African Journal of Biotechnology

Volume 15 Number 35, 31 August 2016

ISSN 1684-5315

Academic Journals

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Vol. 15(35), pp. 1855-1863, 31 August, 2016
DOI: 10.5897/AJB2015.15051
Article Number: ADEDD9360229
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Pharmacological properties of cashew (*Anacardium occidentale*)

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Received 15 October, 2015; Accepted 3 August, 2016

***Anacardium occidentale* L. is a tree native to Brazil, which is rich in phenolic lipids. Nowadays, the cashew bark (Cashew Nut Shell Liquid) has received great attention in the pharmaceutical industry, due to its economy, abundance and important chemical compounds. Net of cashew nut shell is classified according to the method of production of: (1) net of the shell of natural cashew nut (60-65% anacardic acid; 15-20% cardol and 10% of cardanol) and (2) liquid from the technical cashew nut shell (60-65% of cardanol, 15-20% cardol and 10% of polymeric material). This work aims to report the pharmacological properties of liquids from cashew nut shells. Results suggest that both liquids have antifungal, antibacterial, antiparasitic, anti-tumor, antiulcerogenic, molluscicides, antimutagenic and antioxidant activities. Natural cashew nut liquid is non-genotoxic, whereas technical liquid is genotoxic in prokaryotes and eukaryotes, although there is no evidence of their mutagenic effects on eukaryotic cells. In conclusion, the excellent antioxidant and non-mutagenic activities of cashew nut shell liquid (CNSL) provide opportunities for CNSL in the cosmetic and/or pharmaceutical industries, but continuous study is needed to allow safe and efficacious preparations.**

Key words: Cashew liquid, cosmetics, pharmacological, pharmaceutical, preparation.

INTRODUCTION

The Anacardiaceae family has 76 genera divided into five tribes (Anacardiaceae, Dobineae, Rhoeae, Semecarpeae and Spondiadeae) covering about 600 species (Correia et al., 2006). *Anacardium occidentale* is an abundant

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tree in the Northeast of Brazil and the states of Piauí, Ceará and Rio Grande of Norte. It represents 90% of cashew production in Brazil. This species is evident for its antioxidant (Melo-Cavalcante et al., 2003), antigenotoxic, antimutagenic (Melo-Cavalcante et al., 2011), antiulcerogenic (Behravan et al., 2012), anti-inflammatory (Olajide et al., 2004), antibacterial, antifungal and larvicides (Behravan et al., 2012) activities. Also, it is a tree rich in anthocyanins, carotenoids, ascorbic acid (vitamin C), flavonoids and other polyphenols as well as mineral components. The bark and leaves are used in folk medicine (Konan and Bacchi, 2007).

In addition, the true fruit of cashew releases a liquid rich in phenolic compounds, known as liquid from chestnut shell- cashew nut shell liquid (CNSL). The components of cashew nut shell liquid depend on the method of production and are classified into two general categories: natural CNSL (LCCI) and technical CNSL (CTCL) liquids. LCCI contains 60-65% of anacardic acid, 15-20% cardol, 10% cardanol and trace amount of metilcardol, while CTCL contains 60-65% of cardanol, 15-20% cardol, and trace amount of polymeric methyl-cardol l material (Kumar et al., 2002). Both of the liquids also contain trace amount of phytosterols, triacontane and others (Andrade et al., 2011).

Though the pharmacological activities reported for CNSL is linked to pharmaceutical consumption, more analysis is needed, especially of its genotoxic effects. Assessment of genotoxic and carcinogenic potentials in drug development is crucial before approving and making it available in the market as pharmaceutical products.

Thus, this review aimed to take a picture of *A. occidentale*, highlighting its chemical compositions and related biological activities.

MORPHOLOGY AND GEOGRAPHICAL DISTRIBUTION OF *A. OCCIDENTALE*

A. occidentale has a height of 5-10 m, but in clay land can reach up to 20 m. It has a crooked trunk of 25-40 cm in diameter. The leaves are oval, obovais, leathery, glabrous; rosy when young; it has vináceas flowers, arranged in terminal panicles (Lorenzi, 2008). According to Gomes (2010), cashew tree is spread around the world, between latitudes 27ºN in Southern Florida and 28ºS of South Africa; and also in low latitude regions, near the equator, between the parallel 15ºN and 15ºS, in coastal areas, typically tropical South America, Africa and Asia. *A. occidentale* is common among the Northeastern states such as Ceará, Piauí and Rio Grande do Norte (Lubi and Thachil, 2000). The family Anacardiaceae covers over 70 genera in which more than 600 species are distributed in tropical, sub-tropical and temperate regions in the world (Engels et al., 2012). The family is rich in important secondary metabolites with varieties of interesting biological activities (Abu-Reidah et al., 2015).

CHEMICAL COMPOUNDS PRESENT IN *A. OCCIDENTALE* AND ITS BIOLOGICAL ROLES

Both yellow and red fruits of *A. occidentale* possess ferulic acid, caffeic acid, sinapic acid, gallic acid, and ellagic myritine (Moo-Huchin et al., 2015). Flavonoid contents in yellow and red cashew may be 12.1 ± 0.3 and 6.4 ± 0.4 mg/g, respectively. The compound, camferol-3-O-glucoside is the major constituent in both varieties, followed by camferol-3-O-arabinofuranoside and quercetin-3-O-glucoside (Shukri and Alan, 2010). The extract of cashew fibers has 11 carotenoids in which auroxantins and β- criptoxantins account for about 50% (Abreu et al., 2014).

In the phytochemical analysis of cashew leaves, it is reported that it has (E) -β-ocimene, α-copaene and δ-cadienol; while the fruits contain palmitic, oleic acids, furfural, 4-hydroxydodecanoic acid, lactone, (E) -hexenal, (Z) -hex-3-enol and haxadecanol (Maia et al., 2000). Cashew is rich in anacardic acid, cardanol and cardol along with other alkyl phenolic compounds (Trevisan et al., 2006). It is also evident to have monomeric phenols, flavonoids, glycosides such as myricetin and quercetin hexoside, pentoside, rhamnosides and glycosidic anthocyanidins (Michodjehoun-Mestres et al., 2009). The leaves are rich in alkaloids, essential oils, tannins (Ayepola and Ishola, 2009), saponins, cardenolides and others (Onasanwo et al., 2012). In addition, hydrolysable tannins, phenols, flavones, flavonols, xanthones, chalcones, catechins (Santos et al., 2013), terpenoides and other phenolic compounds (Doss and Thangavel, 2011) have also been reported. Cashew shells also contain a significant amount of gallic acid (345.16 ± 16.24 mg) (De Abreu et al., 2013) and their leaves contain cardanol, cardol (Leitão et al., 2013) and palmitate, oleate, linoleate sitosterol,, sitosterol, stigmasterol, 3-O-β-D-galactopyranoside sitosterol, 3-O-β-D-galactopyranoside stigmasterol, 3-O-β- D-glucopyranoside. Stem bark is used for a mixture of sisterol anacardic acids (mono- and diene), alkaloids, tannins and anacardic acids (Chaves et al., 2010).

A. occidentale is known for its analgesic and gastroprotctive activities. The cashew nut extract at a dose of 200 mg/kg was found to have non-ulcerogenic effect on rats (Behravan et al., 2012). A similar activity was observed with the hydroethanolic extract of cashew leaves, where tannins were suggested as being responsible for moieties (Konan and Bacchi, 2007). Vanderlinde et al. (2009) reported that the acetone extract of cashew stem bark in rodents contains antibodies, and has anti-inflammatory and antinociceptive effects. The dichloromethane extract of cashew leaves is also suggested to have an analgesic effect on rats (Onasanwo et al., 2012). The traditional medicine practitioners in Amazon Region are still using cashew for the treatment of diarrhea, dermatitis, headache, and infectious diseases (Lizcano et al., 2010). One study

reported that the methanol extract of cashew stem bark at a dose 200 mg/kg protected mice from lipopolysaccharides induced septic shock (Olajide et al., 2004).

Boiled extract from the new leaves of cashew has for wound healing property (Mazzetto et al., 2009), while the adult leaf extract inhibits the action of the enzyme tyrosinase, demonstrating a therapeutic potential for skin pigmentation problems (Abdul et al., 2008). A recent study showed anti-ulcer actions by the hydro-ethanolic extract of cashew (0.1%), leaving the increased gastric acid section. This demonstrates its anti-*Helicobacter pylori* effect (Ajibola et al., 2010). The aqueous extract of leaves of *A. occidentale* showed hypoglycaemic activity in streptozotocin induced diabetic rats at a dose of 175 mg/kg, where repeated administration of this dose (twice/day) significantly reduced the blood glucose level ($p < 0.01$) by 43% in diabetic rats (Sokeng et al., 2001).

Petroleum ether and ethanolic extracts of cashew leaves showed antimicrobial activity against *Bacillus subtulis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherchia coli*, *Candida albicans* and *Aspergillus niger* (Dahake et al., 2009; Doss and Thangavel, 2011; Onasanwo et al., 2012); the latter extract had more effect on Gram-positive bacteria (Doss and Thangavel, 2011). The CNSL derivative, 2-hydroxy-6-pentadecylbenzamide was more active against *S. aureus*, *E. coli* (Pokharkar et al., 2008), *A. flavus*, *Fusarum* sp., *A. fumigafus*, *A. flavus* and *A. niger* (Kannan et al., 2009). The action of anacardic acid (6-pentadecylsalicylic acid) alone and in combination with methicillin was investigated against methicillin-resistant *S. aureus* (Muroi and Kubo, 1996; Tan and Chan, 2014). There are also reports that anacardiac acid and its newly synthesized benzylamine analogs are antibacterial (Kubo et al., 1999; Reddy et al., 2012) and contain anacardiac acid used against *H. pylori* (Castillo-Juarez et al., 2007).

CNSL at 2000 mg/kg also suggested its anti-*Aedes aegypti* with the median lethal concentration (LC_{50}) by 90% (Guissoni et al., 2013). The salt, sodium anacardate was also found effective against the same species (Farias et al., 2009). However, Oliveira et al. (2011) suggested cardanol ($LC_{50} = 8.20 \pm 0.15$ ppm) as a strong larvicidal agent than the anacardic acid (LC_{50} 12.4 ± 0.10 ppm).

The leaf extract (25-250 mg/ml) as well as processed juice (cajuína) inhibits 1,1-dipheny-picrylhydrazyl (DPPH) radicals (Queiroz et al., 2011). CNSL and its compounds were also proved to have antioxidant potential (Andrade et al., 2011; Oliveira et al., 2011). The antioxidant activity order observed for the cashew components was: CNSL > cardanol = hydrogenated cardanol and alkylated> hydrogenated cardanol (Lima et al., 2008).

Cashew pulp juice and methanol extract of stem bark (500-2000 µg/ml) have antigenotoxic activity against *Salmonella typhimurium* and Chinese hamster lung fibroblasts (V79 cells), respectively (Barcelos et al., 2007a). According to Melo-Cavalcante et al. (2005), cashew pulp (cajuína) protected *S. typhimurium* (TA102) from damage induced by aflatoxin B1 (AFB1), while the methanolic extract of bark (500-2000 µg/ml) of Chinese hamster (V79) in doxorubicin (0.75 mg/ml) induced damage (Barcelos et al., 2007b). In addition, there are reports for anticlastogenic (*in vivo*) (Melo-Cavalcante et al., 2011) and antimutagenic potentials of the cajuína in *S. typhimurium* TA98 (Chen and Chung, 2000). The latter one may relate to its tannic acid (Chen and Chung, 2000).

Toothpastes without fluoride used by children containing cashew and mango were tested for their antimicrobial activity; they significantly inhibited *Streptococcus mutans*, *S. sobrinus* and *Lactobacillus acidophilus* (Carvalho et al., 2011). Otherwise, the inhibition of the microorganism, *S. mutans* and the formation of its biofilm open the door for its application in dental caries (Furtado et al., 2014). The aqueous extract of cashew has hypoglycemic activity (Alexander-Lindo et al., 2004).

Anacardic acid and lunasin, derived from cashew and now seen as having anti-cancer properties, arrest the cell cycle at S mitotic phase (Hsieh et al., 2011). Otherwise, caspase-independent apoptosis inhibition in pituitary adenoma and lung adenocarcinoma cells (Seong et al., 2013) and histone acetyltransferases and nuclear factor kappa B (NF-κB) may be a potential target in chemotherapy (Sung et al., 2008). There are other evidences for its anticancer activity (Schultz et al., 2010; Wu et al., 2011; Huang et al., 2014); where the benzamide derivatives, 2-isopropoxy-6-pentadecyl-N-pyridin-4-ylbenzamide, 2-ethoxy-N-nitrophenyl) -6-pentadecylbenzamide and 2-ethoxy-6-pentadecyl-N-pyridin-4-ylbenzamide strongly inhibit HeLa cell lines (Chandregowda et al., 2009).

An alcohol or its metabolites may lead to hyperacetylation of histone thus overexpression of factor GATA4, which is linked to cardiac malfunctions (Wang et al., 2012). In a recent study, the anacardic acid in pregnant female rats at a dose of 5 mg/kg (intraperitoneal) produced an inhibitory effect on histone H3K9 hyperacetylation induced by alcohol. In addition, a reduced acetylation in the promoter region of the GATA4 fetal hearts of mice was also reported. It was also observed that the abortion rates, stillbirths and intestinal timpanismos decreased in mice, thus demonstrating the cardio-protective activity of anacardic acid (Peng et al., 2014).

Aurora kinase enzymes play an important role in chromosome segregation and cell division. They are of three types: A, B and C (Bischoff and Plowman, 1999). Deregulation of aurora kinase can result in mitotic abnormalities and genetic instability leading to defects in centromere function in chromosome alignment, and cytokinesis (Fu et al., 2007). In several types of cancer, there is a relationship with overexpression of kinase A and B (Murata-Hori and Wang, 2002). Through a virtual

Table 1. Biological activities found in the LCC and its constituents.

Activities	CNSL and its constituents	References
Genotoxic	CNSL	Oliveira Galvão et al., 2014
Antimicrobial	Derived from CNSL (2-hydroxy-6-pentadecylbenzamide), anacardic acid	Pokharkar et al., 2008; Kannan et al., 2009; Tan and Chan, 2014.
Antioxidant	LCCI, CTCL, alkylated and hydrogenated cardanol, cardanol, anacardic acid and its derivative (benzyl amine), urea and thiourea derivative anacardic acid	Andrade et al., 2011; Abreu et al., 2014.
Anticholinesterase	LCCI	Andrade et al., 2011
Larvicidal	CTCL, LCCI, anacardic acid	Andrade et al., 2011; Oliveira et al., 2011; Guissoni et al., 2013.
Sunlight protector (UV_A and UV_B)	LCCT	Romeiro et al., 2006.
Anti-*Helicobacter*	CNSL	Kubo et al., 1999.
Antitumor /anticancer	Anacardic acid, cardol	Stepanenko et al., 2004; Sung et al., 2008; Tocco et al., 2009; Schultz et al., 2010; Wu et al., 2011; Teerasripreecha et al., 2012; Huang et al., 2014; Peng et al., 2014.
Mutagenic and genotoxic	Anacardic acid	Alam-Escamilla et al., 2015;
Non-cytotoxic and genotoxic	Anacardic acid pendacylsalicylic	Alam-Escamilla et al., 2015;
Non-mutagenic	Anacardic acid and anacardic acid methyl ester	Carvalho et al., 2011;
Anti-dermatitis	Cardanol, cardol and anacardic acid	Diogenes et al., 1995
Non-genotoxic	Cardol	Navarro et al., 2014;
Anticholinesterase	Cardol	Oliveira et al., 2009.

evaluation, it was found that anacardic acid could be fitted into the aurora kinase enzyme A and B; and thus, could activate the aurora kinase A-mediated phosphorylation of histone H3 by modifying the structure of the enzyme and increasing its activity (Kishore et al., 2008). The drug sildenafil (VIAGRA) is a potent inhibitor of 5-fosfadiesterase (Terrett et al., 1996). This is the key enzyme used for the regulation of smooth muscle tone, playing an important role in erectile dysfunction (Beavo and Reifsnyder, 1990). A sildenafil analog was synthesized from anacardic acid (Paramashivappa et al., 2002).

However, the resorcinolic lipid (cardol) was also reported for its antimicrobial (Kubo et al., 1999), antitumor, molluscicide, tyrosinase inhibitory (Zhuang et al., 2010), and liposome formation (Przeworska et al., 2001) activities. In addition, it can prevent and repair damage done to DNA (Stepanenko et al., 2004). A recent study indicated that a new resorcinolic lipid, 3-heptyl-3,4,6-trimethoxy-3H-isobenzofuran-1-one (AMS35AA) alone produced neither genotoxic nor mutagenic effects in mice (Navarro et al., 2014). Otherwise, both cardanol and cardol exhibited antiproliferative properties with LC_{50} ranging from 41.3 to 52.4 mg/ml and 43.8 to 53.5 µg/ml in cancer cell lines (Teerasripreecha et al., 2012).

Nowadays, cardol has gained interest (Kubo et al., 1994) along with other natural compounds such as coumarin (Finn et al., 2005) for their inhibitory activity against tyrosinase (Tocco et al., 2009), a multifunctional enzyme that has copper involved in melanin biosynthesis. Tyrosine catalyzes the ortho-hydroxylation of tyrosine to dopaquinone, which spontaneously polymerizes to melanin. Melanogenesis inhibitors are used to whiten the skin of patients treated with pigmentation disorders, such as overproduction of melanin (Hartong et al., 2006) and Addison's disease (Pandya and Guevara, 2000).

Oliveira et al. (2011) found the anticholinesterase activity of CNSL constituents, although cardol, cardanol, carbachol and anacardic acids were previously reported for their cholinesterase inhibitory activity (Rosenberry et al., 2008). Some of the biological activities of LCC and its chemical compounds are presented in Table 1.

OTHER FEATURES AND PROCESSING

India is the pioneer in the CNSL production. Unlike Brazil, their method of processing is semi-automatic, with lower performance generating a lot of CNSL as a byproduct. The cashew agribusiness in Brazil comprises 12 companies (8 in Ceará, 3 in Rio Grande do Norte and 1 in Piauí) focusing on the export of cashew kernels. They

have the capacity to process up to 360 tons of brown, 70,000 tons of almonds and 45,000 tons of CNSL per year (Mazzetto et al., 2009). CNSL is a product considered as having very low value (Rios Façanha et al., 2007). It is purchased for oil processing and then resold at high prices, because of its widespread use in the production of resins and polymers as in the USA and India. However, Ceará State was the only one still standing out in the export of CNSL in the years 2012 and 2013, despite the large bundle in exports in 2013. Among the major producers of Chestnut in the Northeast, Piauí is the only one who did not export LCC (Sindicato Das Indústrias Do Açúcar E De Doces E Conservas Alimentícias Do Estado Do Ceará-Disponível, 2014).

Cashew fruit (nut) is in the form of rim that grows on the end of the pseudo called cashew (Rozas-Muñoz et al., 2012). The cashew nut is characterized as having almond, cotyledon and a liquid. The former one is the edible part, widely consumed as snacks to accompany drinks or ingredients for confectionery and bakery products. The cashew nut contains rich amount of tannins, mono- and polyunsaturated fatty acids, proteins, sugars (Venkatachalam and Sathe, 2006) and others such as (+) - catechin, (-) - epicatechin, β-carotene, lutein, and α-tocopherol (Trox et al., 2011). According to Gómez-Caravaca et al. (2010), anacardic acid is the main component present in raw LCCI and CNSL roasted in extracting the press cold, then followed by cardol, 2-methyl cardol and cardanol. The latter one exists in high amount in the roasted oil. However, the phenolic compounds vary by the roasting temperature applied (Chandrasekara and Shahidi, 2011).

The cashew nut is formed by three protective fabrics: Epicarp outer integument, spongy flesh, whose alveoli can be filled by the CNSL; and cored by inner cavity (Ogunsina and Bamgboye, 2014). The liquid from it is considered as viscous, dark, caustic with long chain saturated and unsaturated phenols, which is a mixture of meta-alkylphenols variably with unsaturated benzene rings (18-27%/nut) (Lomonaco et al., 2009; Velmurugan et al., 2014). The cashew nut trade began in the early 1920s when India was a pioneer in the processing and marketing as an industry. Today, India is the largest cashew producer in the world with a production of 665,000 tons/year (Anonymous, 2009). The CNSL and almond allow for a range of industrial applications including synthetic polymers (Lubi and Thachil, 2000) and bioactive compounds (Paiva et al., 2000).

CONTENTS OF CASHEW AND THE EXTRACTION PROCESSES OF CASHEW LIQUIDS

There are several processes for the production of CNSL: Cold extraction (by presses), solvent extraction, thermal-mechanical process (temperature approximately 190°C) by which CNSL and residual cake were obtained by 18 and 55%, respectively. The husks are heated to 80°C and subjected to pressing subsequently to obtain LCC and a residual cake. When CNSL is subjected to a decarboxylation at 180°C with 15 rpm agitation in a variable time, the anacardic acid turns to cardanol. This liquid is called technical CNSL (CTCL) (Mele and Vasapollo, 2008). Then, the CNSL is filtered and stored in metal drums or tanks (Paiva et al., 2000). The cold LCCI obtained by this process contains anacardic acid, cardanol, cardol and polymeric materials by 62, 6.99-60, 10-23 and 30%, respectively (Lochab et al., 2014); while LCCT is evident to have cardanol (56.24%) and cardol (59.9%) along with other constituents such as phytosterol (10.68%), β-sitosterol (9.22%), stigmasterol (1.46%), triacontanes (4.66%) and anacardic acid (1.79%) (Andrade et al., 2011). The vacuum pyrolysis extraction (temperature: up to 500°C and pressure: at 720 mm of Hg) primarily produces cardanol and cardol. In the extraction with supercritical carbon dioxide (SC-CO_2), it was found that the fraction contained mostly cardanol (70-90%) with traces amount of anacardic acid and cardol. The highest percentage of cardanol (85%) is obtained with 300 bars at 60°C using the SC-CO_2 extraction (Patel et al., 2006).

The phenolic lipids ($C_{15}H_{31}$) are either saturated and unsaturated (Suresh and Kishanprasad, 2005) and the anacardic acid has a carboxylic acid grouping in the ortho position, differing from the other phenolic lipids (Patel and Bandyopadhyay, 2006). According to Bloise et al. (2012), the cardanol consists of a rich amount of phenolic lipids: 3-n-pentadecylphenol (20-30%), 3- (pentadeca-8-enyl) phenol (70 -80%), 3- (pent-deca-8,11-dienyl) phenol (5%), and 3- (pentadeca-8,11,14-trienyl) phenol (< 5%) (Patel and Bandyopadhyay, 2006). The cardol (5-n-pentadecylresorcinolic acid) has a similar structure of tocopherol and a long chain of saturated hydrocarbons or lacks it (Kozubek and Tyman, 1999; Patel and Bandyopadhyay, 2006; John and Vemula, 2006). Among the phenolic compounds, trienes constitute the greatest amount followed by monoenes, dienes (Nishiyama et al., 2000). There are also evidences of xantoproteins, carbohydrates, sitosterol, stigmasterol, β-amyrin lupeol, catechin, epicatechin minerals (e.g. - Na, Mg, P and Ca) and vitamins (e.g. - A, B2, B6 and B12) in the caju chestnut husk (Kannan et al., 2009). However, the climate, geography, botany, origin and the extraction processes also have effect on the chemical composition of the CNSL (Gedam and Sampathkumaran, 1986; Rodrigues et al., 2006; Ologunde et al., 2011).

INDUSTRIAL APPLICATIONS OF THE NET BARK OF CASHEW NUTS

Cardanol is applicable to prepare surfactants, gel, nanotubes and nanofibres; unlike carbon nanotubes, it provides integrity in internal and external surfaces, thus the opportunity for the delivery of biomolecules and therapeutic agents (Balachandran et al., 2013). Otherwise

it has non-aggressive odor, low volatility, high boiling point (Mazzetto et al., 2009), excellent stability at very low temperature (-70°C), good thermal insulation and stability (Attanasi et al., 1996).

The CNSL is now been one of the valuable sources for bio-fuel and can be used directly in diesel engine (Velmurugan et al., 2014; Vallinayagam et al., 2014). Cardanol has also been used as an antioxidant. Derivatives of polymers and resins (Jaillet et al., 2014) can be incorporated as anti-corrosion, waterproof, flame retardants, surface coating, and rubber friction modification materials (Chuayjuljit et al., 2007). The resins obtained from the CNSL are considered as flexible and have greater solubility in organic solvents, thus may be used as resistance to bases and acids (Sadavarte et al., 2009). Cardanol are also used for reducing brittleness and improving the flexibility of the blades (Blazdell, 2000). Both cardol and cardanol have been used in various applications such as adhesives, fuel additive (Suwanprasop et al., 2004; Vasapollo et al., 2011), plasticizing (Alexander and Thachil, 2010), cleaning, disinfectants, germicides and health care for workers (Prabhakaran et al., 2001). Seeds, nirmali (Srimurali et al., 1998) impregnated with zirconium coconut shell (Sathish et al., 2007) and clays have been used as adsorbents for the removal of fluoride from water (Alagumuthu and Rajan, 2010). Evidence shows that United States and Britain during the World War II used CNSL as an insulator of high voltage cables (Gomes, 2010).

Although there are several reports on the biological activity of CNSL and in many non-clinical *in vivo* and *in vitro* trials, its actual cytogenetic activity mechanism prior to recommendation for pharmaceutical formulations and/or cosmetic application is yet to be found out.

CONCLUSION

This review favored the understanding of the similarities between chemical compounds and their biological activities isolated from *A. occidentale*. The reported activities were antioxidant, anti-inflammatory, anti-diarrheal, antinoceptive, anticancer, antimicrobial, antitumor and antimutagenic, which are important for pharmaceutical and cosmetic formulations. Although, a number of in *vivo* and *in vitro* non- / pre-clinical studies were observed during this revision, in order to ensure safety, profile of the chemical moieties isolated from cashew prior to mass manufacturing is crucial. Thus it should be emphasized that this study may be helpful in that noble path-length.

Conflict of interest

The authors have declared that there is no conflict of interests.

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academicJournals

Vol. 15(35), pp. 1864-1870, 31 August, 2016
DOI: 10.5897/AJB2016.15305
Article Number: CAF5B1D60231
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Aflatoxins: A silent threat in developing countries

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Received 25 February, 2016; Accepted 12 August, 2016

Several mycotoxins are known to contaminate crop produce and processed forms but aflatoxins are the most common. They are mainly produced by fungi belonging to the genera *Aspergillus* and *Penicillium*. Cereals and their products which constitute the staples in most developing countries are particularly vulnerable to attack by aflatoxigenic fungi. Despite the potential health risk posed to animals and humans, many people in developing countries are oblivious of the ability of aflatoxins to cause cancer and other debilitating diseases. This review therefore examines the various types of aflatoxigenic fungi and toxins, their occurrence in foodstuffs, their harmful effects, economic losses caused, regulation including the tolerable limits set by various national and international agencies and how their effects can be minimized or eliminated. Since developing countries are less resourced, there is the need for their developed counterparts and international agencies to offer them financial and technical support, to enable them to embark on education, research and other activities and ultimately minimize contamination in their products.

Key words: Aflatoxicosis, fungi, regulation, standards, toxins.

INTRODUCTION

In developing countries, cereals which constitute the staples are susceptible to fungal infections which result in mycotoxin contamination due to poor agronomic and postharvest practices. Mycotoxins are toxic secondary metabolites produced by fungi in agricultural products that are susceptible to mould infestation and can be classified according to their fungal origin, chemical structure and biological activity (Okello et al., 2010). They are commonly produced by fungi belonging to the genera, *Aspergillus*, *Fusarium* and *Penicillium*. The Food and Agriculture Organisation estimates that one quarter of the world's food crops are affected by mycotoxins (CRA, 2011). Mycotoxin production and contamination are unavoidable and depend on a variety of environmental factors in the field and or during storage, which makes it a unique challenge to food safety (Park and Stoloff, 1989). Their occurrence in food is mainly as a result of direct contamination of agricultural commodity and their survival of food processing to some extent. Over 200 mycotoxins have been reported but only those occurring naturally in foods are of significance in food safety.

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Aflatoxins are the most widely studied and dangerous mycotoxins (Okello et al., 2010). They were not well known until
the 1960s when turkey poults died in East Anglia after being fed on pelleted feed containing groundnut meal which was shown to be a toxic constituent (Moss, 2002). Although, aflatoxin contamination is a serious problem in developing countries, it is most common in African, Asian and south American countries with warm and humid climates (Dohlman, 2004). Darwish et al. (2014) reported that aflatoxins are the most common mycotoxins (43.75%) in Africa followed by fumonisin (21.87%), ochratoxins (12.5%), zearalenone (9.38%), deoxynevalenol (6.25%) and beauvericin (6.25%). They reported high levels of aflatoxin in samples collected from several African countries including South Africa, Lesotho, Egypt, Tunisia, Morocco, Sudan, Tanzania, Zambia, Uganda, Kenya, Ethiopia, Nigeria, Ghana, Benin, Mali, Togo and Bourkina Faso.

TYPES OF AFLATOXIGENIC FUNGI AND THEIR TOXINS

Aflatoxins are produced by *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus nomius*, *Aspergillus ochraceoroseus*, *Aspergillus pseudotamarii*, *Aspergillus bombycis*, a species with the imperfect stage, *Emericella venezuelensis* and *Aspergillus niger* (Moss, 2002). Sowley and Baalabong (2013) isolated some aflatoxigenic fungi namely, *A. niger*, *A. flavus* and *A. ochraceus* from grains stored by indigenous methods with *A. flavus* which is the most common producer of aflatoxin (Bankole and Adebanjo, 2003) as the most frequent. According to Ruiqian et al. (2004), *A. flavus* and the closely related species, *A. parasiticus* have a world-wide distribution and normally occur as saprophytes in soil and many kinds of decaying organic matter. Aflatoxigenic fungi produce four major aflatoxins: B1, B2, G1 and, G2 plus two additional metabolic products, M1 and M2, that are of significance as direct contaminants of foods and feeds (Bankole and Adebanjo, 2003; Okello et al., 2010). However, Aflatoxin B1 produced by *A. flavus* and *A. parasiticus* is the major and most common toxin in food; it is among the most potent genotoxic and carcinogenic aflatoxins (EFSA, 2013; Schmalle III and Munkvold, 2015). Aflatoxin M1 is a major metabolite of aflatoxin B1 in humans and animals, which may be present in milk from animals fed with aflatoxin B1 contaminated feed (EFSA, 2013) and may subsequently contaminate other dairy products such as cheese and yogurt (Augusto, 2004).

OCCURRENCE OF AFLATOXINS IN FOODSTUFFS

Aflatoxins have been detected in several foodstuffs of plant and animal origin. They can occur in foods, such as groundnuts, treenuts, maize, rice, figs and other dried foods, spices and crude vegetable oils and cocoa beans, as a result of fungal contamination before and after harvest (EFSA, 2013). Milk, eggs and meat products are sometimes contaminated because of the animal consumption of aflatoxin-contaminated feed. However, the commodities with the highest risk of aflatoxin contamination are maize, peanuts and cotton seed (Anonymous, 2014a).

Maize which is a major staple in most developing countries can easily be contaminated with aflatoxins which have been detected at varying levels. For instance in Ethiopia, Ayalew (2010) detected aflatoxin in 88% of maize samples with concentrations below 5 µg kg^{-1}, except in one sample which had 27 µg kg^{-1}. In Benin, aflatoxin B1 level up to 14 g kg^{-1} and aflatoxin G1 level up to 58 g kg^{-1} were detected in stored maize (Bankole and Adebanjo, 2003). Kpodo (1996) reported that all the maize samples collected from silos and warehouses in Ghana contained aflatoxins at levels ranging from 20 to 355 g kg^{-1}, while fermented maize dough collected from major processing sites contained aflatoxin levels of 0.7 to 313 g kg^{-1}. Hennigen and Dick (1995) detected aflatoxins B1 and G1 in concentrations that varied from 12 to 906 µg kg^{-1} in 34.8% of samples collected from silos and 10 to 14 µg kg^{-1} in 23% of samples collected from maize farms in Rio Grande do Sul, Brazil. Sekiyama (2005) also detected aflatoxins in 3.2% of maize-based food samples in Brazil. Maize samples from Indian communities in which there was an outbreak of aflatoxicosis were contaminated with aflatoxin (0.000625 to 0.0015.6 g kg^{-1}) and the affected people were suspected to have consumed between 2000 and 6000 µg kg^{-1} of aflatoxins daily for a month (Reddy and Raghavender, 2007).

Apart from maize, groundnut is another crop which is widely cultivated, consumed locally and also exported by most developing countries. Just like maize, it is vulnerable to attack by aflatoxigenic fungi. Bankole and Adebanjo (2003) reported that groundnuts cultivated in Northern Nigeria were contaminated with aflatoxin levels up to 2000 g kg^{-1}. Philips et al. (1996) reported that a contaminated groundnut meal used to feed dairy cattle had aflatoxin as high as 3000 µg kg^{-1}. All weanimix samples collected from the Ejura-Sekyedumase district of Ghana, were contaminated with 83.34% above the 20 µg kg^{-1} limit for aflatoxin set by the U.S. Food and Drug Administration (Kumi et al., 2014).

Fresh milk which is often consumed in developing countries without treatment poses a high risk to consumers. Iqbal et al. (2014) reported that aflatoxin M1 was found above the measurable level (0.004 µg l^{-1}) in 64 and 52% of milk samples from urban and rural farmhouses, respectively, in Pakistan. According to them, 99.4% of all samples analysed exceeded the EU limit of 0.05 µg l^{-1}. In Turkey, Polat and Gul (2014) also detected

a mean level of 0.03 µg l^{-1} of aflatoxin in milk produced by animals which were fed with contaminated feed.

