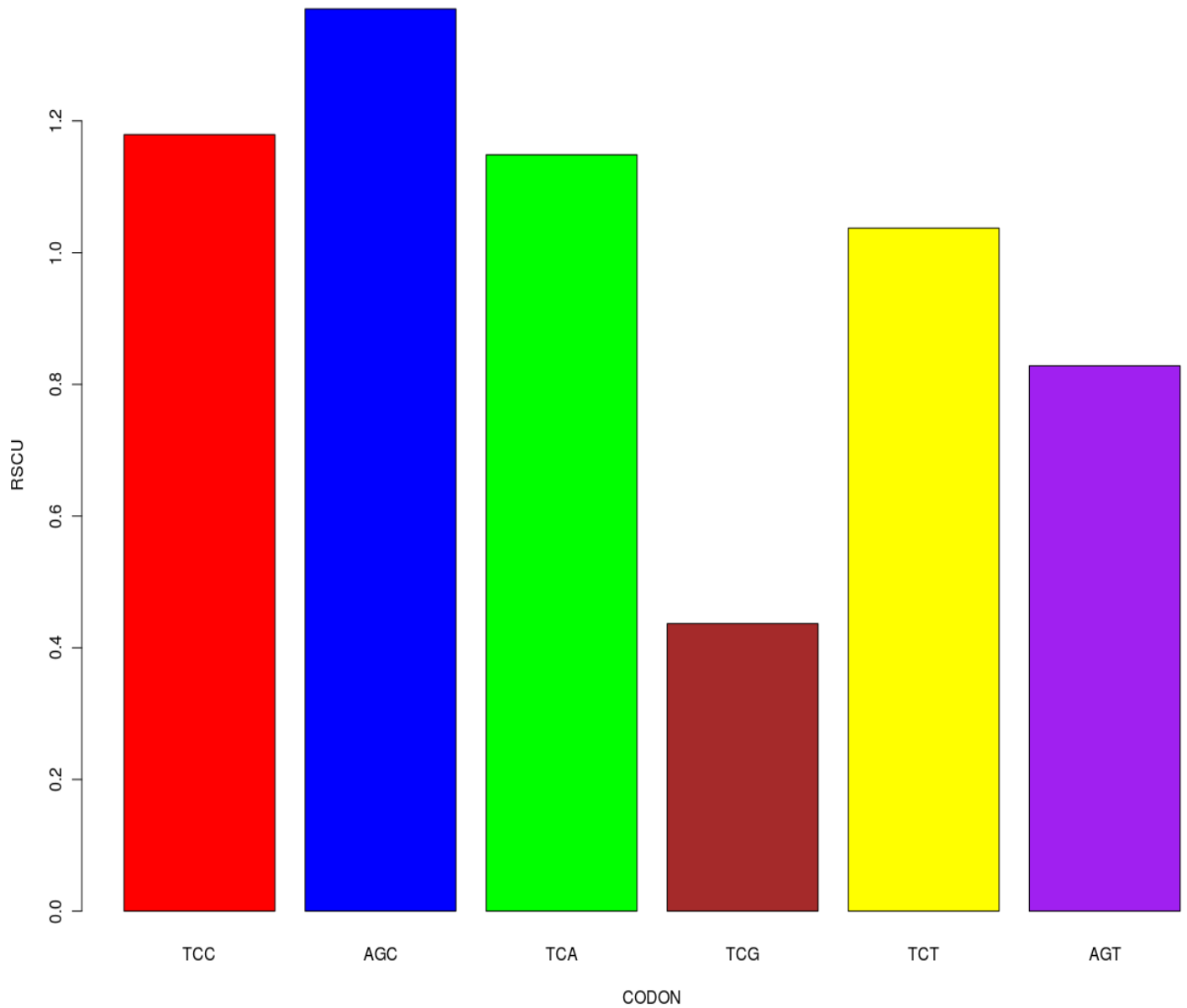
Figure 1. The relative synonymous codon usage of serine showing codon AGC and TCG as the most and the list preferred codons respectively.

Table 3. The correlation between the genome composition factors and the selected codon indices.

RSCU'	gCAI	GC% Content	CDS Length	GC3 Ratio	GC3 Counts	CODON counts
RSCU'	0.725***	0.796***	-0.840***	0.162***	-0.733***	-0.847***
gCAI	1	-0.355***	-0.773***	-0.321***	-0.823***	-0.777***
GC% Content		1	0.066***	0.914***	0.367***	0.066***
CDS Length			1	0.008***	0.931***	0.999***
GC3 Ratio				1	0.338***	0.008***
GC3 Counts					1	0.931***
CODON counts						1

*Denotes level of significance

Table 4. The coefficients of the multivariate regression analysis explaining the genomic composition factors affecting genomic codon adaptation index.

Coefficients	Estimates	Std. error	t- value	p-value
Intercept	4.153e-02	1.308e-03	31.750	< 2e-16
GC_CONTENT	-9.768e-02	3.240e-03	-30.149	< 2e-16
CDS_LENGTH	1.179e-06	2.474e-07	4.764	1.91e-06
GC3_RATIO	-5.018e-02	1.823e-03	-27.531	< 2e-16
GC3_COUNTS	-1.117e-05	1.273e-06	-8.774	< 2e-16

Table 5. The coefficients of the multivariate regression analysis explaining the genomic composition factors affecting the genomic RSCU (RSCU').

Coefficients	Estimates	Std. error	t- value	p-value
Intercept	-1.225	5.763e-04	-21.247	< 2e-16
GC_CONTENT	-6.091e-02	1.461e-03	-41.690	< 2e-16
CDS_LENGTH	-1.002e-06	1.114e-07	-8.997	< 2e-16
GC3_RATIO	2.078e-02	8.206e-04	25.318	< 2e-16
GC3_COUNTS	4.997e-06	5.724e-07	8.730	< 2e-16

reports in organism such as yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Populus tremula and Silene latifolia* (Qiu et al., 2011). Previous studies have shown that metabolic systems prefer to express those genes that are less costly (Hahn and Kern, 2005). Moreover, there have been reports of longer genes having higher expression level, CAI values and higher codon usage bias in some unicellular genomes, specifically *E. coli* (Stoletzki and Eyre-Walker, 2007). These contradicting reports (positive and negative correlation) indicate there are no universal rules about gene length and codon bias. In this study, the longer genes had lower gCAI values or lower codon usage bias. The general consensus is that there should exist a positive correlation between gCAI values and gene length, which could be explained by selection of the preferred codons to avoid errors during translation. Since the cost of producing a protein is proportional to its length, selection in favor of codons which increase accuracy should be greater in longer genes, and long genes should therefore have higher synonymous codon bias.

