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Review

Role of microorganisms in biodegradation of food additive Azo dyes: A review

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Food additives Azo dyes are synthetic compounds added to foods to impart color and improve their properties. Some azo dyes have been banned as food additives due to toxic, mutagenic, and carcinogenic side effects. Long exposure to foods containing azo dye leads to chronic toxicity. Some microorganisms are capable to degrade these dyes and convert them to aromatic amines. In human body, microbiota can play a vital role in biodegradation of azo dyes by producing azo reductase. Aromatic amines are toxic, water-soluble and well absorbed via human intestine. In the current study, the role of microorganisms in biodegradation of six dyes related to azo group was discussed. These dyes are: Tartrazine E102, Sunset Yellow E110, Ponceau E124, Azorubine E122, Amaranth E123, and Allura Red E129 which are classified as the most harmful food additive dyes.

Key word: Food additive, azo dyes, microorganisms, azo reductase, aromatic amines.

INTRODUCTION

Food additives are synthetic compounds added to food for many proposes such as maintaining the product from deterioration or improving its safety, freshness, taste, texture or appearance (WHO, 2012). Color additives are extensively used in food, cosmetics, and drugs (Macioszek and Kononowicz, 2004). Colorants are widely used for giving attractive coloring properties; every year industrial factories produce about 8 million tons of food colorants. Azo dyes are one of the most famous dyes used in coloring food, cosmetics, pharmaceutical products, and in the textile industry (Lorimer et al., 2001). Azo dyes are organic compounds containing an azo group (-N=N-), but some dyes have two (diazo), three (triazo) or more (Benkhaya et al., 2020; Bell et al., 2000). These dyes are aromatic compounds that are usually stable and highly water soluble.

In the USA and European countries, some azo dyes have been banned as food additives due to toxic, mutagenic, and carcinogenic side effects (Chung, 2000).

Azo dyes can cause many diseases such as, edema of the face, tongue, neck, larynx and pharynx, contact dermatitis, respiratory disease, lacrimation, hypertension, exophthalmos, upon ingestion, blindness, rhabdomyolysis, skin irritation, chemosis, vomiting gastritis, vertigo and acute tubular necrosis supervene (Young and Yu, 1997).

Some microorganisms are capable of utilizing azo dyes and degrade them, but intermediate compounds are toxic, mutagenic, and carcinogenic (Houk et al., 1999, Helal et al., 2000; IARC, 1982). However, azo dyes do not accumulate in the human body cells; they are metabolized in the liver by azo reductase and excreted out in the urine (Hassan and Elnmer, 2017).

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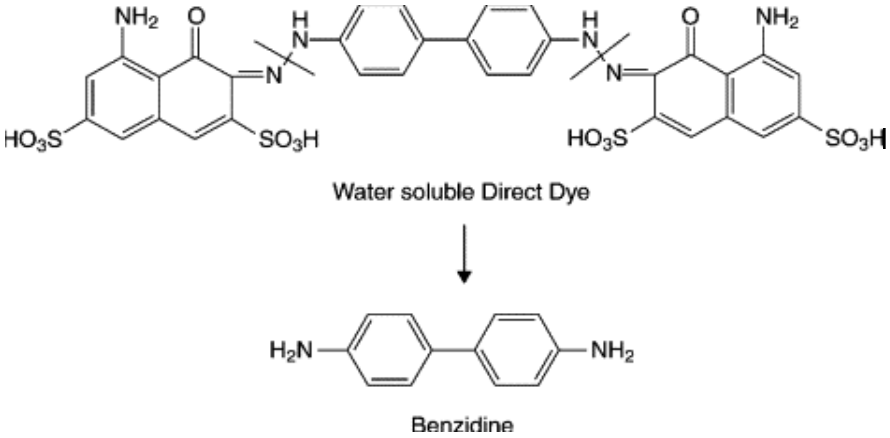

Figure 1. Reduction of azo dye to Benzidine.

The danger of these compounds is not due to the dye itself, but to intermediate products produced through biodegradation of the dyes (Varjani et al., 2020). Reduction of azo group (N=N linkage) leads to production of aromatic compounds which are more toxic and known for causing mutations and carcinogenic diseases (Puvaneswari et al., 2006; Alabdraba and Bayati, 2014).

In some cases, chronic toxicity affects cellular viability which leads the cell to absorb the dye instead of decolorizing it (Chen, 2002).

For example, 1,4-diamino benzene is intermediate compound and a major component of azo dye, it releases when azo dyes degrade (Figure 1). Benzidine causes human and animal tumors and contact allergen. Human intestinal microform, skin microflora, and environmental microorganisms can degrade azo dyes .Reduction of azo compounds can also occur by human liver azoreductase, or by non-biological factors (Chang, 2016).

Non-biological factors such as temperature, pH, oxygen level and structure and concentration of dye help in biodegradation process (Ajaz et al., 2019; Varjani et al., 2020).

A lot of studies discussed the capacity of these microbes to degrade food additive azo dyes; the aim of this study is to focus on:

(i) Chemical structure, properties and safety of different azo dyes that are used as a food additive such as Tartrazine E102, Sunset Yellow E110, Ponceau E124, Azorubine E122, Amaranth E123, and Allura Red E129 classified as the most dangerous additive dyes base on their side effects on human being.
(ii) Role of microorganisms in biodegradation of additive food azo dyes.

TARTRAZINE (E102)

Tartrazine (E102) is one of the most famous artificial colors; it is also referred to as FD&C yellow #5. The molecular weight is 534.3, and the formula is $C_{16}H_9N_4Na_3O_9S_2$. Figure 2 shows chemical structure of Tartrazine.

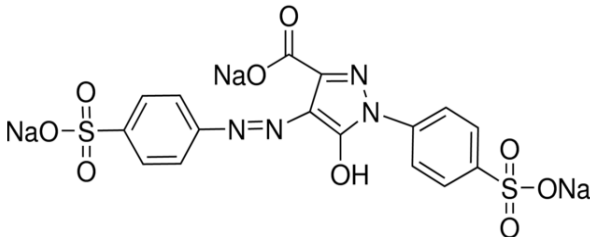

Figure 2. Chemical structure of Tartrazine.

The acceptable daily intake (ADI) is defined as the amount of a chemical that can be taken daily for an entire lifetime without any adverse reaction. The ADI of Tartrazine is up to 7.5 mg/kg bodyweight (FAO,WHO, 1964). It is used as a food additive to improve properties and give the color for food products.

Many reports recorded that Tartrazine can cause chronic urticarial and asthma (Lockey, 1959). Researches have also mentioned that Tartrazine dyes can cause some problems in children such as allergies, hyperactivity, learning impairment, irritability and aggressiveness as results of eating them from some foods like sweets and ice cream (Romieu, 2005).

A lot of studies discussed the ability of microorganisms to degrade Tartrazine. Some studies focus on human intestinal microflora in degradation of TZ. Human intestinal microflora is a group of microbes found in the small intestine (Sherwood et al., 2013).

Human gut microbes can produce azoreductase enzymes that degrade azo dyes, Azoreductases breakdown azo bonds (R-N = N-R') and produce colorless aromatic amines (Guillou et al., 2016). Aromatic amines dissolve in water and are absorbed easily via human intestine (Bomhard and Herbold, 2005).