Nonconventional food sources can also be contaminated with aflatoxin. For instance, in Nigeria, aflatoxins were detected in bush mango seed samples from which *A. flavus* was isolated (Adebayo-Tayo et al., 2006). The concentration of aflatoxins B1 and G1 which were detected in the bush mango samples ranged between 0.2 and 4.0 µg kg^{-1}, and 0.30 and 4.20 µg kg^{-1}, respectively.

HARMFUL EFFECTS OF AFLATOXINS

People in most developing countries are ignorant about the harmful effects of aflatoxins. This is supported by N'dede et al. (2012) report that the majority of the respondents in Benin did not have any information on aflatoxin contamination of peanut and its harmful health effects on human and animals. Awuah et al. (2008) also reported that the menace caused by aflatoxins was not well appreciated by Ghanaians because it has never been considered as a serious enough issue to merit an awareness campaign. Over four billion people in developing countries are repeatedly exposed to aflatoxins, contributing to greater than 40% of the disease burden in these countries (Schmalle III and Munkvold, 2015). The impact of aflatoxin on health has been supported by experiments in China and African countries which have a high incidence of the hepatitis B infection where dietary exposure to aflatoxin was prevalent. Exposure to aflatoxin is widespread in West Africa, probably starting in utero, and blood tests have shown that very high percentage of West Africans are exposed to aflatoxins. In a study carried out in the Gambia, Guinea Conakry, Nigeria and Senegal, over 98% of subjects tested positive to aflatoxin markers (Wild, 1996).

Serious fatalities can result from the consumption of contaminated produce due to the toxic nature of aflatoxins. For instance, a serious fatality occurred in India in 1974 when nearly 1000 people fell ill while over 100 died following the consumption of contaminated maize (Moss, 2002). The health conditions caused by aflatoxins are varied and depend on the level and length of exposure. Some of the health problems posed by aflatoxin contamination include aflatoxicosis, cancer, infertility, hepatocellular carcinoma, liver cirrhosis, nephropathy, immunodeficiency, anaemia, stunting, underweight in humans and nutritional interference (William et al., 2004; Darwish et. al., 2014).

Among the health problems caused by aflatoxin contamination, aflatoxicosis is one of the most serious ones. This is confirmed by Bommakanti and Waliyar (2012) report that the condition has been experienced in many countries including China, India, Thailand and in several African countries, which are all in the developing world. In Kenya, aflatoxicosis accounted for the death of 125 out of several hundred people who became severely ill from the consumption of food contaminated with aflatoxins (Lewis et al., 2005; Strosnider et al., 2006). The Malaysian state of Perak experienced an outbreak of aflatoxicosis in 1988 which killed 13 children who consumed noodles contaminated with up to 3 mg of aflatoxin (Mohd-Redzwan et al., 2013).

Cancer is another serious condition resulting from the consumption of food contaminated with aflatoxins. Among aflatoxins B1, B2, G1, and G2 which have been listed as group I carcinogens and are said to be the cause of hepatotoxicity in developing countries (CRA, 2011), aflatoxin B1 is the most potent and commonly occurring and has also been recognized as a teratogen, mutagen, hepatocarcinogen, immunosupressant and a potent inhibitor of protein synthesis. According to Augusto (2004), aflatoxin M1, just as toxic as aflatoxin B1 is listed as a Group 2B carcinogen by the International Agency for Research on Cancer. Groopman et al. (1988), also reported that epidemiological, clinical and experimental studies have revealed that exposure to large doses (>6000 mg) may cause acute toxicity with lethal effect, whereas exposure to small doses for prolonged periods is carcinogenic.

Apart from humans, aflatoxins are highly toxic to livestock and poultry (Cassel et al., 2012). Consumption of low concentrations by animals sensitive to aflatoxins can lead to death in 72 h and at nonlethal levels, the health and productivity of animals fed contaminated feed are seriously impaired (Cassel et al., 2012). In 1966, the first outbreak of aflatoxicosis in India occurred in the Mysore state resulting in the death of 2219 chicks. A worse incident occurred in the Chittoor district of Andhra Pradesh in 1982 resulting in heavy mortality in chicks with one hundred percent mortality in commercial farms.

FACTORS THAT PREDISPOSE CROPS TO AFLATOXIN CONTAMINATION

Field and postharvest practices can predispose crop produce to aflatoxin contamination. The risk of contamination is greater in developing countries where peasant farmers who constitute the majority face financial challenges and have little or no access to improved technology. The factors that influence mycotoxin production are either biological (biotic), environmental (abiotic) or nutritional (Diener and Davis, 1966; Okello et al., 2010). Some of the biotic factors include cultivar susceptibility and growth stage, insect and bird damage and presence of other fungi or microbes and strain variation in the fungus while abiotic factors include mechanical damage, moisture, temperature, pH and other crop stresses such as drought, soil type, suitability of substrate, excessive rainfall, gaseous exchange and

gaseous environment and preservatives and crowding of plants (CAST, 1989; CRA, 2011; Suttajit, 1989; Robens, 1990; William et al., 2004). Nitrogen stress is another biotic factor which can also predispose crops to aflatoxin contamination. Most of the factors enumerated above are beyond the control of farmers in developing countries. For instance, unpredictable rainfall which is worsened by climate change makes crops grown in developing countries more prone to water stress and therefore a higher risk of aflatoxin contamination. Also, due to lack of access to improved technology, farmers in developing countries cannot test soils to determine their physicochemical characteristics before cropping.

ECONOMIC LOSSES

Developing countries suffer most from impact of enforcement of regulation by European and international agencies, particularly the former which is a major importer of agricultural commodities from developing countries. The economic losses to developing countries are varied. The losses do not only arise from crop and livestock losses but also from costs associated with regulatory compliances (CRA, 2011). For instance, Bankole and Adebanjo (2003) reported that as a result of regulation, exports of agricultural products particularly groundnuts from developing countries had dropped considerably resulting in major economic losses to producing countries. Losses from rejected shipments and lower prices for inferior quality can devastate developing country export markets (Bhat and Vasanthi, 2003). In 2011, Argentina, China, India and South Africa experienced 37, 60, 136 and 12 rejections, respectively (Codex Alimentarius Commission, 2014). The World Bank predicted that, policy change by the EU will reduce by 64% imports of cereals, dried fruits and nuts from African countries like Chad, Egypt, Gambia, Mali, Nigeria, Senegal, South Africa, Sudan and Zimbabbwe, and thus cost African countries about US$670 million in trade per year (Bankole and Adebanjo, 2003).

Wu et al. (2011) reported that, the magnitude of the economic impacts of the health consequences associated with consumption of aflatoxin-contaminated food in developing countries is not known due to a lack of good data. According to them, the quantification of economic losses and estimation of the effects of aflatoxin on health will encourage Health Ministries to enforce standards and provide crucial advocacy to benefit the rural poor, such as improving their level of education about aflatoxin exposure. The toll of the effects on human health includes the cost of mortality, the cost of productive capacity lost when people die prematurely, the cost of morbidity, losses resulting from hospitalization and the cost of health care services, both public and private. There is intangible cost of pain, suffering, anxiety and reduction of the quality of life (Bhat and Vasanthi, 2003).

According to Otsuki et al. (2001), compliance requirements on exporters impose costs on developing countries, such as the cost of upgrading production systems, processing and storage equipment, and quality control stations. The FAO has also highlighted a number of compliance problems which include lack of funds allocated to research on aflatoxins, scarcity of highly trained and experienced personnel, inadequate facilities for safe aflatoxin research, lack of maintenance of laboratory facilities and inadequate infrastructure (FAO-WHO, 1997). Contamination of maize, a staple in developing countries reduces its economic value which can result in large monetary losses and lead to the removal of large amounts from the market as a result of stringent regulatory limits (Riley and Norred, 1999).

REGULATION AND TOLERANCE LIMITS

Due to the potential health hazards for humans, threshold levels of aflatoxins in commodities have been established worldwide (Augusto, 2004). In 2003, FAO reported that 15 countries were known to have specific mycotoxin regulations but by 2011, the number had risen to 99 with some having specific regulations for aflatoxin B1 or M1 in milk (CRA, 2011). For majority of the African countries, specific mycotoxin regulations seem to be lacking but several of these countries recognize that they have problems due to mycotoxins and that regulations should be developed (FAO, 2003). The maximum limits for aflatoxins in foodstuffs are the single most commonly established mycotoxins limits worldwide. The limits for aflatoxins may be controlled as the total aflatoxins referring to the sum of aflatoxin B1, B2, G1, G2 and or aflatoxin B1 (Kubo, 2012).

The limits or standards set by various national and international agencies are varied as shown by the ensuing examples, some of which consider only total aflatoxins while others consider both total and aflatoxin B1. The European Union aflatoxin tolerance standards are 2 μg kg^{-1} aflatoxin B1 and 4 μg kg^{-1} total aflatoxins (B1, B2, G1 and G2) for peanuts, nuts, dried fruits and cereals for direct human consumption (Augusto, 2004). It appears European standards are more stringent than those of the United States which has an action level of 20 μg kg^{-1} for human food except milk (FAO, 1996; Schmalle III and Munkvold, 2015). In Europe, the maximum levels of aflatoxin M1 in milk meant for adult consumption and milk meant for infants or baby food production are 0.050 and 0.025 μg kg^{-1}, respectively (Iqbad et al., 2014) while that for the United States is 0.05 μg kg^{-1} (Augusto, 2004).

Food production systems in developing countries do not favour the implementation of international regulations such as those set by Codex Alimentarius Commission to

regulate the amounts of aflatoxin in food (William et al., 2004). As a result, there is a higher risk of exposure in developing countries because where there is trade, the least contaminated foods and feeds are exported and the more highly contaminated products are retained at home for consumption. It is therefore not surprising that African countries are greatly concerned about the standards imposed on their exports. This situation was aggravated by the use of different regulations by various developed countries, but these concerns were partly addressed through the harmonization of aflatoxin standards by the European Union, which eventually took effect in April 2002 (EU Commission Regulation No. 466/2001, 2001). As part of the harmonisation process, in 1997, the maximum acceptable limit of 10 μg kg^{-1} for groundnuts subject to further processing and 4 μg kg^{-1} in groundnuts intended for direct consumption were amended in 1998 to 15 μg kg^{-1} (8 μg kg^{-1} for aflatoxin B1) for the groundnut subject to further processing and 4 μg kg^{-1} (2 μg kg^{-1} for aflatoxin B1) for groundnuts intended for direct consumption (EU Commission Regulation No. 1525/98, 1998a). Although, the harmonization of aflatoxin standards in EU member countries seemed to have alleviated the situation, developing countries faced greater challenges because the new EU standards were more stringent than those set by Codex Alimentarius Commission, the United States and other countries such as Australia. For instance, while EU standards for total aflatoxins in groundnuts for processing and consumption are 15 and 4 μg kg^{-1}, respectively, those set by Australia are 15 and 5 μg kg^{-1}. The USA has an even lower standard of 20 μg kg^{-1}. The Codex Alimentarius standard is more considerate because it has no separate standard for aflatoxin B1 based on the assumption that 70% or about 10 μg kg^{-1} of the total aflatoxin level of 15 μg kg^{-1} is accounted for by aflatoxin B1. Fortunately, the EU regulation on aflatoxins agrees with existing Codex Alimentarius maximum aflatoxin level, but the EU standards cover more products and have separate maximum levels for aflatoxin B1 (Anonymous, 2010).

PREVENTION AND MANAGEMENT OF AFLATOXIN CONTAMINATION

According to Suttajit (1989), prevention of aflatoxin contamination can be primary, secondary or tertiary. The primary prevention is considered as the most important and most effective for reducing fungal growth and mycotoxin production. Some of the primary prevention practices include development of plant varieties resistant to fungi, lowering moisture content of seed after harvest and during storage, storing commodities at low temperature, application of fungicides and preservatives and control of insect infestation in stored bulk grains with approved insecticides. Secondary prevention includes re-drying of products, removal of contaminated seeds, inactivation or detoxification. Tertiary prevention involves complete destruction of contaminated products and detoxification or destruction of mycotoxins to the minimum level. Some methods of preventing aflatoxin contamination include education and extension, rapid drying, physical separation, smoking, use of plant products, biological control, detoxification, seminars and workshops, adoption of good agronomic practices, early harvesting, sanitation, use of improved storage structures, synthetic chemicals, resistant varieties and fumigation (Bankole and Adebanjo, 2003). Bhat and Vasanthi (2003) proposed good agricultural practices such as rotating crops, irrigating to eliminate drought stress, controlling weeds, cultivating mould-resistant stocks and introducing biocontrols such as non-mycotoxigenic fungal strains. They also suggested that drying rapidly by mechanical means and keeping crops dry, sorting out contaminated nuts by physical means; sorting by color, and washing with water, the use of chemical methods of detoxification such as ammoniation, application of chemicals like oltipraz and chlorophyllin, physical sorting of contaminated grains or nuts and change of diets by individuals to avoid risky foods such as maize could reduce exposure to aflatoxins. Cassel et al. (2012) recommended that aflatoxin contamination can also be prevented by keeping storage and feeding facilities clean. According to them, aflatoxin contaminated feed can be tolerated by some livestock particularly older animals but the risk becomes greater with increasing levels of contamination. They maintained that feed additives including organic acids like propionic, sorbic and benzoic acids and their salts such as calcium propionate, potassium sorbate and copper sulphate inhibit mould growth in feed. Minerals such as zeolite and bentonite as well as hydrated sodium calcium aluminosilicate (HSCAS) can protect animals by binding to any aflatoxin that may be present in feed. The Codex Alimentarius Commission (2014) also recommended the implementation of good agricultural practices (GAP) and good manufacturing practices (GMP) by producers.

Control of mycotoxin in Africa is a matter of importance not only for health implications, but also for improvement of the economy in the affected countries. According to Darwish et al. (2014), a number of strategies for reduction and control of mycotoxins have been considered in different African countries. These include prevention of mould growth in crops and other feedstuffs, decontamination of mycotoxin-contaminated foods and continuous surveillance of mycotoxins in agricultural crops, animal feedstuffs and human food. Other control measures that have been tried in some African countries include segregation of contaminated peanuts in Malawi, detoxification of peanut meal for export in Senegal, regulation of mycotoxins in animal feed according to the susceptibility of the animal species in Zimbabwe,

selection of peanut varieties less susceptible to aflatoxin contamination in Bourkina Faso and improvement in produce-handling practices during the 1960s in Nigeria and the 1990s in The Gambia (Bhat and Vasanthi, 2003). According to Cassel et al. (2012), time of harvest is important in influencing the occurrence and levels of aflatoxin. For instance, harvesting maize above 20% moisture content followed by rapid drying to at least 14% within 24 to 48 h of harvest checks the growth of *Aspergillus* spp. and minimizes aflatoxin production. Chulze (2010) reported that it is possible to control aflaoxins in stored commodities by controlled atmospheres, preservatives or natural inhibitors; the use of antioxidants and essential oils is possible but the cost can be prohibitive on a large scale.

In recent times, there have been initiatives aimed at controlling aflatoxins in developing countries, especially Africa. One of such initiative is the Partnership for Aflatoxin Control in Africa (PACA), which is based on a Memorandum of Understanding that was signed between the African Union Commission and Mars Incorporated, aimed at sharing food safety resources and expertise to control aflatoxins in food crops which constitutes a significant threat and a major deterrent to use of key African raw materials in global supply chains (African Union Commission, 2015). Another initiative is the aflatoxin control in maize and peanuts project, which is aimed at developing and implementing holistic strategies to address aflatoxin contamination in maize and peanuts including developing and scaling up biological control technology interventions to improve the health and income of farmers and their families and generate wealth in the crop value chain (African Agricultural Technology Foundation, 2015). The project is funded by Bill and Melinda Gates Foundation and African Agricultural Technology Foundation (AATF) through the IITA and UK aid from the UK government, respectively.

CONCLUSION

It is obvious that impoverished and less privileged people of developing countries stand an even greater risk of further impoverishment and starvation, if stringent measures are not applied for the management of aflatoxin contamination. Implementation of recommended prevention and control strategies could make food more expensive and less affordable, since farmers will have to invest in drying and storage equipment among others. Their plight is worsened by the absence of laboratories for testing foods which are economically and financially inaccessible. However, it will be better to ensure that contamination levels are minimal to safeguard the health of people in developing countries whose lifespan is relatively short. The plight of people in developing countries is worsened by the fact that international bodies like the World Health Organisation (WHO) do not consider aflatoxin as a high-priority risk; hence, little attention is paid to the health issues resulting from the consumption of contaminated food.

Developed countries and international agencies such as the FAO and WHO should provide the necessary financial and technical assistance to enable developing countries to carry out research and education. This would ultimately inure to the benefit of developing countries in terms of increased foreign exchange earnings, from the sale of products that meet required standards and better health through the consumption of safer food, devoid of or containing minimal levels of aflatoxins.

Conflict of interests

The author has not declared any conflicts of interests.

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academicJournals

Vol. 15(35), pp. 1871-1876, 31 August, 2016
DOI: 10.5897/AJB2016.15450
Article Number: F690D2E60233
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Phylogenetic analysis of 23S rRNA gene sequences of some *Rhizobium leguminosarum* isolates and their tolerance to drought

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Received 4 May, 2016; Accepted 3 August, 2016

The phylogenetic relationships among thirteen *Rhizobium leguminosarum* bv. *viciae* isolates collected from various geographical regions were studied by analysis of the 23S rRNA sequences. The average of genetic distance among the studied isolates was very narrow (ranged from 0.00 to 0.04) and the studied isolates formed two main groups based on cluster analysis. The isolates were tested for their growth on YMA medium supplemented with concentrations 10, 20 and 30% of polyethylene glycol plus control. All isolates exhibited good drought-tolerant efficiencies at 10% PEG. While most of the isolates could not tolerate up to 20% PEG, isolates of Rlv6, Rlv9, Rlv12 and Rlv13 tolerated up to 20% PEG.

Key words: *Rhizobium leguminosarum*, 23S rRNA gene, phylogenetic tree, diversity and drought tolerance.

INTRODUCTION

Rhizobia are gram-negative bacteria that can establish a symbiotic relationship with the roots of leguminous plants and form root organ called nodule, wherein bacteria is hosted and nitrogen is fixed. The classification of the bacteria belonging to the genus of *Rhizobium* was based on plant infection abilities for a long time (Van Berkum and Eardly, 1998). Recently, The rRNA genes are very useful for investigating the phylogenetic relationships among bacteria since their evolution are slow and their gene products performs central functions in the bacterial cell (Pulawska et al., 2000). 16S rRNA gene was the most frequently used in microbial phylogeny studies (Kolbert and Persing, 1999, Ismail et al., 2013; El-Zanaty et al., 2014). Previous studies showed that the 23S rRNA gene contains more genetic information and better diagnostic sequence stretches than the 16S rRNA gene. Moreover, the progress in sequencing tools and the decrease in their costs make the 23S rRNA powerful tool to elucidate the taxonomic relationships (Pulawska et al., 2000; reviewed in Hunt et al. (2006) and Pei et al. (2009). Wolde-meskel et al. (2005) investigated the genetic diversity among 195 rhizobial strains isolated from root nodules of 18 agroforestry species growing in diverse zones in southern Ethiopia using PCR–RFLP of the ribosomal operon (16S rRNA gene, 23S rRNA gene and the internal transcribed spacer (ITS) region between

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them).

The environmental stresses such as salinity, drought and heavy metals can suppress the growth and symbiotic characteristics of most rhizobia (Duzan et al., 2004; Gálvez, 2005). Drought is the main abiotic factor that affects survival and growth of soil microorganisms. Moreover, drought stress has negative effects on plant growth and metabolisms (Ehsanpour and Amini, 2003; Aydi et al*.,* 2008; Fall et al., 2011). It was suggested that drought limits nodulation through its effects on persistence and survival of rhizobia in the soil, root-hair colonization and infection by rhizobia (Zahran, 1999; Vriezen et al*.,* 2007; Mhadhbi et al*.,* 2008). Therefore, isolation of rhizobia strains tolerant to stresses like drought is necessary for efficient nitrogen fixation and improving plant productivity especially in the water limited areas (Abd El-Halim et al., 2001; Abdel-Salam et al., 2002; Diouf et al., 2007). Athar and Johnson (1996) reported that nodulation, growth and nitrogen fixation in alfalfa can be improved by inoculating plants with competitive and drought tolerant rhizobia. In addition, it was demonstrated that, selecting stress-tolerant cultivars and stress-tolerant rhizobia could be a rational strategy to improve the yield of legumes in stressed environments (Ben Rhomdhane et al., 2007; Meuelenberg and Dakora, 2007; Mhadhbi et al., 2008). Therefore, the objectives of this study were to: 1) analyze the phylogenetic relationships among thirteen isolates of *Rhizobium leguminosarum* bv. *viciae* obtained from various geographical regions in Egypt by analysis of the 23S rRNA sequences; 2) characterize the isolates of *R. leguminosarum symbiovar vicia faba* according to their tolerance to drought.

MATERIALS AND METHODS

Rhizobium isolation

Rhizobial isolates used in this study were isolated from nodules of *Faba bean* plants nodules growing in different Egyptian regions (Table 1) as described previously by Hewedy et al. (2014).

Table 1. Nomination and location of studied *Rhizobium* isolates.

Isolate	Geographical origin
Rlv1	Quesna City, Menoufia Governorate
Rlv2	Banha City, Qalyoubia Governorate
Rlv3	Sadat City, Menoufia Governorate
Rlv4	Zefta City, Gharbia Governorate
Rlv5	Beni-Suef City, Beni-Suef Governorate
Rlv6	Suez City, Suez Governorate
Rlv7	El-Tor city, South Sinai Governorate
Rlv8	Al Arish City, North Sinai Governorate
Rlv9	Rafah City, North Sinai Governorate
Rlv10	New Al Menia City, Menia Governorate
Rlv11	Moshtohor City, Qalyoubia Governorate
Rlv12	Cairo City, Cairo Governorate
Rlv13	Ismailia City, Ismailia Governorate

DNA extraction

The bacterial cultures of isolates were grown in Yeast Extract Manitol medium (YEM) and extraction of total DNA was performed using GeneJET™ Genomic DNA Purification kit (Fermentas, Vilnius, Lithuania) following manufacturer's instructions. Samples were kept at -20°C until using in PCR reactions.

PCR amplification of 23S rRNA

The amplification of 23S rRNA partial gene(s) of tested isolates was done using 23S forward and reverse primers designated from NCBI site (http://www.ncbi.nlm.nih.gov/). The forward primer was 5′TGG GCA CTG TCT CAA CGA ′3**,** while the reverse primer was 5′GGA TAG GGA CCG AAC TGT CTC 3. The polymerase chain reaction (PCR) was performed in 50-µL reaction volume containing 100 ng DNA, 50 µL PCR Master Mix (Fermentas, Lithuania) and 20 µM of forward and reverse primers. Amplifications were performed with the following PCR conditions: i) initial denaturation at 95°C for 5 min, ii) 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s and 10 min final extension at 72°C.

23S rRNA partial gene sequencing

The PCR products for the thirteen isolates were used in sequencing of the 23S rRNA gene from both strands using the same primers used in PCR amplification and Big Dye Terminator v3.1 cycle sequencing kit at Bioneer (Daejeon, Korea).

Drought tolerance

The effect of drought on rhizobia-growth was examined in YMA medium supplemented with concentrations 10, 20 and 30% of polyethylene glycol (PEG 6000). One hundred microliters of suspension (1×10^{-8} cfu/ml) of overnight grown cultures of studied isolates were transferred to petri dishes containing YMA medium supplemented with the previous PEG concentrations. After incubation at 28°C for three days, the bacterial growth was determined by counting the number of colonies formed.

Data analysis

The reference 23S rRNA gene of *R. leguminosarum* (AF207785.1) was retrieved from NCBI site (http://www.ncbi.nlm.nih.gov/). The alignment, genetic diversity and phylogenetic analyses were conducted using MEGA version 6 (Tamura et al., 2013).

Two ways ANOVA was used to analyze the data of drought tolerance at 0.05 levels. Descriptive statistics, GLM and Duncan test at 0.05 were calculated by SAS computer program (SAS, 2004).

RESULTS AND DISCUSSION

Amplification and sequencing of 23S rRNA gene

This work presents study on the genetic diversity of

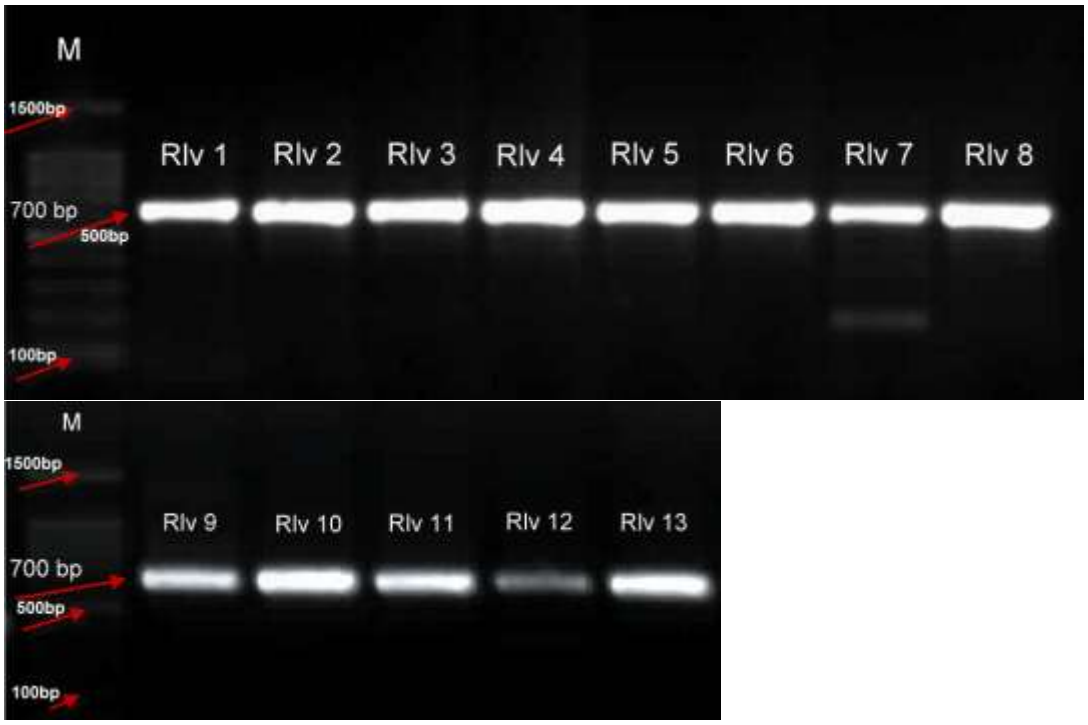

Figure 1. Partial amplification of 23S rRNA gene in *Rhizobium leguminosarum symbiovar. Viciae* Isolates (From Rlv1 to Rlv13); M, GelPilot 100 bp Plus Ladder (Qiagen, cat. no. 239045).

thirteen isolates of *R. leguminosarum* bv. *Viciae* collected from different *Vicia faba* fields and representing several governorates in Egypt (Table 1). Since, the structure of ribosomes is largely conserved among the three kingdoms of life organisms and the horizontal gene transfer events are unlikely to occur in the highly constrained rRNA genes, these advantages make the phylogenetic analysis and taxonomic classification of cellular organisms based on rRNA genes very ideal (Pei et al., 2009). The 23S rRNA partial gene was amplified using 23S rRNA primers which were designed on NCBI site. All of isolates yielded a single-fragment about 700 bp as indicated in Figure 1. After amplification, the same primers were used for partial sequencing of 23S rRNA gene. The alignment was done for all isolates sequences with the reference 23S rRNA gene of *R. leguminosarum* (AF207785.1) published in NCBI (http://www.ncbi.nlm.nih.gov/). The genetic diversity was estimated based on the number of base pairs substitution per site among all isolates using mega program version 6 (Tamura et al., 2013) as shown in Table 2. The phylogenetic relationship analysis using UPGMA method, divided the isolates into two principal groups (Figure 2); the first group included the isolates Rlv6, Rlv8 and Rlv9, while the second group contained isolates of Rlv1, Rlv2, Rlv3, Rlv4, Rlv5, Rlv7, Rlv10, Rlv11, Rlv12 and Rlv13. Although, the tested isolates were collected from different geographic zones in Egypt, the values of genetic distances among these isolates were very low and ranged from 0.00 to 0.04 (Table 2). This may be due to the high conserved nature of the 23S rRNA gene sequences (Pei et al., 2009), so the sequences variability is limited and, second, it is possible that these isolates have originated from the same genetic background and the human activities like soil and plant transfer limited the genetic diversity of these isolates (Ismail et al., 2013).

Analysis of drought tolerance

The rhizobial isolates were tested for their drought tolerance in YMA medium supplemented with increasing concentrations of PEG 6000 (10, 20 and 30%) and evaluated as tolerant and sensitive based on the number of colonies formed. In general, all isolates showed good drought-tolerant efficiencies at 10% PEG, while most of them could not tolerate up to 20% PEG (Table 3). These results are in agreement with a previous study which showed that most rhizobial strains, which nodulate important crops, are very sensitive to drought and abiotic factors such as high salt, pH, and temperature (Rehman and Nautiyal, 2002). The results showed that nine isolates (Rlv1, Rlv2, Rlv3, Rlv4, Rlv5, Rlv7, Rlv8, Rlv10 and Rlv11) could not tolerate 20% PEG concentration and were considered as sensitive to drought stress. In contrast, the isolates Rlv6 of Suez, Rlv9 of Rafah, Rlv12 of Cairo and Rlv13 of Ismailia succeeded to grow on 20% of PEG and are considered as tolerant. It was shown that

Table 2. Genetic distance divergence between isolates sequences based on base substitution, analysis conducted using the Maximum Composite Likelihood model using MEGA6 program (Tamura et al., 2013).

Isolate	Rlv1	Rlv2	Rlv3	Rlv4	Rlv5	Rlv6	Rlv7	Rlv8	Rlv9	Rlv10	Rlv11	Rlv12	Rlv13
Rlv1	0.00												
Rlv2	0.01	0.00											
Rlv3	0.00	0.01	0.00										
Rlv4	0.01	0.01	0.01	0.00									
Rlv5	0.00	0.01	0.00	0.01	0.00								
Rlv6	0.04	0.04	0.04	0.03	0.04	0.00							
Rlv7	0.00	0.01	0.00	0.01	0.00	0.04	0.00						
Rlv8	0.04	0.04	0.04	0.03	0.04	0.00	0.04	0.00					
Rlv9	0.04	0.03	0.04	0.02	0.04	0.01	0.04	0.01	0.00				
Rlv10	0.00	0.01	0.00	0.01	0.00	0.04	0.00	0.04	0.04	0.00			
Rlv11	0.01	0.00	0.01	0.01	0.01	0.04	0.01	0.04	0.03	0.01	0.00		
Rlv12	0.01	0.02	0.01	0.02	0.01	0.03	0.02	0.03	0.02	0.01	0.01	0.00	
Rlv13	0.00	0.01	0.00	0.01	0.00	0.04	0.00	0.04	0.04	0.00	0.01	0.01	0.00

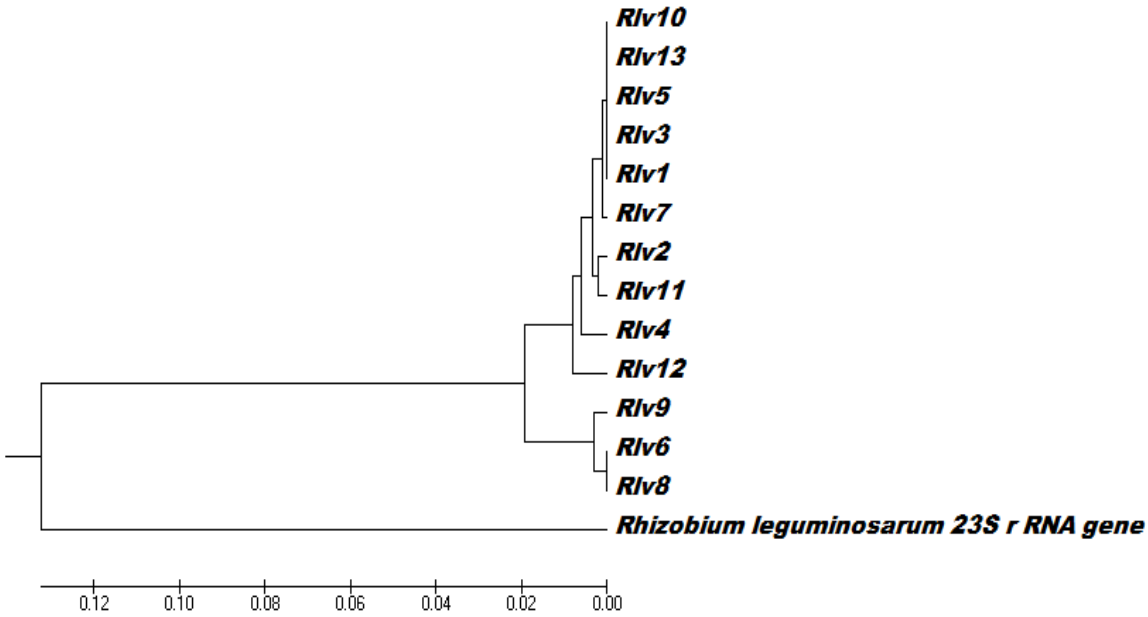

Figure 2. Phylogenetic relationship between 13 rhizobial isolates using UPGMA method and MEGA6 program based on 23S rRNA gene sequences data.

Table 3. Rhizobial isolates growth (Number of colonies) in YMA medium supplemented with increasing doses of PEG 6000 ranged between 10 to 30%.