In such genes, by using optimal codons, translation is faster whereby ribosomes move faster along the mRNA and are released quickly to be available to translate other mRNA (Zhao et al., 2007). The use of optimal codons increases the accuracy of translation by reducing translational errors that can occur. The errors include missense in which an incorrect amino acid is incorporated into the growing peptide chain and nonsense in which the peptide synthesis terminates prematurely by incorporating stop codons. It is believed that both missense and nonsense errors that produce non- and misfunctional proteins respectively, are costly to the cell because they consume amino acids and energy both in their production and during breakdown (Stoletzki and Eyre-Walker, 2007). Besides, missense errors may have other serious consequences, for example, a missense error in a DNA polymerase may temporally increase the mutation rate (Ninio, 1991).

Pig genome just like other mammals is found to vary greatly in base composition between different genomic regions. In vertebrates, such as mammalian and birds, one of the most striking features of their genomes is the difference in G+C contents isolated regions called isochore structures. In pig there exists heterogeneity in G+C content that results in variation in codon usage bias as was revealed elsewhere (Hershberg and Petrov, 2010).

Having a relatively high GC content, we expected the pig preferred codons to mirror the genome composition. GC rich organisms tend to have GC rich optimal codons, while AT rich organisms tend to have AT rich optimal codons. This observation is manifested in RSCU as most preferred codons end with G or C albeit with some ending with A or T. This phenomenon is dependent on the isochore structure of the pig genome that we confirmed by observed variation in GC content. The data analyzed provide evidence for the mutational bias hypothesis. In our view the codon bias is skewed towards the AT ending codons as was revealed by inverse correlation between gCAI and GC3s. Indeed, AT rich genes were shorter in length and could imply efficient protein translation to minimize energy consumption. We also suspect that

other factors besides mutation bias may have contributed to codon usage. Amongst the other factors, we hypothesize that selection for preferred codons is affected by the abundance of tRNA in the cells or the ones that bind those tRNAs with optimal binding strength (Ikemura, 1985; Kanaya et al., 2001). We could not confirm this due to lack of information on tRNA . However, this hypothesis has been proven in other organisms like *E. coli, B. subtilis and C. cerevisae.* In that study cellular tRNAs correlates positively and closely to tRNA gene copy numbers; by extension this suggests that in these species there is correlation between optimal codon use and tRNAs abundance. However such correlation was not found in studies involving *D. melanongaster* and humans (Kanaya et al., 2001).

The positive correlation observed between GC content, GC3 and gene length explains the computed low codon bias. This is because long genes tend to have more G and C, abundant G or C at the third codons which are negatively related to gCAI. The nucleotide composition factors only play significant but minor roles in shaping the codon usage in the pig genome as revealed in low R^2 value and statistical interpretation exhibited in multivariate regression analysis. These statistical inferences are clear indication that the pig genome is so complex and molecular functions are controlled by several factors.

Conclusions

We confirm the existence of codon usage bias in the porcine genome which might suggest there is weak selection of preferred codons for translation accuracy. The codon usage bias is influenced slightly by nucleotide composition factors among others.

Conflict of interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Sodicity tolerant polyembryonic mango root stock plants: A putative role of endophytic bacteria

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The importance of extracellular enzyme producing endophytic bacteria and their ability to elicit 'induced systemic tolerance' against abiotic stress (sodic soil) has been documented. However, the performance of these microbes under various abiotic stresses, especially saline-sodic conditions is less understood and formed the focus of this investigation. The diversity of endophytic bacteria associated with leaves, stems and roots of sodicity tolerant polyembryonic mango root stock (GPL-1 and ML-2), grown at the sodic soil experiment farm (shivery farm), Central Soil Salinity Research Institute, Regional Research Station, Lucknow, India was investigated. In this study, we isolated 16 bacterial endophytes through natural selection from saline sodic soils, analysed extracellular enzyme activity, performed molecular profiling and phylogenetic analysis based on 16S rDNA sequences. Results indicate that the isolates belonged to four major phylogenetic groups: low G+C Gram positive bacteria, Firmicutes, Proteobacteria and Bacteroidetes. Endophytic bacteria from the phylum Firmicutes were predominant in the root portion 60.0% (Na^+ - 7.72 ± 0.05; K^+- 2.08 ± 0.85) and stem portion 75.0% (Na^+ - 5.79 ± 0.05; K^+- 13.58 ± 0.53), respectively. Most isolates that exhibited extracellular enzymatic activity in 3.0 M NaCl concentration belonged to the genus *Bacillus* sp., *Bacillus clausii*, *Bacillus pumilus* and *Bacillus licheniformis* (CSR-M-06, CSR-M-08, CSR-M-09 and CSR-M-16) exhibited the stronger activities in extracellular enzymes such as amylase, protease, cellulase and lipase than other isolates.

Key words: Endophytic bacteria, sodicity tolerance, polyembryonic mango, extracellular enzyme activity.

INTRODUCTION

Mango (*Mangifera indica* L.), one of the 73 genera of the family Anacardiaceae in order Sapindales, is amongst the most important tropical fruits of the world. It is more sensitive to salinity at early growth stages which led to identification of salt tolerant true to type polyembryony rootstocks ML-2, GPL-1 and ML-6 (Damodaran et al., 2013). Tolerance to sodicity in salt sensitive crops like gladioulus and banana may be epigenetic or may be through a response of the plant to the endophytic population that reside in the root zone of the crop and produce stress tolerant enzymes like superoxy dismutase (SOD), catalase, peroxidise, polyphenol oxidase and other phenol compounds (Damodaran et al., 2014). Bacterial endophytic species are common inhabitants of a wide range of plant species and reside within cells (Jacobs et al., 1985), in the intercellular space (Patriquin

and Dobereiner, 1978), or in vascular systems (Bell et al., 1995) of a plant. Bacterial endophytes seem to be ubiquitous in plant tissues, having been isolated from flowers, fruits, leaves, stems, roots and seeds of various plant species (Kobayashi and Palumbo, 2000). Significant variations in the populations of both indigenous and introduced endophytes have been reported. These variations are attributed to plant source, plant age, tissue type and environment (Zinniel et al., 2002). The potential to use salt tolerant endophytes to remediate salt polluted soils has attracted considerable interest (Idris et al., 2004). Bacterial endophytes can stimulate contaminant disappearance by the accumulation and transformation of sodium (Na^+).

Moore et al. (2006) describes the diversity of endophytes found in poplar trees, growing at a phyto-remediation field site contaminated with toluene. Germaine et al. (2004) demonstrated that within the diverse bacterial communities found in poplar trees several endophytic strains were present that had the potential to enhance phyto-remediation of volatile organics and herbicides. For this, several bacterial species possess genes responsible for resistance to salt and have evolved a variety of mechanisms to reduce heavy metals stress (Alonso et al., 2000; Van Houdt et al., 2009; Khan et al., 2009).