Perez-Diaz and McFeeters (2009) investigated the ability of *Lactobacillus casei* and *Lactobacillus paracasei* in a modification of the azo dye, Tartrazine. They found 14 other lactic acid bacteria (LAB) that are capable of removing the food coloring Tartrazine.

Oranusi and Njoku (2005) discussed the ability of *Streptococcus faecalis* and *Escherichia coli* isolated from human intestinal microflora in biotransformation of food dyes (Tartrazine and Quinoline yellow). Isolated bacteria were maintained in media containing these dyes. Decolorization in aerobic conditions was higher than anaerobic conditions. Microorganisms can produce cytoplasmic flavin reductases and redox equivalents by metabolism of soluble starch and transfer electrons to the chromophoric group of the dyes.

Fungi also can degrade food additive dyes and remove their toxicity. Das and Das (2017) studied the effect of twelve types of fungi (*Irpex lacteus, Aspergillus species, Penicillium geastrivorus, Datronia sp., Myrothecium roridum, Polyporus arcularius, Fomitopsis feei, Pleurotus ostreatus, Trametes versicolor, Trametes hirsuta, fomes fomentarius* and *Ganoderma lucidum)* on decolorization and detoxification of different types of toxic azo dyes. Results showed the capacity of these fungi in biodegradation of different food additives. Toxicological assays by using daphnids showed a significant reduction of toxicity after dye decolorization of 12 types of fungi.

AZORUBINE E122

Azorubine or carmoisine is a synthetic azo dye; it is red food color and very soluble in water. The ADI is 4 mg/kg/day. The molecular weight is 502.431 and the formula is $C_{20}H_{12}N_2Na_2O_7S_2$. Figure 3 shows the chemical structure of AZ.

Azorubine shows possible effects on human health such as allergic reactions, rashes, skin swelling and hyperactivity, while some researches have shown no evidence of carcinogenic or mutagenic.

Kiayi et al. (2019) investigated the ability of *Saccharomyces cerevisiae* ATCC 9763 in degradation of Azorubine. Azorubine (Carmoisine) was removed from the aqueous medium after incubation with *Saccharomyces* for seven hours under anaerobic conditions. Results of spectrophotometry and chromatography confirmed biodegradation products of carmoisine into aromatic amines.

In the study of Au and Dzulkafli (2012), it was found that *P. ostreatus* has the capability in decolorizing of four different food dyes: Carmoisine Red, Tartrazine Yellow, Brilliant Blue, and Fast Green. UV-Spectrophotometer data confirmed complete decolorization of 10 ppm food dyes by *P*. *ostreatus*. *P. ostreatus* recorded percentages of degradation of the dyes with Carmoisine (81.34%), Tartrazine (45.36%), Fast Green (28.99%) and Brilliant Blue (19.82%) during aerobic conditions.

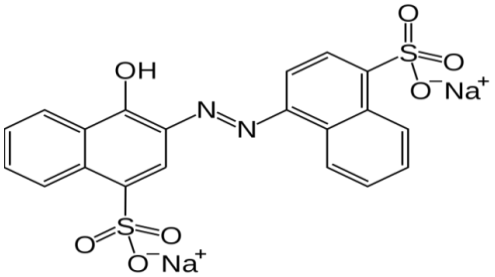

Figure 3. Chemical structure of Azorubine.

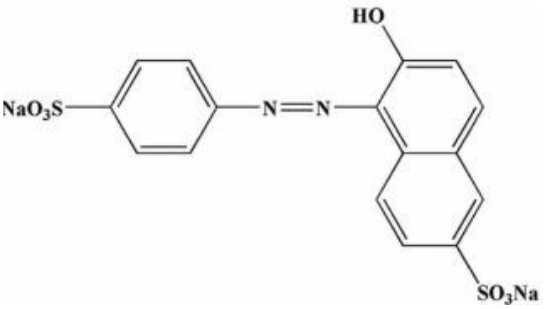

Figure 4. Chemical structure of sunset yellow.

SUNSET YELLOW E110

Sunset Yellow is a synthetic azo dye used for coloring foods, it is also known as FD&C Yellow 6; its formula ($C_{16}H_{10}N_2Na_2O_7S_2$,), Molecular weight is 452.38 g/mol, chemical structure is shown in Figure 4. The ADI is 4 mg/kg bw/day.

Sunset Yellow may cause anxiety migraines, eczema, immunosuppression and asthma (Sarıkaya et al., 2012).

Muntholib et al. (2015) studied the ability of *Actinobacillus* sp. in degradation of sunset yellow dye and p-cresol. Bacterial inoculum in the growth phase and stationary phase were tested to degrade 70 ppm of sunset yellow in M9 medium. Changes in the dye concentration were measured by using a spectrophotometer at a wavelength of 482 nm. Results show *Actinobacillus* sp. inoculum in stationary phase is more effective in degrading sunset yellow (11.88%) compared to the percentage of growth phase inoculum (2.02%).

In the study of Elbanna et al. (2017), 120 lactic acid bacterial strains and 10 bacterial intestinal isolates were examined to study their effect on degradation of sunset yellow (E110) and carmoisine (E122). High-performance liquid chromatography (HPLC) data of sunset yellow (E110) and carmoisine (E122) under anaerobic conditions showed degradation products by human intestinal bacteria that they are chemically classified as toxic aromatic amines.

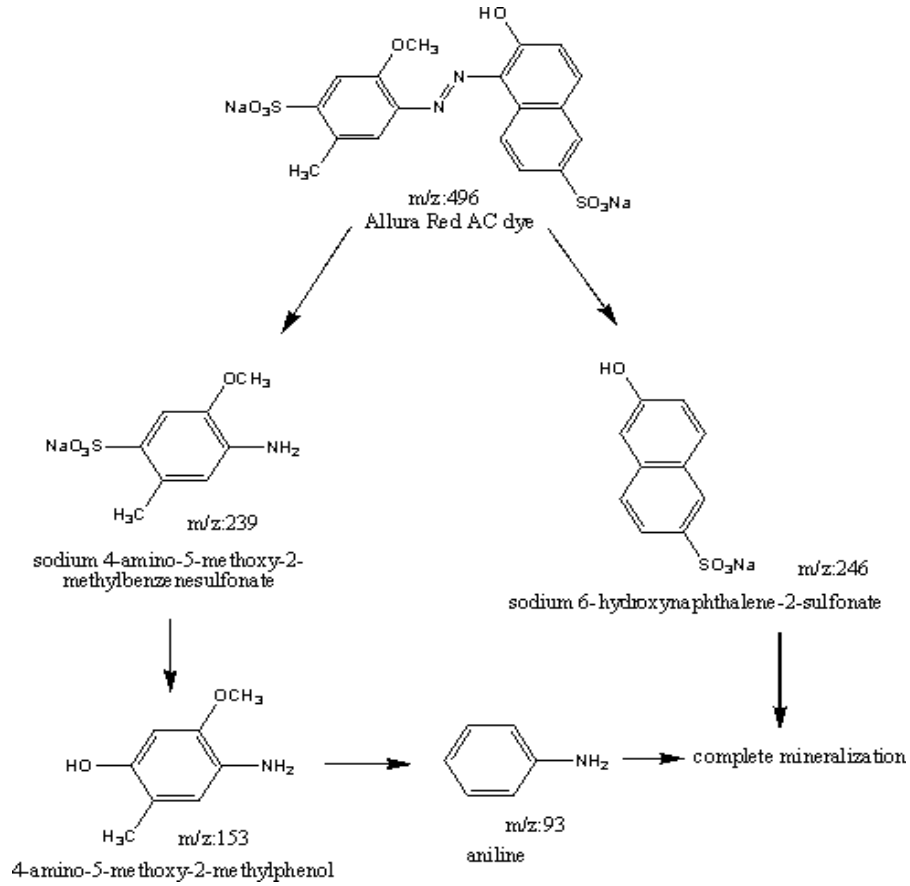

Figure 5. Proposed mechanism of ARAC by Ochrobactrum anthropi HAR08 (Kale and Thorat, 2014).