Isolate	YMA medium supplemented with PEG 6000			
	Control (0%)	10%	20%	30%
Rlv1	72.66^{de}	36.33^{c}	0.00^{c}	0.00
Rlv2	111.33^{ab}	63.33^{ab}	0.00^{c}	0.00
Rlv 3	70.66^{de}	50.00^{bc}	0.00^{c}	0.00
Rlv4	99.33^{bc}	54.00^{bc}	0.00^{c}	0.00
Rlv 5	92.33^{bcd}	59.66^{abc}	0.00^{c}	0.00
Rlv 6	112.66^{ab}	67.00^{ab}	2.33^{b}	0.00

Table 3. Contd.

Rlv7	57.66^{e}	47.00^{bc}	0.00^{c}	0.00
Rlv8	84.66^{cd}	56.00^{abc}	0.00^{c}	0.00
Rlv9	96.66^{bc}	56.33^{abc}	3.66^{b}	0.00
Rlv10	98.66^{bc}	68.00^{ab}	0.00^{c}	0.00
Rlv11	82.00^{cd}	53.66^{bc}	0.00^{c}	0.00
Rlv12	96.00^{bc}	63.33^{ab}	3.33^{b}	0.00
Rlv 13	131.00^{a}	80.00^{a}	8.00^{a}	0.00

* Means within classification followed by different letters are significantly different (Duncan, 0.05 level).

the isolate RLV13 of Ismailia gave the best growth in presence of PEG then the isolates; Rlv6 of Suez, Rlv9 of Rafah, Rlv12 of Cairo respectively Table 3. The failure of rhizobia isolates to grow at the highest doses of PEG is due to PEG reduces water availability by binding water molecules without penetrating the cell wall (Rehman and Nautiyal, 2002; Rasanen et al., 2004; Cytryn et al., 2007; Abdel-Salam et al., 2010). Moreover, the osmotic stress can affect negatively on Rhizobia by changing their morphology, survival in soil, root-hair colonization, infection and dehydration of cells (Niste et al., 2013). On the other hand, the success of isolates Rlv6, Rlv9, Rlv12 and Rlv13 to grow on 20% of PEG may be due to acquiring some mechanisms or pathways for drought tolerance through their growth in water limited soils. These results are supported with previous review suggesting that Rhizobia having some key tolerance mechanism/pathways against certain abiotic stresses (Gopalakrishnan et al., 2015) and with other observations illustrated the roles of rhizobia in adaptation of crops to various abiotic stresses (Yang et al., 2009; reviewed by Grover et al., 2010).

Conflict of interests

The authors have not declared any conflict of interest.

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academicJournals

Vol. 15(35), pp. 1877-1882, 31 August, 2016
DOI: 10.5897/AJB2016.15399
Article Number: 86E37DD60235
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Resistance of corn genotypes to fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Received 13 April, 2016; Accepted 17 August, 2016

The objective of this study was to evaluate resistance mechanisms in 12 corn genotypes (transgenic hybrids: 30A91 PW, 20A78 HX, Impacto VIP 3, 20A55 HX, NS90 PRO 2, Maximus VIP 3, BX 1293YG, RB 9004 PRO, Feroz VIP 3, LG 6036 PRO; conventional: AG 1051 and variety: AL Bandeitante). Attractiveness, aversion to feeding, and antibiosis were evaluated via free and no choice tests in the laboratory. Attractiveness was evaluated at 1, 5, 10, 15 and 30 min and 1, 2, 6, 12 and 24 h by counting the number of larvae that fed on each genotype. The preference for feeding was determined by quantifying the leaf area of each genotype consumed. Antibiosis was determined by assessing biological parameters of the fed caterpillars in relation to each genotype. The biological parameters evaluated were (a) Larval stage: the viability of the larval stage and weight of larvae at ten days; and (b) pupal stage: the viability and weight of pupae at 24 h of age. After emergence, the moths were fed and evaluated to assess the longevity of the adults and the total life cycle. The transgenic genotypes NS90 PRO2, Maximus VIP 3, Feroz VIP 3 and Maximus VIP 3 elicited an aversion and/or an antibiosis reaction from fall armyworm (FAW). The transgenic genotypes HX 20A55, 30A91 PW, LG 6036 PRO, 20A78 HX and BR 9004 PRO showed a moderate resistance to FAW. The conventional genotypes AG 1051 and AL Bandeirante were highly susceptible to FAW and the transgenic genotype BX 1293 YG was susceptible to FAW.

Key words: Integrated pest management, transgenic crop, plant resistance to insects, *Zea mays*.

INTRODUCTION

Many insects cause damage to corn and often damage the entire plant from the roots to the shoots. The fall armyworm (FAW) *Spodoptera frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae) is a polyphagous species and is considered the primarily pest of this crop (Fernandes et al., 2003; Lima et al., 2006; Mendes et al., 2011). The damage arises from the continual consumption of leaf tissue by the first instars through older larvae, which often cause extensive defoliation and the complete destruction of the plant. Plant cutting and feeding on the tassel

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and ear has also been observed. The reduction in the productivity of corn caused by FAW can reach 60%, depending on the genotype and growth stage of the plant where the damage was caused (Cruz et al., 2008).

The control of FAW has been achieved mainly using chemical insecticides that have an adverse effect on the environment and may promote the selection of resistant insects (Lima et al., 2006). The use of host plant resistance (HPR) has been studied in recent years and has the potential for use as a strategy to control to FAW in corn crops. HPR is a desirable tactic because it is compatible with other control methods and often exhibits synergistic effects with insecticides and natural enemies (Azevedo et al., 2002; Janini et al., 2011; Jesus et al., 2014).

The resistance mechanism in corn plants occurs via antibiosis when the negative effects of a resistant plant affect the biology of the insect pest utilizing the plant as a host (Smith, 2005). The effects of an antibiotic plant may range from mild to lethal and are the result of either chemical or morphological plant defenses. Antixenosis is characterized by the presence of morphological or chemical plant factors that adversely alter insect behavior. As a result, the insect may search for an alternate host plant. The plant responds to pest damage by building a tolerance, producing new vegetative and reproductive structures (Smith, 2005; Seifi et al., 2013).

Antibiosis has been shown in AM 013, RO 009 and MA 002 corn genotypes, where there was lower larval viability; and antixenosis was found in RR 168 and PA 110, where lower leaf consumption by FAW occurred (Lima et al., 2006). Cunha et al. (2008) observed antibiosis in BRS Missões - B and BR 111 VI Sel. Dent C, which affected the larval stage of FAW.

The advancement of biotechnology has led to the development of genetically modified (GM) plants, which can now be considered an additional strategic component of Integrated Pest Management programs, or IPMs (Betz et al., 2000). In Brazil, the use of plants modified with the *Bacillus thuringiensis* (B*t*) gene, which is expressed by the Cry protein, has been the primary tactic employed to control FAW in corn crops (Waquil et al., 2002). However, the improper application of this strategy comes with certain risks; as with other strategies, failure to observe the refuge rules and the absence of pest monitoring can lead to the selection of resistant insects (Storer et al., 2010).

Given the importance of FAW and the scarcity of information about how HPR is used to control this pest, this study aimed to evaluate the resistance in conventional and transgenic corn genotypes to FAW.

MATERIALS AND METHODS

The experiment was conducted at the Agricultural Entomology Laboratory of the Goiano Federal Institute - Campus Urutaí - GO (temperature 25 ± 2°C, relative humidity 60 ± 10%; photophase 14 h light). 12 corn genotypes were evaluated (transgenic hybrids: 30A91 PW, 20A78 HX, Impacto VIP 3, 20A55 HX, NS90 PRO 2, Maximus VIP 3, BX 1293YG, RB 9004 PROVTPRO, Feroz VIP 3 and LG 6036 PRO; a conventional hybrid: AG 1051 and the variety: AL Bandeirante). Seeds of these genotypes were sown in 5 L pots in a greenhouse to obtain leaves for the maintenance and preparation of laboratory trials.

To obtain FAW larvae, pairs of moths were kept in polyvinyl chloride (PVC) cages 10 cm in diameter and 21.5 cm in height. These cages were lined with paper to provide a location for oviposition, and they were capped with "voiale". Cotton balls soaked in a 10% honey solution were kept in the cages to feed the moths. The oviposits were collected daily, separated and placed in 100 mL plastic containers containing 5 g of artificial diet. These containers were kept in the room described in the previous paragraph. The artificial diet was prepared according to Kasten Junior et al. (1978).

Larvae were separated during the second instar (approximately 4 mm) and placed into individual 50 mL plastic containers with 5 g of artificial diet. These containers were covered with acrylic (2.5 cm in diameter) and kept in a room (temperature 25 ± 2° C, relative humidity 60 ± 10%; photophase 14 hours) until pupae formation. The pupae were sexed, and males and females were transferred to cylindrical plastic tubes ($Ø$ = 10.5 cm, h = 15 cm) after emergence to continue the insect colony.

Antibiosis between *S. frugiperda* and corn genotypes

Newly hatched FAW caterpillars were separated into individual Petri dishes 6 cm in diameter with humidified filter paper and corn leaves (30 days old), which was then closed using a polyethylene film. Fresh food was provided daily.

The following biological parameters were evaluated: (a) Larval stage: the viability of the larval stage and weight of larvae at ten days; and (b) pupal stage: the viability and weight of pupae at 24 h of age. After emergence, the moths were not fed, and the longevity of the adults was evaluated. For this experiment, a completely randomized design with 20 repetitions was adopted.

Aversion to feeding and attractiveness under no- and free-choice test conditions

The attractiveness of the corn to FAW larvae in the second instar was assessed for each genotype 25 days after emergence. Leaf disks were cut into 2.5 cm diameter disks and distributed in a circular manner in a Petri dish (14 cm in diameter) over moistened filter paper.

During the attractiveness free choice feeding test, 12 caterpillars (2^{nd} instar) were released at the center of the Petri dish. The attractiveness of each foliar disk to the caterpillars was evaluated by counting the number of disks fed upon at 1, 3, 5, 10, 15, and 30 min and 1, 2, 6, 12 and 24 h after release. When 80% of the leaf area of one of the genotype leaf disks had been consumed, the experiment ended. The experimental design adopted was a randomized block with ten repetitions.

The attractiveness no choice test followed the same methodology described previously, except that one genotype was made available in each Petri dish (6 cm diameter). To evaluate whether there was an aversion to feeding, two leaf disks (2.5 cm in diameter) were removed equidistant from the leaves. One was offered to the insects and the other, known as the aliquot, was oven-dried at 60°C for 48 h. The amount of dry matter consumed by the FAW larvae was determined by taking the difference between the weight as measured before the experiment and the remaining portion of the disk post-experiment. A completely randomized design with 20

Table 1. Weight of larvae (10 days - g) and pupae (24 h - g), larval and pupal viability (%), adult longevity (days), cycle (days) to *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in different corn genotypes.

Genotypes[1]	Events	Larva		Pupa		Adult	Cycle
		WEI	VIA	WEI	VIA	LON	
20A55 HX	TC1507	0.03^{b}	10.0^{c}	0.12^{ab}	5.00^{c}	-	-
20A78 HX	TC1507	0.01^{b}	0.00^{c}	-	0.00^{c}	-	-
30A91 PW	MON89034. TC1507	0.01^{b}	5.00^{c}	-	5.00^{c}	-	-
AG 1051	Convencional	0.27^{a}	85.0^{a}	0.18^{a}	85.0^{a}	4.06	26.00 ^{b}
AL Bandeirante	Convencional	0.25^{a}	90.0^{a}	0.16^{ab}	90.0^{a}	4.12	25.12 ^{b}
BX 1293 YG	MON810	0.06^{b}	45.0^{b}	0.16^{ab}	45.0^{b}	4.00	31.85 ^{a}
Feroz VIP 3	BT11. MIR162	-	0.00^{c}	-	0.00^{c}	-	-
Impacto VIP 3	BT11. MIR162	-	0.00^{c}	-	0.00^{c}	-	-
LG 6036 PRO	MON89034	0.03^{b}	0.00^{c}	-	0.00^{c}	-	-
Maximus VIP 3	BT11. MIR162	-	0.00^{c}	-	0.00^{c}	-	-
NS90 PRO 2	MON89034	-	0.00^{c}	-	0.00^{c}	-	-
RB 9004 PRO	MON89034	0.02^{b}	10.0^{c}	0.12^{b}	10.0^{c}	3.0	31.50 ^{a}
F Test	-	18.74**	37.63**	4.12**	40.49**	0.42^{NS}	26.92**
P value	-	<0.0001	0.0000	0.0064	0.0000	0.7375	0.0001

[1]Means followed by the same letter in the column are not significantly different by the Scott-Knott test at 5% probability. NS = non significant, ** = significant at 1%. Weight of larvae and pupae (WEI), larval and pupal viability (VIA) and adult longevity (LON).

repetitions was adopted.

Statistical analysis

The data were subjected to an analysis of variance, or Fisher's exact test, followed by Tukey's test (5% probability) using the software SISVAR version 5.1 (Ferreira, 2011).

RESULTS AND DISCUSSION

Antibiosis between *S. frugiperda* and corn genotypes

The corn genotype consumed influenced the development of FAW at all life cycle stages except the adult stage (Table 1). Caterpillars that consumed AG 1051 (0.27 g) and AL Bandeirante (0.25 g) exhibited higher body weights. Caterpillars that feed on the leaves of the 30A91 PW (0.01 g), 20A78 HX (0.01 g), 20A55 HX (0.03 g), LG 6036 PRO (0.03 g), BX 1293 YG (0.06 g) and RB 9004 PRO (0.02 g) genotypes had the lowest weights. The NS90 PRO 2, Maximus VIP 3, Feroz VIP 3 and Impacto VIP 3 genotypes caused 100% larval mortality in less than 10 days after the caterpillars had hatched.

Those caterpillars that were fed on conventional genotypes showed the highest larval weights, and the caterpillars that were fed on transgenic genotypes had the lowest larval weights. This may be an indication of the cumulative detrimental effect of the altered corn on FAW at this phase, which resulted in the death of the larvae within 10 days. This can be explained by the inclusion of Cry genes in the genome of these genotypes. A Cry protein from *B. thuringiensis* is capable of forming crystals containing endotoxins that have an insecticidal effect on lepidoptera larvae (Schnepf et al., 1998).

These data are similar to those reported by Williams et al. (1997) and Buntin et al. (2001), who observed that FAW that fed on GM corn genotypes exhibited lower weights than those that fed on conventional genotypes. The same authors observed that larvae that fed on GM corn also showed prolongation of larval development compared with caterpillars that fed on conventional corn. Mendes et al. (2011) found that even when larvae were fed hybrid Bt corn and survived, the caterpillars' biomass was reduced compared those fed non-Bt corn.

The larval viability of FAW was also influenced by corn genotypes. When fed on AG1051 and AL Bandeirante leaves, the caterpillars showed the highest viability indices, 85 and 90%, respectively. When the caterpillars were fed the NS90 PRO 2, Maximus VIP 3, LG 6036 PRO, Feroz VIP 3 Impacto VIP 3 and 20A78 HX genotypes, they did not reach the next stage.

This impact may be the result of antibiosis, or a preference for other food types, particularly given the presence of the toxic proteins Cry1A 105 and Cry2Ab2 (PRO and PRO 2), Cry1F (HX), Cry1Ab and VIP 3Aa20 (VIP 3), which has deleterious effects on FAW. Similar results were obtained by Fernandes et al. (2003) and Michelotto et al. (2011), who concluded that Bt corn negatively impacted FAW at the larval phase.

The caterpillars fed the AG 1051 genotype showed the greatest pupal weight (0.18 g), whereas those that fed on RB 9004 PRO had the lowest weight (0.12 g). However, the 20A55 HX, BX 1293 YG and AL Bandeirante genotypes did not yield significantly different weights than AG 1051 or RB 9004 PRO.

Table 2. Average number of third-instar larvae of *S. frugiperda* (Lepidoptera: Noctuidae) attracted to corn genotypes and leaf mass consumed (mg) in free choice test.

Genotypes[1]	Events	Time in minutes					
		1	3	5	10	15	30
20 A 55 HX	TC1507	0.00^{b}	0.00^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.00^{b}
20 A 78 HX	TC1507	0.60^{b}	0.60^{a}	0.60^{a}	0.60^{a}	0.20^{b}	0.00^{b}
30 A 91 PW	MON89034. TC1507	0.20^{b}	0.20^{a}	0.10^{b}	0.00^{b}	0.00^{b}	0.00^{b}
AG 1051	Convencional	0.20^{b}	0.10^{a}	0.30^{b}	0.10^{b}	0.30^{a}	0.10^{b}
AL Bandeirante	Convencional	0.50^{b}	0.50^{a}	0.30^{b}	0.20^{b}	0.40^{b}	0.20^{a}
BX 1293 YG	MON810	0.20^{b}	0.20^{a}	0.30^{b}	0.30^{b}	0.40^{a}	0.40^{a}
Feroz VIP 3	BT11. MIR162	0.50^{b}	0.50^{a}	0.50^{a}	0.50^{a}	0.30^{a}	0.40^{a}
Impacto VIP 3	BT11. MIR162	0.50^{b}	0.40^{a}	0.60^{a}	0.80^{a}	0.70^{a}	0.30^{a}
LG 6036 PRO	MON89034	1.10^{a}	0.70^{a}	1.20^{a}	1.10^{a}	0.70^{a}	0.40^{a}
Maximus VIP 3	BT11. MIR162	0.40^{b}	0.40^{a}	0.70^{a}	0.80^{a}	0.50^{a}	0.40^{a}
NS90 PRO 2	MON89034	0.20^{b}	0.20^{a}	0.20^{b}	0.00^{b}	0.00^{b}	0.00^{b}
RB 9004 PRO	MON89034	0.30^{b}	0.30^{a}	0.40^{b}	0.10^{b}	0.00^{b}	0.00^{b}
Teste F	-	2.47**	1.54NS	2.42*	3.82**	3.05**	2.38*
P valor	-	0.0087	0.1281	0.0099	<0.0001	0.0013	0.0115

Genotypes[1]	Events	Time in hours					CON
		1	2	6	12	24	
20 A 55 HX	TC1507	0.10^{a}	0.00^{b}	0.00^{b}	0.00^{b}	0.30^{b}	10.40^{a}
20 A 78 HX	TC1507	0.20^{a}	0.00^{b}	0.30^{a}	0.20^{b}	0.30^{b}	09.90^{a}
30 A 91 PW	MON89034. TC1507	0.00^{a}	0.00^{b}	0.00^{b}	0.10^{b}	0.20^{b}	09.70^{a}
AG 1051	Convencional	0.30^{a}	0.60^{a}	0.40^{a}	0.70^{a}	1.60^{a}	11.00^{a}
AL Bandeirante	Convencional	0.10^{a}	0.30^{b}	0.40^{a}	0.40^{a}	0.90^{a}	07.80^{a}
BX 1293 YG	MON810	0.00^{a}	0.00^{b}	0.00^{b}	0.10^{b}	0.30^{b}	09.10^{a}
Feroz VIP 3	BT11. MIR162	0.20^{a}	0.20^{b}	0.50^{a}	0.20^{b}	0.20^{b}	06.30^{a}
Impacto VIP 3	BT11. MIR162	0.20^{a}	0.70^{a}	0.20^{b}	0.30^{b}	0.30^{b}	04.10^{a}
LG 6036 PRO	MON89034	0.30^{a}	0.20^{b}	0.50^{a}	0.30^{b}	1.20^{a}	06.10^{a}
Maximus VIP 3	BT11. MIR162	0.20^{a}	0.30^{b}	0.30^{a}	0.20^{b}	0.50^{b}	03.60^{a}
NS90 PRO 2	MON89034	0.00^{a}	0.00^{b}	0.00^{b}	0.10^{b}	0.10^{b}	05.40^{a}
RB 9004 PRO	MON89034	0.00^{a}	0.10^{b}	0.10^{b}	0.00^{b}	0.40^{b}	07.00^{a}
Teste F	-	1.09^{NS}	3.33**	2.29*	2.29*	4.98**	1.53^{NS}
P valor	-	0.3717	0.0004	0.0148	0.0149	<0.0001	0.132

[1]Means followed by the same letter in the column are not significantly different by the Scott-Knott test at 5% probability. NS = non significant, * = significant at 5%, ** = significant at 1%. Mass consumed (CON).

The lower weight and reduced viability of FAW pupae that were fed the transgenic genotype may be due to the presence of the Bt gene, which expresses toxic proteins. This is similar to Fernandes et al. (2003), where the weights of FAW pupae that fed on conventional corn were significantly higher than those that fed on GM corn MON810 (Cry 1 AC).

The genotypes in the present study influenced the viability of FAW pupae, and the caterpillars that fed on the conventional genotypes AL Bandeirante (90%) and AG 1051 (85%) had the highest pupal viability. The 20A55 HX (5%), 30A91 PW (5%) and RB PRO 9004 (10%) genotypes elicited the lowest viability. Of the intermediate results, the genetically modified BX 1293 YG genotype (45%) elicited the highest pupal viability. Caterpillars that fed on NS90 PRO 2, Maximus VIP 3, LG6036 PRO, Feroz VIP 3, Impacto VIP 3, and 20A78 HX did not yield values for larval viability because they failed to complete this stage. Of the adults that did emerge, no significant differences were observed for this parameter.

Aversion to feeding and attractiveness in the free and no choice tests

The results obtained from the attractiveness free and no choice tests are presented in Table 2. The attractiveness of FAW (2nd instar) to the various genotypes varied significantly except for at the 3 min and 1 h sample points.

Table 3. Average number of third-instar larvae of *S. frugiperda* (Lepidoptera: Noctuidae) attracted to corn genotypes and leaf mass consumed (mg) in no choice test.

Genotypes[1]	Events	Time in minutes					
		1	3	5	10	15	30
20 A 55 HX	TC1507	0.25^{b}	0.15^{c}	0.15^{b}	0.30^{b}	0.25^{b}	0.15^{b}
20 A 78 HX	TC1507	0.60^{a}	0.20^{c}	0.25^{b}	0.20^{c}	0.20^{b}	0.20^{b}
30 A 91 PW	MON89034. TC1507	0.30^{b}	0.00^{c}	0.25^{b}	0.35^{b}	0.45^{a}	0.50^{a}
AG 1051	Convencional	0.80^{a}	0.80^{a}	0.45^{a}	0.80^{a}	0.75^{a}	0.75^{a}
AL Bandeirante	Convencional	0.45^{a}	0.50^{b}	0.55^{b}	0.65^{a}	0.60^{a}	0.60^{a}
BX 1293 YG	MON810	0.40^{b}	0.30^{c}	0.30^{b}	0.40^{b}	0.40^{a}	0.30^{b}
Feroz VIP 3	BT11. MIR162	0.50^{a}	0.30^{c}	0.75^{a}	0.80^{a}	0.55^{a}	0.45^{a}
Impacto VIP 3	BT11. MIR162	0.60^{a}	0.30^{c}	0.50^{a}	0.55^{a}	0.45^{a}	0.45^{a}
LG 6036 PRO	MON89034	0.20^{b}	0.15^{c}	0.05^{b}	0.00^{c}	0.10^{b}	0.10^{b}
Maximus VIP 3	BT11. MIR162	0.35^{b}	0.25^{c}	0.45^{a}	0.40^{b}	0.40^{a}	0.35^{b}
NS90 PRO 2	MON89034	0.15^{b}	0.20^{c}	0.10^{b}	0.05^{c}	0.20^{b}	0.25^{b}
RB 9004 PRO	MON89034	0.55^{a}	0.40^{c}	0.60^{a}	0.50^{a}	0.35^{b}	0.30^{b}
Teste F	-	3.28**	4.64**	4.71**	7.13**	3.21**	3.49**
P valor	-	0.0003	0.0000	0.0000	0.0000	0.0004	0.0002

Genotypes[1]	Events	Time in hours					CON
		1	2	6	12	24	
20 A 55 HX	TC1507	0.10	0.10	0.25	0.25	0.20^{b}	10.45
20 A 78 HX	TC1507	0.15	0.20	0.30	0.15	0.35^{b}	09.60
30 A 91 PW	MON89034. TC1507	0.20	0.20	0.25	0.25	0.35^{b}	11.64
AG 1051	Convencional	0.35	0.45	0.25	0.40	0.70^{a}	14.10
AL Bandeirante	Convencional	0.20	0.20	0.10	0.25	0.70^{a}	10.80
BX 1293 YG	MON810	0.10	0.10	0.35	0.10	0.40^{b}	07.70
Feroz VIP 3	BT11. MIR162	0.20	0.30	0.20	0.05	0.40^{b}	07.50
Impacto VIP 3	BT11. MIR162	0.30	0.15	0.30	0.10	0.20^{b}	08.90
LG 6036 PRO	MON89034	0.15	0.15	0.40	0.15	0.45^{b}	06.40
Maximus VIP 3	BT11. MIR162	0.15	0.25	0.25	0.10	0.35^{b}	07.90
NS90 PRO 2	MON89034	0.05	0.20	0.10	0.05	0.05^{b}	09.00
RB 9004 PRO	MON89034	0.20	0.15	0.35	0.35	0.40^{b}	13.40
Teste F	-	0.94^{NS}	1.14^{NS}	0.88^{NS}	1.73^{NS}	3.29**	0.9377^{NS}
P valor	-	0.4939	0.3254	0.5556	0.0665	0.0003	0.5049

[1]Means followed by the same letter in the column are not significantly different by the Scott-Knott test at 5% probability. NS = non significant, * = significant at 5%, ** = significant at 1%. Mass consumed (CON).

The Feroz VIP 3, Impacto VIP 3, LG 6036 PRO and Maximus VIP 3 genotypes were more attractive to FAW from 5 to 30 min after release. Over time, the caterpillars became stimulated by conventional genotypes AG 1051 and AL Bandeirantes, as shown from 6 to 24 h after the release of the caterpillars.

This lower preference of the FAW larvae for the transgenic genotypes may be associated with insect perception of the Bt protein in food or damage to the microvilli in the caterpillars' gut, which is caused by the endotoxin present in the protein crystals of Bt (Schnepf et al., 1998). Boiça Junior et al. (2012) also observed an initially greater attractiveness of *Alabama argilacea* (Lepidoptera: Noctuidae) on the cotton cultivar NuOpal (GM), and the cultivar FMX 966 (conventional) was more attractive to this caterpillar over time. Berdegué et al. (1996) and Stapel et al. (1998) studied the feeding of diets with or without the added protein Cry 1A (b) to *Spodoptera exigua* (Lepidoptera: Noctuidae) and observed a greater preference for the diet without the toxin. This is evidence that the aversion to specific genotypes is related to the presence or absence of a toxin in the diet of the caterpillar.

The attractiveness of specific genotypes to FAW in the no choice test was significantly different at 1, 3, 5, 10, 15 and 30 min and 24 h after the release of the insects (Table 3).

In the free choice test, the Feroz VIP 3 and Impacto VIP 3 genotypes were more attractive to FAW from 5 to 30 min. Although the conventional genotypes AG 1051 and AL Bandeirante were attractive to the caterpillars at all sample points, especially during the early periods, the

results show that they were most attractive 6 to 24 h after the release of the caterpillars. This proves that corn genotypes with inserted Bt genes can be used as an IPM strategy to control FAW, particularly in conjunction with other control tactics.

Williams et al. (1997) found that the leaf area of corn consumed by FAW was significantly lower in transgenic hybrids. Waquil et al. (2002) reported that the 2722 IMI hybrid expressing the Cry 1F toxin is resistant to FAW and has a degree of resistance immunity, and a hybrid expressing the Cry 1A (b) toxin and natural resistance was moderately resistant to FAW larvae.

Others mechanisms that may reveal the cause of this resistance may be involved, as Williams et al. (1998) data show. These researchers conducted tests to determine which mechanisms supported corn's resistance to FAW and observed that morphological characteristics such as hardness of the grains and leaves are factors involved in the expression of resistance. May be these genotypes also have this characteristic, which contributed to the manifestation of resistance to FAW in this study.

Conclusions

The transgenic NS90 PRO2, Maximus VIP 3, Feroz VIP 3 and Impacto VIP 3 genotypes elicited an aversion and antibiosis reaction from FAW. The transgenic HX 20A55, 30A91 PW, LG 6036 PRO, 20A78 HX and BR 9004 PRO genotypes were moderately resistant to the insect, whereas the conventional AG 1051 and AL Bandeirante genotypes were highly susceptible and the transgenic BX 1293 YG genotype was susceptible to FAW.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors want to thank the Brazilian Council for Scientific and Technological Development - CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq) for productivity in research grant conseded for the last author.

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Vol. 15(35), pp. 1883-1891, 31 August, 2016
DOI: 10.5897/AJB2016.15237
Article Number: 0B2C49E60237
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity assessment of farmers' and improved potato (*Solanum tuberosum*) cultivars from Eritrea using simple sequence repeat (SSR) markers

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Received 26 January, 2016; Accepted 21 June, 2016

Sixty three potato clones (51 farmers' and 12 varieties) from Eritrea, 18 and 12 varieties from Kenya and Rwanda, respectively were characterized using 12 highly polymorphic simple sequence repeat (SSR) markers. The study was designed to assess the genetic diversity and varietal distinctness among the different samples. In total, 91 alleles ranging between 2 (STM1053) to 13 (STM0031) alleles per marker were scored. All but 97.8 SSR markers were highly polymorphic with an average PIC value of 0.87 (0.51 to 0.98). All of the 51 farmers' cultivars were clearly distinct from each other. Samples from Eritrea showed the highest genetic diversity as explained by the diversity index (h). The principal coordinate analysis (PCoA) revealed that the local farmers' Eritrean samples are different from the Kenyan, Rwandese and even the imported varieties. Genetic distance analysis generated three clusters correlating with the PCoA findings. Cluster I consisted of 45 samples with 6 sub-clusters; Cluster II consisted of 29 samples with a majority (26) from Eritrea while cluster III consisted of 19 samples. Potato materials from Eritrea appeared to cluster separately from the other samples, which reflects a contribution from the Tuberosum germplasm prominent in temperate regions, unlike from the Andigenum germplasm for Kenyan and Rwandan potato materials. Most of the Eritrean samples in cluster I are farmers' cultivars with intermediate maturity, good performance and better tuber quality characteristics. Cluster II contains mainly the imported variety from Eritrea characterized by late emergence and late maturity. The Kenyan and Rwandese were grouped mainly in Cluster III. In summary, the farmers' cultivars are distinct from the Kenyan and Rwandese materials and represent more genetic diversity than the varieties imported into Eritrea. This finding is of interest to national breeding program to use the farmer's materials as source of genetic variation for traits of interest.

Key words: Potato, simple sequence repeat (SSR), principal coordinate analysis (PCoA), cluster analysis, Eritrea, multivariate.

INTRODUCTION

Potato *(Solanum tuberosum* L*)* is the fourth most important food crop in the world, after maize, wheat, and rice. The crop plays a significant role in human nutrition worldwide, where more than 320 million tons of potatoes

are produced annually on 20 million hectares of land (Poczai et al., 2010). Being one of the major food staple crops in the Eastern African countries, potato is rated among the choice crops for food security and income generation for resource-poor farmers (Kyamanywa et al., 2011).

Likewise, potato is one of the most important and widely grown vegetable crops in Eritrea. Over 70% of the population lives in rural areas and relies on subsistence agriculture (NEPAD, 2005), where the potato crop plays an important economic role as it is cultivated by small scale and subsistence farmers. The crop is generally believed to have entered into Africa around the turn of 20th century (FAO, 2008). It is also assumed that the Italian colonizers introduced the crop to Eritrea around the same time. During the long tradition of potato cultivation in Eritrea, farmers adopted a number of landraces. We define here a landrace as potatoes produced by farmers under a local name with no known origin. Although farmers are now growing recently introduced varieties from Europe, they still recognize the important values of landraces such as good resistance to various stresses and market value, but these landraces are unfortunately low in yielding (Biniam et al., 2014).

The landraces and imported potatoes varieties have not been genetically characterized, which would help to understand their genetic diversity and distinctness. This knowledge is important to better orient potato breeding and germplasm conservation (Liao and Guo, 2014). For a long time, breeding was largely done based on morphological descriptors (Yada and Tukamuhabwa, 2010) but with the new molecular technologies in breeding programs with more diverse genetic resources, including those bearing known alleles of interests can be utilized (Nováková et al., 2010).

Molecular markers are useful tools for genetic diversity assessment, and classification of genetic materials (Tiwari et al., 2013; Spooner et al., 2007). Several types of molecular markers have been applied for potato genetic characterization with each having its own pros and cons (Nováková et al., 2010). Simple sequence repeat (SSR) markers are particularly useful, since they are highly polymorphic, represent co-dominant markers (Tiwari et al., 2013; Ghislain et al., 2009; de Galarreta et al., 2007; Chimote et al., 2007; Barandalla et al., 2006), have low operational costs, and are highly reproducible (Favoretto et al., 2011; Spooner et al., 2007). To that effect, they have been used extensively in potato and other crops for various breeding and diversity studies (Ghislain et al., 2009; Kandemir et al., 2010). Ghislain et al. (2004) recommended 18 highly informative and user friendly SSRs markers after screening 156 SSRs for their characterization power in potato.

According to Raker and Spooner (2002), the high level of polymorphism and heterozygosity explained by SSR markers in potato make them also useful tools for measuring genetic differences between closely related taxa. Several other studies demonstrated that five or six pairs of SSR markers were sufficient to distinguish many potato cultivars (Zhuk et al., 2008). It was reported by Berg and Hamrick (1997) that a sample size of 30 to 50 using 10 to 20 markers can successfully characterize the genetic diversity in the population. The authors added that adding more individuals or loci will not appreciably change the standard statistics of genetic variability.

This study reports the assessment of genetic diversity and relatedness among farmers's potato and introduced varieties from Eritrea and compares it to other potato germplasm from Kenya and Rwanda using 12 SSR markers. The results will benefit potato farmers, breeders and germplasm conservationists.