Recently, production of enzymes such as amylase, proteases, cellulose and lipase by salt tolerant endophytic bacteria under adverse environments has become a major field of investigation (Alqueres et al., 2007). This interest is due to the possibility of these microorganism producing enzymes with biotechno-logically potential antifungal agents (*Cryptosporiopsis cryptocandina, quercine*), besides producing factors of plant growth, toxins and enzymes (Araújo et al., 2002; Strobel, 2003; Azevedo, 1998; Stierle et al., 1993). Several reports confirmed that the activity of salt tolerant bacterial enzymes remained stable at high salt concen-trations (Prakash and Jaiswal, 2009). However, extracel-lular enzymes like amylase, lipase, cellulose and protease are produced by different microorganisms via *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens, Pseudomonas* (Devi et al., 2010; Bassabrani et al., 2012), *Halobacillus* sp. (Amoozegar et al., 2003) and *Bacillus dipsosauri* (Deutch, 2002) but production of this enzyme by salt tolerant plant bacterial endophytic strains. Previous studies on the occurrence of endophytic bacteria in sodicity tolerant polyembryonic mango root stock or other parts of the plants, on sodium uptake pattern and PGPR traits of microorganisms have been conducted (Damodaran et al., 2013; Kannan et al., 2014). However, there are no reports on the occurrence and diversity of extracellular enzyme producing endophytic bacteria in sodicity tolerant polyembryonic mango root stock grown in salt affected sodic soils.

MATERIALS AND METHODS

Sites and isolation of endophytic bacteria

Endophytic bacteria were isolated from surface-sterilized sodicity tolerant polyembyonic mango root stock ML-2 and GPL-1 (the plants can grow normally at the level of pH-9.53 and electrical conductivity EC- < 4.0). The rhizosphere soil total Na^+ and K^+ concentrations of GPL-1 and ML-2 were 21.20 meq / L; and 0.126 meq / L respectively. Two tolerant polyembryonic mango plants were collected from the experimental farm of Central Soil Salinity Research Institute Regional Research Station, Lucknow, India, located at 260 47′45′′ to 260 48′13′′ N latitude and 800 46′7′′ to 800 46′32′′ E longitude, lying in the Central part of Indo-Gangetic plain, in June 2012. Roots, stems and leaves from each plant were separated and washed extensively first in several changes of 0.01 M EDTA and then in distilled water to remove any non-specifically bound Na^+ / K^+ and dried to constant weight. Dried plant tissues were ground into a fine power, and digested with HNO_3:H_2SO_4:$HClO_4$ (10:1:4). Digested material was diluted to 10 ml and the sodium (Na^+) / potassium (K^+) content was analyzed with the help of a flame photometer (Furman, 1975). Plant samples were washed with tap water followed by three rinses with deionized water and then separated into roots, stems and leaves. Healthy plant tissues were sterilized by sequential immersion in 75% (v/v) ethanol for 2 min, and 1% mercuric chloride for 1 min and washed three times with sterile water to remove surface sterilization agents. To confirm the surface disinfection process was successful, water from the final rinse was plated on Nutrient agar. Plant tissue materials (0.2 g) were ground by a mortar and pestle in the presence of 5 ml of sterile distilled water. Serial dilutions were spread on plates containing 0.1 to 3.0 M range $NaCl_3$ supplement of nutrient agar medium with or without $NaCl_3$. The medium was found to be most suitable for the isolation of sodium tolerant bacteria in preliminary experiments. $NaCl_3$ resistant colonies were picked randomly and purified by streaking three to four times on the same media. Sixteen endophytic bacterial isolates growing well on sub culturing were finally selected and stored on slants for further study.

Analysis of extracellular enzyme activity

Endophytic bacterial isolates were analysed for production of four enzymes under (0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 M) NaCl stress, that is, protease, amylase, cellulase and lipase by plate methods. Proteolytic activities of the cultures were studied in a medium containing skimmed milk (HiMedia, Mumbai). Zones of precipitation of paracasein around the colonies in the next 48 h were taken as evidence of proteolytic activity. The presence of amylolytic activity was determined using starch agar medium (HiMedia, Mumbai). After inoculation of endophytic bacteria and incubation at $28\,°C$ for 5 days, the plates were flooded with 0.3% I^2 and 0.6% KI solution and a clear zone around the growth indicated hydrolysis of starch. For cellulase activity, mineral-salt agar plate 5 containing 0.4% $(NH_4)_2SO_4$, 0.6% NaCl, 0.1% K_2HPO_4, 0.01% $MgSO_4$, 0.01% $CaCl_2$ with 0.5% carboxymethylcellulose and 2% agar (HiMedia, Mumbai) were surface inoculated. Iodine solution was used to detect cellulase activity as described.

Growth pattern of effective isolates at different NaCl concentrations

Growth of salt tolerant bacteria was measured at different NaCl concentration (0.1, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 M). L-Broth having different NaCl concentrations was prepared and autoclaved. For preparation of inoculum, bacterial cells harvested from overnight

culture were washed and resuspended in sterile distilled water to adjust the OD at 106 cells per ml at 600 nm. 100 µl of inoculum was given in all media having different NaCl concentrations and incubated at 37°C on shaker with 150 rpm shaking for 24 h. Bacterial growth was monitored at 600 nm (Spectrophotometer S200, R & M Marketing, England). Three replicates per strain were taken for each salt concentration.

Vigour index studies

Plant growth promoting (PGP) activities of the current strains were assessed using seedling vigour index by pot culture studies in saline sodic soils of pH 9.40, electrical conductivity (ECe) 6.72 dSm^{-2}; sodium (Na$^+$) 21.20 meq / L. The vigor index was calculated by using the formula as described by Abdul-Baki and Anderson (1973).

Vigor index= Percent germination x Seedling length (shoot length + root length)