ALLURA RED AC (E129)

Allura red (E129) is synthetic diazo colorant and one of the most widely used dyes in food, drug, paper, cosmetic and textile industries. It is also known as FD&C Red 40. The formula is $C_{18}H_{14}N_2Na_2O_8S_2$ and molecular weight is 496.42 g/mol. The ADI is 0 to 7 mg/kg body weight (bw)/day.

Kale and Thorat (2014) studied the ability of *Ochrobactrum anthropi* (HAR08) in decolorizing of azo dye Allura Red AC. Percentage of biodegradation of (ARAC) recorded 95% in nutrient medium within 24 h. Degradation of Allura red was confirmed by FTIR spectroscopy and GC-MS techniques. It found that dye completely mineralized. Pathway of biodegradation of ARAC dye has been proposed Figure 5.

AMARANTH E123

Amaranth has been classified as an ionic dye. It can be applied to food, leather, paper, wood, synthetic and natural fibers (Anjaneya et al., 2013; Shahmoradi et al., 2011). It is known also as FD&C Red No. 2. Its formula is $C_{20}H_{11}N_2Na_3O_{10}S_3$ and molecular weight 604.473 g/mol. Chemical structure is shown in Figure 6. The ADI is 0.15 mg/kg / day body weight (bw)/day. It can cause acute and chronic toxicity. Amaranth dye can cause allergic, respiratory diseases, and human and animal tumors (Mittal et al., 2005). This dye could be mutagenic agent, genotoxic and carcinogenic (Jabeen et al., 2013; Jadhav et al., 2013). Considering its potential for hazardous toxicity, amaranth dye has been banned in many countries (Jadhav et al., 2013; Karkmaz et al., 2004).

Basu and Kumar (2015) studied the effect of food colorant on hemoglobin protein. They found significance conformational interaction changes between the dye and the protein by using 3D fluorescence techniques and FTIR. The interaction of amaranth with hemoglobin may enable realizing the toxic effects of azo dyes.

Amaranth dye is degraded by human intestinal microflora and its toxic or carcinogenic effects may derive from the microorganisms' degradation products (Ahmad and Kumar, 2011). Chan et al. (2012) investigated pathways metabolism of Amaranth dye by microaerophilic–aerobic consortium bacteria as

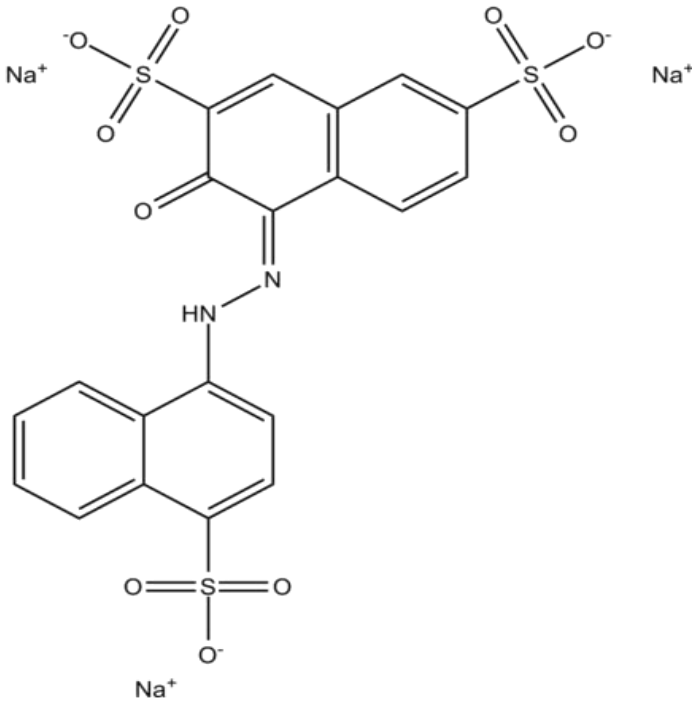

Figure 6. Chemical structure of Amaranth.

intermediates. During biodegradation of Amaranth dye by *C. freundii* and *E. cloacae* in aerobic conditions, azo linkage breakdown and produce intermediates that mineralized through metabolic pathways including benzoyl-CoA, protocatechuate, salicylate, gentisate, catechol and cinnamic acid. The steps of biodegradation of Amaranth by isolate NAR-2 are: Azo reduction, deamination, desulfonation and aromatic ring cleavage.

PONCEAU E124

Ponceau is widely used in coloring of some food such as soups, wine, cider sauces, preserves, etc. Its formula is $C_{20}H_{11}N_2Na_3O_{10}S_3$, molecular weight is 604.47 g/mol. The chemical structure is shown in Figure 7. The ADI is 0.7 mg/kg /day. It affects human health and causes urticarial, rhinitis and asthma.

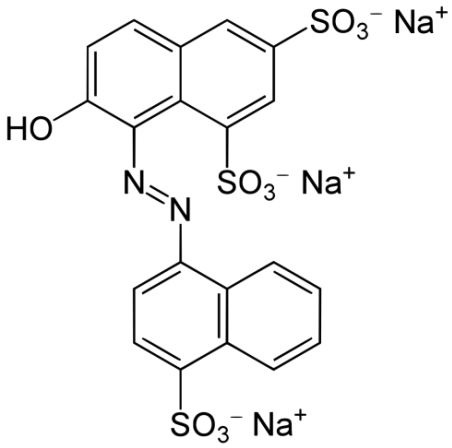

Figure 7. Chemical structure of Ponceau.

Masarbo et al. (2019) studied the decolorization of Ponceau 4R by 3 bacterial stains *Bacillus* sp. AK1, *Lysinibacillus* sp. AK2 and *Kerstersia* sp. VKY1 individually and in consortia. Spectrophotometry and chromatography analysis confirmed the products of biodegradation of Ponceau 4R and the formation of 4-aminonaphthalene-1-sulphonic acid and 5-amino-6-hydroxynaphthalene-2, 4-disulphonic acid as the products of azo bond breakage.

Cheng et al. (2016) studied the ability of sixty-three strains of white-rot fungi to degrade four types of textile azo dyes; Direct Blue 71 (C.I. 34140), Orange G (C.I. 16230), Ponceau 2R (C.I. 16450), and Biebrich Scarlet (C.I. 26905). Isolate *Coriolopsis* sp was only able to degrade four dyes with optimization of parameters such as temperatures, pH, nitrogen and carbon sources.