MATERIALS AND METHODS

Plant material

A total of 63 potato samples from Eritrea and 30 samples from the International Potato Center (CIP Sub-Saharan regional office) comprising 18 from Kenya and 12 from Rwanda were analyzed. The samples from Eritrea were collected from farms in Zoba Debub (27) and Zoba Maekel (24), with additional varieties from the National Agricultural Research Institute, NARI (12). The samples from Eritrea were grown in pots in a greenhouse located at NARI Halhale, Eritrea while those from CIP were grown in the greenhouse located at the Biosciences eastern and central Africa (BecA) –ILRI hub in Nairobi Kenya. Two weeks after germination, a fresh leaf from each sample was collected for DNA extraction.

DNA extraction and PCR

Extraction of DNA was done from fresh tender leaves with a combination of the modified Cetyl-trimethyl Ammonium Bromide (CTAB) (Semagn, 2014) and the QIAGEN DNeasy mini kit methods. The quantity and quality of the extracted DNA was determined using the Nanodrop® 2000C spectrophotometer and agarose (0.8%) gel electrophoresis stained with GelRedTM (Biotium USA) (25 µl/L). Genomic DNA was normalized to a final concentration of 20 ng/µl after which it was subjected to PCR amplification using a set of 12 fluorescently labelled SSR markers (Table 1) as described by Ghislain et al. (2009). The PCR conditions were set as follows: Denaturation at 94°C for 5 min; 35 cycles consisting of a denaturation at 94°C for 30 s, annealing (55 to 60°C depending on the markers) for 1 min, and extension at 72°C for 1 min and a final extension at 72°C for 20 min.

Quality of the amplified PCR products were determined using 2% agarose gel electrophoresis after 45 min at 70 V, and observed using Syngene bio-imaging gel documentation. High quality amplified PCR products were pooled based on the florescent dye used and analyzed by capillary electrophoresis on ABI PRISM 3730 (Applied Biosciences).

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Table 1. Characteristics of the 12 SSR markers used in the study.

Locus	Motif	Forward	Reverse	Size (bp) range	Dye	Annealing T (ºC)	Chromosome position
STG0016	$(AGA)_n$	AGCTGCTCAGCATCAAGAGA	ACCACCTCAGGCACTTCATC	137-174	PET	56	I
STM5114	$(ACC)_n$	AATGGCTCTCTCTGTATGCT	GCTGTCCCAACTATCTTTGA	297-325	VIC	55	II
STM1053	$(TA)_n$ $(ATC)_n$	TCTCCCCATCTTAATGTTTC	CAACACAGCATACAGATCATC	170-196	6-FAM	55	III
STI0012	$(ATT)_n$	GAAGCGACTTCCAAAATCAGA	AAAGGGAGGAATAGAAACCAAAA	183-234	NED	56	IV
STI0032	$(GGA)_n$	TGGGAAGAATCCTGAAATGG	TGCTCTACCAATTAACGGCA	127-148	PET	60	V
STI0004	$(AAG)_n$	GCTGCTAAACACTCAAGCAGAA	CAACTACAAGATTCCATCCACAG	83- 126	PET	56	VI
STM0031	$(AC)_n$ ... $(AC)_n$ (GCAC) $(AC)_n$ $(GCAC)_n$	CATACGCACGCACGTACAC	TTCAACCTATCATTTTGTGAGTCG	168-211	NED	60	VII
STM1104	$(TCT)_n$	TGATTCTCTTGCCTACTGTAATCG	CAAAGTGGTGTGAAGCTGTGA	178-199	VIC	60	VIII
STM1052	$(AT)_n$ GT $(AT)_n$ $(GT)_n$	CAATTTCGTTTTTTCATGTGACAC	ATGGCGTAATTTGATTTAATACGTAA	214-263	NED	55	IX
STM1106	$(ATT)_n$	TCCAGCTGATTGGTTAGGTTG	ATGCGAATCTACTCGTCATGG	151-214	VIC	60	X
STM0037	$(TC)_n$ $(AC)_n$ AA $(AC)_n$ $(AT)_n$	AATTTAACTTAGAAGATTAGTCTC	ATTTGGTTGGGTATGATA	87-133	6-FAM	55	XI
STI0030	$(ATT)_n$	TTGACCCTCCAACTATAGATTCTTC	TGACAACTTTAAAGCATATGTCAGC	94- 137	6-FAM	56	XII

Data analysis

Fragment analysis from the raw data generated on the ABI PRISM 3730 was done using the GeneMapper v4.1 to determine and score allele peaks. Most genetic analysis software is designed for diploid organisms and therefore, their use for ploidy crops like potato required conversion of co-dominant SSR markers (up to 4 alleles per locus) into dominant markers (presence and absence of each allele) as described by Kubik et al. (2009). Data was converted into binary (0/1) results using the ALS binary software. AlleloBin was used to determine the exact allele call size. The quality index from the AlleloBin was interpreted as follows: 0.00 to 0.30: no inspection required; 0.31 to 0.40: binning likely good; 0.41 to 0.45: binning or sizing poor; > 0.45: binning and sizing unacceptable (Idury and Cardon, 1997). GenAlex version 6.4 (Peakall and Smouse, 2012) was used to calculate genetic distances matrix among the populations which further yielded the Principal Coordinate Analysis (PCoA) to display graphic distribution of populations. GenAlex was also used to calculate Analysis of Molecular Variance (AMOVA) to compute the differences of variance among the populations and unbiased genetic identity among populations based on 999 permutations. DARwin 6.0 (Perrier and Jacquemoud, 2006) was used to construct a cluster tree by estimating dissimilarity indices based on the binary data (simple allele matching). The genetic similarity matrix of the populations was calculated using Jaccard coefficient after which a dendrogram was generated using Unweighted Pair-Group Method using Arithmetic averages (UPGMA) based on the estimates of genetic similarity. Power-Marker version 3.25 (Liu and Muse, 2005) was used to calculate Polymorphic Information Content (PIC) to estimate the power of each of the markers in explaining variation among the populations as well as to generate PCoA at the population level.

RESULTS

Allele profile

Analysis of the data using AlleloBin yielded a total of 91 amplified alleles, of which 33 alleles (36%) were rare (≤5%) allele. The number of allele per locus ranged between 2 (STM1053) to 13 (STM0031) with an average of 8 alleles per marker (Table 2). The quality index of the markers ranged from 0.089 (STM1053) to 0.5 (STM0032) with an average of 0.322. There was a moderately negative correlation between the motif size (SSR repeats length) and the allele number amplified (r = -0.31); and PIC (r = -0.37). There was also a moderate positive correlation between the allele number scored and PIC value (0.58). On average, 78% of the 93 potato samples shared a common major allele at any given locus ranging from 46 (STM0031) to 100% (STM1053 and STI0030) common alleles at each locus. The SSR markers used were highly informative and polymorphic as observed from their PIC value (Table 2). The lowest PIC value was 0.51 (STM1053) while highest was 0.98 (STM0037). All of the landraces were found to be distinct from one another.

Potato germplasm structure

Mean number of observed and expected heterozygosity was calculated using the data set (93 clones by 12 SSR markers) to estimate the structure of the various populations (Table 3). Samples from Eritrea showed highest genetic diversity as explained by the diversity index (h). Moreover, highest number of different (private) alleles was observed from Eritrean samples, Zoba Maekel (1.45). The observed genetic diversity ranged between 0.19 (Kenya and Rwanda) to 0.21 (Zoba Debub). The mean expected diversity for the total of samples was 0.21 indicating about

Table 2. SSR marker allele analysis.

Marker	Total no of allele	Gene diversity	PIC	Repeat length	Quality index	Abundant allele (%)	Rare allele (<=5%)
STG0016	9	0.94	0.93	3	0.166	160 (69)	145 148 166 184
STM5114	6	0.89	0.88	3	0.255	321 (79)	318 324
STM1053	2	0.58	0.51	3	0.089	195 (100)	None
STI0012	7	0.91	0.91	3	0.152	195 (62)	213
STI0032	5	0.95	0.95	3	0.501	135 (73)	144
STI0004	10	0.87	0.86	3	0.386	103 (73)	91 100 115 118 124 130
STM0031	13	0.93	0.93	2	0.444	164 (46)	150 184 190 230
STM1104	6	0.87	0.86	3	0.413	194 (76)	191 200 203 206
STM1052	7	0.95	0.94	2	0.456	232 (54)	None
STM1106	6	0.81	0.80	3	0.130	181 (76)	184 217
STM0037	8	0.98	0.98	2	0.335	94 (98)	104 106
STI0030	12	0.94	0.94	3	0.344	109 (100)	85 91 94 100 106 121 136
Mean	8	0.89	0.87	-	0.322	-	-

Table 3. Populations estimation based on number of different alleles, polymorphism (%), and diversity level.

Population	N	Na (SE)	%P	uh (SE)	h (SE)
Zoba Debub	27	1.33 (0.10)	65.9	0.22 (0.02)	0.21 (0.02)
Zoba Maekel	24	1.45 (0.09)	72.5	0.21 (0.02)	0.20 (0.02)
NARI	12	1.12 (0.11)	56.0	0.22 (0.02)	0.20 (0.02)
Kenya	18	1.32 (0.10)	65.9	0.20 (0.02)	0.19 (0.02)
Rwanda	12	0.28 (0.10)	62.6	0.21 (0.02)	0.19 (0.02)
Mean	-	1.30	64.6	0.21	0.20
SE	-	0.05	2.7	0.01	0.01

N = sample number; Na = number of different alleles; %P = polymorphic percentage; uh = unbiased expected diversity; h = observed diversity; SE = standard error.

21% of the samples were expected to be heterozygous at a given locus. There was slight increase in the value of Nei's unbiased estimate of expected genetic diversity (uh) as compared to the observed diversity (h) across all markers for each population.

Principal Coordinate analysis (PCoA)

Analysis of PCoA showed that the first 3 axes explained a cumulative 27.7% of the variation among the populations which grouped them into three clusters (Figure 1).

The Eritrean (Maekel and Debub) samples were widely scattered in all the four coordinates. This can be ascribed to the fact that potato seed tubers are imported to the country from different sources with no standard importation system. Most of the Kenyan and a few Rwandese samples were grouped together. The PCoA analysis provided an alternative way to view the genetic similarity and diversity among and within the populations. PCoA analysis of only the Eritrean population indicated that there was no clear distinction between the samples of different origins but samples from NARI clustered together in the middle (Figure 1).

A separate PCoA test at population level gave a total of 92.5% variation by the first three axes with the first and second axes being 49.3 and 28.7%, respectively. The analysis differentiated the populations distinctly where Kenya and Rwanda clustered together in the fourth quadrant. Zoba Debub and Zoba Maekel clustered together in the second quadrant while NARI grouped separately in the first quadrant (Figure 2).

Analysis of molecular variance

To assess and quantify the diversity level and the genetic

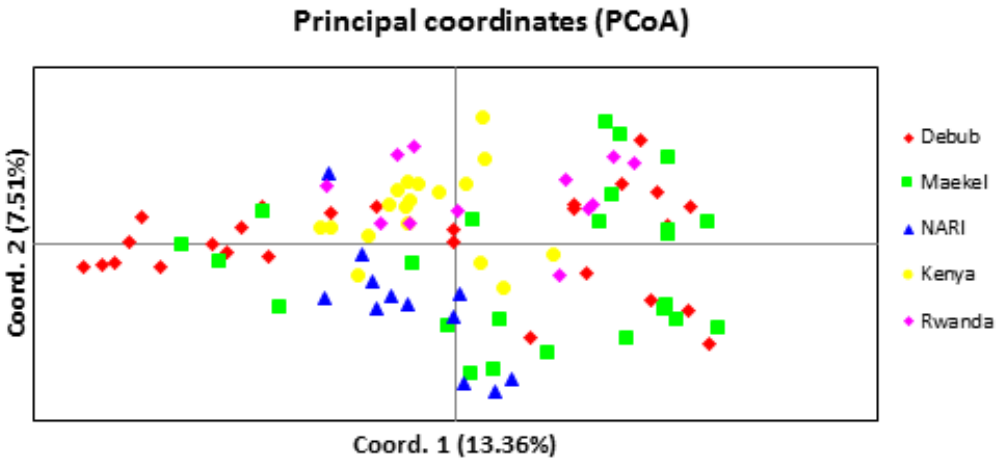

Figure 1. Principal coordinate analysis of the potato samples from Eritrea, Kenya and Rwanda.

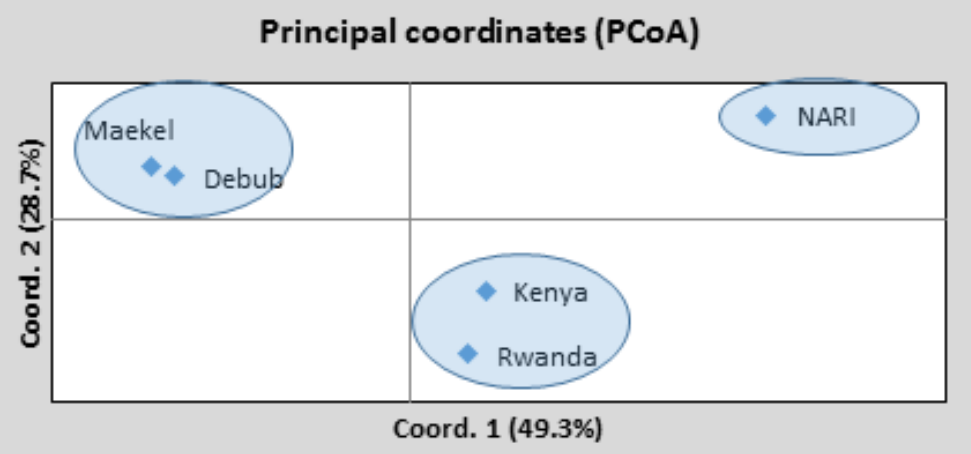

Figure 2. PCoA of the populations based on genetic distance matrix (NeiP) using Coordinate 1 and 2.

Table 4. Pairwise sample matrix of Nei unbiased genetic distance.

Variable	Zoba Debub	Zoba Maekel	NARI	Kenya	Rwanda
Zoba Debub	0.000	-	-	-	-
Zoba Maekel	0.011	0.000	-	-	-
NARI	0.033	0.024	0.000	-	-
Kenya	0.032	0.036	0.036	0.000	-
Rwanda	0.032	0.028	0.048	0.018	0.000

relationship among the 93 samples, an analysis of molecular variance (AMOVA) was done. AMOVA showed that there was a significant variation (p=0.001) among and within populations. About 92% of the variation was observed within population while the remaining 8% variation was observed among population.

The pairwise sample matrix among the potato samples was determined using the unbiased Nei's genetic distance. The genetic distance matrix index among population ranged from 0.011 to 0.048 (Table 4). The results indicate that there is relatively close relationship between the two Eritrean samples Zoba Debub and Zoba

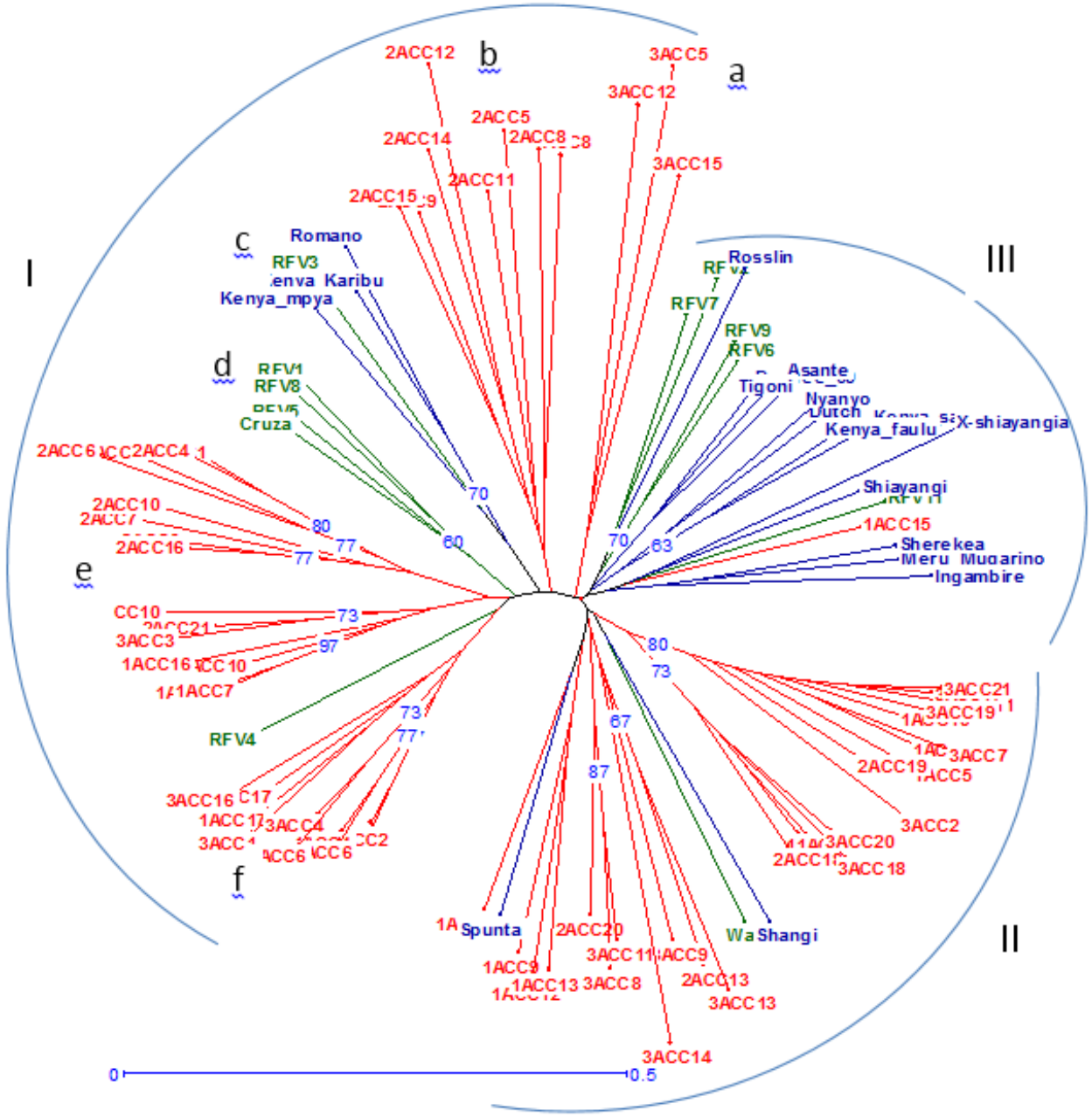

Figure 3. Unrooted UPGMA dendrogram of the 3 potato populations from Eritrea, Kenya and Rwanda on the 93 *Solanum tuberosum* sample. Bootstrap values ≥ 50% from 100 replications are indicated above the nodes.

Maekel as well as between the Kenyan and Rwandese populations.

This conclusion was further supported by the previous PCoA analysis. The highest variation was noted between NARI and Rwandese samples (0.048).

Cluster analysis

The dendrogram tree generated by UPGMA analysis revealed similar findings as those of the PCoA analysis. The samples were clustered into three main distinct clusters with the majority of Kenyan and Rwandese samples grouping together (Figure 3). The largest was Cluster I consisting of 6 sub clusters (a, b, c, d, e, and f). Sub cluster “a” contains 3 samples from Eritrea; while sub cluster “b” had 8 from Eritrea, cluster “c” contains 3 from Kenya and 1 from Rwanda. Sub cluster “d” consists of 4 from Rwanda.

Moreover, sub cluster “e” and “f” consisted of 15 from

Eritrea and 10 from Eritrea and one from Rwanda, respectively. Cluster II consists of 29 samples with a majority (26) being from Eritrea; 2 from Kenya and 1 from Rwanda. Cluster III consists of 19 samples with 13 from Kenya; 5 from Rwanda and 1 from Eritrea. For most of the Eritrean samples, there was no sharp relationship in clustering according to their geographic origin. The tree in Figure 3 shows bootstrap (60 to 97) values at the inner node indicating the shared similarity between the different samples in the specified bootstrap replications. Eritrean samples in Cluster I belong to the intermediate maturity and good performance group with better tuber quality whereas most of the Eritrean samples in Cluster II are from NARI characterized by late emergence and late maturity.

DISCUSSION

The total amount of data point generated by the 12 SSR markers on the Eritrean, Kenyan, and Rwandese populations is within the range of the several studies reported previously. The 12 SSR markers amplified a total of 91 alleles with a mean of 8 allele per locus.

In previous similar allele scoring studies, great variation of results have been reported. Chimote et al. (2007) found a total of 123 amplified alleles using 4 SSR markers with an average of 34 alleles per marker. Moreover, Rocha et al. (2010) reported a total of 136 polymorphic fragments amplified using 20 primers with an average of 6.8 per primer. On average, 2 to 14 alleles per locus were amplified with an average of 6.67 (Muthoni et al., 2014).

Similarly, Favoretto et al. (2011) found a total of 46 alleles amplified using 10 SSR markers. Carputo et al. (2013) reported a total of 46 alleles using 12 SSR markers with an average of 3.8 alleles per locus while Solano et al. (2013) reported an average of 9.16 per locus where 64 alleles were amplified using 7 SSR markers. Recently, Muhinyuza et al. (2015) reported a total of 84 alleles amplified using 13 SSR markers. This high amplification level in potato is ascribed partially to the ploidy level of the crop. Moreover, the wide range of variation in the report by authors is associated to the different SSR markers used.

Similar to other studies, we observed higher polymorphism for short repeat motifs. Previously, Madhusudhana et al. (2012) reported significant negative correlation between repeat motif and repeat number ($r = -0.44$); allele number ($r = -0.39$) and PIC values ($r = -0.38$). On the other hand, Solano et al. (2013) reported a positive correlation between allele number and SSR motif length while Sajib et al. (2012) reported that there were no correlations between the number of allele detected and the number of SSR repeats present in a particular locus. However, de Flamingh et al. (2014) suggests that complexity, rather than repeat length alone, influence amplification success. Our results coincide with the majority that the shorter the motif length the more alleles are amplified. In addition, Muthoni et al. (2014) and Muhinyuza et al. (2015) reported positive and strong correlations between number of alleles and PIC.

The PIC value of each SSR markers was within the range of several reports with an average of 0.87. PIC value describes the discriminatory power of the markers between the samples. Moreover, according to Muhinyuza et al. (2015) PIC effectively demonstrates the power of SSR markers in measuring genetic variation among potato cultivars. The value reported by several studies in potato varied depending on the SSR marker used and samples tested. In the current study, the PIC value ranged between 0.51 to 0.98. Our finding is slightly lower than the report by Lioa and Gua (2014), but relatively higher than reports of Ghislain et al. (2006), Rocha et al. (2010), Muthoni et al. (2014), Favorreto et al. (2011), Solano et al. (2013), D'hoop et al. (2010) and Muhinyuza et al. (2015) in potato.

The PCoA analysis showed that there was no distinct relationship between the samples and their geographic origins within the Eritrean materials. This result can be ascribed to the fact that samples are freely moving from Zoba Debub to Zoba Maekel by farmers and thus the same germplasm is available everywhere. This result is in contrast with those of Solano et al. (2013) who reported that samples were clustered in accordance to their geographical origin in Chile. The later may illustrate the difference in how farmers obtain their seed, which in Chile is largely through specialized seed producers.

The study clustering analysis generated three groups of potato samples pertaining to different populations. This result is supported by the PCoA analysis discussed previously. Cluster I was mainly dominated by the Eritrean landraces with distinct characteristics from the newly introduced varieties. The latter are mainly grouped in Cluster II, whereas the majority of Kenyan and half of the Rwandese are found in cluster III. The relatively low unbiased Nei genetic distance between the Eritrean samples (Zoba Debub and Zoba Maekel) could be attributed to the free seed movement within the country.

Previously, Liao and Guo (2014) reported that among 85 potato cultivars from Yunnan China studied using 24 SSR markers there was relatively low genetic diversity as explained by the genetic similarity matrix. Earlier, it was also reported by Gebhardt et al. (2004) that high genetic similarity was noted as a result of narrow genetic base in European cultivated potatoes. The Eritrean populations were found to be different from the Kenyan and Rwandese. This result was also supported by the PCoA analysis and Nei unbiased genetic distance matrix.

Conclusion

Genetic diversity assessment is essential for the

characterization of the distinctiveness of field and germplasm collection samples, which can help to identify new parents for breeding programs as well as the most interesting samples for conservation of useful germplasm. SSR markers provided a clear molecular characterization and genetic diversity assessment between potato samples. The samples from Eritrea showed some degree of distinctness from the samples of Kenya and Rwanda.

However, Eritrean landraces were markedly more genetically diverse than the new introduced varieties confirming the observations by farmers. The taxonomic origin of the Eritrean potato germplasm is not known, but this study results seem to indicate that it could be more of a Tuberosum origin from European germplasm rather than an Andigenum origin from CIP breeding materials released in the tropical zone (Kenya and Rwanda). The study main conclusion is that the potato Eritrean landrace germplasm presents more genetic variation than newly introduced varieties, and can therefore be exploited for potato breeding.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors would like to express their utmost gratitude to the African Biosciences Challenge Fellowship (ABCF) program at the Biosciences eastern and central Africa (BecA-ILRI-hub) for funding the laboratory work. The ABCF is funded by the Australian Department for Foreign Affairs and Trade (DFAT) through the BecA-CSIRO partnership; the Syngenta Foundation for Sustainable Agriculture (SFSA); the Bill & Melinda Gates Foundation (BMGF); the UK Department for International Development (DFID) and; the Swedish International Development Cooperation Agency (Sida). Furthermore we are thankful to Japanese International Cooperation Agency (JICA) in collaboration with Eritrean Higher Education Commission for funding the field work and sample collection. We also gratefully acknowledge CIP regional staff in Nairobi for their cooperation in providing reference potato samples.

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Vol. 15(35), pp. 1892-1898, 31 August, 2016
DOI: 10.5897/AJB2016.15558
Article Number: 159109C60239
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Efficiency and response of conilon coffee genotypes to nitrogen supply

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Received 7 July, 2016, Accepted 18 August, 2016

The objective of the study was to differentiate genotypes with higher efficiency and responsiveness to nitrogen supply, to understand how the nitrogen supply can impact the dry matter allocation and the accumulation of this nutrient in the different plant compartments of genotypes of conilon coffee, cultivated under contrasting conditions of nitrogen availability in the soil. The plants were cultivated during 150 days in pots containing 10 kg of soil, in greenhouse. The experiment was set up in a 13×2 factorial scheme, following a completely randomized design (CRD) with three replications. The factors were: 13 genotypes and two levels of nitrogen fertilization (0 and 100% of the N recommended level). The N supply increased between 70 and 210% of the total dry matter and between 360 and 680% of the concentration of N content in leaves of the genotypes of conilon coffee. It was possible to observe that the expression of the genotypes was modulated by the availability of N in the soil, since they presented different behaviors in the studied environments (with 0 or 100% of N supply in the soil). The genotypes CV-03, CV-07 and CV-08 were classified as non-efficient and non-responsive, while the genotypes CV-01, CV-04 and CV-09 of conilon coffee were classified as efficient and responsive.

Key words: Alpha parameter, *Coffea canephora* (Pierre ex A. Froehner), mineral nutrition.

INTRODUCTION

Among the species of the genus *Coffea*, the species *Coffea arabica, Coffea canephora* and *Coffea liberica* have been widely cultivated for beverage production (Ramalho et al., 2013). During the last decade, the cultivation of *C. canephora* has been greatly contributing to the increase in the worldwide production of coffee, which has been increasing the need for scientific and technologic advances for the management of genotypes

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of this diverse species.

There is wide intra and interspecific variability among genotypes of *C. canephora*, mainly in characteristics as growth rate, drought tolerance, ripening cycle and crop yield (Ferrão et al., 2008; Fonseca et al., 2004; Marraccini et al., 2012; Martins et al., 2015a; Rodrigues et al., 2012); conferring to conilon coffee, a great possibility of exploration, by means of identifying genetic material adapted to diverse conditions of soil and weather (Martins et al., 2015b). Therefore, the need to understand aspects of the nutritional demand and efficiency of genotypes to use and convert the taken nutrients is undeniable (Martins et al., 2013a; Partelli et al., 2014); however, the breeding programs have not been considering the nutritional efficiency as a selection criterion to identify superior genotypes of conilon coffee worldwide.

Differences in the nutritional efficiency among genotypes of conilon coffee have been reported in several studies (Martins et al., 2013a, 2015a, 2015b). Scientific results indicate that N and P supply in the soil can cause alterations in the biomass allocation patterns, making it possible to discriminate genotypes by their tolerance to the deficit of these nutrients in the soil (Colodetti et al., 2014; Martins et al., 2013b, 2015b). This diverse behavior suggests the possibility of selection of genotypes of *C. canephora* to contrasting soil conditions, especially for areas with low natural fertility, which would be passive of exploration using genotypes of rapid growth and low nutritional demand.

Nitrogen is the second mostly required nutrient by *Coffea* species, being the main component of enzymes, amino acids, proteins, nucleotides, hormones and chlorophyll molecules, among other compounds of the plant metabolism (Carelli et al., 2006). Additionally, nitrogen nutrition is decisive in the protection against the photoinhibition of photosynthesis when the coffee plants are cultivated under high irradiances, promoting and reinforcing the protection mechanisms (Carelli et al., 2006; Ramalho et al., 2000). It is estimated that approximately 75 kg of N are exported per hectare, during one cycle, only by the flowers of *Coffea* spp. (Laviola et al., 2008; Malavolta et al., 2002), and the amount may vary according to the ripening cycle (Partelli et al., 2014), making the study of nutritional efficiency and responsiveness an economic, social and environmental need for all coffee producing regions (Fageria, 1998).

The objective of the study was: (i) to understand how the nitrogen supply can impact the dry matter allocation and the accumulation of this nutrient in the different plant compartments of genotypes of conilon coffee; (ii) to evaluate the behavior of genotypes of conilon coffee cultivated under contrasting conditions of nitrogen availability in the soil; and (iii) to differentiate genotypes with higher efficiency and responsiveness to nitrogen supply.

MATERIALS AND METHODS

Experimental design

The experiment was conducted in greenhouse, installed in the experimental area of the Centro de Ciências Agrárias of the Universidade Federal do Espírito Santo (CCA-UFES), located at municipality of Alegre, in Espírito Santo State, with a latitude of 20°45' S, a longitude of 41°33' W and altitude of 277.41 m.

The experiment was set up in a 13×2 factorial scheme, following a completely randomized design (CRD) with three replications. The factors were: 13 clones of the clonal cultivar "Vitória Incaper 8142" (CV-01, CV-02, CV-03, CV-04, CV-05, CV-06, CV-07, CV-08, CV-09, CV-10, CV-11, CV-12, and CV-13) and two levels of nitrogen fertilization (0 and 100% of the N recommended level, according to Lani et al. (2007)). The experimental unities were constituted by one seedling per pot.

Soil preparation

Soil was collected at the experimental research area of the CCA-UFES, from a site with wavy topography and covered by pasture vegetation (*Brachiaria* species), from 10 to 40 cm depth, eliminating the first 10 cm of the soil profile to evade the effect of organic matter, more present in this superficial layer. A sample was collected and subjected to chemical and physical analyses (Table 1), and the soil was classified as dystrophic red-yellow clay loam (Embrapa, 1997). The soil was dried in shadow, homogenized using a 2.0 mm mesh sieve, separated in samples of 10 dm^3, standardized by weight and packed in sealed plastic pots (14 L). The soil fertility was corrected using liming (2.19 g per pot, 100% of real power of full neutralization) to increase the level of Ca^{++} and elevate the base saturation, following the recommendation of Prezotti et al. (2007).

Multiplication of clones and cultivation

After the soil preparation, conilon coffee plantings provided by Instituto Capixaba de Pesquisa, Assistência Técnica e Extensão Rural (Incaper), produced at Fazenda Experimental de Marilândia (Marilândia-ES) by vegetative propagation (cutting), were selected at their 120 days of development, presenting two pairs of leaves and good phytosanitary and nutritional aspects. The plantings, from each specific genotype, were accommodated in the pots, identified and cultivated with the different levels of nitrogen fertilization.

Fertilization with the other nutrients, beside nitrogen, was performed following the actual recommendation for the crop in the Espírito Santo State (Prezotti et al., 2007). The irrigation of the pots was performed daily, keeping the soil humidity near the adequate level for the initial growth of coffee plants. The phytosanitary management and spontaneous plants removal was conducted manually, whenever required.

Nitrogen fertilization

The levels of nitrogen fertilization used in the study were chosen based on recommendations proposed by Lani et al. (2007), using the levels of 0 and 100% aiming to effectively differentiating the responses to the nutritional efficiency for nitrogen. For this end, 0.00 or 1.56 g of N were applied to the pots, following the treatments, in the form of urea salt (NH_2CONH_2), diluted in distilled water and applied to the soil. This solution was applied 10 cm away from the plant crown and was parceled into five applications: the

Table 1. Physical and chemical characteristic of the soil used as substrate.

Characteristic	Value
Sand (g kg^{-1})[1]	386.73
Silt (g kg^{-1})[1]	36.61
Clay (g kg^{-1})[1]	576.66
Soil density (kg dm^{-3})[2]	1.15
pH[3]	5.96
P (mg dm^{-3})[4]	6.1
K (mg dm^{-3})[5]	79
Ca ($cmol_c$ dm^{-3})[6]	0.22
Mg ($cmol_c$ dm^{-3})[6]	0.46
Al ($cmol_c$ dm^{-3})[7]	0
H+Al ($cmol_c$ dm^{-3})[8]	1.98
Sum of bases ($cmol_c$ dm^{-3})	0.87
Potential CEC ($cmol_c$ dm^{-3})	2.85
Effective CEC ($cmol_c$ dm^{-3})	0.87
Base saturation (%)	30.59
Aluminium saturation (%)	0
Organic matter (g kg^{-1})[9]	13.7

[1]Pipette method (slow mixing); [2]Graduated cylinder method; [3]pH in water (relation 1:2.5); [4]Extracted by Mehlich-1 and determined by colorimetry; [5]Extracted by Mehlich-1 and determined by flame photometry; [6]Extracted with 1 mol L^{-1} potassium chloride and determined by titration; [7]Extracted by oxidation, humid route, with potassium dichromate in sulfuric medium, and determined by titration (Embrapa, 1997).

first one on the first day and the others periodically at 30, 60, 90 and 120 days after planting.