Molecular profile of endophytic bacterial

Bacterial DNA from the elite strains showing higher extra cellular activities were extracted according to the method given by Araujo et al. (2002), with amplification of 16S rDNA being performed in a 25 µl reaction mixture, which consisted of 25 ng genomic DNA, 10× reaction buffer with 15 mM MgCl$_2$, 2.5 mM each of dNTPs, 0.2 mM bacterial universal primer (27F-AGAGTTTGATCMTGGCTCAG) (1492R-GGYTACCTTGTTACGACTT), and 1 unit of Taq DNA polymerase (Bangalore Genei Pvt Ltd, India). The reaction was carried out in a thermal cycler (PTC-200, MJ Research Inc, USA). The DNA amplification program was set to a 5 min initial denaturation at 94°C followed by 30 cycles of 1 min at 94°C for denaturation, 1 min at 54°C for annealing, 2 min at 72°C for extension, and ended with a final 10 min extension at 72°C. The reaction products were separated by running 5 µl of the polymerase chain reaction mixture in 1.2% (w/v) agarose gel and staining the bands with ethidium bromide (Sambrook et al., 1989). The Restriction Fragment Length Polymorphism (RFLP) assay involved restriction enzyme digests and the 16S rRNA gene PCR products using five different enzymes. Reactions were performed in a 30 µl reaction mixture, which consisted of 10 µl of each PCR product, 2 µl of 10× Fast digest green buffer, 17 µl nuclease free water and 1 µl (1FDU) for restriction enzyme cleavage by either *Alu*I, *Hae* III, *Eco*RI, *Msp*I or *Bam*HI (Fermentas, USA). Digestions were performed for 5 min at 37°C to ensure that complete fragmentation was achieved. Digested DNA fragments were analysed in a 1.5% (w/v) agarose gel with 10 mg / ml of ethidium bromide and photographed on a gel documentation system. For cluster analysis, the data were converted to a binary matrix, where the digit 1 or 0 represented the presence of or absence of DNA band in the gel. NTSYSpc 2.20 (Exeter software, New York) was used to analyze the fingerprinting. The data were analysed using the neighbor-joining tree construction method to generate the Dice similarity coefficient. Similarity coefficients were used to construct the dendrogram using the Dice coefficient in clustering (Damodaran et al., 2012). Statistical stability of the branches in the cluster was estimated by bootstrap analysis using Winboot software program (Yap and Nelson, 1996). The principle co-ordinate analysis was performed with NTSYS software with the Dice (1945) coefficient.

Sequencing and phylogenetic analysis

Purified PCR products from the 16S rRNA genes of pure culture isolates representing all major RFLP patterns were sequenced on an ABI3730 automated sequencer (Applied Biosystems, USA) with primers 27 f and 1492 r. All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and the 16S rDNA similarity sequences searches were performed using the BLASTN tool in the NCBI website. These sequences were aligned using the multiple sequence alignment program, CLUSTAL X (Thompson, 1996). The method of Jukes and Cantor (1969) was used to calculate evolutionary distances. A phylogenetic dendogram was constructed by the neighbor-joining method and tree topologies were evaluated by performing bootstrap analysis of 1,000 data sets using MEGA 3.1 (Molecular Evolutionary Genetic Analysis).

Nucleotide sequence accession numbers

The sequence obtained in this study was deposited in the GeneBank nucleotide sequence database under the accession numbers (KC433665 and KC433667).

RESULTS

Na$^+$, K$^+$ partitions and bacterial endophytes in different parts of sodicity tolerant mango root stock

Na$^+$ and K$^+$ concentration in plant tissues are shown in Table 1. Na$^+$ concentration in the roots were significantly higher than that of stem and leaves and K$^+$ concentration of root parts were significantly lower than that of other parts. There were significant differences between the Na$^+$ and K$^+$ concentrations in the roots of salt tolerant polyembryonic mango root stock (GP-1 and ML-2) and salt sensitive polyembryonic mango root stock (GTP-2). It is worth noting that GPL-1 and ML-2 polyembryonic root stock accumulated significantly higher Na$^+$ and lower accumulation K$^+$ in its (7.72 ± 0.05 meq / l 2.08 ± 0.85 meq / l) dry weight. A total of forty NaCl-resistant-endophytic isolates were obtained, from GPL-1 and ML-2 root stock. Endophytic bacterial counts of the two sodicity tolerant polyembryonic mango root stock (GPL-1 and ML-2) ranged from 4.4 × 10^4 to 9.6 × 10^4 fresh weight (Tables 1 and 2). It was very noticeable that NaCl-resistant-endophytic bacterial from GPL-1 root stock attained 10^4 cfu g^{-1} with fresh weight of 2.6 and ML-2 root stock with fresh weight of 2.31 ± 0.20 × 10^4 cfu g^{-1}, respectively. It was also observed that endophytic NaCl tolerant bacterial were nil or 0.00 in stem and leaves of the GPL-1, ML-2 root stock.

In-vitro growth response of endophytes with effect on different concentration of NaCl

Endophyte isolates were grown in the presence of different concentrations (0.1 to 3.0 M NaCl in L-Broth) of NaCl is presented in Figure 1. All the isolates were able to grow in medium containing 1.0 M and more NaCl. They could tolerate 2.0 to 3.0 M NaCl in solid medium and 2.5 to 3.0 M NaCl in the liquid medium (L-Broth). However, growth was less at 2.0 and 2.5 M NaCl. In liquid medium most of the isolates could respond up to 3.0 M NaCl while some could grow up to the range of 2.5 (CSR-M-08, CSR-M-09, CSR-M-06, CSR-M-16) and

Table 1. The number of total and sodium chloride resistant-endophytic bacteria and Na^{+}, K^{+} concentration of different tissue in sodicity tolerant polyembryonic mango root stock.

Sample	Total bacteria[a] (10^4 cfu g^{-1})	NaCl-resistant bacteria[b] (10^4 cfu g^{-1})	Na^{+} concentration in plant tissue (meq / l)	K^{+} concentration in plant tissue (meq / l)
Sodicity tolerant polyembryonic mango (*Mangifera indica*) root stock (GPL-1)				
Root	9.0 ± 0.05	2.6 ± 0.57	7.72 ± 0.05	2.08 ± 0.85
Stem	4.4 ± 0.10	00 ± 00	3. 78 ± 0.12	9.78 ± 0.56
Leaves	4.6 ± 0.15	00 ± 00	1.58 ± 0.50	3.45 ± 0.96
Sodicity tolerant polyembryonic mango (*Mangifera indica*) root stock (ML-2)				
Root	9.6 ± 0.76	2.31 ± 0.20	7.04 ± 0.04	2.32 ± 0.24
Stem	5.7 ± 0.26	00 ± 00	5.79 ± 0.05	13.58 ± 0.53
Leaves	5.4 ± 0.11	00 ± 00	2.17 ± 0.33	2.28 ± 0.38
Sodicity sensitive polyembryonic mango (*Mangifera indica*) root stock (GTP-2)				
Root	00 ± 00	00 ± 00	18.72 ± 0.09	24.82 ± 0.11
Stem	00 ± 00	00 ± 00	4.24 ± 0.16	1.32 ± 0.17
Leaves	00 ± 00	00 ± 00	4.29 ± 0.02	1.40 ± 0.32

[a]Average (± standard deviations) of the cfu from three repetitive plating NA medium. [b]Average standard deviations) of the cfu from three repetitive plating NA medium + 2.5 M of NaCl.

Table 2. Enzyme activities by endophytic bacteria isolated from sodicity tolerant polyembryonic mango root stock (GPL-1 and ML-2).