Omar (2008) studied the ability of green algae, diatoms, and cyanobacteria strains in degradation of Tartrazine and Ponceau. The results show that reduction of color depends on number of azo group in the dyes and the type of strain of algae. Biodegradation of azo dyes is related to activity of Azo reductase which is responsible

for reduction of azo linkage and produce aromatic amines.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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An investigation of meristem culture in *Anchote* [*Coccinia abyssinica* (Lam.) Cong.] under the influence of plant growth regulators and media strength

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Considering the potential benefit of meristem culture in the mass production of quality propagation materials, an experiment was conducted to investigate meristem culture in *KUWE* (G1) and 223098 (G2) genotypes of anchote. Concentration levels of 6-benzylamimopurine (BAP) with and without gibberellic acid (GA_3) and naphthalene acetic acid (NAA) for shoot induction while BAP and kinetin (Kn) for shoot multiplication were evaluated. Full and half nutrient-strength of Murashige and Skoog medium (FMS and HMS) with sole application of indole butyric acid (IBA) and indole acetic acid (IAA) were tested for root induction. About 80% (G2) and 71% (G1) of meristems induced shoots at 1 and 0.5 mg/L BAP, respectively. The highest shoot number (6.52), in G2, was recorded at 0.5 mg/L BAP while 0.5 mg/L BAP + 0.5 mg/L Kn produced 6.32 shoots in G1. FMS + 0.5 mg/L IBA was effective in 100% root induction in both genotypes. Survival rates of 58% (G1) and 42% (G2) were obtained. Generally, 0.5 mg/L BAP (G1) and 1 mg/L BAP (G) are best for shoot induction while 0.5 mg/L BAP (G2) and 0.5 mg/L BAP+0.5 mg/L Kn (G1) for multiple shoot development with best root formation on FMS+0.5 mg/L IBA.

Key words: Direct regeneration, genotype, meristem explants, MS media, plant growth regulators (PGRs).

INTRODUCTION

Anchote [*Coccinia abyssinica* (Lam.) Cong.] is an annual trailing vine belonging to the Cucurbitaceae family. It is an endemic species found both cultivated and in the wild in Ethiopia and best known and grown principally for its tuberous root even though its tender leaves and immature fruits are also widely used as food (Demel et al., 2010; Ermias et al., 2011). The tuber of *anchote* is the richest in protein, calcium and iron contents as compared to other common and widespread root tubers (Habtamu and Kelbessa, 1997; Habtamu et al., 2013). The low content of anti-nutritional factors also reflects the desirable quality of the tuber. Though not well investigated, the edible leaves and fruits of *anchote* have been indicated containing high nutritive composition,

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even better than the commonly used tubers (Desta, 2011; Girma and Dereje, 2015). *Anchote* has been used as folklore medicine to heal the bone fracture, backache, displaced joints and other diseases such as gonorrhea, tuberculosis, and cancer (Dawit and Estifanos, 1991; Amare, 2003).

Anchote is traditionally cultivated in some districts of Western, Southwestern and Southern parts of Ethiopia, areas where *anchote* is widely domesticated, on altitude between 1300 and 2800 m.a.s.l with 762 to 1016 mm annual rainfall (Amare, 2003). The average yield at farmers level has been estimated from 10 to 20 t/ha (Amsalu et al., 2008) which is significantly low than the maximum tuber yield (76-80 t/ha) obtained under experimental condition (Desta, 2011; Daba et al., 2012). Since *anchote* is commonly propagated by seed, the high out-crossing nature of *amchote* flowers (Edwards et al., 1995) has been challenging the production sustainability and breeding of the crop. As a result, the scarcity of quality propagation material coupled with lack of improved variety is among the most important constraints in *anchote* production. In fact, such a bottleneck could be undoubtedly improved through applying plant cell, tissue and organ culture technique called "*meristem culture*".

Culturing of an organized tissue in the form of very small shoots or meristems is the most valuable application of plant cell, tissue and organ culture in order to produce plenty of axenic and genetically stable propagation materials of elite varieties (Alam et al., 2004; Badoni and Chauhan, 2009; Rani and Raina, 2000; George and Debergh, 2008). However, meristem culture is a complex process that is affected by multiple plant endogenous factors including phytohormones and the culture environment.

Folla et al. (2013) conducted direct plantlet regeneration experiment from nodal and shoot tip explants of *anchote* using 6-benzylamimopurine (BAP) and kinetin for culture establishment, BAP and indole acetic acid (IAA) for shoot multiplication and half nutrient-strength MS medium supplemented with indole butyric acid (IBA), IAA and naphthalene acetic acid (NAA) for root induction. Similarly, Jane et al. (2016) conducted nodal culture experiment in *anchote* using BAP, kinetin and thidiazuron (TDZ) for micro-shoot formation, while IBA and NAA on half nutrient-strength of MS medium for root induction. Shekhawat et al. (2014), in *Coccinia indica*, have demonstrated the effect of BAP and kinetin on the nodal bud break and shoot multiplication while IAA and IBA on the root induction. However, a research report on meristem culture of *anchote,* broadly in *Coccinia* species, has not been found.

The exact condition required to initiate and sustain plant cells in culture or to regenerate intact plants from cultured cells has been found to depend on many factors, of which most important are: genotype, explants, composition of basic medium and plant growth regulators (Reed, 1999; Slater et al., 2003; Loyola-Vargas and Vazques-Flota, 2006; George and Debergh, 2008). Due to variation with such factors, there was found no method that can be universally recommended with a new species of interest. Therefore, this research work was initiated to investigate meristem culture in *KUWE* and 223098 genotypes of *anchote* through regulating plant growth regulators and nutrient-strength of the medium thereby to find out the best media composition for each developmental stage of meristem culture.

MATERIALS AND METHODS

Plant and experimental site

Seeds of two *anchote* accessions, *KUWE* (G1) and accession number 223098 (G2), each from different genotype cluster (Desta, 2011) were collected from Debre Zeit Agricultural Research Centre (DZARC). Mother plants were established in a greenhouse using pots filled with 2:1:1 ratio of soil, sand and compost, respectively. The experiment was conducted at the Plant Tissue Culture Laboratory of National Agricultural Biotechnology Research Centre (NABRC), Ethiopian Institute of Agricultural Research (EIAR), which is located at about 44 km far to the west of Addis Ababa, the capital city of Ethiopia.

Media preparation

The Murashige and Skoog (MS, 1962) basal nutrients with 3% sucrose were used as the basic components of the media. The pH of the medium was adjusted to 5.8 upon addition of plant growth regulators. Then, agar type I was added at a rate of 4 g/L while boiling to solidify the media. The prepared media were autoclaved for 20 min at a temperature of 121°C and 15 psi pressure and left for three days in the culture room before use to check for any contamination.

Shoot bud surface sterilization

Apical shoot buds of about 1 to 2 cm were collected from greenhouse grown plants at their full vegetative stage. Washing twice with tap water and once with the addition of liquid detergent and 1 to 2 drops of Tween-20 was done to remove surface dust and reduce the level of surface contaminants. Further surface disinfection was done under the laminar airflow hood by treating shoot buds with 70% ethanol for 30 s followed by 1% Clorox bleach (NaOCl) for 10 min based on the results of the preliminary experiment conducted to optimize shoot bud surface sterilization of anchote. Upon the completion of each surface disinfection treatment, shoot buds were rinsed three to four times with sterilized double distilled water to remove the effect of surface sterilizing agents.