Nutritional evaluation and grouping by responsiveness

After 150 days of cultivation, the plants were cut, separating the stems, leaves and roots. These parts were removed from the pots, washed, weighted and dried in shadow, further separately packed in paper bags and taken to laboratory stove, with forced air circulation, at a temperature of 65°C, until a constant weight was obtained to determine the dry matter.

To determine the shoot dry matter (SDM) and the root dry matter (RDM), the plant material was weighted on analytical scale (precision: 0.001 g), obtaining results on grams per plant. As SDM is the sum of the dry matter of leaves, stem and branches, total dry matter (TDM) was obtained by the sum of the SDM to the RDM.

The dry matter of each vegetal component was milled in a Wiley electric mill, equipped with stainless steel sieve having a mesh size of 0.42 mm, until the material became a homogeneous power, and packed in paper bags for chemical analysis of nutrient content (Silva, 1999). The determination of N content in each vegetal component was done according to the semimicro Kjedahl method (Malavolta et al., 1997). The α parameter was calculated as reported by Fox (1978), in order to classify the clones according to nutritional efficiency and responsiveness to nitrogen fertilization. The α parameter represents the ratio between the difference of SDM accumulated in each level of nitrogen fertilization ($SDM_{100\%}$ - $SDM_{0\%}$) and the difference of the amount of applied nitrogen (1.56 g to 0.00 g per pot) adapted by Martins et al. (2013a).

The results of α parameter for each clone were set in the ordinates axis and the means for SDM were set in the abscissas axis, arranging four quadrants that allowed the classification of the clones in 4 groups: ER, efficient and responsive; ENR, efficient and non-responsive; NER, non-efficient and responsive; NENR, non-efficient and non-responsive.

Statistical analyses

The data was subjected to analysis of variance ($p \leq 0.05$), using SISVAR statistic software (Ferreira, 2011) and when the sources of variation were significant, the Tukey test ($p \leq 0.05$) was used to compare the results of nitrogen levels and Scott-Knott test ($p \leq 0.05$) was used to group clones of homogeneous behavior.

RESULTS AND DISCUSSION

The analysis of variance presented significant effect of the interaction between genotypes of conilon coffee and the different scenarios of N supply in the soil (Table 2). This fact indicates that the dry matter accumulation (Table 3) and the N content (Table 4) of coffee plants were mutually influenced by the genetic constitution and nitrogen supply. Similar results have been evidenced by the same genotypes cultivated in environments with different levels of phosphorus supply (Martins et al., 2013a, 2013b).

Supply of de N influence the dry matter accumulation and leaf nitrogen

The N supply in the soils implicated increase of dry matter (RDM, SDM and TDM) and N content (CR, CSP and CT) of the genotypes (Tables 3 and 4), for example, higher N supply caused a gain of 60% over the total dry matter (Table 3) and 76% of the leaf content of the nutrient (Table 4) for the genotype CV-09. These results suggest that genotypes of conilon coffee may be highly exigent in N, which has been related in past decades for the genus *Coffea* (Carelli et al., 2006; Catani and Moraes, 1958) and specifically for genotypes of conilon coffee (Colodetti et al., 2015).

Overall, the adequate N supply caused increase of RDM, SDM and TDM (Table 3), as well as of CR, CSP and CT (Table 4) for all genotypes. This fact may be explained by a higher production of structural carbohydrates and sugars, and by the stabilization of the nitrate assimilation rate which inhibits limitations of root growth over the shoots, designating more energy for the metabolic processes (Carelli et al., 2006; DaMatta et al., 1999).

In contrast, the unsatisfactory performance of the genotypes when cultivated in the environment with low N availability's (Tables 3 and 4) may be explained by the

Table 2. Mean squares, coefficients of variation (CV) and overall means of dry matter (g/plant) of roots (RDM), shoots (SDM) and total (TDM); and N content (mg/plant) of root (CR), shoots (CSP) and total (CT), for genotypes of conilon coffee grown in environments with discriminating levels of N supply.

Variation source or parameter	df[1]	RDM	SDM	TDM	CR	CSP	CT
Genotypes (G)	12	25.90*	180.76*	263.18*	0.003*	0.010*	0.018*
Levels of N (N)	3	424.12*	5617.47*	8137.74*	0.310*	6.329*	9.422*
Interaction G*N	36	24.69*	84.48*	160.03*	0.002*	0.007*	0.013*
Residue	104	0.86	3.25	4.46	0.0001	0.0006	0.001
Overall Means		15.39	29.22	44.62	0.17	0.40	0.57
CV (%)		6.05	6.17	4.73	7.03	6.37	4.89

*Significant at 5% probability by F test. [1]degrees of freedom.

Table 3. Mean values of dry matter (g/plant) of roots (RDM), shoots (SDM) and total (TDM) for genotypes of conilon coffee grown in two levels of N supply (0 and 100% of the recommended values for the crop, respectively N1 and N2).

Genotype	RDM		SDM		TDM	
	N_1	N_2	N_1	N_2	N_1	N_2
CV-01	10.37^{bB}	18.59^{bA}	16.55^{aB}	42.42^{aA}	26.92^{aB}	61.01^{bA}
CV-02	12.79^{aB}	22.09^{aA}	15.42^{aB}	38.15^{bA}	28.21^{aB}	60.24^{bA}
CV-03	9.62^{bB}	16.48^{cA}	9.65^{bB}	34.74^{cA}	19.28^{bB}	51.23^{dA}
CV-04	12.19^{aB}	18.07^{bA}	12.20^{bB}	40.87^{aA}	24.39^{aB}	58.94^{bA}
CV-05	12.83^{aB}	15.20^{cA}	15.50^{aB}	35.28^{cA}	28.33^{aB}	50.49^{dA}
CV-06	11.95^{aB}	18.90^{bA}	10.66^{bB}	39.59^{bA}	22.61^{bB}	58.49^{bA}
CV-07	9.57^{bB}	16.09^{cA}	11.21^{bB}	31.15^{dA}	20.78^{bB}	47.24^{eA}
CV-08	13.56^{aB}	16.60^{cA}	11.34^{bB}	30.34^{dA}	24.90^{aB}	46.94^{eA}
CV-09	8.59^{bB}	17.25^{cA}	12.84^{bB}	37.03^{bA}	21.43^{bB}	54.28^{cA}
CV-10	10.74^{bB}	16.42^{cA}	12.38^{bB}	39.87^{bA}	23.12^{bB}	56.29^{cA}
CV-11	9.30^{bB}	16.00^{cA}	12.88^{bB}	39.00^{bA}	22.18^{bB}	55.00^{cA}
CV-12	9.40^{bB}	19.85^{bA}	11.13^{bB}	43.93^{aA}	20.54^{bB}	63.79^{aA}
CV-13	12.09^{aB}	18.45^{bA}	13.91^{aB}	30.69^{dA}	26.00^{aB}	49.14^{dA}

Means followed by the same letter in each variable, uppercase letters in lines (Tukey) and lowercase letters in columns (Scott-Knott), are not different ($p < 0.05$).

imposition caused by this low supply, which results in decrease of chlorophyll concentration and Calvin-cycle enzymes, which by itself leads to a lower capacity of carbon assimilation and increase the sensibility for photo-inhibition (Carelli et al., 2006; Ramalho et al., 1998, 1999, 2000), compromising the whole carbohydrate accumulation and dry matter production (Amaral et al., 2001).

Differential responsiveness of genotypes to N supply

The genotypes presented different behavior regarding the N supply in the soil, resulting in distinct groups of homogeneous means for dry matter (RDM, SDM and TDM) and N content in the plant tissues (CR, CSP and CT) (Tables 3 and 4).

Overall, the genotypes CV-01, CV-02, CV-04 and CV-13 presented higher accumulation of dry matter (Table 3), while the genotypes CV-01 and CV-05 presented higher N accumulation in the plant tissues (Table 4), when grown with low supply of N. For the environment with adequate N supply, the genotype CV-02 developed high accumulation of dry matter and N content, while CV-04 presented only high N content (Table 4).

Several reports describe a genetic control of the efficiency to use nutrients and this expression occurs in conformity with the level of availability of nutrients in the soil, which implicates the determination of heterogenic groups between cultivars of the same species, or even inside the same cultivar (Carelli et al., 2006; Martins et al., 2013a, 2015a, 2015b; Neto et al., 2016; Rodrigues

Table 4. Mean values of N content (mg/planta) of roots (CR), shoots (CSP) and total (CT) for genotypes of conilon coffee grown in two levels of N supply (0 and 100% of the recommended values for the crop, respectively N1 and N2).

Genotype	CR		CSP		CT	
	N_1	N_2	N_1	N_2	N_1	N_2
CV-01	0.09^{bB}	0.22^{dA}	0.16^{aB}	0.78^{aA}	0.25^{aB}	1.00^{bA}
CV-02	0.11^{bB}	0.32^{aA}	0.13^{aB}	0.76^{aA}	0.23^{bB}	1.08^{aA}
CV-03	0.09^{bB}	0.29^{bA}	0.09^{cB}	0.68^{bA}	0.18^{bB}	0.97^{cA}
CV-04	0.17^{aB}	0.26^{bA}	0.12^{bB}	0.78^{aA}	0.29^{aB}	1.05^{aA}
CV-05	0.13^{aB}	0.24^{cA}	0.15^{aB}	0.70^{bA}	0.27^{aB}	0.94^{cA}
CV-06	0.12^{aB}	0.27^{bA}	0.10^{cB}	0.66^{cA}	0.22^{bB}	0.93^{dA}
CV-07	0.11^{bB}	0.19^{fA}	0.11^{bB}	0.61^{cA}	0.22^{bB}	0.80^{eA}
CV-08	0.13^{aB}	0.21^{eA}	0.10^{cB}	0.57^{dA}	0.23^{bB}	0.78^{eA}
CV-09	0.09^{bB}	0.24^{cA}	0.11^{bB}	0.66^{cA}	0.21^{bB}	0.90^{dA}
CV-10	0.12^{aB}	0.23^{dA}	0.10^{cB}	0.69^{bA}	0.22^{bB}	0.92^{dA}
CV-11	0.11^{bB}	0.18^{fA}	0.12^{bB}	0.71^{bA}	0.23^{bB}	0.89^{dA}
CV-12	0.08^{bB}	0.24^{cA}	0.10^{cB}	0.74^{aA}	0.18^{bB}	0.98^{cA}
CV-13	0.09^{bB}	0.19^{fA}	0.12^{bB}	0.56^{dA}	0.22^{bB}	0.75^{eA}

Means followed by the same letter in each variable, uppercase letters in lines (Tukey) and lowercase letters in columns (Scott-Knott), are not different ($p < 0.05$).

et al., 2015).

The additive and non-additive effects of the genetic control are linked directly to the accumulation and allocation of dry matter and nutrients in the green photosynthetic tissues (Colodetti et al., 2015; Martins et al., 2013b, 2015b; Neto et al., 2016), which may partially explain the occurrence of differential behavior among genotypes of conilon coffee as function of the N supply in the soil (Tables 3 and 4).

Furthermore, the modulation of biomass production and N content in leaves of genotypes of conilon coffee imposed by the availability of N in the soil may be governed by different traits. Revisiting other studies, it is possible to observe that different performances of genotypes can be attributed to morphology, architecture and diameter of the root system for environments characterized by having low availability of nutrients (Amaral et al., 2011; Colodetti et al., 2015; Martins et al., 2013b). However, in environments with ideal nutritional supply, the differential performance of the genotypes seems to be governed by traits related to the ripening cycle, with evidences that early genotypes may be more efficient in the allocation of biomass and nutrients (Martins et al., 2015b; Partelli et al., 2014), but without evidences of changes in tolerance indexes.

Efficiency and responsiveness of genotypes to N

The genotypes CV-01, CV-04 and CV-09 were classified as efficient and responsive (ER), presenting satisfactory development in soils with low supply of N and also a significant increase in dry matter when cultivated in soils with adequate N supply (Figure 1). The genotypes CV-02, CV-05 and CV-13 were classified as efficient but non-responsive (ENR), indicating that these genotypes do not respond as much to the increase in the nitrogen supply, but are efficient to grow in conditions of low availability of this nutrient (Figure 1).

The genotypes CV-06, CV-10, CV-11 and CV-12 are non-efficient but responsive, indicating that these genotypes may not tolerate low supply of N, but present considerable gain as function of the increased supply of N in the soil. The genotypes CV-03, CV-07 and CV-08 were classified as non-efficient and non-responsive (Figure 1).

Revisiting results of other nutritional studies, it is possible to observe that the genotype CV-04 was also characterized as efficient and responsive to P (Martins et al., 2013a) and tolerant to low supplies of N and P in the soil (Colodetti et al., 2014; Martins et al., 2015b), demonstrating that this genotype may express desirable characteristics in terms of nutritional efficiency for both nutrients. This is in addition to the other agronomic traits, indicating that this genotype may be explored by breeding programs aiming to improve the nutritional efficiency of future cultivars.

Another intriguing observation is that CV-08 was classified in opposite groups regarding the nutrition with N and P, being characterized as NENR for N (Figure 1) and intolerant to the deficit of N in the soil (Colodetti et al., 2014), but ER for P (Martins et al., 2013a) and tolerant to the deficit of P in the soil (Martins et al., 2015b). This fact is indicative that nutritional efficiency

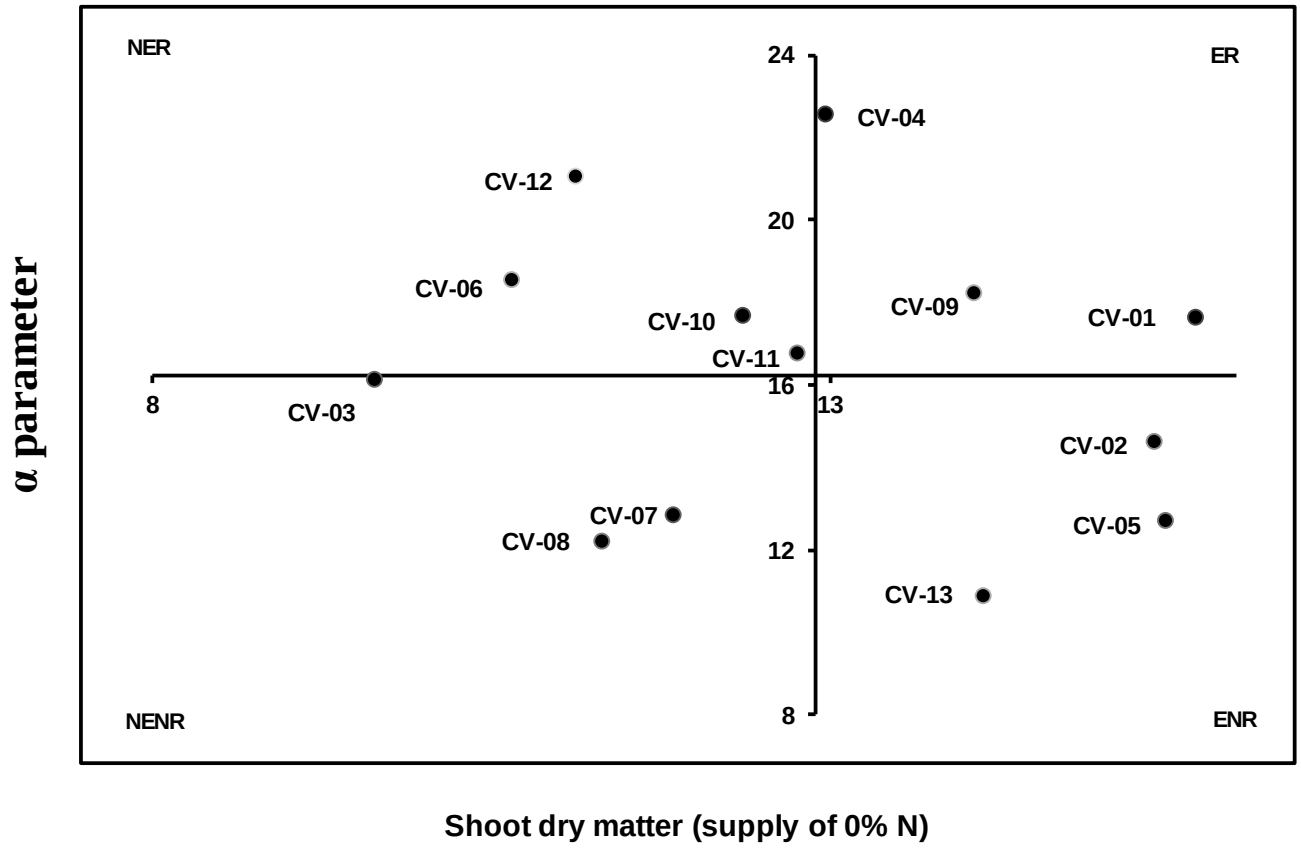

Figure 1. Classification of conilon coffee genotypes according to nutritional efficiency and responsiveness to nitrogen supply. (ER: Efficient and responsive; ENR: efficient and non-responsive; NER: non-efficient and responsive; and NENR: non-efficient and non-responsive).

may present specificities related to the uptake and use of each nutrient in isolate routes and in different magnitudes; making it possible for the same genotype (same morphology and architecture of roots), to present different capacities to acquire and metabolize specific nutrients.

Furthermore, it was possible to notice correlation between the use efficiency of N with the classification of the genotypes regarding their ripening cycle. Save for some exceptions, genotypes of late cycle presented a tendency of being classified as efficient and genotypes of early cycle presented a tendency to be classified as responsive (Martins et al., 2015b).

Conclusion

The nitrogen supply modulates the production of dry matter and nitrogen accumulation for conilon coffee genotypes. The conilon coffee genotypes CV-01, CV-04 and CV-09 are efficient and responsive to nitrogen fertilization.

ACKNOWLEDGEMENT

The authors acknowledged the supported by Ufes, Incaper and CBPD-Café to support the project; CAPES (LSM – doctor scholarship), CNPq (LDM – post-doc scholarship; MAT - scholarship of research productivity), and FAPES (LDM – post-doc scholarship and project financial support; WNR – post-doc scholarship).

Conflicts of interests

The authors declared that they have no conflict of interests.

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Vol. 15(35), pp. 1899-1912, 31 August, 2016
DOI: 10.5897/AJB2015.15172
Article Number: 745B4A560241
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Phosphate solubilization and multiple plant growth promoting properties of rhizobacteria isolated from chickpea *(Cicer aeritinum L.)* producing areas of Ethiopia

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Received 16 December 2015; Accepted 2 March, 2016

Chickpea is one of the major legume crops widely grown in Ethiopia. The low availability of phosphorus in soil is among the stresses that constrain the production of this crop in the country. However, there are rhizobacteria capable of solubilizing insoluble forms of phosphorus in soil and make it available to the plant. Thus, this study was aimed at isolation and characterization of phosphate solubilizing bacteria from chickpea rhizosphere. Fifty phosphate solubilizing bacterial strains were isolated from the soil samples, characterized biochemically and identified by 16S rDNA sequences analysis. The results indicate the presence of genera *Acinetobacter, Bacillus*, *Brevibacillus*, *Burkholderia*, *Empedobacter, Enterobacter*, *Pseudomonas*, *Ralstonia, Sphingomonas* and *Stenotrophomonas*. Phosphate solubilizing efficiencies of the strains were analyzed using different insoluble phosphorus sources and the results show that most isolates released a substantial amount of soluble phosphate from tricalcium phosphate, rock phosphate and bone meal. Screening for multiple plant growth promoting attributes showed that 44 and 18% of them were capable of producing indole acetic acid and inhibiting the growth of *Fusarium oxysporum* under *in vitro* conditions, respectively. A direct impact of several strains (*Bacillus flexus* (PSBC17), *Pseudomonas fluorescence* (PSBC33), *Enterobacter* sp. (PSBC35), *Enterobacter sakazaki* (PSBC79) and *Enterobacter* sp. (PSBC81)) on the growth of chickpea in pot culture has been demonstrated by the increase in the number of root nodules, shoot dry matter, nitrogen and phosphorus concentration of shoot. Based on the results, we conclude that chickpea rhizosphere harbor phosphate solubilizing bacteria which are diverse in taxonomy and phosphate solubilizing efficiencies. Thus, consecutive studies should focus on field studies on those strains due to their potentially high importance for the phosphorus nutrition of crops in this area and in this context for the improvement of the sustainability of crop production in the country.

Key words: Plant growth promoting rhizobacteria (PGPR), indole acetic acid (IAA), rhizosphere soil, rock phosphate, bone meal.

INTRODUCTION

Chickpea (*Cicer aeritinum* L.) is one of the major food legume crops grown widely in tropical, sub-tropical and temperate regions of the world. It is also widely grown in Ethiopia over an area of 208,388.6 ha (Central Statistical Agency (CSA), 2011).

This crop is an important source of dietary protein for the majority of the Ethiopian population. In addition, chickpea restores and maintains soil fertility and therefore, grown in rotation with major cereals in traditional low-input agricultural systems. This is due to its ability to fix dinitrogen in association with root nodule bacteria belonging to the genus *Mesorhizobium* (Nour et al., 1994). However, its yield has remained rather low due to several stresses which prevail under Ethiopian edaphoclimatic conditions (Keneni et al., 2011; Jida and Assefa, 2012).

Phosphorus (P) is one of the key macronutrients required for growth and development of plants. Most Ethiopian soils are acidic and mainly characterized by low available P (Beyene, 1982; Mamo et al., 1988). This is partly due to acid mediated P-fixation in the soil. Hence, low available P in soil is one of the major soil-related stresses that limit the production of chickpea and other pulse crops in the country (Tilaye, 1985). The most common method of improving P limitation and hence increasing crop production is application of chemical fertilizer. This is highly limited by its increasing cost and for this reasons its application in chickpea production is particularly low in Ethiopia (Jida and Assefa, 2012) causing an urgent need for economically feasible and ecologically acceptable P-fertilizer sources. Thus, using microbial biofertilizer for improving the availability of P in the rhizosphere of plant represents an attractive and practically applicable alternative to the use of chemical fertilizers (Naik et al., 2008).

Bacteria that colonize the rhizosphere and enhance plant growth are referred to as plant growth promoting rhizobacteria (PGPR). Phosphate solubilization that makes the P available in soil for plant growth is considered as one of the important attributes of PGPR (Chen et al., 2006), since phosphate solubilizing bacteria (PSB) are capable of converting insoluble phosphate to soluble orthophosphate ions which can be taken up and utilized by plants (Pal, 1998; Rodriguez and Fraga 1999; Chen et al., 2006; Vyas and Gulati, 2009). Previous studies already showed the presence of phosphate solubilizing bacteria (PSB) in the rhizosphere of different agriculturally important crops and their pivotal role in converting of insoluble P to the orthophosphate (Pal, 1998; Peix et al., 2001a; Rajapaksha and Senanayake, 2011).

Several studies revealed that rhizobacteria particularly from genera *Agrobacterium, Bacillus, Burkholderia, Enterobacter, Erwinia, Pseudomonas, Rhizobium, Mesorhizobium, Pantoea* and *Serratia* are known to be highly efficient in solubilizing insoluble soil phosphate into available inorganic phosphate (Illmer and Schinner, 1995; Rodriguez and Fraga, 1999; Naik et al., 2008; Castagno et al., 2011). It has also been documented that different species of PSB isolated from Ethiopian soils were able to release phosphate from insoluble P sources such as tri-calcium phosphate, hydroxyl apatite, rock phosphate and old bone (Muleta et al., 2013; Keneni et al., 2010; Jida and Assefa, 2012). Recently, emerging evidences indicated that, besides increased P uptake, the production of phytohormones such as indole acetic acid (IAA) and suppression of different soil borne phytopathogens by PSB could also play an imperative role in plant growth promotion (Peix et al., 2001b; Ponmurugan and Gopi, 2006; Naik et al., 2008; Shahab et al., 2009). Several reports demonstrated that inoculation of PSB which exhibited multiple plant growth promoting activities significantly improved plant growth and yield under glasshouse and field conditions (Peix et al., 2001a; Shahab et al., 2009; Yadav et al., 2010).

In view of their multiple plant growth promoting attributes, PSB could maintain soil quality and health. Therefore, studies committed to isolation, characterization and selection of efficient PSB with different plant growth promoting properties from chickpea rhizosphere soils are highly desirable to design strategies to use those native strains for the development of inoculant technologies for organic agriculture. Despite the benefits that PSB could present to legume cultivation, the information about indigenous rhizobacterial strains with these properties from the rhizosphere of chickpea in Ethiopian soils is very limited. Thus, the aim of this study was to isolate and characterize phosphate solubilizing bacteria from chickpea rhizosphere and evaluate their plant growth promoting activities under glasshouse conditions.

MATERIALS AND METHODS

Study sites and soil samples collection

Soil samples were collected from 36 chickpea grown fields found in Amhara, Oromia and Tigray Regional States of Ethiopia (Figure 1) with altitude range from 1,526 (Alamata) to 2,840 masl (Sheno) in October, 2009 (Table 1). About 3 kg of soil samples were excavated from 15 to 20 cm depth from each site, collected into sterile

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Figure 1. Soil sample collection sites.

polyethylene bags and carefully transported to Applied Microbiology Laboratory, Addis Ababa University. The soil samples were stored at 4°C until further analysis.

Isolation and enumeration of phosphate solubilizing bacteria

Each soil sample was air-dried and filled into surface sterilized plastic pots. Chickpea seeds (*Akaki* cultivar) were surface sterilized with 95% ethanol and 3% sodium hypochlorite solution for 10 s and 3 min, respectively. The seeds were rinsed five times with sterilized distilled water and allowed to germinate on water agar (1%) surface for three days at 25°C. Five pre-germinated seeds were planted in each pot. The seedlings were thinned down to three 5 days after emergence (DAE). All pots were situated in the greenhouse and watered every three days.

Forty five days after planting (DAP) chickpea seedlings were selected from each pot and uprooted carefully with their rhizosphere soil. 10 g of rhizospheric soil with adhered roots were transferred to flasks containing sterilized 90 ml saline solution of 0.85% w/v NaCl. The flasks were incubated on a gyratory shaker at 120 rpm for 30 min followed by serial dilution to 10^6. Aliquots (0.1 ml) of the appropriate dilutions were plated on Pikovskaya medium (Pikovskaya, 1948). All plates were incubated at 30°C for 5 days. The number of phosphate solubilizing bacteria found in each soil samples was determined using the plate count method. Colonies with a large halo zone and different morphologies were selected and purified by re-streaking on Pikovskaya (PK) medium. The persistence of their phosphate solubilizing ability was confirmed by three successive subcultures in the same medium. Pure isolates were maintained on PK agar slants at 4°C and 50% (v/v) glycerol at -20°C. Isolates were designated as phosphate solubilizing bacteria from chickpea (PSBC) 01- PSBC135 (Table 1).

Phosphate solubilization efficiency of PSB strains

The PK agar plates were spot inoculated with 10 µl of bacterial culture grown in PK broth to the exponential phase. After incubation at 30°C for 5 days, formation of a clear zone around the spot was checked and their solubilization indices (SI) were calculated according to Edi-Premono et al. (1996). Based on their SI along with the abundance, isolates were selected and used for quantitative analysis of phosphate solubilization efficiency in PK broth. 100 ml of PK broth without phosphorus sources was dispensed in a 250 ml Erlenmeyer flask and supplemented with tricalcium phosphate (TCP), Egyptian rock phosphate (RP) or bone meal (BM) that contained equivalent amount of P as sole P source. The flasks were inoculated with 100 µl bacterial culture grown in PK broth to exponential phase. Uninoculated PK broths supplemented with the aforementioned insoluble P sources were included as controls. The flasks were incubated at room temperature on a gyratory shaker at 120 rpm for 12 days. 5 ml subsamples were withdrawn from each treatment on day 0, 4, 8, and 12 for pH change and soluble P analysis. The sample was centrifuged at 15,000 g for 15 min and the supernatant was used for the analysis. The amount of

Table 1. Abundance and distribution of phosphate solubilizing bacteria in chickpea producing areas of Ethiopia.

Isolation sites	Regional state of isolation site	Altitude of isolation site (masl)	Abundance of PSB (CFU/g) in soil isolation site soil	Number of isolates selected	Isolates obtained from each site
Galessa	A	2017	3.7×10^{4de}	1	PSBC01
Alamata	T	1526	1.5×10^{5bcde}	1	PSBC02
Robe	T	1658	6.9×10^{4cde}	1	PSBC05
Woldya	A	2074	1.3×10^{5bcde}	1	PSBC06
Aja	A	2023	5.6×10^{5a}	2	PSBC07,41
Debre Selam	A	1896	6.1×10^{4cde}	1	PSBC10
Alem Tena	O	1637	5.9×10^{4cde}	1	PSBC13
Chirameda	A	1747	3.5×10^{4de}	1	PSBC14
Sirnka	A	1843	3.6×10^{4de}	2	PSBC15, 16
Angut Michael	A	1850	1.1×10^{5cde}	2	PSBC17,18
Maksagnt	A	1978	6.1×10^{4cde}	2	PSBC28,29
Yetinora	A	2437	7.8×10^{4cde}	2	PSBC30,31
Lalibela	A	2138	4.8×10^{4de}	2	PSBC33,40
Olankomi	O	2378	2.2×10^{5bc}	1	PSBC35
Debre Libanos	O	2594	2.2×10^{5bc}	2	PSBC42,46
Dibulko	A	1992	4.8×10^{4de}	1	PSBC45
Ginchi	O	2378	2.9×10^{3e}	2	PSBC61,62
Ambo	O	2170	4.0×10^{3e}	1	PSBC63
Fiche	O	2748	1.9×10^{5bc}	1	PSBC67
Tikana	T	1942	5.9×10^{4cde}	2	PSBC68,126
Fogera	A	1931	4.8×10^{4de}	1	PSBC69
Alshin	T	2082	3.1×10^{4de}	1	PSBC70
Bilbila	A	2069	3.4×10^{4de}	1	PSBC71
Amber	A	2454	6.0×10^{4cde}	1	PSBC72
Gurura	O	1906	2.3×10^{3e}	2	PSBC74,79
Goro	O	1832	2.9×10^{3e}	1	PSBC81
Teji	O	2065	1.4×10^{5bcde}	2	PSBC86,89
Asgori	O	2078	4.0×10^{3e}	1	PSBC97
Itacha	A	2134	4.6×10^{4de}	1	PSBC99
Ilala	A	1924	6.2×10^{4cde}	1	PSBC108
Goha Tsion	O	2517	4.6×10^{4de}	1	PSBC109
Mojo	O	1774	3.7×10^{4de}	1	PSBC123
Obbi	O	2108	5.9×10^{3ce}	1	PSBC125
Sandafa	O	2554	1.5×10^{5bcde}	2	PSBC131,132
Sheno	O	2840	ND	2	PSBC133,134
Chole	O	2612	4.8×10^{4de}	2	PSBC135

ND, not determined, PSB abundance data are average of triplicates and data in the same column followed by the same letter do not differ significantly at p=0.05 using Duncan's Multiple Range Test (DMRT). A, Amahara; O, Oromia; T, Tigray.

solubilized P was determined following the phospho-molybdate method (Murphy and Riley, 1962) and the amount of solubilized P obtained from the control was deducted from their respective treatments.

Morphological, biochemical and API characterization of the isolates

The isolates were characterized using the following tests: Gram reaction (KOH test) (Gregorson, 1978); Oxidative/Fermentative (O/F) test (Huge and Leifson, 1953); endospore formation test and Analytical Profile Index (API) biochemical test kits assisted by API computer software (bioMèrieux, France) following the manufacturer's instruction. Gram positive, endospore forming rods were identified to species level using API 50CH test kits. Gram negative rods with fermentative reaction in the O/F test were identified using the API 20E test strips while Gram negative rods with oxidative reaction in O/F test were identified by API 20NE test kits. Moreover, isolates were subjected to 16S rDNA sequence analysis based on the result of API identification system.

Identification by 16S rDNA sequence analysis

Genomic DNA extraction

All isolates were streaked on Luria Bertani (LB) agar media and incubated for 24 h at 30°C to get single colonies. A single colony of each isolate was picked using a sterile tooth pick and suspended in 30 µl sterile H_2O in 50 µl reaction tubes. The DNA from Gram negative bacteria was extracted using the thermal denaturation method (Mohran et al., 1998). All tubes were incubated at 95°C for 10 min. The DNA of Gram positive isolates was extracted by consecutive heat and freeing (for 3 min at 65°C followed by freezing for 3 min at -70°C), the cycles were repeated three times. All the tubes were centrifuged at 13,000 *g* for 2 min and stored at 4°C until further analysis.

Polymerase chain reaction (PCR) amplification of 16S rDNA and sequencing

2 µl of the extracted DNA was used as a template for PCR amplification. The 25 µl PCR reaction generally contained 0.4 µl 10 mM dNTP, 2.5 µl 10× PCR buffer, 2.5 µl 25 mM $MgCl_2$, 0.2 µl (5 units/µl) Taq polymerase, 1 µl (10 mM) of forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1 µl (10 mM) of reverse primer 1492r (5'-GGTTACCTTGTTACGACTT-3'). The PCR program included a denaturation step of 5 min at 95°C, followed by 30 cycles of 95°C for 30 s, 50°C for 45 s, 72°C for 1.30 min, and a final extension step of 10 min at 72°C. PCR products were sanger-sequenced using the forward primer 27f at the Institute of Clinical and Molecular Biology, University of Kiel**.** The sequences were edited using Bioedit (http://www.mbio.ncsu.edu/BioEdit/). A BLAST search on the NCBI Gene Bank database (www.ncbi.nlm.nih.gov/) was used to identity of the isolates.