Sample	Plant tissue parts	Strains (closest relative sequence)	% similarity in BLAST match	Phylum	Hydrolytic enzyme activity			
					Amylase	Protease	Cellulase	Lipase
Sodicity tolerant polyembryonic mango (*Mangifera indica*) root stock (GPL-1)	Root	CSR-M-01 (*Agrobacterium tumifaciens*)	99	P	-	-	-	-
		CSR-M-02 (*Oceanobacillus sp*)	99	P	-	-	-	-
		CSR-M-08 (*Bacillus clausii*)	100	F	++	++	++	++
		CSR-M-09 (*Bacillus pumilus*)	99	F	+	++	+	++
	Stem	CSR-M-05 (*Bacillus coagulans*)	99	F	-	-	-	-
		CSR-M-12 (*Bacillus pumilus*)	98	F	+	+	+	-
	Leaves	CSR-M-10 (*Unculture organism*)	99	B	-	-	-	-
Sodicity tolerant polyembryonic mango (*Mangifera indica*) root stock (ML-2)	Root	CSR-M-04 (*Ocenobacillus caeni*)	99	P	-	-	-	-
		CSR-M-06 (*Bacillus sp*)	100	F	+++	++	+	+
		CSR-M-07 *(Brevibacillus borstelensis*)	99	F	+	-	+	+
		CSR-M-14 (*Agrobacterium fabrum*)	96	P	-	-	-	-
		CSR-M-15 (*Bacillus subtilis*)	99	F	-	-	-	-
		CSR-M-16 (*Bacillus licheniformis*)	99	F	+++	++	++	+
	Stem	CSR-M-03 (*Psuedomonas aeroginosa*)	99	P	-	+	+	-
		CSR-M-11 (*Bacillus amloliquefaciens subsp*)	99	F	-	-	-	-
	Leaves	CSR-M-13 (*Bacillus lehensis*)	99	P	-	-	-	-

- No production; + 0.3–0.5 cm; ++ 0.6–0.9 cm; +++>1 cm. F=Firmicutes; P=Proteobacteria; B=Bacteroidetes.

3.0 M (CSR-M-06, CSR-M-08, CSRM-09, CSR-M-16).

Extracellular enzyme production by bacterial endophytes under different concentration of NaCl

Sixteen (16) newly isolated endophytic bacterial were screened for production of extracellular enzymes. The method of radial diffusion in solid media with supplement of 0.1 to 3.0 M of sodium chloride concentration indicated that actively hydrolysing enzymes with higher salt concentration could be detected in qualitative form by direct correlation of the diameter of the zone of substrate hydrolysis and colony growth. This practical tool facilitates and speeds the selection and comparison of the enzymatic production of different isolates. The enzyme biosynthesis capacity of isolated bacterial endophytes
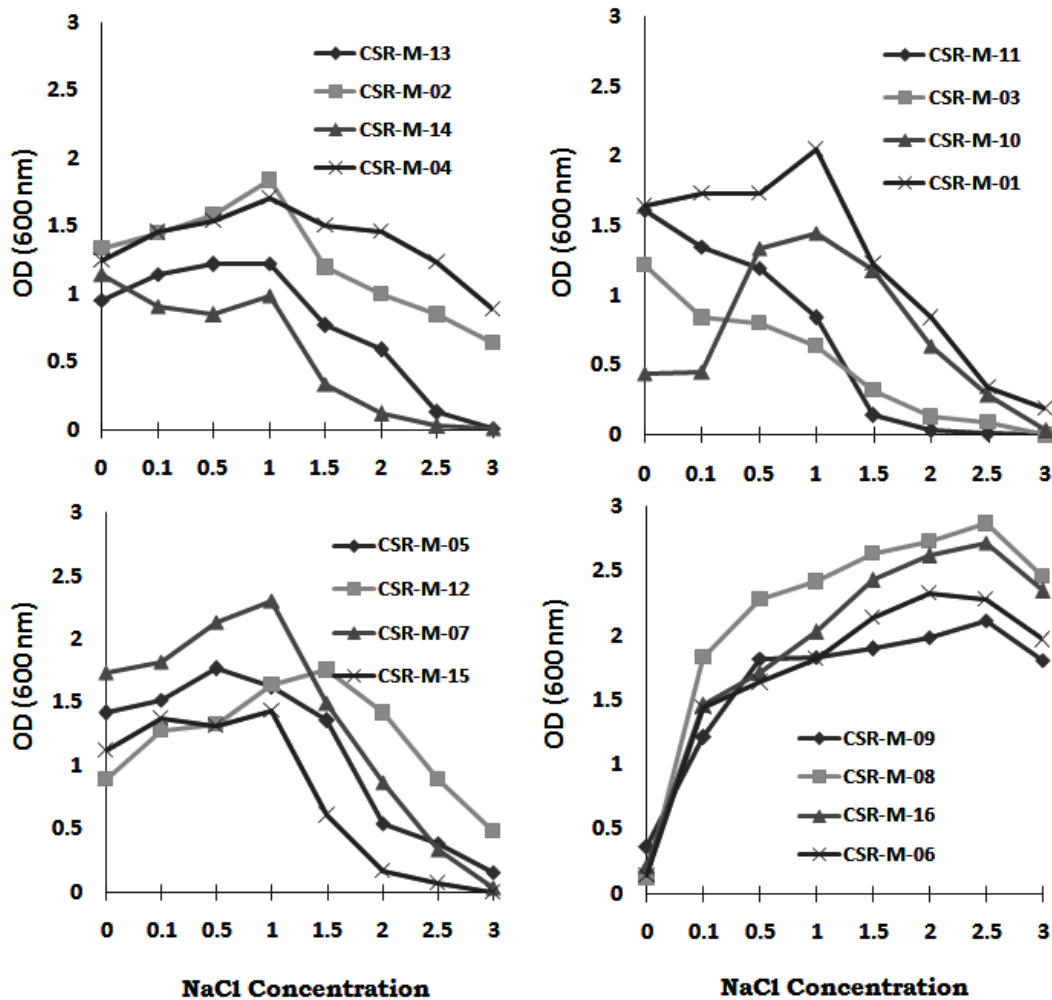
Figure 1. Growth response of endophytic bacterial isolates to different concentration of NaCl.

was shown in Table 2. Four isolates (CSR-M-08, CSR-M-09, CSR-M-06 and CSR-M-16) showed positive hydroly-tic activity from sodicity tolerant polyem-bryonic mango root stock (GPL-1 and ML-2), two isolates from root of GPL-1 root stock (CSR-M-08 and CSR-M-09) and the other two isolates (CSR-M-06, CSR-M-16) from root of ML-2 mango root stock. The highest amylase, protease, cellulase and lipase activity was recorded from bacterial endophytes isolated from the root portion of both GPL-1 and ML-2. The zones of substrate hydrolysis ranged from 0.3 to >1.0 cm, respectively, and among the four most hydrolytic isolates, amylase production was found to be superior.