Meristem establishment

Meristems (≤ 1 mm size) consisting of the meristematic dome with one to two leaf primordia were isolated using sterile hypodermic needle and scalpel under a dissecting microscope (Olympus) as described by Alam et al. (2004). Isolated meristems were quickly transferred to culture jars containing 50 ml volume of sterilized MS media supplemented with BAP (0.5, 1, 2 and 4 mg/L) alone and in combination with 0.1 mg/L GA_3 and 0.01 mg/L NAA. Data on the

number of days to shoot response, total response of meristems, shoot induction frequency and callus induction frequency was collected to evaluate shoot induction response of meristems.

Shoot multiplication

Normal and healthy meristem derived shoots (≥ 1 cm length) were isolated and inoculated on to freshly prepared media containing different concentrations of BAP (0.25, 0.5 and 1 mg/L) and kinetin (0.25 and 0.5 mg/L) singly and in combination. Data on the number of shoot length, leaf number and number of nodes was collected at the fourth week of culturing on the multiplication media.

Root induction

Normal and healthy shoots of about 2 cm and more in length were selected and inoculated on full and half nutrient-strength of the MS medium (FMS and HMS) supplemented with IBA and IAA individually, each at the same concentration levels (0.5, 1, 2 and 4 mg/L). Data on the root induction frequency, number of roots per shoot and root length was recorded at the fourth week of culturing on the rooting media.

Culture condition

Cultures were treated with hormone-free medium for a week whenever transferring them to a different type and combinations of plant growth regulators (PGRs) to avoid their carry-over effect. Hormone-free medium was used as a control treatment for all the developmental stages of meristem culture investigated in this experiment. All culturing processes were undertaken in the aseptic condition of the laminar air flow hood. Unnecessary callus developed at the base of shoots was trimmed with the help of surgical blade before shoots subjected to multiplication and root induction treatments. All cultures were kept in a growth room under 16 h light (2700 lux light intensity) and 8 h dark cycle at a temperature of 25 ± 2°C. To facilitate growth, the cultures were transferred to fresh media of the same combination at every four weeks interval.

Acclimatization procedures

Acclimatization was done by transplanting healthy root-formed shoots in a pot filled with sterilized sand, soil and compost in a ratio of 2:1:1, respectively. Roots were rinsed several times with tap water to remove the attached agar before planting them onto the prepared soil medium. Pots with plantlets were placed in a greenhouse by covering them with a plastic sheet. The cover was gradually removed and the survived plantlets were counted after four weeks of acclimatization.

Design and data analysis

All the factorially combined treatments were arranged in Completely Randomized Design (CRD). Each data was collected from a total of 20 sample units, five observations per culture jar replicated four times. Data of all the quantitative parameters were subjected to statistical analysis with SAS computer statistical tool (version 9.1). Least Significant Difference (LSD) test was conducted to compare treatment means, which revealed a significant difference of F-test in the analysis of variance (ANOVA), at $p \leq 0.05$ probability level.

RESULTS AND DISCUSSION

Effect of plant growth regulators on meristem culture establishment

Meristems showed their first growth response by increasing in size with greenish white color and became greener as they continued their growth and development of shoots (Figure 1A to C). Meristems of both genotypes established with lower concentrations of BAP alone and/or in combination with GA_3 responded to shoot within less than 10 days of incubation. An increase in the number of days to shoot induction response with a decrease in shooting frequency was observed at the higher levels of BAP as well as in all treatments containing NAA. This is mainly due to unnecessary callus development at the base of meristems (Figure 1D), which suppressed the development of shoots. Though the callusing potential of BAP and NAA was investigated earlier in nodal culture of *Coccinia grandis* (Thiripurasundari and Rao, 2012), such a phenomenon was investigated in different plant species: *Telfairia occidentalis* (Adesoye et al., 2012), potato (Yasmin et al., 2011), sweet potato (Alam et al., 2010), and tomato (Ishag et al., 2009). Geleta and Tileye (2011), on the other hand, obtained maximum shoot induction frequency in sweet potato meristem culture with the combination of BAP, NAA and GA_3 which argue with the present findings in *anchote*.

Genotypes varied in their maximum shoot induction potential and in their requirement for optimum BAP concentration. The highest shoot induction frequency was obtained in G2 at 1 mg/L BAP while maximum shoot induction frequency in G1 was obtained at 0.5 mg/L BAP (Data not shown). The effectiveness of BAP alone at a lower concentration in the initiation medium of eggplant meristem culture (Sharmin et al., 2008) and shoot culture in other genotype of *anchote* (Folla et al., 2013) was reported, which are in agreement with the current finding. The variation between genotypes may be due to their totipotentiality and endogenous level of PGRs as stated by Piqueras and Debergh (1999). Keeping the variation between genotypes, the current flinging is even better than the maximum shoot induction frequency obtained by Folla et al. (2013) in shoot culture of *anchote*.

Effect of genotype and cytokinins on shoot multiplication

Interaction of genotype, BAP and kinetin resulted in a significant ($p \leq 0.05$) variation on the multiplication and development of meristem derived shoots (Table 1). Genotypes were not significantly different in the production of maximum shoot number. However, maximum shoot number in G1 was obtained in a medium supplemented with 0.5 mg/L BAP + 0.5 mg/L kinetin, while in G2, 0.5 mg/L BAP alone was effective in the

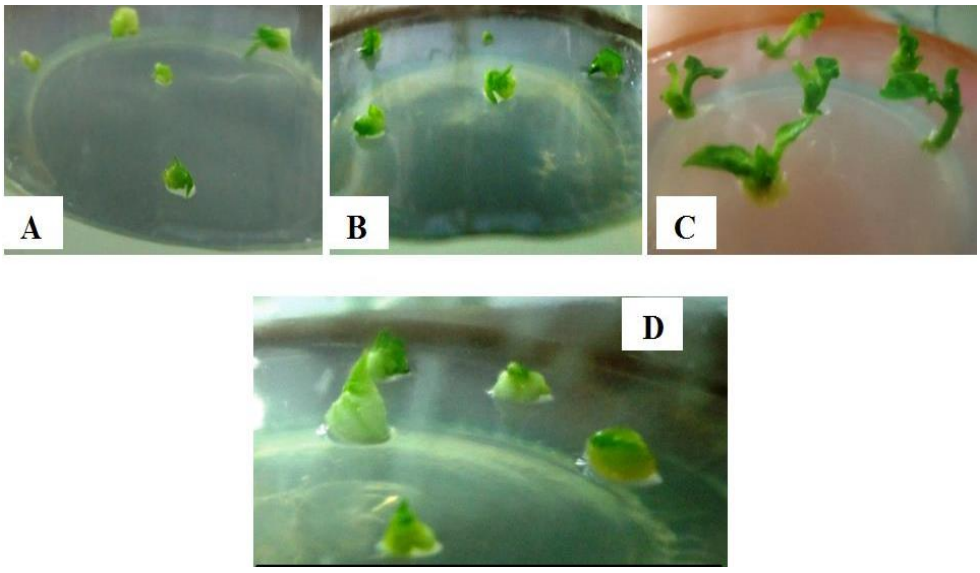

Figure 1. Trends in shoot induction. Commencement of shoot development at 8th day of initiation showing green color with the development of tiny leaves (A: G2= 1 mg/L BAP and B: GI=0.5 mg/L); normal shoots obtained after four weeks of culture with no callus formation (C: GI=0.5 mg/L); unnecessary callus developed at the base of meristems which suppressed shoot development, taken at 4th week of culture (D: G2= 4 mg/L BAP + GA3 + NAA).