Phylogenetic analysis

Sequence data were multiple aligned using Clustal W and compared with available sequences of bacterial lineage from the NCBI database. A phylogenetic tree was constructed by using Neighbor-Joining method from distance matrices on MEGA4 program (Tamura et al., 2007). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and were given in units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

Screening in vitro antagonistic activity against Fusarium oxysporum

The *in vitro Fusarium oxysporum f.sp. ciceri* (FOC) growth inhibition activity of the PSB isolates was tested using the dual culture technique (Landa et al., 1997). 20 µl of bacterial culture grown in nutrient broth to the exponential phase was equidistantly spotted on the margins of nutrient agar (NA) plates amended with glucose (1.0%) and incubated at 30°C. A 4 to 5 mm diameter agar disc from potato dextrose agar (PDA) cultures of the fungal pathogens was placed at the center of the NA plate inoculated with the bacterial isolate and incubated at 30°C for 5 days. The radii of the fungal colony towards and away from the bacterial colony were measured and *in vitro* mycelia growth inhibition % was calculated according to Idris et al. (2008).

Assay for Indole-3-acetic acid (IAA) production

Isolates were grown in LB broth supplemented with 5 mM L-tryptophan for 48 h and their IAA producing ability was detected according to Bric et al. (1991). Quantitative analysis of IAA was performed using the method of Loper and Scroth (1986). The culture were centrifuged at 10,000 *g* for 15 min and 2 ml supernatant was transferred into a tube to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5M $FeCl_3$ in 50 ml 35% (v/v) $HClO_4$) were added. The mixture was incubated at room temperature for 25 min. The tubes were observed for the development of pink color and their absorbance was measured at 530 nm. The IAA concentration was determined against standard curve constructed from different concentrations of indole-3-acetic acid.

Response of chickpea to the inoculation with PSB isolates under glasshouse conditions

Sterilization, germination, inoculation and planting of chickpea

Bacterial isolates that showed high phosphate solubilization efficiency in PK broth were selected for their *in vivo* growth promotion test under greenhouse conditions. Chickpea seeds were surface sterilized, germinated and transplanted to plastic pots as described before. Prior to seedling transplantation, germinated seedlings were inoculated with PSB culture grown in PK broth and adjusted to 10^8 cells per seed. Inoculated seedlings were transferred to each pot containing 3 kg soil with the following physical and chemical properties (pH: 6.0, total N: 0.025%, organic carbon: 0.975 %, total P: 1667 ppm, available P: 2.99 ppm, available K: 25.33 ppm, cation exchange capacity: 50 $Cmolkg^{-1}$ and loamy texture). The soil sample was analyzed at the laboratory of Addis Ababa Environmental Protection Agency.

The pots were arranged in completely randomized block design and irrigated with distilled sterilized water every three days. Uninoculated P-fertilized (20 mg kg^{-1}as KH_2PO_4) pots were included as positive controls (PC) and uninoculated non P-fertilized pots as negative controls (NC). After sixty days all seedlings were carefully uprooted and washed under gently flowing tap water. The nodules were counted, and shoot dry weight was measured after drying for 48 h at 70°C. Total N and P concentration analysis of the shoot was carried out using the micro-kjeldahl and phospho-molybdate methods (Murphy and Riley, 1962), respectively at the laboratory of Addis Ababa Environmental Protection Agency.

Data analysis

The data sets were statistically analyzed using analysis of variance (ANOVA) and the treatment means were compared relatively to the controls following Duncan's multiple range test (DMRT) (Duncan, 1955). The correlation between different parameters was evaluated using Pearson's correlation coefficient on SPSS V.15 (SPSS Inc.2000).

RESULTS AND DISCUSSION

Isolation, enumeration and identification of PSB isolates

The abundance of PSB ranged from 2.3×10^3 to 5.6×10^5 CFU g^{-1} of rhizosphere soil from Alamata or Sandfa and Gurura respectively (Table 1). The PSB ≥ 3.1×10^4 in most (83%) of the samples, indicating that those

bacteria are generally highly abundant in the chickpea rhizosphere soils collected from the producing areas of Ethiopia. The population of PSB varied among the samples from different sites. This variation might be attributed to many factors such as soil nutrient status, pH, moisture content, organic matter composition and certain soil enzyme activities (Ponmurugan and Gopi, 2006).

Similarly, several studies indicated that the proportions of such bacteria were very high in the rhizosphere of agriculturally important crops, such as maize, sorghum, rice, barley and chickpea, and thus they could play a crucial role in P nutrition of the crops (Ponmurugan and Gopi, 2006; Rajapaksha and Senanayake, 2011).

Fifty PSB isolates with SI ≥1.40 were selected from 36 soil samples (Table 1). All of them were identified to the genus and species level by API biochemical tests and 16S rDNA sequence analysis. The results revealed the presence of diverse groups of phosphate solubilizing bacteria in the rhizosphere of chickpea. They were dominated by Gram negative rhizobacteria which accounted for 86% of the isolates (Table 2). Gram positive isolates were only 14%, indicating that they represent rather a minority among phosphate solubilizing bacteria in the investigated rhizosphere.The rhizospheres of many agriculturally important crops favor more Gram negative rhizobacteria than Gram positive (Muleta et al., 2009). Furthermore, 8 genera were identified using biochemical tests. Those genera were identified as *Burkholderia* (28%), *Pseudomonas* (20%), *Bacillus* (12%), *Enterobacter* (8%), *Stenotrophomonas* (4%), *Brevibacillus* (2%), *Sphingomonas* (2%), and *Ralstonia* (2%). Interestingly, members of the genus *Pseudomonas* were dominated by *P. fluorescence* (80%) while all isolates of the genus *Burkholderia* were identified as *B. cepacia*. The other isolates, however showed a low identification level (<90%) which might be due to the limitations of using biochemical characteristics to identify environmental isolates with a diverse nature.

To confirm the biochemical characterization, the 16S rDNA sequence analysis was also employed (Table 2). Based on the abundance of a particular group, biochemical test results and PGP activity 20 isolates were selected for sequence analysis. The presence of biochemically identified genera such as *Pseudomonas, Bacillus, Ralstonia,* and *Burkholderia* have been confirmed while two genera (*Acinetobacter* and *Empedobacter*) have been identified additionally. The disagreements between API biochemical test kits and 16S rDNA analysis results could be due to several reasons such as culture conditions and a diverse nature of environmental isolates.

Gram negative isolates exhibited a higher diversity with regard to the detected genera and species compared to the gram positive isolates. Isolates PSBC13, PSBC67, PSBC68 and PSBC69 showed 99% sequence similarity to *Pseudomonas lini* (EU221401), PGPR isolated from wheat rhizosphere while PSBC41 was found to be 99% similar to *Pseudomonas corrugate* (HQ242748) phosphate solubilizing bacteria. On the other hand, PSBC97 showed 100% sequence similarity with the denitrifying bacterium *Ralstonia pickettii* (HE575958) isolated from soil whereas PSBC02 showed 99% similarity with *R. pickettii* strain QL-A6 (HQ267096) obtained from tomato rhizosphere. Isolate PSBC05 was found to be 99% similar to *Burkholderia phytofirmans* (HQ242761) phosphate solubilizing rhizobacteria isolated from P-rich soil while PSBC07 and PSBC99 showed 99% similarity with *Burkholderia terricola* (FM209484) salt tolerant bacteria isolated from faba bean nodules. Isolates PSBC06 and PSBC89 were found to be 99% similar with *Acinetobacter lwoffii* (FN393792) and *A. lwoffii* (EF204280) obtained from different environmental samples, respectively while PSBC123 showed 99% similarity with *Acinetobacter johnsonii* (EU594557), an endophytic bacteria obtained from sugar beet. Isolates PSBC40 and PSBC63 were found to be 99 and 100% similar with *Empedobacter brevis* (EU293154) isolated from soil, respectively. Several studies indicated that different species from genus *Pseudomonas, Ralstonia, Burkholderia, Flavobacterium* and *Acientobacter* are efficient phosphate solubilizing bacteria (Rodriguez and Fraga, 1999).

Gram positive isolates PSBC31 and PSBC71 were found to be 99% similar with *Bacillus subtilis* (EF656456) endophytic bacteria from wheat and antagonistic to wheat sharp eyespot disease and *B. subtilis* (HM027569) soil-borne PGPR, respectively whereas PSBC28 showed 99% similarity with soil *B. subtilis* (HM027569). Isolate PSBC17 showed 99% similarity with *Bacillus flexus* (JQ660603) obtained from verimcompost. Moreover, the partial 16S rDNA sequences of the isolates were multiple aligned with the nearest neighbors and other relevant bacterial sequences, and their identity and evolutionary history was inferred by constructing phylogenetic tree (Figure 2). The phylogenetic tree showed the clustering of PSBC isolates with their respective genera with high bootstrap support values.

Qualitative analysis of phosphate solubilizing efficiency of the isolates

The SI of each PSB isolate is depicted in Table 1. Phosphate solubilizing efficiency of these isolates was qualitatively determined on PK agar medium using SI as measure of their efficiency and the result showed that there were significant ($p \leq 0.05$) variations among the isolates. The SI of the isolates ranged from 1.40 (PSBC126) to 3.06 (PSBC02). This indicates that PSBC02 was the most efficient phosphate solubilizing bacteria on PK agar medium. Generally, the highest SI was produced by Gram negative isolates indicating that they are more efficient in solubilization of TCP on PK agar medium which has previously been observed

Table 2. Phosphate solubilization index and identification of phosphate solubilizing bacteria isolated from chickpea rhizosphere.

Isolate	SI± SD	Gram reaction	O/F test	Endospore test	API identification	16S rDNA sequence identification
PSBC01	1.7±0.10^{j-n}	-	O	-	*Pseudomonas fluorescence*	ND
PSBC02	3.06±0.42^{a}	-	O	-	UI	*Ralstonia pickettii (JX979097)*
PSBC05	2.25±0.25^{d-f}	-	O	-	UI	*Burkholderia phytofirmans (JX979098)*
PSBC06	1.57±0.15^{k-n}	-	O	-	UI	*Acinetobacter lwoffii (JX979099)*
PSBC07	2.66±0.34^{bc}	-	O	-	*Burkholderia cepacia*	*Burkholderia terricola (JX979100)*
PSBC10	1.54±0.19^{l-n}	-	O	-	*Burkholderia cepacia*	ND
PSBC13	2.28±0.25^{d-f}	-	O	-	*Pseudomonas* sp.	*Pseudomonas lini (JX979123)*
PSBC14	1.44±0.06^{n}		O	-	*Burkholderia cepacia*	ND
PSBC15	2.88±0.18^{ab}	-	O	-	*Stenotrophomonas maltophila*	"
PSBC16	2.83±0.17^{a-c}	+	O	+	*Bacillus* sp.	"
PSBC17	2.53±0.38^{b-e}	+	O	+	*Bacillus* sp.	*Bacillus flexus (JX979101)*
PSBC18	1.94±0.07^{f-j}	-	O	-	*Burkholderia cepacia*	ND
PSBC28	2.85±0.17^{a-c}	+	O	+	*Bacillus* sp.	*Bacillus subtilis (JX979102)*
PSBC29	1.65±0.13^{j-m}	+	O	+	*Bacillus stearothermophilus*	*Bacillus subtilis (JX979103)*
PSBC30	2.48±0.13^{c-e}	-	O	-	*Burkholderia cepacia*	ND
PSBC31	1.91±0.10^{g-k}	+	O	+	*Brevibacillus brevis*	*Bacillus subtilis (JX979104)*
PSBC33	2.20±0.20^{e-g}	-	O	-	*Pseudomonas fluorescence*	ND
PSBC35	2.50±0.10^{c-e}	-	F	-	*Enterobacter* sp.	"
PSBC40	1.90±0.10^{g-l}	-	O	-	UI	*Empedobacter brevis (JX979105)*
PSBC41	1.45±0.04^{n}	-	O	-	*Pseudomonas fluorescence*	*Pseudomonas corrugate (JX979106)*
PSBC42	1.52±0.14^{mn}		O	-	*Burkholderia cepacia*	ND
PSBC45	2.00±0.07^{f-j}	-	O	-	*Burkholderia cepacia*	"
PSBC46	2.12±0.13^{f-h}	-	O	-	*Burkholderia cepacia*	"
PSBC61	2.12±0.12^{f-h}	-	O	-	UI	"
PSBC62	2.06±0.17^{f-i}	-	O	-	UI	"
PSBC63	2.50±0.25^{c-e}	-	O	-	UI	*Empedobacter brevis (JX979107)*
PSBC67	2.07±0.12^{f-i}	-	O	-	*Pseudomonas fluorescence*	*Pseudomonas lini (JX979108)*
PSBC68	1.69±0.27^{j-n}	-	O	-	*Pseudomonas fluorescence*	*Pseudomonas lini (JX979124)*
PSBC69	1.83±0.11^{h-m}	-	O	-	*Pseudomonas aeruginosa*	*Pseudomonas lini (JX979109)*
PSBC70	1.51±0.15^{mn}	-	O	-	*Pseudomonas fluorescence*	ND
PSBC71	2.08±0.29^{f-h}	+	O	+	*Bacillus sp.*	*Bacillus subtilis (JX9110)*
PSBC72	2.07±0.12^{f-i}	-	O	-	*Pseudomonas fluorescence*	ND
PSBC74	2.62±0.13^{b-d}	-	O	-	*Burkholderia cepacia*	"
PSBC79	2.58±0.14^{b-d}	-	F	-	*Enterobacter sakazaki*	„
PSBC81	1.43±0.07^{n}	-	F	-	*Enterobacter* sp.	-
PSBC86	1.95±0.25^{f-j}	-	O	-	*Sphingomonas paucimobils*	-
PSBC89	1.43±0.06^{n}	-	O	-	UI	*Acinetobacter lwoffii (JX979111)*
PSBC97	1.87±0.08^{g-m}	-	O	-	*Ralstonia pickettii*	*Ralstonia pickettii (JX9112)*
PSBC99	1.46±0.05^{n}	-	O	-	*Burkholderia cepacia*	*Burkholderia terricola (JX979113)*
PSBC108	1.52±0.17^{mn}	-	O	-	*Stenotrophomonas maltophila*	ND
PSBC109	1.86±0.13^{g-m}	-	O	-	*Burkholderia cepacia*	-
PSBC123	1.46±0.12^{n}	-	O	-	UI	*Acinetobacter johnsonii (JX979126)*
PSBC125	1.81±0.15^{h-m}		O	-	*Pseudomonas fluorescence*	ND
PSBC126	1.40±0.16^{n}	-	O	-	*Burkholderia cepacia*	-
PSBC131	1.80±0.17^{h-m}	+	O	+	*Bacillus sp.*	-
PSBC132	1.85±0.11^{g-m}	-	O	-	*Burkholderia cepacia*	-
PSBC133	1.66±0.13^{j-n}	-	O	-	UI	-
PSBC134	1.44±0.06^{n}	-	O	-	*Burkholderia cepacia*	-
PSBC135	1.43±0.05^{n}	-	O	-	UI	-

ND, not determined; O, oxidative; F, fermentative; UI, showed low identification level (<90%) by API computer software; +, Gram positive or positive for the test; -, Gram negative or negative for the test; parenthesis: accession number in NCBI Gene bank; means in the same column followed by the same letter do not differ significantly at p=0.05 using Duncan's Multiple Range Test (DMRT)

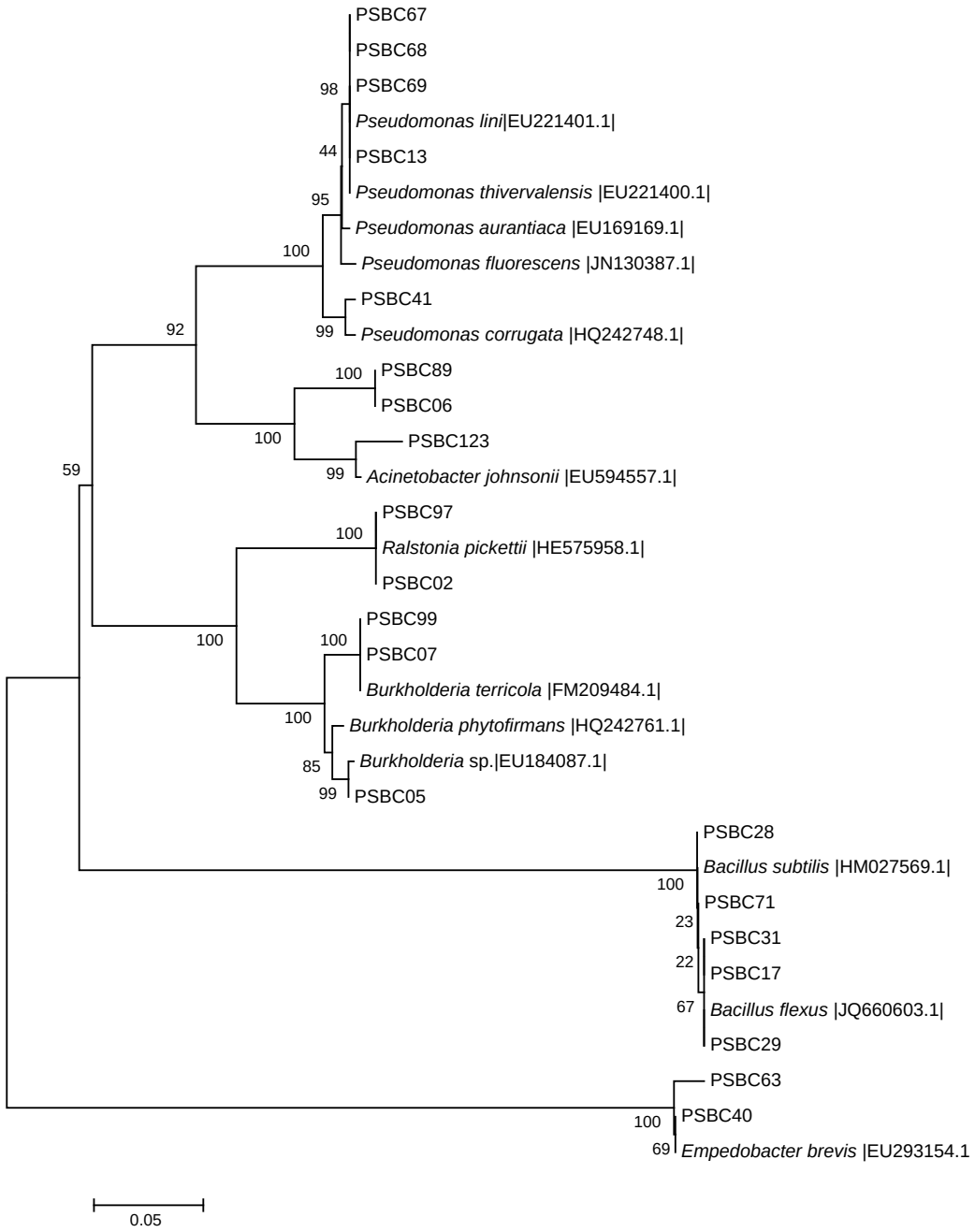

Figure 2. Neighbour-Joining phylogenetic tree of 16S rRNA gene sequence showing the positions of different PSBC strains. Bootstrap values are shown at branching point.

(Muleta et al., 2013).

Quantitative analysis of phosphates solubilization efficiency of the PSB isolates

The quantitative analysis of insoluble P sources solubilization efficiency of selected isolates was carried out in PK broth using TCP, RP and BM as sole P sources. All tested isolates released a larger amount of soluble phosphates into the medium until the last sampling day (Table 3). The amount of soluble P was found to vary significantly between the isolates and sampling days. After 12 days of incubation, the highest amount of P was released by PSBC35 (379 µg ml^{-1}), indicating that this isolate is the most efficient TCP solubilizer (Table 3). On the other hand, PSBC29 was found to be poor in solubilization of TCP as it released

Table 3. TCP and rock phosphate solubilization efficiency of PSB isolates from chickpea rhizosphere.

Isolate	$Ca_3(PO4)_2$						Rock phosphate						Bone meal					
	4th day		8th day		12th day		4th day		8th day		12th day		4th day		8th day		12th day	
	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE	pH	P (µg ml^{-1}) ±SE
PSBC05	5.2	13±8^{c}	5.8	174±9^{b-d}	6.3	145±12^{c}	4.2	28±1^{b}	4.7	42±1^{c}	4.9	44±2b	-	-	-	-	-	-
PSBC17	4.8	26±10^{c}	4.9	200±12^{b-d}	5.9	207±9^{b}	5.1	14±1^{cd}	5.3	53±2^{a}	5.6	56±1^{a}	6.0	155±1^{b}	6.1	161±6^{c}	6.2	162±3^{c}
PSBC28	4.8	30±4^{c}	5.7	172±44^{b-d}	5.9	161±53^{c}	4.1	10±1^{d}	4.2	7±1^{h}	4.5	7±1^{f}	-	-	-	-	-	-
PSBC29	4.9	15±6^{c}	4.7	70±20^{e}	5.9	137±44^{c}	4.6	14±1^{cd}	5.0	20±1	5.1	14±3^{e}	-	-	-	-	-	-
PSBC31	5.0	21±7^{c}	5.3	148±42^{c-e}	5.9	148±58^{c}	5.8	12±4^{cd}	5.8	15±1^{f}	5.8	15±1^{e}	-	-	-	-	-	-
PSBC33	4.6	41±4^{c}	5.1	295±39^{a}	5.8	315±50^{a}	4.1	36±3^{a}	4.1	38±3^{d}	4.9	38±1^{c}	6.1	119±7^{d}	6.1	115±1^{e}	6.1	120±4^{e}
PSBC35	5.4	120±32^{b}	5.8	263±42^{a}	6.1	379±54^{a}	4.9	31±3^{ab}	5.8	46±4^{b}	6.1	57±3^{a}	6.1	124±2^{c}	6.1	131±3^{d}	6.3	139±1^{d}
PSBC79	5.6	171±70^{a}	5.8	238±44^{ac}	6.2	346±28^{a}	4.3	32±2^{ab}	4.6	34±3^{e}	5.0	33±3^{d}	5.0	33±11^{f}	5.5	182±8^{b}	5.7	195±1^{b}
PSBC81	4.9	48±10^{c}	5.2	193±12^{b-d}	6.1	299±48a	4.1	35±9^{a}	4.7	38±1^{d}	5.0	35±4^{cd}	6.0	211±5^{a}	6.4	217±3^{a}	6.5	232±1^{a}
PSBC97	5.0	53±14^{c}	5.6	110±42^{c-e}	6.0	143±37c	6.9	17±1^{c}	5.6	12±1^{g}	6.1	15±1^{e}	4.6	66±2^{e}	5.0	87±9^{f}	4.8	102±4^{f}

SE, standard error; TCP, tricalcium phosphate, the data is average of triplicates. Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by Duncan's Multiple Range Test (DMRT).

only 137 µg ml^{-1} of soluble P after 12 days. However, a slight decline was found for PSBC5 and PSBC31 after the 8th day of incubation. During solubilization the isolates showed a sharp decline of pH from neutral to acidic until the 4th day of incubation (Table 3). The acidic pH ranged from 4.8 (PSBC33) to 5.6 (PSBC79). After the 4th day all isolates except PSBC29 exhibited progressively increasing pH of the growth medium until the 12th day of incubation. The correlation analysis showed that there was statistically significant inverse relation between the concentration of soluble P and concomitant drop in pH of the growth medium (r = -0.82), suggesting that acid production is the mechanism of TCP solubilization.

Similarly, selected isolates were tested for their rock phosphate solubilization ability. The amount of soluble phosphorus released and corresponding pH change of the medium varied among isolates and sampling dates (Table 3). The highest amount of soluble P was 57 µg ml^{-1} which was released by PSBC35, while the least was 7 µg ml^{-1} recorded for PSBC28 on 12th day of incubation. The amount of soluble P was increased gradually up to the 12th days of incubation for most isolates (PSBC17, PSBC5, PSBC33 and PSBC31) while the other isolates showed a slight decrease. There were sharp drops of pH from 7.0 to acidic pH values during RP solubilization. The lowest pH was noted on 4th day for all isolates except PSBC97. Then, the pH of the growth medium increased gradually until the 12th day. The correlation analysis showed that there was an inverse relation (r = -0.34) between soluble P and an associated drop in pH of the culture medium suggesting that acidification of the growth medium as mechanism of rock phosphate solubilization.

The amount of soluble P released from the finely ground old bone (BM) and corresponding pH drop of the medium by the PSBC isolates is shown in Table 3. All tested isolates showed a very high BM solubilization ability with different levels of efficiency. There was a significant variation among isolates in terms P solubilization and concomitant pH change of the media. Isolate PSBC81 was more efficient in solubilizing BM (233 µg ml^{-1}) than the other isolates, followed by PSBC79 (195 µg ml^{-1}). The concentration of soluble P increased progressively with days of incubation. Similar to other P sources, a drop in pH was observed over the course of BM solubilization in all isolates. On the 4th day drops in pH ranging from 4.6 to 6.1 were noted for isolates PSBC97 and PSBC33, respectively. Then, pH of the medium was increased gradually until the last sampling day. A positive correlation (r = 0.27) was observed between the concentration of soluble P and decrease in pH which was,

however, not statistically significant. This is in line with the result of the previous study (Keneni et al., 2010) suggesting that mechanisms other than acid production were involved in the dissolution of BM. Hence, further studies on the solubilization mechanisms of BM are highly recommended.

All PSB isolates recovered from chickpea rhizosphere soils showed considerably higher solubilization ability of different insoluble inorganic phosphates in broth culture. In general, the highest amount of P was released from TCP followed by BM after 12 days of incubation. The lowest dissolution and sharpest drop in pH was obtained during solubilization of RP. Likewise, a previous study (Keneni et al., 2010) showed that phosphate solubilizing microorganisms mobilized more P from insoluble inorganic salts than from naturally occurring phosphate sources such as rocks and bone meal which are made of apatite.

All isolates displayed a significant drop in pH of the culture media during the solubilization of all tested insoluble phosphate sources. Several studies indicated that the drop in pH is due to the production of organic acids during bacterial growth (Chen et al., 2006; Muleta et al., 2013; Vyas and Gulati, 2009). The decreasing pH and the production of organic acids resulted in solubilization of a considerable amount of soluble P (Chen et al., 2006). The results of the present study established a clear relationship between pH drop and P solubilization. These results are consistent with the report of earlier studies (Illmer and Schinner, 1995; Whitelaw, 2000; Naik et al., 2008; Vyas and Gulati, 2009) which showed that solubilization of insoluble phosphate sources were mediated specifically by the decreasing pH of the medium. It has been well documented that mineral phosphate solubilization by phosphate solubilizing bacteria strains results from the release of low molecular weight organic acids (Illmer and Schinner, 1995; Kim et al., 1997; Vyas and Gulati, 2009), probably as an interacting effect of their hydroxyl or carboxyl groups that may chelate the cations bound to the phosphate, thereby converting it into soluble forms (Kpomblekou and Tabatabi, 1994; Kim et al., 1997). Gram negative isolates displayed better performance in solubilization of different insoluble inorganic P sources compared to Gram positive phosphate solubilizing bacterial isolates in the study which is in line with the results of previous studies (Muleta et al., 2013; Tripura et al., 2007). The fluctuations and differences in insoluble P sources solubilization efficiency observed among the isolates could be attributed to differences in the types and the amount of organic acids produced (Vyas and Gulati, 2009). Alternatively, it could also be explained by the differences of in rate of P release and immobilization (Muleta et al., 2013; Prasanna et al., 2011), when cells in the culture immobilize more phosphate for microbial biomass production, the corresponding values decrease, creating such fluctuations (Muleta et al., 2013). From our the observation in this study, a decrease in soluble P and concomitant increase in the pH values of the growth medium indicates re-utilization of available P (Rashid et al., 2004; Tripura et al., 2007). In addition, the excreted organic acids could be reused by the isolates for their own metabolism (Tripura et al., 2007) which is highly associated with an exhaustion of the original carbon source (Rashid et al., 2004) since phosphate solubilization is a complex process depending on many factors such as nutritional, physiological and growth conditions of the culture (Reyes et al., 1999).

The results indicate that PSB isolates native to Ethiopian soils exhibited high potential for RP solubilization. The dissolution of rock phosphate to a significant extent by these indigenous PSB isolates would give the opportunity to utilize rock phosphate deposits found in different parts of the country (Muleta et al., 2013) as cheap P fertilizers. Earlier studies in Ethiopia (Bekele and Hofner, 1993) indicated successful use of rock phosphate under greenhouse conditions. Thus, inoculation of native PSB isolates with superior rock phosphate solubilization ability along with the application of rock phosphate could improve the productivity of chickpea and other crops. Furthermore, the superior BM solubilization of these isolates would also help to use the waste accumulated around the abattoirs found in different urban areas of the country as P fertilizer along with PSB inoculants. This could reduce the dependence on the very expensive chemical fertilizers and enable a shift towards sustainable crop farming.

Multiple PGP characteristics of PSB isolates from chickpea rhizosphere

All PSB isolates were screened for multiple PGP characteristics such as IAA production and inhibition of *F. oxysporum* growth under *in vitro* conditions (Table 4). The results indicate that in addition to their phosphate solubilization activity, the PSB isolates exhibited different PGP properties. Dual culture assay indicated that 18.4% of the isolates inhibited the growth of *F. oxysporum* under *in vitro* conditions (Table 4). These isolates belonged to the genera *Pseudomonas* (44.4%), *Burkholderia* (22.2%) and *Acientobacter* (11.1%). All antagonistic isolates from *Pseudomonas* group were identified as *Pseudomonas fluorescence*. Several studies have reported that phosphate solubilizing bacterial isolates obtained from different agriculturally important crops rhizosphere were antagonistic to different fungal phytopathogens such as *Fusarium* species, *Phytium* species, *Rhizoctonia* species and others under *in vitro* conditions (Peix et al., 2001b; Naik et al., 2008). In Ethiopia, chickpea production has been constrained by wilt and root rot caused by different fungal plant pathogens (Ahmed and Ayalew, 2006). These diseases have been responsible for major yield losses (Ahmed and Ayalew, 2006) and thus isolates which inhibited the growth of the respective pathogens

Table 4. Multiple PGP characteristics of PSB isolated from chickpea rhizosphere.

Isolate	FOC inhibition (%)± SE	IAA (µg ml^{-1}) ± SE
PSBC01	41.2±1.3^{b}	-
PSBC02	-	17.6±0.8^{d}
PSBC05	38.5±1.1^{c}	-
PSBC06	-	12.3±1.1^{f}
PSBC13	-	19.2±1.0^{d}
PSBC14	-	19.7±1.2^{d}
PSBC16	-	21.4±0.2^{d}
PSBC17	-	61.2±1.2^{a}
PSBC28	-	20.5±0.7^{d}
PSBC29	-	16.4±0.8^{e}
PSBC31	-	16.3±0.6^{e}
PSBC33	-	37.7±1.2^{b}
PSBC39		25.8±0.2^{c}
PSBC40	-	22.1±1.4^{d}
PSBC61	-	18.2±0.9^{d}
PSBC62	25.6±1.5^{e}	-
PSBC67	-	19.4±1.4^{d}
PSBC68	40.3±1.8^{b}	13.4±1.3^{ef}
PSBC69	44.2±1.6^{a}	16.8±1.5^{d}
PSBC70	45.8±1.9^{a}	18.7±1.6^{d}
PSBC71	-	19.6±1.3^{d}
PSBC72	-	14.6±0.4^{e}
PSBC79	-	15.5±0.7^{e}
PSBC81	-	58.0±2.0^{a}
PSBC97	-	18.6±1.5^{d}
PSBC123	26.3±2.1^{e}	14.3±0.6^{e}
PSBC125	-	-
PSBC126	-	15.2±1.0^{e}
PSRB134	40.5±1.4^{b}	-
PSRB135	30.3±2.1^{d}	-

IAA, indole acetic acid; SE, standard error; +, positive for the test; -, negative for the test; FOC, *Fusarium oxysporum f.sp. cicero.* Means in the same column followed by the same letter do not differ significantly at p=0.05 using Duncan's Multiple Range Test (DMRT).

would give dual advantage as biofertilizer and biocontrol agents. Consequently, such isolates are highly recommended for greenhouse and field tests.

Further, 49.0% of the PSB isolates were shown to be capable of producing IAA equivalent substance, indicating that this ability is common among rhizosphere bacteria. The majority of these isolates were members of genus *Pseudomonas* (12.3%) and *Bacillus* (27.2%) while all the rest belonged to the genera *Ralstonia, Empedobacter, Acientobacter, Burkholderia* and *Enterobacter*. The highest amount of IAA equivalent substance produced by PSBC17 was 61 µg ml^{-1} whereas the lowest was by PSBC68 (14 µg ml^{-1}) (Table 4). The most capable strains belonged to the genera *Bacillus* and *Enterobacter*. Similarly, several previous studies demonstrated that PSB isolates from different crops rhizosphere were found to exhibit different IAA production ability (Ponmurugan and Gopi, 2006; Vikram et al., 2007; Naik et al., 2008; Shahab et al., 2009; Yadav et al., 2010). These studies indicated that most of the isolates produced considerably higher amount of IAA. The large variation in the amount of IAA produced by different strains has been attributed to the variability in the metabolism of different strains of PSB (Leinhos and Vacek, 1994). Such isolates were found to be excellent in promoting plant growth under greenhouse experiments (Shahab et al., 2009; Yadav et al., 2010). Thus, these isolates could provide additional plant growth promoting activities apart from solubilizing insoluble P sources and hence further studies are required to explore the exact contribution of IAA production in the promotion of plant growth.

Table 5. Effects of inoculation of PSB strains on growth of Chickpea.