Screening of bacterial isolates for rice seedling vigour under sodic soil

Among the 16 isolates tested for their efficacy to improve the vigour index of rice seedlings under sodic soils (Table 3), interestingly, CSR-M-16 isolate showed the highest germination of 93.33% with a vigour index of 4675.83 while the vigor index of CSR-M-8, CSR-M-06 and CSR-M-09 isolates were on par (4393.57, 4260.45 and 3992.14, respectively) and these also showed range of 86.33 to 91.66% germination. Apart from this the isolates CSR-M-8, CSR-M-06 and CSR-M-09 also showed higher germination percentage of >86% and vigor index. The root length of 30 days old seedling for all treatment ranged from 4.3 to 13.6 cm, while higher value (13.6, 12.8, 12.3 and 12.1 cm) was recorded in seedling of CSR-M-16, CSR-M-06, CSR-M-08 and CSR-M-09. The lowest was found in control seedling (3.7 cm). Shoot length significantly higher (36.5, 35.6, 35.5 and 34.0 cm) was observed in seedling of CSR-M-16, CSR-M-08, CSR-M-06 and CSR-M-09. The smallest length (8.3 cm) was found in control seedling. Root dry weight ranged from length 0.07 to 0.44 g where higher (0.44, 0.38, 0.38, 0.34 g) root weight was found in seedling of CSR-M-06, CSR-M-09, CSR-M-16 and CSR-M-08 treatment. The
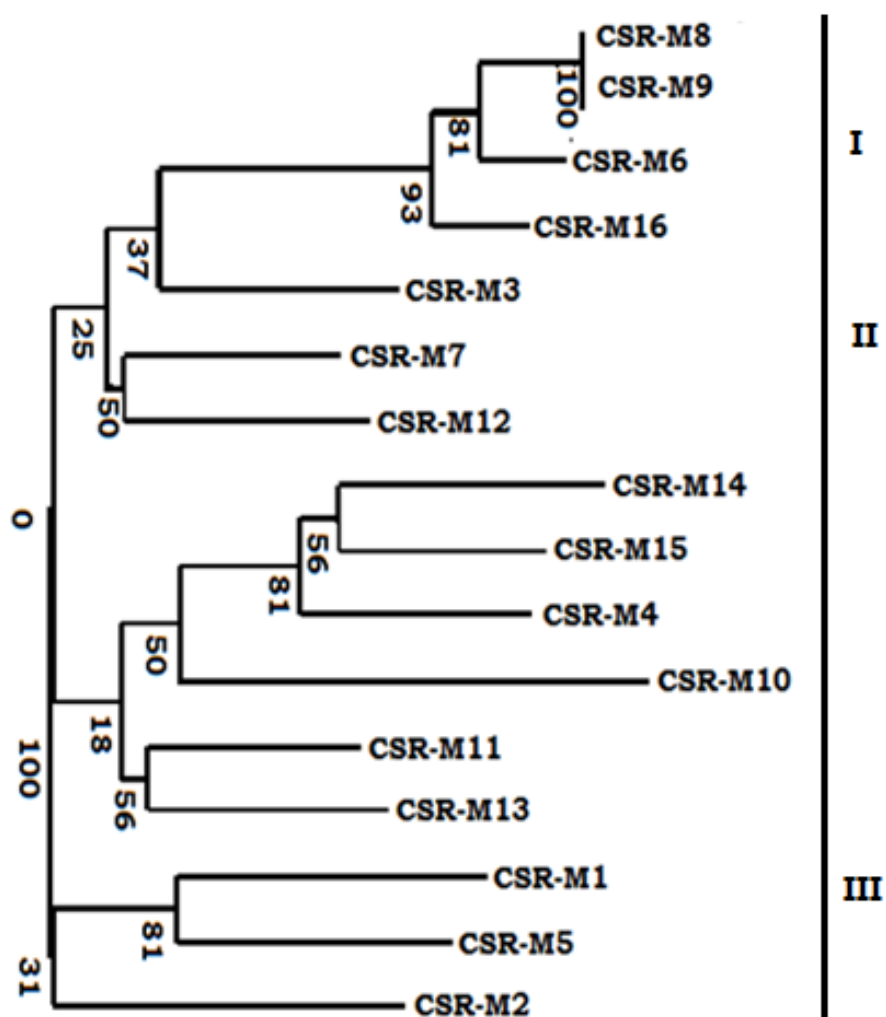
Figure 2. Dendrogram of the diversity of bacterial endophytes of sodicity tolerant polyembryonic mango accessions (GPL-1 and ML-2) based on RFLP markers and generated using neighbor joining algorithm.

lowest was observed in seedling of CSR-M-13 (0.07 g) treatment. Similarly significantly higher shoot dry weight (1.86, 1.75, 1.68 and 1.67 g) was found in seedling of CSR-M-09, CSR-M-16, CSR-M-06 and CSR-M-08 treatment.

PCR-RFLP finger printing

Endophytic bacterial isolates obtained from the rhizosphere of the salt tolerant mango rootstocks were found to be gram-negative predominantly. Of the 40 isolates discovered, 16 were used to assess genetic diversity by PCR-RFLP using 16S rRNA gene universal primer 27F and 1492R. The primers amplified a single band of 1.5 Kb in all the endophytic isolates and no inter or intraspecific variation was observed among the 16 endophyte strains. A set of five different restriction

enzymes (*Alu*I, *Hae*III, *Eco*RI, *Msp*I and *Bam*HI) were used for RFLP analysis. The banding pattern generated with enzyme *Alu*I, *Hae*III and *Msp*I showed greater polymorphism compared to the other enzymes and were chosen to construct the dendrogram and principle coordinate.