production of multiple shoots. Application of BAP alone at a concentration of 1 mg/L in G2 and 0.5 mg/L in G1 was also satisfactory in the production of multiple shoots as compared to the control treatments, which produced only a single shoot. This result indicates the effectiveness of BAP than kinetin for multiple shoot production from meristem-derived shoots of *anchote*. Supportive results were reported by Bhujyan (2013) in potato varieties. Though, genotypic variation in cassava (Mapayi et al., 2013), eggplant (Sharmin et al., 2008) and sweet potato (Geleta and Tileye, 2011) shoot multiplication, in response to a given cytokinins, has been well demonstrated. Folla et al. (2013) obtained a maximum of about four shoots at fourth week on multiplication medium; where the number of shoots per explant increased to 10 as the culture time increases. Similarly, Shekhawat et al. (2014) in nodal culture of *C. indica* obtained an increased number of shoots through subsequent sub-culture. In the present experiment, however, a maximum of about six shoots were obtained within four weeks of culture duration. This clearly shows the culture time duration factor, apart from explant variation which was responsible for an increased rate of shoot multiplication.

Most of the media supplemented with kinetin produced longer shoots and higher numbers of leaf and node regardless of the genotype difference (Table 1). The positive effect of kinetin on shoot elongation in *Stevia rebaudiana* has been reported recently by Sridhar and Aswath (2014). Moreover, the negative effect of BAP on plantlet height was demonstrated by Sanavy and Moeini (2003) in potato cultivars. G1 was found superior to G2 in leaf and node number regardless of the type and concentration of cytokinins used. A genotypic difference in potato meristem culture has been reported by Al-Taleb et al. (2011) in terms of shoot numbers, shoot length and number of leaves of meristem-derived shoots.

Effect of genotype, media nutrient-strength and rooting hormones on root induction

Interaction of genotype, MS media nutrient-strength, rooting hormone and their concentrations resulted in a significant ($p \leq 0.05$) variation on the root induction and development parameters (Table 2). Full nutrient-strength of MS medium with a lower concentration of IBA was found effective in terms of root formation in both genotypes; 0.5 mg/L is the best, which resulted in 100% root formation. Al-Taleb et al. (2011) also reported related findings in different genotypes of potato. In contrary, Jane et al. (2016) and Folla et al. (2013) obtained maximum root induction frequency, in other genotypes of *anchote*, on half nutrient-strength of MS medium supplemented with lower concentration of IBA. Full nutrient-strength of MS medium supplemented with 1 and 2 mg/L IAA as well as half nutrient-strength of MS medium supplemented with 0.5 mg/L IAA also resulted in 100% root induction frequency in G2. However, roots formed in these media were found morphologically abnormal (Figure 2A and B) as compared to the normal morphological appearance roots (Figure 2C and D) obtained from full nutrient-

Table 1. The mean value of shoot multiplication parameters for *KUWE* (G1) and 223098 (G2) genotypes of anchote as affected by the interaction of various concentration levels of 6-Benzylamimopurine (BAP) and Kinetin (Kn).

Genotype	Treatments		Mean values ± SD			
	BAP (mg/L)	Kn (mg/L)	ShN	ShL (cm)	LN	NN
G1	0	0	1.00 ± 0.00^{p}	3.72 ± 0.12^{de}	4.32 ± 0.17^{defg}	3.20 ± 0.14^{ef}
		0.25	2.35 ± 0.06^{m}	4.15 ± 0.13^{bc}	4.17 ± 0.09^{fgh}	3.60 ± 0.22^{bcd}
		0.5	2.30 ± 0.08^{mn}	4.07 ± 0.09^{bc}	5.37 ± 0.17^{a}	3.70 ± 0.08^{bc}
	0.25	0	3.55 ± 0.13^{ef}	2.97 ± 0.05^{fg}	4.57 ± 0.13^{cde}	2.70 ± 0.22^{hi}
		0.25	3.50 ± 0.08^{fg}	2.80 ± 0.22^{fg}	4.30 ± 0.22^{efg}	3.60 ± 0.22^{bcd}
		0.5	3.82 ± 0.12^{cd}	3.57 ± 0.34^{e}	4.60 ± 0.08^{cd}	4.12 ± 0.17^{a}
	0.5	0	4.02 ± 0.17^{c}	1.30 ± 0.08^{n}	3.62 ± 0.12^{jk}	2.32 ± 0.13^{j}
		0.25	3.10 ± 0.22^{hi}	4.27 ± 0.12^{b}	4.40 ± 0.22^{cdef}	3.72 ± 0.05^{b}
		0.5	6.32 ± 0.12^{a}	2.05 ± 0.24^{ij}	4.27 ± 0.13^{fg}	3.42 ± 0.25^{de}
	1	0	3.67 ± 0.12^{def}	2.77 ± 0.17^{g}	3.27 ± 0.25^{lm}	2.67 ± 0.09^{i}
		0.25	3.00 ± 0.08^{ij}	2.20 ± 0.14^{hi}	4.67 ± 0.21^{bc}	3.40 ± 0.18^{de}
		0.5	2.65 ± 0.24^{kl}	1.60 ± 0.18^{lm}	3.07 ± 0.17^{mn}	2.40 ± 0.29^{j}
G2	0	0	1.00 ± 0.00^{p}	3.07 ± 0.09^{f}	3.67 ± 0.22^{ij}	2.72 ± 0.26^{hi}
		0.25	1.97 ± 0.12^{o}	5.92 ± 0.33^{a}	4.92 ± 0.12^{b}	3.47 ± 0.05^{cd}
		0.5	2.07 ± 0.09^{o}	3.90 ± 0.22^{cd}	4.17 ± 0.36^{fgh}	2.82 ± 0.17^{ghi}
	0.25	0	3.30 ± 0.29^{gh}	2.42 ± 0.42^{h}	3.50 ± 0.35^{jkl}	2.32 ± 0.17^{j}
		0.25	2.12 ± 0.09^{no}	1.97 ± 0.05^{ijk}	4.10 ± 0.08^{gh}	2.92 ± 0.09^{gh}
		0.5	3.30 ± 0.22^{gh}	1.70 ± 0.22^{klm}	3.57 ± 0.26^{jk}	2.77 ± 0.22^{hi}
	0.5	0	6.52 ± 0.33^{a}	1.87 ± 0.05^{jkl}	3.60 ± 0.27^{jk}	2.37 ± 0.05^{j}
		0.25	2.32 ± 0.05^{mn}	1.42 ± 0.12^{mn}	3.37 ± 0.09^{kl}	1.87 ± 0.19^{k}
		0.5	3.72 ± 0.05^{de}	4.12 ± 0.33^{bc}	3.92 ± 0.17^{hi}	3.02 ± 0.05^{fg}
	1	0	4.72 ± 0.21^{b}	1.60 ± 0.22^{lm}	3.47 ± 0.13^{jkl}	2.00 ± 0.08^{k}
		0.25	2.50 ± 0.08^{lm}	4.02 ± 0.17^{bc}	3.45 ± 0.21^{jkl}	2.05 ± 0.13^{k}
		0.5	2.80 ± 0.14^{jk}	1.22 ± 0.05^{n}	2.85 ± 0.17^{n}	1.57 ± 0.09^{l}
CV %			4.89	6.99	5.00	5.78

Means with the same letter within the column are not significantly different ($p \leq 0.05$); ShN: Shoot Number, ShL: shoot Length, LN: leaf number; NN: node number.

strength of MS medium supplemented with 0.5 mg/L IBA. This might be associated with the development of unnecessary callus. Unnecessary callus formation was observed at an elevated concentration of IBA and almost at all concentrations of IAA, regardless of the variation in nutrient-strength of the MS medium. Such a phenomenon was also observed by Shekhawat et al. (2014) in *C. indica* and Folla et al. (2013) in other genotypes of *anchote*.