Treatments	Nodule number plant^{-1} ±SE	Shoot dry weight(g) Plant^{-1}±SE	Total Nitrogen (%) of shoot± SE	Phosphorous concentration in shoot(mg/g SDW) ± SE
UIA	25.0±2.6^{d}	0.75±0.02^{f}	1.34±0.07^{d}	1.38±0.059^{c}
P+	32. 7±4.0^{c}	1.09±0.22^{cd}	2.02±0.48^{b}	1.55±0.140^{c}
PSBC17	32. 7±2.5^{c}	0.92±0.02^{def}	1.44±0.05^{d}	1.33±0.051^{d}
PSBC33	33.0±3.6^{c}	0.89±0.06^{ef}	1.63±0.12^{cd}	1.36±0.031^{d}
PSBC35	41. 7±2.6^{b}	1.41±0.16^{ab}	2.48±0.19^{a}	1.75±0.261^{bc}
PSBC81	44.7±2.5^{ab}	1.47±0.15^{a}	2.03±0.02^{bc}	1.42±0.158.5^{c}
PSBC79	32.3±3.2^{c}	0.92±0.03^{def}	2.43±0.15^{ab}	2.01±0.49^{ab}

SE, standard error. The data is average of triplicates. Numbers in the same column followed by the same letter do not differ significantly at p= 0.05 by Duncan's Multiple Range Test (DMRT); SDW, shoot dry weight; UIA, uninoculated control; P+, soluble p fertilizer treated.

Response of chickpea to inoculation PSB with different P sources

The effects of inoculation of PSB isolates on chickpea growth parameters such as the number of nodules, nodule dry weight, shoot dry matter yield and, N and P concentration of shoot were investigated under glasshouse conditions. During this experiment, *B. flexus* (PSBC17), *P. fluorescence* (PSBC33), *Enterobacter sp.* (PSBC35), *Enterobacter sakazaki* (PSBC79) and *Enterobacter sp.* (PSBC81) were used as inoculants (Table 5).

Shoot dry matter yield of the plant increased significantly by inoculation of all isolates except PSBC33 when compared to the uninoculated control. Isolate PSBC81 and PSBC35 were the most effective ones in terms of increasing shoot dry weight of chickpea with an increase in shoot dry weight by 96 and 88%, respectively over the uninoculated control (Table 5). These increases were significantly higher ($p \leq 0.05$) compared to shoot dry weight of P fertilized plants. Though it is difficult to production of growth promoting IAA equivalent substances by the all the isolates used as inoculants contributes to their stimulatory effects on plant growth. Likewise, Yadav et al. (2010) reported that most of PGPR isolates resulted in a significant increase in shoot length, root length and dry matter production of shoot and root of chickpea seedlings under growth chamber conditions.

The number of nodules was one of the parameters measured and used to evaluate the effect of different PSB isolates inoculation. All tested isolates significantly improved nodulation of chickpea over the uninoculated control. PSBC81 was the most efficient in inducing nodulation of chickpea followed by isolate PSBC35, increasing the number of nodules by 78.8 and 66.8%, respectively. These superior increments were also significantly higher than the number of nodules obtained from P fertilized plants. Inoculation of all isolates improved dinitrogen fixation detected as an increase in N concentration in shoot over the uninoculated control. However, only PSBC35, PSBC79 and PSBC81 resulted in a significant increase in N concentration compared to the uninoculated control. The highest concentration of N was obtained in plants inoculated with PSBC35 followed by PSBC79 with N concentrations of 2.48 and 2.43%, respectively.

The study demonstrated that the increase in nodule number is positively correlated with the increase in shoots dry weight (r=0.93) and N content (r=0.70). Peix et al. (2001b) have previously established similar correlation among the growth parameters in common bean inoculated with the phosphate solubilizing strain of *Burkholdera cepacia.* It has been reported that available P deficiency in soils could also have a deleterious effect on symbiotic interaction between rhizobia and legume crops like chickpea, thus affecting its growth and productivity (Gyaneshwar et al., 2002). Several studies have demonstrated that inoculation of seedlings with PSB isolates increased the level of available P in the rhizosphere soil (Sundara et al., 2002; Vyas and Gulati, 2009). In this way, inoculation of chickpea with selected phosphate-solubilizing bacteria improves nodulation and dinitrogen-fixation processes of this legume crop.

Likewise, inoculation effects of PSB isolates on the concentration of P in shoot of chickpea have been shown. Inoculation of PSBC79, PSBC81 and PSBC35 significantly increased the concentration of P in chickpea shoot over the uninoculated control (Table 5). In contrast, the P concentration of PSBC33 and PSBC17 inoculated plants did not vary significantly from uninoculated control plants. The highest concentration (2.01 mg) of P was obtained in PSBC79 inoculated plant whereas the lowest (1.16 mg) was recorded in PSBC33 inoculated plants. Though the effect of PSB inoculation on the level of available P was not determined, results obtained suggests that they improved the availability of soluble P in the rhizosphere soil and thus, the P uptake of the plant. Several studies have revealed that inoculation of PSB isolates increased the available P in soil (Sundara et al., 2002; Vyas and Gulati, 2009) and concomitantly improved P uptake of the plant (Peix et al., 2001a; Peix et al., 2001b; Vikram and Hamzehzarghani, 2008; Castango et

al., 2011).

Conclusion

In general, the results of the study showed that chickpea rhizosphere soils from different producing areas of Ethiopia harbor considerably high numbers of PSB isolates. The study has also demonstrated that those PSB isolates belong to different bacterial genera: *Acinetobacter, Bacillus, Brevibacillus, Burkholderia, Chryseomonas, Enterobacter, Empedobacter, Pseudomonas, Ralstonia, Sphingomonas* and *Stenotrophomonas.* The PSB strains were able to release significantly higher amount of soluble P from different insoluble phosphates sources such as TCP, RP and BM. The results also revealed phylogenetic and biochemical diversity of PSB with multiple plant growth promoting traits. Greenhouse experiment showed that inoculation of chickpea with PSB resulted in significant increase in nodule number, dry matter yield, N and P uptake of the plant. Moreover, the study indicated that these strains are of particular interest for future research and need to be tested in the field under different agroecological conditions. The diversity and PGP characteristics study of PSB from rhizosphere soil will be useful to design strategies to use these strains as inoculants in sustainable agriculture. Thus, consecutive studies should focus on field studies on those strains due to the potentially high importance for the nutrition in this area and in this context for an improvement of the sustainability of land use in Ethiopia.

Conflict of interests

We do not have any conflict of interest.

ACKNOWLEDGEMENTS

The first author would like to thank Addis Ababa University, Wollega University, Institute für Allgemeine Mikirobiologie, Christian-Albrechts-Universität Kiel and German Academic Exchange Service (DAAD) for financial support.

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Vol. 15(35), pp. 1913-1919, 31 August, 2016
DOI: 10.5897/AJB2012.8384
Article Number: D7BC4E160243
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of packaging and chemical treatment on storage life and physicochemical attributes of tomato (*Lycopersicon esculentum* Mill cv. Roma)

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Received 20 December, 2012; Accepted 17 August, 2016

Fresh fruits and vegetables are inherently more liable to deterioration under tropical conditions characterized by high ambient temperatures and humidity. In determining the effects of chemical treatment on tomatoes (*Lycopersicon esculentum* Mill cv. Roma), fruits purchased at turning stage of ripening were packaged in low density polyethylene bags (60 µ) containing wooden dust particles moistened with 400 ppm potassium permanganate solution. Samples were treated with hot water dip, boric acid (H_3BO_3) dip at 1000 ppm, $CaCl_2$ dip at 10,000 ppm, a combination of H_3BO_3 and calcium chloride treatment as well as control. Results of chemical treatment showed increase in weight loss, pH, and a slight increase in moisture content. Total soluble solids and titratable acidity of samples showed a steady decrease, with data on physicochemical qualities collected at 7 days interval. Fruits stored with hot water and combination of H_3BO_3 and $CaCl_2$ treatments showed higher keeping quality. Shelf life elongation treatments used at tropical ambient temperature of 30 ± 2°C was able to preserve tomato fruits for 21 days from spoilage and microbial attack while retaining its colour and other physicochemical properties.

Key words: Tomato, shelf life, packaging, respiration, pretreatment.

INTRODUCTION

Post-harvest loss of fruits and vegetables are a matter of concern especially for countries whose economy is based on agriculture. Horticultural crops due to high moisture content are inherently more susceptible to deterioration especially under high temperature conditions. Consequently, postharvest losses of fruits and vegetables are extremely high in Nigeria (30 - 50%), exacerbated by poor marketing, distribution and storage facilities (Aworh, 2009). Tomato (*Lycopersicon esculentum* Mill) is one of the very perishable fruits and it changes continually after harvesting (Babitha and Kiranmayi, 2010). Depending on the humidity and temperature, it ripens very quickly,

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ultimately resulting in poor quality as the fruit becomes soft and unacceptable. Upon harvest, many processes affecting quality loss sets in, thus limiting storage life by factors such as transpiration, postharvest diseases, increased ripening and senescence. Unlike other chilled perishable foods, fresh produce continues to respire after harvesting (Padmini, 2006). Respiration is affected by the intrinsic properties of fresh produce as well as various extrinsic factors, including ambient temperature.

Packaging fresh and unprocessed fruits and vegetables poses many challenges for packaging technologists in Nigeria and Sub-Saharan Africa. The quality of fresh produce is markedly dependent on growing conditions, minimizing bruising and other damage during harvesting and processing (Galic et al., 2011). Theoretically, an ideal package has a perfect chemical inertia that allows the food product to hold its original characteristics. But in reality, there are interactions between the package and the food product. These interactions depend not only on the package system itself, but also on the intrinsic nature of the preserved food product (Casp et al., 1999). With the high rate of spoilage of harvested food produce exacerbated by increased respiration and ethylene production in tropical regions of the globe, producers are faced with the challenge of shelf life extension of these produce. Several authors have reported the use of pretreatment with different compounds either in isolation or in combination with other preservation methods (Wang et al., 2010; Workneh et al., 2012; Tigist et al., 2013) for extension of storage life of fresh harvested produce.

Tomatoes and other fruits and vegetables in Nigeria are commonly transported at long distance haulage either loose or packed in raffia baskets, jute bags, fiberboard cartons and other improvised containers made from metal, plastic and wood. The traditional basket of the 'inverted cone' design offers little protection to perishable produce (Aworh, 2009). Since they invariably have no handles, the produce is compressed each time they are lifted as the pressure is transmitted inwards. Their rough surfaces puncture the produce accelerating decay and physiological breakdown. Primary modes of tomato transportation to processing facilities and retail outlets include road and rail with road transportation being the more preferred mode of transport for the harvested produce (Olayemi et al., 2010; Sibomana et al., 2016). This research therefore seeks to develop an appropriate method of polyethylene packaging and to determine the best chemical treatment required for storage life elongation of indigenous tomato varieties, with minimal or no loss in quality at ambient conditions.

MATERIALS AND METHODS

Experimental site and materials

The research was conducted at the food processing laboratory, Department of Food Technology, University of Ibadan, Ibadan, Nigeria. Indigenous Roma-VF tomato (*L. esculentum* Mill.) used for this research work, were purchased from Shasha, a local market in Ibadan, South West Nigeria. Tomato cultivar was purchased at turning stage of ripening; pink colour extending from blossom end, covering 10 to 30% of fruit (Rubatzy and Yamaguchi, 1997). Tomato fruits were then transported to the laboratory and selected for uniformity of colour, size and freedom from defects. Upon sorting, tomato was gently washed by hand, dried, before being used in subsequent experiments.

Chemical treatment and packaging

Tomato fruit at turning stage of ripeness were divided into five lots, with each lot having a different chemical treatment. Treatments given to samples include sample A, no treatment; sample B, hot water treatment; sample C, hot water and H_3BO_3; sample D, hot water and $CaCl_2$; sample E, hot water, H_3BO_3 and $CaCl_2$ treatment. Hot water treatment was carried out by hot water dip at 55°C for 5 min in a thermostated water bath. H_3BO_3 treatment was carried out by dipping tomato fruits in 1000 ppm boric acid solution for 30 to 60 s, removed and dried (Sammi and Tariq, 2007). $CaCl_2$ treatment was also done by dipping tomato fruits in 10,000 ppm $CaCl_2$ solution for 1 to 2 min, removed and dried (Babitha, 2006; Sammi and Tariq, 2007). For $KMnO_4$ treatment, 400 ppm saturated solution of $KMnO_4$ was prepared; it was then wetted in saw dust, tied in a stay cloth and introduced into the packaged tomato fruits. All treated fruits were packaged in perforated low density polyethylene (LDPE) bags of 60 µ measuring 12 by 9.5 cm, containing saturated solution of $KMnO_4$ which was wetted in saw dust, placed in stay cloth material and then packaged with tomato fruit. Samples were then placed in paper boards, with each packaging material containing two tomato fruits each and placed in the paper board. Packaged samples placed in the paper board were then stored at ambient temperature of 30 ± 2°C.

The stage of ripeness of each tomato fruit was determined using the USDA standard for the classification of matured tomato. Fruits were also evaluated on a 7-point scale by means of visual colour description with score 1, for turning colour stage; 2 for 30% pink; 3 for 70% pink; 4 for 30% light red; 5 for 70% light red; 6 for 30% red; and 7 for 70% red.

Physiological weight loss

The physiological weight loss (PWL) was determined according to the method of Tefera et al. (2007). PWL was calculated for the storage days and converted to percentage of initial weight recorded for each sampling interval. Obtained values of PWL were expressed in percentage with respect to different treatments.

pH and titratable acidity (TA)

Approximately 80 mL of distilled water was added to samples which has already been cut to smaller size and milled. Puree obtained after milling was filtered using a muslin cloth into a beaker. The electrode of the pH meter was then placed in 60 mL of filtrate obtained from sample puree and used for determination of pH values. A pH meter (EDT instrument, model: BA 350) was used for determining the pH.

A total of 50 mL of filtered juice was diluted with 100 mL of distilled water. Diluted juice was placed in a 250 mL conical flask and 4 drops of phenolphthalein indicator added. It was then titrated with 0.1 N NaOH with pH of 8.1 until indicator showed a pink colouration. The appearance of the light pink colour was marked as the end point. TA was calculated from titer values obtained and was expressed as percentage of predominant acid present in fruit. Citric

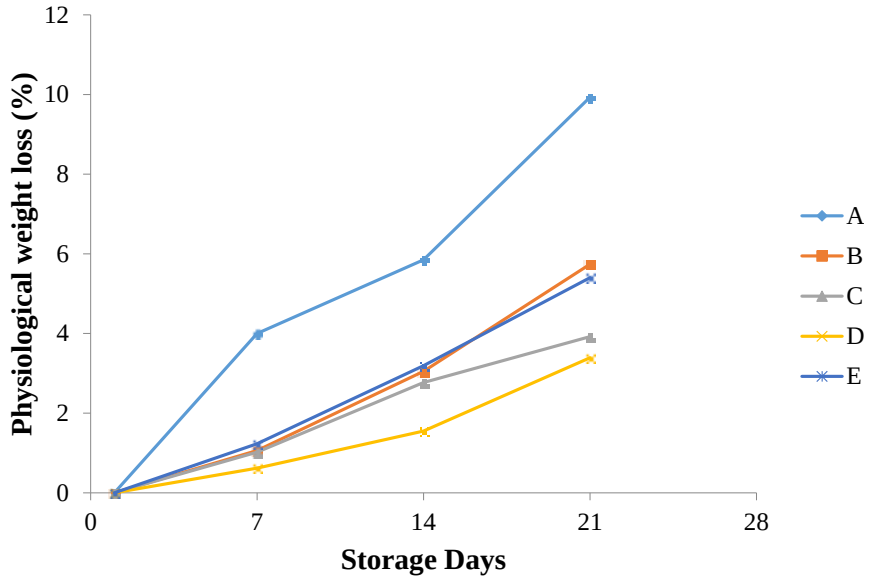

Figure 1. Physiological weight loss (%) of treated fruits. A = sample A; B = sample B; C = sample C; D = sample D; E = sample E.

acid was used as the predominant acid present, with 0.007 used as the citric acid factor.

Total soluble solids (TSS)

TSS was determined using a hand held refractometer, Atago, Japan and according to the methods of Wills and Ku (2001). Samples of different chemically treated fruits were milled with 80 mL of distilled water. A drop of milled samples was placed on the refractometer prism, from which results were taken. Values of TSS taken were expressed as degree (°) Brix.

Moisture content

Moisture content of tomato expressed in percentage was determined by method described by AOAC 925.45 using equation 1 (Horwitz, 2000). Empty crucibles were dried in an oven at 100°C for 30 min and weighed (W1). A total of 10 g of tomato was placed in a crucible, accurately weighed and the combined weight recorded as W2. The crucible was kept in an oven at 100 to 105°C for 6 to 12 h until a constant weight was obtained. The oven dried sample were then placed in a dessicator and allowed to cool. The crucibles were weighed again after cooling (W3).

$$\text{Moisture content (\%)} = \frac{W2-W3}{W2-W1} \times 100 \quad (1)$$

W1 = Initial weight of empty crucible; W2 = weight of crucible + banana flour sample; W3 = final weight of crucible + banana flour sample.

Statistical analysis

Analyses were done in triplicates with results presented as mean values ± standard deviation. Means were compared using analysis of variance with obtained results separated using Duncan multiple comparison test at significant levels of $p < 0.05$. Statistical analysis was done using SPSS 19 for windows (SPSS Inc., Chicago, Illinois) statistical software package.

RESULTS AND DISCUSSION

Results on quality parameters of tomato were analyzed for 21 days and collected at an interval of seven days: Day 1, 7, 14 and 21 of storage period. Obtained results of analysis showed that treatments differed in its effect on quality parameters among various samples examined.

Physiological weight loss (PWL) of treated tomato samples

Loss of weight progressively increased with storage time. Weight loss of fresh tomato is primarily due to transpiration and respiration. Water is lost by transpiration due to differences in vapour pressure of water in atmosphere and tomato surface (Tasdelen and Bayindirli, 1998). Respiration causes weight reduction because a carbon atom is lost from the fruit each time a CO_2 molecule is produced from an absorbed oxygen molecule and evolved into the atmosphere (Bhowmik and Pan, 1992).

PWL was recorded in all samples, with sample A showing the highest loss in weight of 9.93%, recorded on the last day of storage (Figure 1). This was because sample A received no treatment, hence the rate of respiration was not reduced, when compared to other samples with chemical treatment. This result is in agreement with the work of Tefera et al. (2007) who stated that packaging of fruits reduced PWL of mango

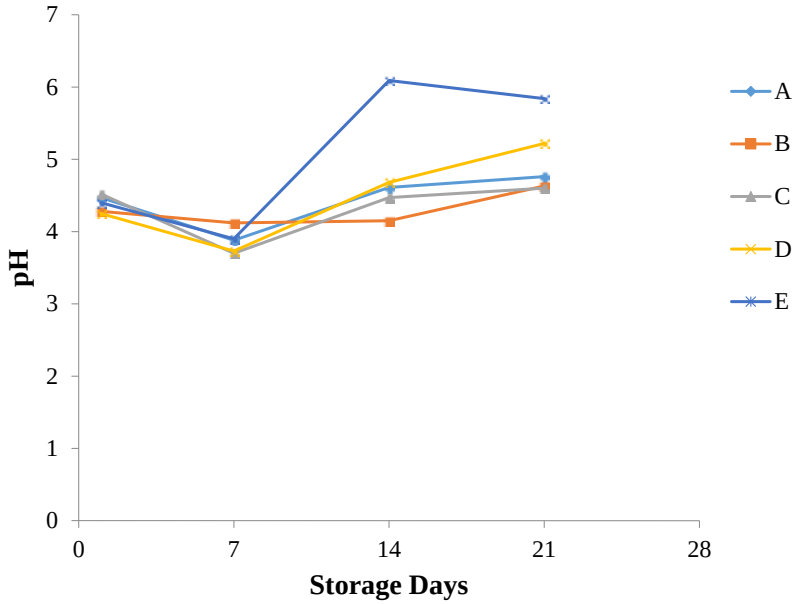

Figure 2. pH of treated tomato fruits. A = sample A; B = sample B; C = sample C; D = sample D; E = sample E.

fruits after 28 days of storage. The observed low weight loss trend in the other samples may be related to water vapour accumulation within the LDPE material during storage. This was as a result of the reduction in O_2 content and an increase in CO_2 content leading to the accumulation of water vapour. Thus packaged and chemically treated tomato samples B, C, D and E showed reduced PWL compared to the unpacked samples. This result agrees with the findings of Workneh et al. (2012) who determined the effects of preharvest treatment, disinfections, packaging and storage environment on quality of tomato. The introduction of potassium permanganate, boric acid and calcium chloride in the packaged fruits contributed in reducing weight loss. Potassium permanganate is said to be an ethylene degrading chemical which degrades ethylene into water and carbon dioxide. Water accumulated within the packaging materials created a high humid environment thereby retarding transpiration and water loss (Thompson, 1994; Roth, 1999).

pH and TA of treated tomato samples

Generally, the pH of fruits increases as fruits undergo ripening. Citric acid has also been shown to be the main acid in tomato juice, with pH of fruit normally between 4.0 and 4.5 (Babitha and Kiranmayi, 2010). From results obtained in this study, the pH of stored tomato fruit increased as the days of storage increased in all treated samples and in control (Padmini, 2006). pH was within range of acid recorded in fruits at day 1 of storage due to the fact that tomato fruit was still at turning stage of ripeness. However, there was recorded decrease in pH (3.70 to 4.12) of samples in day 7 of storage as stored samples became more acidic, before showing an increase in pH on days 14 and 21 of storage respectively (Figure 2). Of all samples analysed, sample E was least acidic (6.09 ± 0.75) at day 14 of storage while sample C was the most acidic (3.70 ± 0.17) at day 7 of storage.

Acidity is often used as an indication of maturity as acid decreases on ripening of fruit. It has also been reported that upon ripening of tomato fruit, malic acid disappears first, followed by citric acid, suggesting the catabolism of citrate via malate. Result of analysis shows that there was a concomitant decrease in TA, from 8.7 to 3.9 across all treated tomato fruit sample as the storage days increased. Sample C showed the least TA of 3.9 ± 0.74 among all samples examined at day 21 of storage. Reduction in TA may be attributed to a decrease in respiration rate caused by low density polyethylene packaging which led to accumulation of moisture in the packaging material. Sample C showed a steady decrease in TA with the exception of day 14 were there was a significant increase before decreasing on day 21 of storage (Figure 3). Sample B showed highest amount of TA which was statistically significant, apart from control. This may be due to its treatment with $KMnO_4$ only thus agreeing with Wills et al. (1981) who stated that $KMnO_4$ contributes to an increase in the CO_2 concentration as ethylene is degraded into CO_2 and water.

Moisture content of treated tomato samples

Water comprises about 80 to 90% of the fresh weight of tomato fruit with the size of the fruit influenced by availability of water to the plant (Babitha and Kiranmayi,

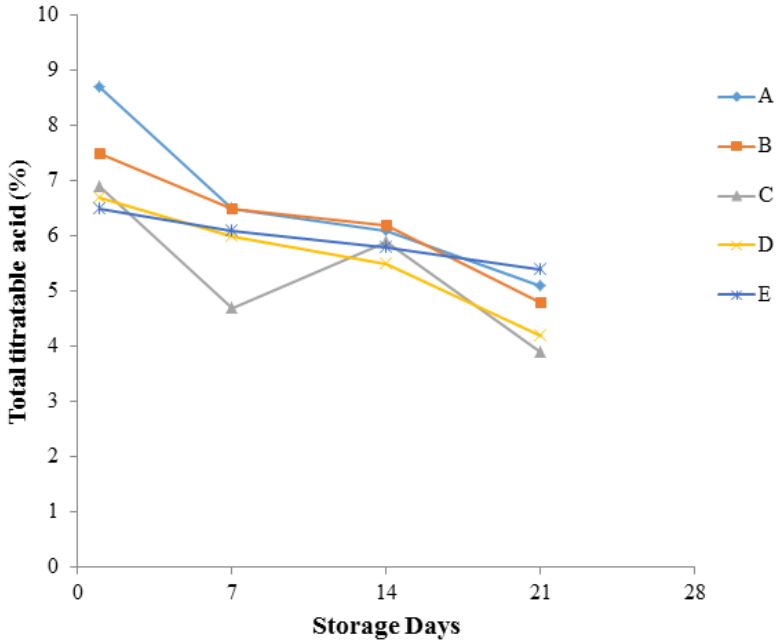

Figure 3. Total titratable acidity (%) of treated fruits. A = sample A; B= sample B; C = sample C; D = sample D; E = sample E.

Table 1. Chemical changes in tomato samples during different days of storage.

Properties	Sample					
	Day	A	B	C	D	E
Moisture content (%)	1	91.20 ± 0.40^{ab}	91.50 ± 0.22^{a}	90.30 ± 0.03^{c}	90.90 ± 0.67^{b}	90.10 ± 0.11^{ab}
	7	91.70 ± 0.09^{a}	90.80 ± 0.09^{c}	90.90 ± 0.01^{bc}	91.30 ± 0.19^{ab}	91.60 ± 0.41^{a}
	14	91.60 ± 0.15^{a}	90.60 ± 0.02^{c}	90.93 ± 0.01^{bc}	91.00 ± 0.66^{bc}	91.50 ± 0.70^{ab}
	21	92.50 ± 0.01^{a}	92.20 ± 0.15^{ab}	91.90 ± 0.84^{b}	91.90 ± 0.20^{b}	92.40 ± 0.82^{a}
TSS (^{o}Brix)	1	3.20 ± 0.01^{ab}	2.40 ± 0.03^{c}	2.70 ± 0.00^{bc}	3.60 ± 0.04^{a}	2.20 ± 0.30^{c}
	7	3.30 ± 0.66^{a}	1.80 ± 0.01^{bc}	0.90 ± 0.00^{d}	2.03 ± 0.10^{b}	1.30 ± 0.12^{cd}
	14	3.00 ± 0.22^{a}	1.00 ± 0.00^{b}	1.20 ± 0.01^{b}	1.30 ± 0.00^{b}	1.10 ± 0.28^{b}
	21	2.50 ± 0.06^{a}	2.50 ± 0.67^{a}	2.00 ± 0.00^{a}	2.10 ± 0.10^{a}	1.90 ± 0.19^{a}
TSS/TA	1	0.37 ± 0.07^{b}	0.32 ± 0.03^{b}	0.39 ± 0.02^{b}	0.54 ± 0.02^{a}	0.34 ± 0.06^{b}
	7	0.49 ± 0.01^{a}	0.28 ± 0.01^{c}	0.19 ± 0.01^{d}	0.42 ± 0.01^{b}	0.22 ± 0.05^{d}
	14	0.49 ± 0.07^{a}	0.16 ± 0.04^{b}	0.20 ± 0.05^{b}	0.24 ± 0.04^{b}	0.19 ± 0.03^{b}
	21	0.49 ± 0.00^{a}	0.52 ± 0.05^{a}	0.51 ± 0.07^{a}	0.50 ± 0.05^{a}	0.35 ± 0.04^{b}

Means in each row with the same alphabet are not significantly different ($p > 0.05$) by Duncan multiple test. Values are means ± standard deviation (n = 3). TSS = total soluble solids; TSS/TA = sugar-acid ratio; sample A = no treatment; sample B = hot water treatment; sample C = hot water and H_3BO_3 treatment; sample D = hot water and $CaCl_2$ treatment; sample E = hot water, H_3BO_3 and $CaCl_2$ treatment.

2010). Ripening in fruits is proceeded by softening, with the resultant effect of increased moisture content of the fruits. There was a slight significant increase in moisture content of samples A, C, D and E (Table 1) as the storage period increased. Sample B showed a steady decline in moisture content on days 7 and 14, due to the hot water treatment which reduced enzymatic activities of ripening and softening of the samples. As observed in all samples during storage days, chemical treatment did not inhibit the action of ripening, cell wall break down and softening of all samples examined. Hence enzymatic action of ripening and cell wall break down continued

during storage with the resultant rise in moisture content as the storage days increased.

Total soluble solids (TSS) of treated tomato samples

In tomato fruit, conversion of starch to sugar is an important index of ripening (Kays, 1997). TSS for sample A was the highest among all five samples examined. This was due to increase in ripening of sample A as a result of no treatment. Treated samples B, C, D and E showed low TSS values due to various chemical treatments which delayed ripening to a certain degree of storage. As reported by the work of Tigist et al. (2013), untreated tomato samples stored at ambient temperature conditions recorded higher TSS values of between 4.23 and 5.22°Brix. Higher TSS values are attributed to the absence of chemical treatment in the samples used for the study as compared to TSS values obtained from samples used in this study. Samples B and E showed low Brix values of 1.0 and 1.1 on day 14 of storage while sample A recorded a significantly high Brix value of 3.3 on day 7 of storage. Sample C showed a drastic decline in TSS on day 7 of storage as ripening was really slowed down, before showing an increase on days 14 and 21 of storage due to the effect of H_3BO_3 treatment on the tomato fruit (Table 1). Result of this study agrees with the work of Wang and Moris (1993) who reported that H_3BO_3 reduces the rate of ethylene and CO_2 production in fruits thereby reducing the rate of respiration and ripening.

Sugar-acid ratio of treated tomato samples

From result of experiment, sugar-acid (TSS/TA) ratio was significantly highest in sample D on day 1 and samples A, B, C and D on day 21 in both treated and untreated tomato samples. TSS/TA ratio increased in day 7 of storage for sample A and remained stable on days 14 and 21 of storage period compared to the treated samples B, C, D and E that showed variations in their TSS/TA ratio. Due to chemical treatment used for storage, samples B, C, D and E showed significantly low rate of senescence in days 7 and 14 (Table 1) of storage period, hence low occurrence of TSS/TA ratio was observed during those storage days. Apart from its use as a maturity index, TSS/TA ratio is employed as ripening index for both tropical and subtropical fruits (Yahia et al., 2011; Guerreiro et al., 2016). According to Fawole and Opara (2013), TSS/TA value plays an important role in fruit taste which is a quality gauge in the processing of juice in the food and beverage industry. The sugar-acid ratio is also used as a better predictor of tomato taste as it involves the specific measurement of sucrose, fructose and glucose contents of the fruit (Beckles, 2012; Sibomana et al., 2016). Flavour characteristics of processed tomato products have also been reported to be influenced by the balance of sugar and acid contents in the fruit (Garcia and Barrett, 2006; Tigist et al., 2013). Sample D was significantly high ($p < 0.05$) on days 1 (0.54 ± 0.02) and 21 (0.50 ± 0.05) of storage period indicating higher percentage of sugar and flavour when compared to other tomato samples.

Conclusion

Tomato samples that were treated exhibited longer storage periods with samples stored using hot water treatment and $KMnO_4$ showing a higher keeping quality. Also samples stored with the combination of $KMnO_4$, H_3BO_3 and $CaCl_2$ equally exhibited high storage ability. These treatments were able to keep the tomato fruits for 21 days without spoilage and recorded little changes in their physicochemical properties. H_3BO_3 treated sample showed high ripening rate compared to other treated fruits while samples B and E showed higher keeping quality. It can therefore be implied from results obtained from this study that the combination of chemical treatments on ripe (turning stage) tomato fruit and packaged in low density polyethylene material can ensure that tomato keeps for 3 to 4 weeks at tropical ambient temperature condition of 30 ± 2°C.

Conflict of interests

The authors have not declared any conflict of interests.

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Vol. 15(35), pp. 1920-1929, 31 August, 2016
DOI: 10.5897/AJB2015.15060
Article Number: A5BC46260247
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Response of chickpea (*Cicer arietinum* L.) to inoculation with native and exotic *Mesorhizobium* strains in Southern Ethiopia

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Received 21 October, 2015; Accepted 12 August, 2016

A series of pot and two consecutive crop-year field experiments were conducted from 2011 to 2012 in Southern Ethiopia to determine the effectiveness of Mesorhizobium strains on two cultivars of chickpea (Shasho and Nattoli). The eight treatments included: Six rhizobial inoculants, the four best indigenous strains (Cp8, Cp41, Cp97 and Cp105); CpNSTC (National Soils Testing Center inoculant); and CpSK (Canadian inoculant), Nitrogen fertilizer and a control. The results from the field and pot experiments indicated that chickpea crop yield can be improved using proper Mesorhizobium inoculation. Inoculation had a pronounced effect on grain yield, yield component, total N uptake, grain protein content, percentage N derived from the atmosphere (%Ndfa) for the seed, and amount of seed N fixed compared to non-inoculated treatments. In the pot experiment, significant difference was recorded among the mesorhizobial strains used with the indigenous strain Cp41 highly effective in shoot dry weight (41%) mg^{-1} plant, grain yield (50%), total N uptake (117%), and %Ndfa (67.9%) followed by CpSK, Cp8 and Cp97. In the second crop-year field experiment, the indigenous *Mesorhizobium* strain Cp41 also proved highly effective in-nodule dry weight (786%) mg^{-1} plant, grain yield (66%), total N uptake (100%), and %Ndfa (53.7%). The maximum seed protein content was recorded during the second crop-year field experiment in Cp41 (20%), followed by N fertilizer added treatment and CpSK (18%). The chickpea indigenous rhizobial strain Cp41, was superior inoculant for almost all parameters. Thus, there are potential advantages to be gained from using efficient rhizobial inoculants under rain fed conditions in Ethiopia.

Key words: Chickpea, rhizobia inoculants, nodulation, growth, yield.

INTRODUCTION

Ethiopia is the top producer of chickpea in Africa. In Ethiopia, chickpea is the third most important grain legume after faba bean (*Vicia faba*) and common bean (*Phaseolus vulgaris* L.) by volume for small-scale farm

production. Chickpea is an essential pulse crop, providing high-quality protein for human nutrition and a source of cash income for farmers. The national average yield of chickpea is 1.7 t ha^{-1} (CSA, 2013), which is far below the potential yield of 4.5 t ha^{-1}. In Southern Ethiopia, the average yield falls below the national average at 1.1 t ha^{-1}. Among increasing population pressures and soil erosion, soil fertility decline is one of the major factors limiting crop yield in Ethiopia. Long standing use of organic residues for fuel and feed has contributed to this decline. To compensate, commercial fertilizers such as diammonium phosphate (DAP) and urea have been used in some parts of the country for about three decades (FAO, 1984). Chickpea however, is usually grown without fertilizer on marginal land and farmers have a mistaken notion that chickpea, being a legume crop, does not need any nutrient support. Nitrogen (N) is the most commonly deficient soil nutrient in Ethiopia, contributing to reduced agricultural yields throughout the country. The use of inorganic N fertilizer is very low among resource-poor farmers, for whom it is prohibitively expensive. Biological nitrogen fixation (BNF) represents a significant potential source of N input in agricultural soils in the country.