Cluster-based dendrogram

The dendrogram showing the genetic relation, extracellular enzyme activity under higher salt concentration among 16 endophytes based on RFLP markers is presented in Figure 2. The dendrogram showed three major clusters. The list of clusters along with the isolate name included in each cluster is also shown in Figure 2. Cluster I (primary cluster) consisted of four salt tolerant endophytes (CSR-M-08, CSR-M-09, CSR-M-06,
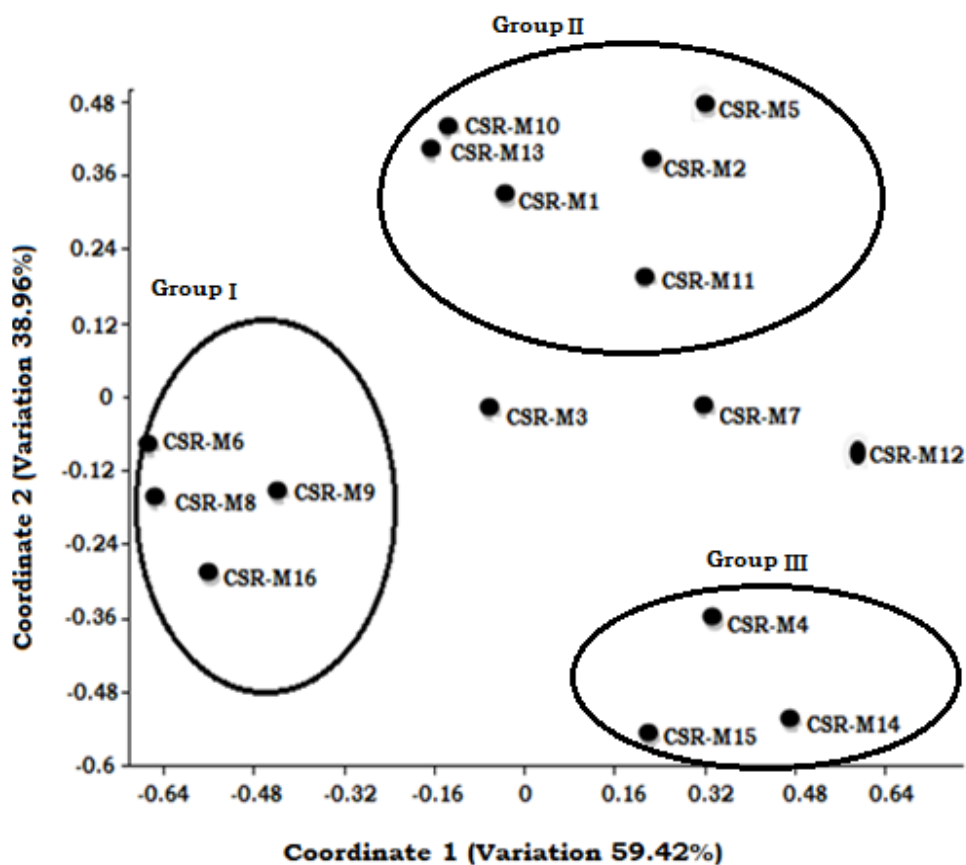
Figure 3. Principal coordinate analysis (PCO 1 Vs PCO2) diversity of bacterial endophytes of sodicity tolerant polyembryonic mango accessions (GPL-1 and ML-2) based on RFLP markers.

CSR-M-16) growing above 2.5 M NaCl and positive for extracellular enzyme activity under higher salt concentration while the moderately salt tolerant isolates CSR-M-03, CSR-M-07, CSR-M-12 up to 1.5 M NaCl showed limited presence of extracellular enzymes like protease and cellulose. Cluster II and cluster III consisted of 12 endophytic bacterial isolates that are negative for extracellular enzyme activity and growth in salt concentration below 0.1 M NaCl. All the isolates of cluster I and II were isolated from roots of the salt tolerant mango rootstocks while the isolates showing limited response to NaCl toxicity are isolated from stem and leaves of the rootstocks.

Principle coordinate analysis

The positioning of 16 bacterial endophytes based on principle coordinate 1 and 2 is given in Figure 3. The scatter plot of Dice similarity showed three major groups of bacterial endophytes belonging to salt tolerant endophytes (group I) and those below 1.0 M NaCl concentration and negative for hydrolysis enzyme activity

(group II, group III). Three bacterial endophytes (CSR-M-03, CSR-M-07, CSR-M-12) separated from the three groups. The principle coordinates 1 and 2 encompassed 59.42 and 38.96% of total variation, respectively.

Confirmation of enzyme producing salt tolerant endophytes

Identification of salt tolerant enzyme producing strains clustered in group I were verified by sequencing the 16S rRNA genes and validated with BLAST (ncbi.nlm.gov.blast/Blast.cgi) database. This method was carried out as follows: DNA was extracted from the isolates by a standard kit (Xcelgen-Germany). Then amplification of 16S rRNA gene (27F-AGAGTTTGATCCTGGCTCAG; 1492R-ACGGCTACCTTGTTACGACTT) was performed by PCR method and eventually the products were sequenced on an ABI 3730xl Genetic analyzer (Applied Biosystems, USA). The results (Figure 4) show that all of the potential strains belonged to the genus *Bacillus*, with the strain CSR-M-06 identified as *Bacillus* sp., CSR-M-08 as

Table 3. Plant promotion activity of rice seedlings following inoculation with endophytic bacteria under sodic soil conditions.

Bacterial Isolate	Root length (cm)	Shoot length (cm)	Root dry weight (g)	Shoot dry weight (g)	% Germination	Vigour index
CSR-M-01	4.3_i	11.2_k	0.16_{de}	0.36_g	33.33_{bc}	516.67_g
CSR-M-02	5.4_g	11.5_j	0.13_{fg}	0.45_{fe}	31.67_{bc}	534.11_{fg}
CSR-M-03	4.6_{hi}	12.1_i	0.12_{gh}	0.14_i	25.00_{cd}	416.67_{gh}
CSR-M-04	5.3_g	15.4_f	0.16_{dfe}	0.39_{fg}	33.33_{bc}	692.22_f
CSR-M-05	4.3_i	11.4_{jk}	0.11_j	0.14_i	26.67_{bcd}	419.56_{gh}
CSR-M-06	12.8_b	35.5_b	0.44_a	1.68_{bc}	88.33_a	4260.45_b
CSR-M-07	8.0_e	20.1_e	0.16_{de}	0.47_{de}	38.33_b	1078.44_e
CSR-M-08	12.3_c	35.6_b	0.34_c	1.67_c	91.66_a	4393.57_b
CSR-M-09	12.1_c	34.0_c	0.38_b	1.86_a	86.66_a	3992.14_c
CSR-M-10	4.6_{hi}	12.5_h	0.15_{fge}	0.26_h	25.00_{cd}	429.17_{gh}
CSR-M-11	5.8_f	12.1_i	0.14_{fge}	0.39_{fg}	23.33_{cd}	418.44_{gh}
CSR-M-12	9.1_d	21.5_d	0.18_d	0.54_d	38.33_b	1172.90_d
CSR-M-13	4.4_{hi}	13.5_g	0.07_j	0.24_h	31.67_{bc}	566.83_{fg}
CSR-M-14	4.4_{hi}	15.3_f	0.13_{fg}	0.15_i	21.67_{cd}	426.83_{fg}
CSR-M-15	4.7_h	11.5_j	0.15_{fe}	0.18_{hi}	18.33_e	297.61_h
CSR-M-16	13.6_a	36.5_a	0.38_b	1.75_b	93.33_a	4675.83_a
Control	3.7_j	8.3_{ll}	0.14_{fge}	0.12_j	6.67_f	80.22_i