There has been observed, generally, an increase in the number of root per shoot with an increase in concentrations of rooting hormones regardless of the variation between genotypes and media nutrient-strength (Table 2). However, a reduction in root length was observed as the number of roots per shoot increases. A higher number of roots at an elevated concentration of rooting hormones may be associated with the formation of callus at the base of cuttings which is responsible for adventitious root formation as described earlier by Jutta et al. (2005). A general reduction in number of roots due to callus development at a lower concentration of rooting hormones was reported by Folla et al. (2013) in other genotypes of *anchote*, which is in contrary with the current findings.

Table 2. The mean values of root induction parameters for KUWE (G1) and 223098 (G2) genotypes of anchote as affected by the interaction of MS medium nutrient-strength (full nutrient-strength (FMS) and half nutrient-strength (HMS)) and various concentration levels of indol butyric acid (IBA) and indol acetic acid (IAA).

MS	Treatment		Mean ± SD					
			G1			G2		
	PGRs	Conc.	RIF (%)	RN	RL (cm)	RIF (%)	RN	RL (cm)
FMS	IBA	0	95 ± 10.00^{ab}	2.87 ± 0.12^{s}	5.57 ± 0.12^{de}	95 ± 10.00^{ab}	8.20 ± 0.22^{l}	6.95 ± 0.26^{a}
		0.5	100 ± 0.00^{a}	4.92 ± 0.09^{p}	7.25 ± 0.13^{a}	100 ± 0.00^{a}	12.47 ± 0.12^{f}	5.17 ± 0.37^{fg}
		1	80 ± 0.00^{cde}	4.10 ± 0.18^{q}	4.37 ± 0.15^{hi}	95 ± 10.00^{ab}	9.50 ± 0.41^{k}	3.80 ± 0.22^{kl}
		2	50 ± 11.55^{hi}	6.32 ± 0.24^{n}	6.22 ± 0.09^{c}	75 ± 10.00^{def}	7.22 ± 0.17^{m}	4.12 ± 0.09^{ij}
		4	60 ± 0.00^{gh}	10.00 ± 0.24^{j}	4.30 ± 0.08^{hi}	70 ± 11.55^{efg}	8.50 ± 0.08^{l}	3.97 ± 0.12^{jk}
	IAA	0.5	95 ± 10.00^{ab}	14.87 ± 0.09^{d}	6.15 ± 0.21^{c}	85 ± 10.00^{bcd}	10.40 ± 0.22^{ij}	3.80 ± 0.33^{kl}
		1	85 ± 10.00^{bcd}	11.00 ± 0.24^{h}	5.60 ± 0.18^{de}	100 ± 0.00^{a}	10.00 ± 0.16^{j}	3.32 ± 0.12^{mn}
		2	95 ± 10.00^{ab}	13.35 ± 0.06^{e}	5.55 ± 0.13^{de}	100 ± 0.00^{a}	10.47 ± 0.12^{i}	3.27 ± 0.09^{mn}
		4	85 ± 10.00^{bcd}	15.60 ± 0.70^{c}	3.35 ± 0.34^{mn}	75 ± 10.00^{def}	15.47 ± 0.42^{c}	2.62 ± 0.34^{pq}
HMS	IBA	0	75 ± 10.00^{def}	3.42 ± 0.05^{r}	6.62 ± 0.17^{b}	85 ± 10.00^{bcd}	4.95 ± 0.13^{p}	4.22 ± 0.12^{hij}
		0.5	45 ± 10.00^{i}	5.67 ± 0.17^{o}	5.37 ± 0.05^{ef}	75 ± 10.00^{def}	5.07 ± 0.09^{p}	3.55 ± 0.13^{lm}
		1	50 ± 11.55^{hi}	7.10 ± 0.22^{m}	5.72 ± 0.17^{d}	60 ± 0.00^{gh}	6.40 ± 0.18^{n}	4.35 ± 0.24^{hi}
		2	45 ± 10.00^{i}	13.27 ± 0.21^{e}	4.50 ± 0.22^{h}	45 ± 10.00^{i}	13.25 ± 0.13^{e}	6.05 ± 0.21^{c}
		4	40 ± 0.00^{i}	5.25 ± 0.64^{p}	2.05 ± 0.10^{rs}	60 ± 0.00^{gh}	2.40 ± 0.08^{t}	0.50 ± 0.08^{t}
	IAA	0.5	95 ± 10.00^{ab}	11.27 ± 0.21^{gh}	4.90 ± 0.08^{g}	100 ± 0.00^{a}	13.30 ± 0.24^{e}	3.07 ± 0.24^{no}
		1	80 ± 0.00^{cde}	14.82 ± 0.17^{d}	2.77 ± 0.17^{op}	90 ± 11.55^{abc}	21.15 ± 0.17^{a}	2.62 ± 0.12^{pq}
		2	60 ± 0.00^{gh}	12.27 ± 0.32^{f}	2.40 ± 0.41^{q}	80 ± 0.00^{cde}	18.67 ± 0.47^{b}	2.40 ± 0.43^{q}
		4	40 ± 0.00^{i}	11.60 ± 0.48^{g}	2.32 ± 0.48^{qr}	65 ± 10.00^{fg}	21.10 ± 0.83^{a}	1.82 ± 0.22^{s}
CV%			10.73	3.01	5.05	10.73	3.01	5.05

Means with the same letter within the column of across the same parameters are not significantly different ($p \leq 0.05$). RIF: Induction frequency, RN: root number; RL: root length.