The major N_2-fixing systems, the symbiotic systems, can play a substantial role in improving the fertility and productivity of low-N soils (Abaidoo et al., 1990). Biological N_2 fixation by rhizobia in legume root nodules is one widely studied mechanism by which plants benefit from association with interacting partners. In rhizobium-legume symbiosis, both plant cultivar and *Rhizobium* strain can affect nodulation (Keneni et al., 2012, Mutch et al., 2003). The bacteria benefit the plants by fixing N_2 in exchange for fixed carbon (C), which is either provided directly to the bacteria or indirectly in root exudates. Generally, it is assumed that a pulse crop well inoculated with the bacteria can fix sufficient quantities of N to eliminate the need for N fertilizer inputs in the crop year (Walley et al., 2006). Nitrogen supplied through BNF is less likely to leach or volatilize in pre-cropping or during cropping than N supplied as inorganic fertilizer (Jensen and Hauggaard-Nielsen, 2003). Depending on cultivar, bacterial strain, and environmental factors, the chickpea and *Mesorhizobium* sub sp. *Ciceri* association can produce up to 176 kg N ha^{-1} annually (Beck et al., 1991). Using high yielding varieties of chickpea along with effective rhizobial strains can enhance yields and minimize the need for nitrogenous fertilizer.

Rhizobial species that produce nodules in chickpea are solely specific to chickpea. Therefore, inoculation with effective strains is advised in soils with a weak or nonexistent bacterial presence (Rupela and Saxena, 1987). The isolation and screening of highly efficient and competitive strains of native rhizobial populations for use as inoculants proves very beneficial. Often the most competitive and persistent inoculant strains in a particular field environment are those isolated from similar environments (Chatel and Greenwood, 1973). One year before this study, 42 effective indigenous Meso*rhizobium* strains were isolated and evaluated at the College of Agriculture, Hawassa University, for their ability to enhance nodulation and biomass yield in locally grown chickpea. A total of 4 Meso*rhizobium* strains were selected for pot and field experimentation on the basis of effective nodule number and biomass yield (unpublished). The objective of the study was to evaluate the effect of the selected strains and commercial inoculants on nodulation and yield in two cultivars of chickpea under greenhouse and field conditions at Ele kebele in Southern Ethiopia.

MATERIALS AND METHODS

A series of greenhouse or pot experiment was conducted in 2011 and two consecutive crop-year field experiments were conducted from mid-August to December in 2011 and 2012 under rain fed conditions in Southern Ethiopia. The experiment was to test the efficacy of *Mesorhizobium* strains inoculation in improving growth, yield, and nodulation of chickpea. The pot experiment and soil chemical analyses were carried out at the soil microbiology laboratory of Hawassa University while the field experiments were conducted at Ele kebele on farmer's field. The plant and seed analyses were done at the Soil Science Department of the University of Saskatchewan (U of S), Canada.

Estimation of indigenous rhizobia nodulating chickpea

Soil samples were collected in the dry season of June 2011 at Ele and Jole Andegna kebeles (Meskan woreda), Huletegna Choroko kebele (Alaba special woreda), and Taba kebele (Damot Gale woreda). The plant infection count or the most probable number (MPN) count was used to determine the number of viable and infective rhizobia (Somasegaran and Hoben, 1994). Ten grams of soil sample were diluted in aseptic conditions in 90 ml sterilized distilled water. One milliliter of the diluted solution was transferred into 9 ml sterilized distilled water up to 10^{-10} and was used to inoculate chickpea seedlings adequately grown in acid-treated and sterilized sand using plastic cups in four replications. Nodule observations were made 45 days after inoculation. Positive and negative nodulation of growth unit were recorded for all dilutions and converted into number of rhizobia g^{-1} of soil using a MPN table.

The enumeration of indigenous rhizobial population by MPN method (Vincent, 1970) revealed that the population size of indigenous rhizobia compatible to this crop varied at different locations, ranging from 0 at Ele and Huletegna Choroko, to 5.8×10^{2} at Taba, and 3.1×10^{4} cell g^{-1} of soils at Jole. With the need for

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Table 1. Average rainfall, maximum and minimum temperature during 2011 and 2012 growing seasons, annual and long-term average (2003-2012) at Butajira, nearby metrological station of the study area.

Year		Rainfall (mm)	[a]Max. T (°C)	[b]Min. T (°C)
2011	July	168.3	23.1	12.0
	August	133.6	23.0	11.5
	September	88.1	24.3	11.9
	October	47.6	25.4	10.7
2012	July	262.0	24.4	12.6
	Annual	981.3	26.2	11.1
	August	236.1	23.9	12.7
	September	217.8	25.8	12.4
	October	98.6	25.5	10.1
	Annual	1523.2	25.6	10.3
10 years (2003-2012)	Annual Average	1146.7	26.1	11.2
Altitude	m above sea level		1950	

[a]Maximum temperature; [b] Minimum temperature.

Table 2. Soil physicochemical characteristics of the 0 to 20 cm soil layer of the experimental sites in Ele at the initiation of the experiments in 2011 and 2012.

Soil characteristic	2011	2012
Texture class [a]	Clay	Clay
pH-H2O (1:2.5)	6.2	6.3
EC ($ms \cdot cm^{-1}$) (1:2.5)	0.078	0.069
Organic carbon (%) [b]	0.74	0.71
Total nitrogen (%) [c]	0.067	0.061
Available P ($mg \cdot P \cdot kg^{-1}$ soil) [d]	2.8	2.6
CEC ($cmolc \cdot kg^{-1}$ soil)[e]	45.1	44.6
Exchangeable bases [f]		
Na ($cmolc \cdot kg^{-1}$ soil)	0.34	0.36
K ($cmolc \cdot kg^{-1}$ soil)	1.29	1.27
Mg ($cmolc \cdot kg^{-1}$ soil)	3.34	3.25
Ca ($cmolc \cdot kg^{-1}$ soil)	22.6	21.8
Micronutrients[g]		
Cu ($mg \cdot kg^{-1}$ soil)	1.33	1.32
Fe ($mg \cdot kg^{-1}$ soil)	8.69	10.32
Mn ($mg \cdot kg^{-1}$ soil)	40.68	41.32
Zn ($mg \cdot kg^{-1}$ soil)	0.89	0.91

[a]Hydrometer; [b]Walklay and Black; [c]Kjeldahl; [d] Olsen; [e&f]Ammonium acetate; [g]diethylene triamine pentaacetic acid (DTPA).

inoculation indicated at three of the locations studied, Ele kebele was selected for the pot and field experimental sites.

Description of the study area

The study was conducted at Ele kebele, about 7 km east of Butajira town and with latitude 08° 12′ N, longitude 38° 27′ E and altitude of 1950 masl. The average annual rainfall from 2003 to 2012 at Butajira, the nearby metrological station is 1146 mm having minimum and maximum temperature of 11.2 and 26.1°C, respectively (Table 1). Before sowing, soil samples were taken from representative points at 0-20 cm depth to make one composite surface soil sample for analysis of soil texture and some chemical properties as results depicted in Table 2, estimated according to the methods described by Van Reeuwijk (2002). A representative soil

sample was collected from this site for pot experiment during field preparation for the 2011 field trial.

Treatments and experimental design

A series of pot experiment and two consecutive crop-year field experiments were conducted from mid-August to December in 2011 and 2012 under rain fed conditions in Ele Kebele. Two chickpea cultivars (Shasho and Nattoli) were used. The eight treatments includes: six rhizobial inoculants, the four best indigenous strains Cp08 (isolated from Mesqan District, Southern Ethiopia), Cp41 (isolated from Bodity District, Southern Ethiopia), Cp97 (isolated from Akaki District, Central Ethiopia), Cp105 (isolated from Dukem District, East Shao, Ethiopia); CpNSTC (National Soils Testing Center inoculant); and CpSK (Canadian inoculant), N fertilizer (20 kg N ha^{-1}, no inoculant) and a control (no fertilizer or inoculation). All plots received the equivalent of 46 kg P_2O_5 kg ha^{-1} as TSP (100 kg TSP). The field and the pot experiments were laid out in complete randomized block design (CRBD) and complete randomized design (CRD) with three replications, respectively. The size of each experimental plot was 4 m × 3 m (12 m^2) with a total of 48 plots. Spacing between chickpea plants, rows, plots, and blocks was 10, 30, 0.5 and 1 m, respectively. For pot experiments, each pot was filled with 4 kg of soil, planted with five seeds per pot, and thinned to three plants at the two leaf stage. All other treatments and management practices were similar to those of the field experiments. Non-nodulating reference chickpea genotype PM233 cultivar from the International Center for Agricultural Research in the Dry Area (ICARDA) (received from the Ethiopian Institute of Agriculture Research, Holleta Research Center) as a reference crop was planted adjacent to the experimental site and in separate pots for assessing percentage N derived from the atmosphere (%Ndfa). Inoculants were prepared from fully grown broth by mixing with ignite based carrier. Seeds were inoculated with the respective rhizobial strains just before planting and kept in shade to maintain the viability of the cells. Seeds were allowed to air dry for a few minutes before planting.

Data collection

The data on nodulation parameters were taken at the mid-flowering stage. Five plants were randomly taken from second border rows on each side of the plot. Nodule number, nodule dry weight, and shoot dry weight were taken from five representative plants per plot. At physiological maturity, plants from the central 6 rows were manually harvested close to the ground surface. Five plants were randomly selected from the central rows of each plot and the number of pods and branches per plant were recorded. The harvested plants were weighed to determine the biomass yield and threshed to determine the grain yield of each plot. Hundred grains were counted to determine 100-grain weight per treatment.

Plant and seed analysis

At physiological maturity, five non-border plants from each plot and non-fixing chickpea cultivar were harvested and separated into straw and grain. These samples were used to determine seed N, straw N, and %Ndfa. The sample materials were oven dried at 70°C to a constant weight and ground to pass through a 2 mm sieve. Nitrogen accumulation in the plant tissue (percent N) was determined using a LECOCNS-2000 carbon, nitrogen, and sulfur analyzer. Ground seed samples were further pulverized to a fine powder in a ball mill and very small portions (approximately 3 mg each sample) of the fine ground samples were pelleted into 6 × 8 mm tin caps. Samples were then analyzed using a Costech ECS4010 elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA, USA) coupled to a Delta V mass spectrometer with a ConFlo IV interface (Thermo Scientific, Bremen, Germany), at the Stable Isotope Facilities, Department of Soil Science, University of Saskatchewan. The total protein content was computed by N content multiplied by a factor of 6.25, in accord with Jackson (1962). Total N per plant (shoot dry weight × N content + grain yield × N seed content) was calculated. The amount of seed N fixed was calculated as (%Ndfa × seed yield × seed N concentration)/100 (Peoples et al., 1995). Percent N derived from the atmosphere (%Ndfa) based on ^{15}N Natural Abundance Method was calculated using the following equation (Unkovich et al., 2008; Bremer and van Kessel, 1990):

$$\%Ndfa = \frac{\delta^{15}N \text{ of reference plant} - \delta^{15}N \text{ of } N_2 \text{ fixing legume}}{\delta^{15}N \text{ of reference plant - B}} \times 100 \quad (1)$$

Where, "δ15N" is:

$$\delta^{15}N = \frac{\text{Atom \% } ^{15}\text{N sample - atom \% } ^{15}\text{N atmosphere}}{\text{Atom \% } ^{15}\text{N atmosphere}} \times 100 \quad (2)$$

Where, the standard was atmospheric N_2 (0.3663 atom % ^{15}N). B is the $\delta^{15}N$ of the N_2-fixing plant grown in N-free medium. The value of B for chickpea was assumed to be −0.7644 (Kyei-Boahen, 2002).

Statistical analysis

Treatment effects were analyzed using the General Linear Model (GLM) procedure of the SAS computer software package (SAS/STAT, version 9.3). Mean values were separated according to Duncan's multiple range test (DMRT) at P = 0.05 (SAS Institute, 2012).

RESULTS AND DISCUSSION

The effect of N fertilization and rhizobial inoculation on nodulation, grain yield, and yield component of chickpea under the pot and field experiments are presented in Tables 3 to 5. The results obtained from the analysis of variance indicated that N fertilization and rhizobial inoculation had significant effects on all studied traits except on plant height.

Nodulation test

The data in Table 3 indicate that inoculation by different strains gave a significantly (P<.001) higher nodule number, nodule dry weight, and shoot dry weight compared to non-inoculated plants of the pot and the two season field experiments. Nodulation was not observed on non-inoculated and N fertilized treatments during the

Table 3. Effect of rhizobial inoculation and N fertilizer application on nodule number (NN), nodule dry weight (NDW), and shoot dry weight (SDW) at 50% flowering stage of chickpea varieties.

Treatment	Pot experiment			2011/2012 Field			2012/2013 Field		
	NN plt^{-1}	NDW mg plt^{-1}	SDW g plt^{-1}	NN plt^{-1}	NDW mg plt^{-1}	SDW g plt^{-1}	NN plt^{-1}	NDW mg plt^{-1}	SDW g plt^{-1}
Strain									
Control	0.0^{c}	0.0^{c}	2.9^{e}	0.0^{f}	0.0^{d}	5.4^{d}	4^{ed}	125^{d}	8^{e}
Nitrogen	0.0^{c}	0.0^{c}	4.6^{a}	0.0^{f}	0.0^{d}	7.6^{a}	3^{e}	37^{e}	13^{cba}
Cp8	69.3^{b}	473. 9^{a}	3.7^{cb}	28.8^{b}	168.6^{b}	6.5^{cba}	20^{c}	263^{c}	12^{dcb}
Cp41	102.9^{a}	497.3^{a}	4.1^{b}	33.7^{a}	233.0^{a}	7.3^{ba}	57^{a}	982^{a}	16^{a}
Cp97	82.5^{b}	438.8^{ba}	3.4^{cd}	14.0^{d}	24.8^{c}	7.1^{cba}	17^{c}	311^{c}	13^{dcba}
Cp105	8.2^{c}	66.3^{c}	3.2^{edc}	6.5^{e}	13.8^{dc}	6.5^{dcba}	10^{d}	304^{c}	12^{edc}
CpSK	68.6^{b}	348.2^{b}	3.7^{cb}	18.5^{c}	26.2^{c}	6.3^{dcb}	28^{b}	584^{b}	14^{ba}
CpNSTC	5.8^{c}	44.2^{c}	3.0^{ed}	6.3^{e}	10.3^{dc}	6.1^{dc}	8^{ed}	151^{d}	9^{ed}
Variety									
Shasho	31.8^{b}	198.3^{b}	3.9^{a}	12.8	56.7	6.9^{a}	16^{b}	322^{b}	15^{a}
Natoli	52.6^{a}	268.9^{a}	3.3^{b}	14.2	62.5	6.3^{b}	20^{a}	368^{a}	10^{b}
Strain	***	***	***	***	***	**	***	***	***
Variety	***	*	***	NS	NS	*	**	*	***
Strain x variety	***	NS	NS	NS	NS	NS	***	***	*
CV	28.21	32.97	10.78	22.97	30.33	13.19	26.72	20.99	20.96

plt, Per plant. Mean values followed by the same letters in each column and treatment showed no significant difference by DMRT (p = 0.05). *,**,***, and N^{S} showed significant differences at 0.05, 0.01, 0.001 probability levels and non-significant differences, respectively.

pot and the 2011/2012 study season field experiments. This result indicates that there had been no history of chickpea production in the experimental field. All tested isolates of chickpea rhizobia showed great variation in their capacity to induce the formation of nodules on host plant roots under lath house and field conditions. The mean nodule number per plant obtained from CpNSTC and Cp41 varied from 5.8 to 102.9, 6.3 to 33.7, and 8 to 57 under pot, first-year field, and second-year field experiments, respectively (Table 3). Similarly, the mean nodule dry weight per plant induced by CpNSTC and Cp41 varied from 44.2 to 497.3, 10.3 to 233, and 151 to 982 mg under pot, first-year field, and second-year field experiments, respectively. Significant differences were observed between varieties in terms of nodule number, nodule dry weight, and shoot dry weight under pot and field experiments. Higher nodule number and nodule dry weight was observed in Nattoli (desi type) than in Shasho (kabuli type) chickpea varieties. In terms of shoot dry weight, however, Shasho recorded higher than Nattoli. This finding agrees with that of Keneni et al. (2012), who reported that the Ethiopian and introduced chickpea germplasm were high in genetic diversity for both symbiotic and agronomic characters.

Maximum shoot dry weight (mg^{-1}plant) in pot experiments were recorded when N fertilizer was applied, with a 59% increase over the uninoculated control. Inoculation with Cp41 showed an increase of 41% in shoot dry weight (mg^{-1}plant) over the uninoculated control. Maximum shoot dry weight (mg^{-1}plant), however, was recorded during the second-year field experiment. When Cp41 was applied; there was a 100% increase over the uninoculated control. Inoculation with CpSK ranked second, with an increase of 75% in shoot dry weight (mg^{-1}plant) over the uninoculated control. There was significant interaction between variety and strains during the 2012/2013 year field experiment. This result is in line with Birhanu and Pant (2012), who reported that chickpea inoculation with *Mesorhizobium* strains gave higher nodule number, nodule dry weight, and biological yield compared to uninoculated plants in Shoa-Robit, Ethiopia.

Grain yield and yield components

Number of pods and branches per plant is an important yield determinant in pulse crops. Number of branches

Table 4. Effect of rhizobial inoculation and N fertilizer application on number of branch (NB), number of pod (NP), and 100-grain weight (100GW) of chickpea varieties.

Treatment	Pot experiment			2011/2012 Field			2012/2013 Field		
	NB plt^{-1}	NP plt^{-1}	100GW (g)	NB plt^{-1}	NP plt^{-1}	100GW (g)	NB plt^{-1}	NP plt^{-1}	100GW (g)
Strain									
Control	6.1	10.7^{c}	23.9^{c}	5.5	33.0^{d}	25.0	6.3^{c}	46^{c}	30.0
Nitrogen	6.8	13.8^{ba}	27.2^{ba}	6.1	63.2^{a}	26.0	10.7^{a}	58^{ba}	30.0
Cp8	5.4	13.9^{ba}	28.1^{a}	5.7	51.0^{cba}	26.8	8.7^{cba}	53^{bc}	29.8
Cp41	6.1	15.8^{a}	28.9^{a}	7.0	56.6^{ba}	26.6	9.7^{a}	68^{a}	30.2
Cp97	5.4	12.6^{bc}	27.2^{ba}	5.8	41.7^{dc}	24.5	9.5^{ba}	63^{ba}	30.4
Cp105	5.9	11.9^{bc}	24.2^{bc}	5.3	40.4^{dc}	24.8	7.1^{cb}	55^{bc}	30.2
CpSK	6.0	12.9^{bc}	28.0^{a}	6.7	49.3^{cba}	25.0	8.5^{cba}	60^{ba}	30.6
CpNSTC	5.7	11.3^{c}	24.7^{bc}	6.2	43.3^{dcb}	24.6	6.8^{c}	54^{bc}	30.3
Variety									
Shasho	7.8^{a}	13.8^{a}	24.1^{b}	5.6^{b}	42.1^{b}	23.2^{b}	9.3^{a}	67^{a}	31^{a}
Natoli	4.0^{b}	12.0^{b}	28.9^{a}	6.4^{a}	52.5^{a}	27.6^{a}	7.5^{b}	47^{b}	29.6^{b}
Strain	NS	***	**	NS	**	NS	**	**	NS
Variety	***	**	***	*	**	***	**	***	***
Strain × variety	NS	NS	NS	NS	NS	NS	NS	NS	NS
CV	14.35	13.38	8.85	17.04	24.05	7.08	23.26	14.77	3.67

plt, Per plant. Mean values followed by the same letters in each column and treatment showed no significant difference by DMRT (p= 0.05). *,**,***,and N^{S} showed significant differences at 0.05, 0.01, 0.001 probability levels and non-significant differences, respectively.

plant^{-1} was not significant under pot experiment and during the 2011/2012 study season field experiment, but during the 2012/2013 study season, field experiment there were significant (P<0.01) differences between treatments (Table 4). The maximum number of branches plant^{-1} (10.7) was recorded in N fertilized treatment followed by Cp41 strain inoculated treatment (9.7), with N fertilizer added and Cp41 strain inoculated treatments yielding an increase of 70 and 54%, respectively, in number of pods plant^{-1} over the uninoculated control.

The application of N fertilizer and rhizobial inoculants significantly (P<0.01) enhanced number of pods plant^{-1} of chickpea under pot and field studies. Number of pods plant^{-1} was increased by 48, 30 and 20% over the control treatments by inoculation of Cp41, Cp8 and CpSK, respectively in pot experiments. Similarly, number of pod plant^{-1} was increased by 48, 37 and 30% over the control treatments by inoculation with Cp41, Cp97 and CpSK, respectively, during the 2012/2013 field experiment. These results are in line with Yadav et al. (2011), who reported that inoculation of seed with *Rhizobium* enhanced nodulation, growth, and yield responses of legumes. The effects of seed inoculation on increasing number of pod plant^{-1} were also observed by Ali et al. (2003). Hundred-grain weight was not significant during the two crop-year field experiments; but in pot experiments, there were significant (P<0.01) differences between treatments. Hundred-grain weight was increased by 21, 18, and 17% over the control treatments by inoculation with Cp41, Cp8, and CpSK, respectively, under pot experimentation.

The straw and grain yield data of chickpea showed that rhizobial inoculation as well as N application significantly (P<0.001) increased the straw and grain yield of the crop in both pot and second-crop year field experiments (Table 5). Grain yields were increased by 50, 28 and 33% over the control treatments by inoculation with Cp41, CpSK, and Cp8, respectively, in the pot experiment. Similarly, grain yields were increased by 66, 53 and 49% over the control treatments by inoculation with Cp41, CpSK and Cp8, respectively, during the 2012/2013 crop-year field experiment. Unexpected drought during the 2011/2012 growing season resulted in no significant difference between treatments on straw and grain yield. These results are in line with Kyei-Boahen et al. (2005), who reported that soil generally increased seed yield over the uninoculated control but the magnitude varied over different seasons depending on the prevailing climatic condition. Increase in straw and grain yield of chickpea with effective *Rhizobium* inoculation has also been reported (Romdhane et al., 2007; Bhuiyan et al., 1998; Gupta and Namdeo, 1996).

Branch and pod number per plant were significantly higher in the Shasho variety than in Nattoli. Although in

Table 5. Effect of rhizobial inoculation and N fertilizer application on straw, grain yield (GY), and plant height (PH) of chickpea varieties.

Treatment	Pot experiment			2011/2012 Field			2012/2013 Field		
	Straw (g/pot)	GY (g/pot)	PH (cm)	Straw (kg/ha)	GY (kg/ha)	PH (cm)	Straw (kg/ha)	GY (kg/ha)	PH (cm)
Strain									
Control	10.4^{d}	8.8^{e}	35.5	2100.0	2167.2	32.5	2625^{c}	1720^{d}	43.6
Nitrogen	17.9^{a}	12.2^{ba}	37.8	2473.4	2207.4	34.5	3385^{a}	2794^{a}	46
Cp8	12.4^{dc}	11.7^{ba}	38.4	2617.6	2543.9	34.7	3171^{ba}	2570^{ba}	44
Cp41	14.9^{b}	13.2^{a}	39.0	2993.5	2597.0	34.6	3382^{a}	2866^{a}	47.4
Cp97	10.8^{d}	10.5^{edcb}	38.3	2425.3	1916.1	32.5	2825^{bc}	2316^{bc}	44.3
Cp105	10.7^{d}	9.6^{ed}	36.7	2691.4	2265.8	35.1	2872^{bc}	2187^{c}	44.1
CpSK	12.9^{c}	11.3^{dcb}	37.3	2438.3	2135.3	34.4	3121^{ba}	2632^{a}	45.4
CpNSTC	10.6^{d}	9.9^{edc}	35.9	2458.7	2177.9	34.6	2568^{c}	2102^{c}	45.6
Variety									
Shasho	14.1^{a}	10.6	40.4^{a}	2215.3^{b}	1722.0^{b}	32.1^{b}	3766^{a}	2430^{a}	45
Nattoli	11.0^{b}	11.1	34.3^{b}	2834.3^{a}	2775.6^{a}	36.1^{a}	2222^{b}	2217^{b}	45
Strain	***	***	NS	NS	NS	NS	***	***	NS
Variety	***	NS	***	**	***	***	***	**	NS
Strain x variety	NS	NS	NS	NS	NS	NS	NS	NS	NS
CV	12.83	13.29	6.32	25.40	25.65	9.75	11.76	10.63	9.28

Mean values followed by the same letters in each column and treatment showed no significant difference by DMRT (p= 0.05). *,**,***,and NS showed significant differences at 0.05, 0.01, 0.001 probability levels and non-significant differences, respectively.

terms of hundred-grain weight, Nattoli recorded significantly higher under pot experimentation (28.9) and during the 2011/2012 study season field experiment (27.6), Shasho yielded significantly higher hundred-grain weight (31) during the 2012/2013 study season field experiment (Table 4).

In terms of grain and straw yield (kg per ha), Nattoli recorded significantly higher during the 2011/2012 study season field experiment, but during the 2012/2013 study season field experiment Shasho yielded significantly higher grain and straw yield as compared to Nattoli (Table 5). This was because during the 2012/2013 study season, there was good rainfall distribution throughout the growing season. These results reveal that the two cultivars differed in their morphology and growth period. Nattoli was an early maturing variety whereas Shasho was a late maturing variety. This indicated that Nattoli would be a suitable cultivar for a short rainy season, whereas Shasho would produce higher grain yields if there is a longer rainy season.

Total N uptake and seed protein content

Total N accumulation in legume plants is one of the best parameters to measure N fixation under experimental conditions. Results on effects of rhizobial inoculation and N fertilizer application on total N uptake (kg/ha) and seed protein content (%) have been presented in Table 6. The rhizobial inoculation and N application significantly (P<.001) increased total N uptake and seed protein concentration in both pot and second-year field experiments. Compared to uninoculated control treatment, total N uptake was increased by 135, 117, 69 and 22% by inoculation with Cp41, CpSK, and CpNSTC, respectively, in the pot experiment. Similarly, total N uptake was increased by 100, 72, 49 and 24% by Cp41, CpSK, N added treatment, and CpNSTC respectively, over uninoculated control treatments during the 2012/2013 field experiment. The maximum seed protein content (23.3%) was recorded in N fertilizer added treatment, followed by Cp41 strain inoculated treatment (23.2%) under pot experimentation. The maximum seed protein content (20%) was recorded in Cp41 strain inoculated treatment, followed by N fertilizer added treatment and CpSK (18%), during the second-year field experiment. Our findings are supported by Aslam et al. (2010), who also reported that inoculants significantly increased grain protein content.

N derived from the atmosphere and N fixed

Results showing effects of rhizobial inoculation and N

Table 6. Effect of rhizobial inoculation and N fertilizer application on total N uptake (TNU), seed protein concentration (SPC), percentage N derived from the atmosphere (%Ndfa) for the seed, and amount of seed N fixed for chickpea cultivars.

Treatment	Pot experiment				Field experiment (2012/2013)			
	TNU (mg/pot)	SPC (%)	Ndfa (%)	N_2 fixed (mg/pot)	TNU (kg/ha)	SPC (%)	Ndfa (%)	N_2 fixed (kg/ha)
Strain								
Control	300.6^{d}	15.5^{c}	-	-	57^{d}	16^{d}	-	-
Nitrogen	708.2^{a}	23.3^{a}	-	-	85^{cb}	18^{b}	-	-
Cp8	429.8^{cb}	17.3^{bc}	62.0^{bc}	128^{bc}	85^{cb}	17^{cd}	40.5^{ab}	41.7^{bcd}
Cp41	654.4^{a}	23.2^{a}	67.9^{a}	261^{a}	114^{a}	20^{a}	53.7^{a}	62.1^{a}
Cp97	445.9^{cb}	20.7^{ba}	56.7^{ab}	161^{cd}	78^{c}	17^{cd}	45.8^{bc}	34.9^{bc}
Cp105	351.1^{cd}	17.3^{bc}	55.7^{bc}	102^{d}	72^{cd}	17^{cd}	37.9^{bc}	32.1^{cd}
CpSK	507.1^{b}	20.5^{ba}	64.5^{ab}	179^{b}	98^{b}	18^{b}	46.9^{ab}	48.8^{b}
CpNSTC	366.2^{cd}	17.3^{bc}	51.5^{c}	89^{d}	71^{cd}	17^{cd}	29.8^{c}	29.1^{d}
Variety								
Shasho	471.2	19.2	59.5	144	89^{a}	17^{b}	41.3	42.1
Nattoli	469.6	19.6	59.9	162	76^{b}	17.9^{a}	43.6	40.8
Strain	***	***	**	***	***	***	**	***
Variety	NS	NS	NS	NS	**	**	NS	NS
Strain x variety	NS	NS	*	NS	NS	*	NS	NS
CV	17.43	16.13	20.53	31.2	14.73	7.27	11.27	18.18

Mean values followed by the same letters in each column and treatment showed no significant difference by DMRT ($p = 0.05$). *,**,***,and N^{S} showed significant differences at 0.05, 0.01, 0.001 probability levels and non-significant differences, respectively.

fertilizer application on N derived from the atmosphere and amount of seed N fixed have been presented in Table 6. Rhizobial inoculation of chickpea showed great variation in N derived from the atmosphere and amount of seed N fixed. The mean percentage N derived from the atmosphere varied from 51.5 to 67.9, induced by isolate Cp41 and CpNSTC, respectively, under pot experiment. Similarly, the highest N derived from the atmosphere (53.7%) was recorded in plants inoculated with strain Cp41, whereas the lowest N derived from the atmosphere (29.8%) was shown by the host inoculated with CpNSTC during the second-year field experiment. Seed inoculation significantly influenced the amount of seed N fixed. It increased from 89 with the CpNSTC inoculant to 261 (mg/pot) with Cp41 inoculant treatments under pot experiments. Similarly, an increase in the amount of seed N fixed, from 29.1 to 62.1 kg/ha was recorded with CpNSTC inoculant and with Cp41 inoculant treatment, respectively during the second-year field experiment. These results were comparable with those of Kyei-Boahen et al. (2005), who reported that the proportion and amount of seed N derived from N_2 fixation were higher for inoculated plants as compared with uninoculated controls across locations. There was no significant difference between varieties regarding N derived from the atmosphere and N fixed. Kyei-Boahen et al. (2002) also found that desi and kabuli chickpea types responded similarly to rhizobial inoculation.

The results of this study indicate that chickpea yield can be improved through proper *Mesorhizobium* inoculation. Inoculation by different strains had a pronounced effect on grain yield, yield component, nodulation, total N uptake, grain protein content, %Ndfa for the seed, and amount of seed N fixed as compared to non-inoculation treatments. Indigenous *Mesorhizobium* strain Cp41 was found to be more significant in its effect for most of the studied parameters, followed by Cp8, CpSK and Cp97, respectively, as compared to the uninoculated control. Indigenous *Mesorhizobium* strain Cp105 and CpNSTC inoculants were found to be ineffective symbiotic nitrogen fixers. This finding agreed with Yadav et al. (2011), in that a *Mesorhizobium* strain indigenous to the growing field locality proved to be a highly effective symbiotic nitrogen fixer for uptake of nutrient content and grain yield of chickpea. Others (Evans, 2005) found indigenous *Rhizobium* strains to be more highly effective symbiotic nitrogen fixers for the uptake of nutrient content and grain yield than introduced commercial inoculants. The current result was also similar with recent report (Tena et al., 2016) that evaluations of rhizobial strains isolated from Ethiopian soils revealed higher rates of N fixation on lentil than the

introduced commercial inoculant. Recently several reports (Chemining'wa et al., 2012; Lamptey et al., 2014; Maleki et al., 2014; Mfiling et al., 2014; Minta and Tsige, 2014; Suryapani et al., 2014) from different countries demonstrated that indigenous rhizobia inoculation improve growth, seed yield, nitrogen fixation and also nutrient up take of legumes. These results indicate that the indigenous chickpea nodulating rhizobial strains used in this study are better adapted to the soil environment and survived in adequate numbers as compared to LtNSTC and LtSK commercial inoculants.

Conclusions

Mesorhizobium strain Cp41, chickpea indigenous rhizobial strain, proved superior in almost all parameters (grain yield, yield component, nodulation, grain protein content, and amount of seed N fixed) as compared to the other inoculants, thus indicating inoculation of chickpea with this particular strain is advantageous in the study area. Chickpea plants that had a longer growth period produced a higher yield (Shasho), compared to plants with a short growth period (Nattoli), when there was adequate moisture during the growing period. This study shows that in locations where rhizobial strains compatible to the target crop are not available, inoculation with appropriate rhizobial inoculants enhance grain yield of the crop. The study, while showing the biodiversity resources available in Ethiopian soils to promote BNF among small-holder producers; it has also indicated the potential that exists to select efficient strains from the indigenous isolates.

Conflict of interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Financial assistance for the research was provided by the Canadian International Food Security Research Fund (CIFSRF) of the Department of Foreign Affairs, Trade and Development and the International Development Research Center (IDRC). The authors extend their thanks to Debrezeit and Holleta Agriculture Research Center for the supply of chickpea cultivars. The authors would also like to express their gratitude to Dr Sheleme Beyene, Mr Molla Mengistu and Mr Tibebu Desalegn for their support during the research period.

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