Values are the means of three replicates. Means in the columns followed by the same subscript letters are not significantly different according to Duncan's multiple range test at P=0.05.
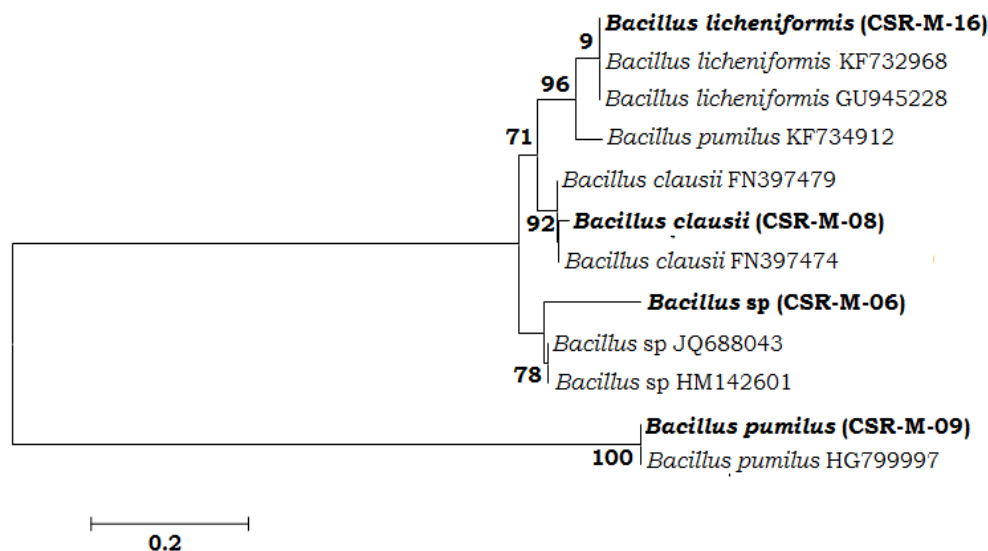
Figure 4. Phylogenetic relationships of the actively hydrolytic enzyme producing sodicity tolerant endphytic bacteria from sodicity tolerant polyembryonic mango root stock (GPL-1and ML-2).

Bacillus clausii, CSR-M-09 as *Bacillus pumilus* and CSR-M-16 as *B. licheniformis.*

DISCUSSION

Although, special attention has been paid to endophytic bacterial isolated from sodicity tolerant polyembryonic mango root stock (GPL-1 and ML-2) in special environments such as sodium and heavy metal contaminated soils (Idris et al., 2004; Barzanti et al., 2007), no information is available about the composition of endophytic bacterial communities present in the two dominant sodium tolerant and accumulating plants naturally growing on salt affected wasteland. The surface sterilization protocol is a critical prerequisite for isolation and identification of the endophytic bacterial that express

plant growth promotion (PGP) traits at high salt concentrations (Kannan et al., 2014). The present study showed that there was a reduction in the bacterial diversity with increased sodium concentration in plant tissue parts. It has been reported earlier that Na$^+$ concentration in tissue parts plays a prominent role in the microbial adaption process as environmental stress leads to reduced bacterial diversity (Borneman et al., 1996). In our study, we have cultured 40 isolates from different tissue portions of polyembryonic mango accessions grown in sodic soils and identified 16 pure cultures for further investigations. The majority of endophytes are identified as *Bacillus sp*. based on 16S rDNA sequencing and validated with BLAST (ncbi.nlm.gov.blast/Blast.cgi) database. A similar process was adopted in many previous characterization studies (Sturz et al., 2000; Kuklinsky-Sobral et al., 2004; Tank and Saraf, 2010).

In this study, 16 bacterial endophytic strains were isolated from both sodicity tolerant polyembryonic mango root stock GPL-1 and ML-2. Out of the 16 endophytes, four (CSR-M-06, CSR-M-08, CSR-M-09 and CSR-M-16) showed tolerance to high (3.0 M) NaCl concentration (Figure 1). It has also been reported previously that bacterial isolated from saline soil are more likely to withstand saline conditions (Upadhyay et al., 2009). Among the isolates, four strains possessed amylase, cellulase, protease and lipase enzyme activity. According to the 16S rRNA sequences the strains belonged to genus *Bacillus* with CSR-M-06 identified as *Bacillus sp*, CSR-M-08 as *B. clausii*, CSR-M-09 as *B. pumilus* and CSR-M-16 as *B. licheniformis* which were able to produce above four enzymes under high NaCl concentrations. The characterized strains showed optimum growth between 2.5 to 3.0 M of NaCl. Therefore, according to the salt tolerance (up to 3.0 M) these strains could be described as salt tolerant microorganisms (Fahimeh et al., 2013; Dave et al., 2006). In general, the hydrolysis enzymes of endophytes appear to be important for the colonization of plant roots with external stress condition (Quadt-Hallmann et al., 1997; Reinhold-Hurek and Hurek, 1998; Sakiyama et al., 2001). This hypothesis is supported by the endophytic bacterial possessing higher activity of amylase, cellulase, protease and lipase enzymes from young radish roots (Weon Taek Seo et al., 2010). Similarly, the presence of cellulolytic and pectinolytic enzymes produced by numerous endophytic bacterial such as *Rhizobium sp.* has been described (Al-mallah et al., 1987). In addition, bacterial enter the interior of the root by hydrolyzing wall-bound cellulose, auxin-induced tumors, water flow and wounds, or where the lateral roots branch (Al-Mallah et al., 1987).

Molecular characterization based on amplification of 16s rDNA revealed the presence of *B. clausii*, *B. pumilus*, *B. licheniformis* and *Bacillus sp*. Variation of 16S rDNA allow the inference of phylogenetic relationship among taxonomically related as well as distinct organisms

(Espinosa-Victoria et al., 2009). Based on Amplified Ribosomal Restriction Analysis (ARDRA), different groups were obtained in the dendrogram and these results find support from the observations made by Chowdhury et al. (2007) who divided the bacterial into various groups based on ARDRA pattern and one representative from each of the 4 groups was selected for sequencing of a 16S rRNA gene fragment so as to compare sequence similarity. Kannan et al. (2014) revealed that apart from normally encountered endophytic microflora, *Bacillus coagulans, Bacillus megatarium, B. pumilus* and *B. subtilis* were encountered. Molecular characterized phyto-ameliorant grasses grown on the patches of undisturbed sodic soil with prominent rhizosphere encountered microflora *B. pumilus* and *B. subtilis*, respectively (Damodaran et al., 2014). In the present study, the bacterial endophyte identified in higher Na$^+$/K$^+$ in the root portion of sodicity tolerant polyembryonic mango root stock belonged to the same group of firmicutes reported previously (Bharathkumar et al., 2008; Islam et al., 2010).

Therefore, this salt tolerant stability and adaptation of salt tolerant strains with capability to produce amylase, cellulase, protease and lipase enzymes (higher than the other strains of other researcher isolates) suggest that these strains could be a good choice for commer-cialisation for cultivation of crops in normal and saline-sodic soil applications. In conclusion, elucidation of the origin of the potential endophytes clarifies influence of these endophytes in reclamation of salt affected soils.

Conflict of interests

The author(s) have not declared any conflict of interests.

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