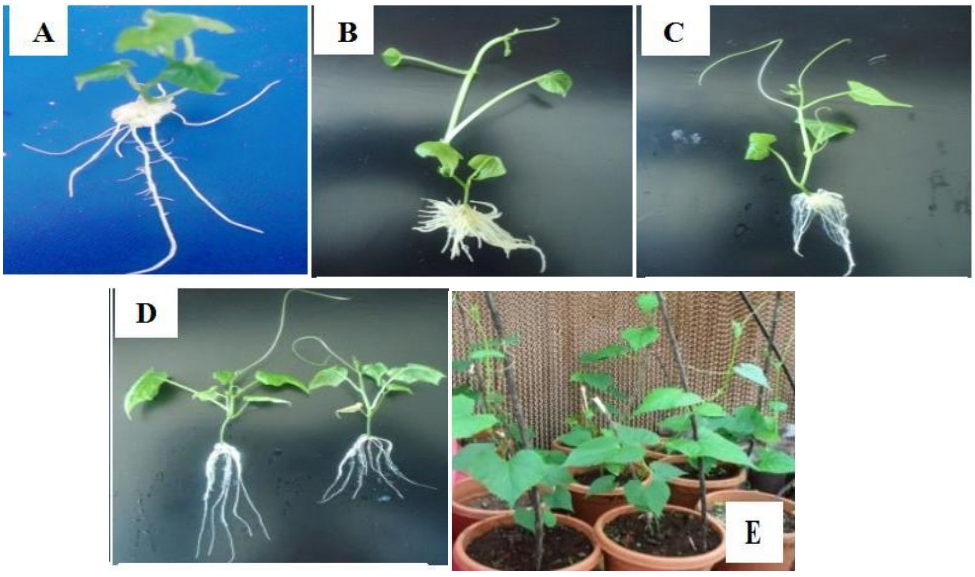

Figure 2. Root and plantlets morphology. Abnormal roots developed in both genotypes due to unnecessary callus formation at the base of the cuttings with the respective media composition (A: G2 = FMS + 4 mg/L IAA and B: G1 = HMS + 4 mg/L IAA); normal morphological appearance of roots of both genotypes with no callus formation (C: G2 = FMS + 0.5 mg/L IBA and D: G1 = FMS + 0.5 mg/L IBA); healthy and normal morphology of acclimatized plants obtained at 4th week of acclimatization (E:).

Acclimatization of plantlets

Meristem-derived plantlets were grown normal and healthy upon acclimatization (Figure 2E). Survival rates obtained in both genotypes indicated low performance with respect to the given soil medium composition (2:1:1 ratio of sand, soil and compost, respectively). However, plantlets of G1 survived better (58%) than plantlets of G2 (42%), after four weeks of acclimatization. Regardless of the genotype difference, Yoseph and Tileye (2013) obtained about 60% survival rate of *in vitro* produced plantlets of anchote, acclimatized on the same potting mixture used in the present study. The low survival rates might be associated mainly with the composition of potting substrates as indicated by Folla et al. (2013) and Jane et al. (2016).

Conclusion

Meristem culture in both *KUWE* (G1) and 223098 (G2) genotypes of *anchote* has been investigated in detail in this study. The optimum media composition has also been determined for each developmental stage of meristem culture. Maximum shoot induction frequency was achieved using 0.5 and 1 mg/L BAP in G1 and G2, respectively. Multiple shoot production was achieved by applying 0.5 mg/L BAP alone and combined application of BAP and kinetin each at 0.5 mg/L, in G2 and G1, respectively. Whereas, full nutrient-strength MS medium supplemented with 0.5 mg/L IBA was found effective in the *in vitro* production of roots, in both genotypes. Plantlets survival rate of 58% in G1 and 42% in G2 were found after a month of acclimatization on 2:1:1 ratio of soil, sand and compost, respectively. The findings of this study can be applied for mass production of quality propagation materials of anchote as well as for any investigation following the technique of meristem culture. However, further experiment might be conducted to improve the survival rate of plantlets during acclimatization.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effects of inoculation with some native arbuscular mycorrhizal fungi on tomato (*Solanum lycopersicum* L.) growth

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In Burkina Faso, tomato (*Solanum lycopersicum* L.) sector plays a very important socio-economic role. However, its production is confronted by many constraints, among which is the soil poverty in mineral elements such as nitrogen and available phosphorus resulting in an increase in the area of land that is sown for this crop and an increase in the use of chemical inputs. In addition, chemical inputs have shown their limits with several environmental negative impacts. Therefore, this study was initiated to help improve sustainable agricultural production. In this study, tomato was grown in the greenhouse and inoculated with three natives' mycorrhizal inocula. The growth parameters were measured at 30 and 60 days after sowing. Above-ground, root and total biomass were assessed at 60 days after sowing. The results showed an improvement in height of 164.44%, in the collar diameter of 75.25%, in above ground biomass production of 540%, root biomass of 1061.97% and a total biomass of 638, 1% after inoculation. This study had shown promising results and merits further investigation *in situ*.

Key words: Tomato, mycorrhizal inoculation, arbuscular mycorrhizal fungi, sustainable agriculture.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.), is an herbaceous plant widely cultivated for its fruit and it represent one of the main food crops in the world. This crop is the second most important vegetable produced and consumed in Western countries (Willcox et al., 2003). With its products, tomatoes are one of the main food sources of carotenoids providing around 80% of the daily intake of lycopene, folate, ascorbic acid, flavonoids, a-tocopherol and potassium in the Western diet (Bramley, 2000; Willcox et al*.,* 2003). In Burkina Faso, tomato is one of the most important vegetable crops. However, its production encounters enormous problems such as the poverty of soils, particularly in phosphorus (P) and nitrogen (N) (Diem et al., 1981; Mikola, 1987), water deficit, wind and water erosion, as well as fungal, bacterial and viral diseases. Its production increased from 1,000 t in 2000 to 12,635 t in 2017 (FAO, 2020). The improvement in this production is linked to the area planted (from 1,000 ha in 2000 to 1,254 ha in 2017) since the yield has remained around 10 t/ha (FAO, 2020). In

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Table 1. Culture substrate physico-chemical characteristics.

Characteristics	Clay (%)	Total silt (%)	Total sand (%)	Total organic matter (%)	Total carbon (%)	Total nitrogen (%)	C/N	Total phosphorus ($mg.kg^{-1}$)	Available phosphorus ($mg.kg^{-1}$)	pH H_2O (p/v: ½ :5)
Values	3.92	5.88	90.2	0.331	0.192	0.016	12	172.52	1.74	6.44

addition to the increase in the cultivated area, there is also the intensive use of chemical fertilizers to correct soil poverty and fight against tomato diseases. This results in huge losses for farmers. In addition, intensive agriculture has shown its limits: Soil and water pollution, the emergence of pesticide-resistant pathogens, high pesticide costs, risks to farmers' health, and weakening ecosystems (Pierre, 2012). Regulations are now in place to limit the use of these inputs. Therefore, maintaining crop productivity through agriculture that substitute chemical inputs use by the mobilization of biological processes is at the heart of the challenges of current agricultural research. Thus, tomato cultivation remains to this day, a major concern because its important contribution to food security and the increase in income of producers, especially in family farming. The current agriculture turns towards an ecological intensification which leans on the promotion of the ecological mechanisms by practices such as crop rotation, the cultural association or the biological control. The use of beneficial microorganisms such as arbuscular mycorrhizal fungi (AMF) is increasingly considered as one of the best sustainable organic farming practices (Haro et al., 2017). It is well established that the tomato forms a symbiosis with arbuscular mycorrhizal fungi (Dodzia et al., 2012; Khalloufi, 2017) while the beneficial effect of mycorrhizal symbiosis on plant growth and production has been the subject of several studies (Haro et al., 2016a, 2012, 2015, 2017, 2016b). Exploiting this symbiosis would be a possibility to improve tomato productivity. Thus, we became interested in the tomato mycorrhizal symbiosis with the aim of evaluating the effect of inoculation of this plant with arbuscular mycorrhizal fungi native to Burkina Faso on its growth.

MATERIALS AND METHODS

Plant and fungal materials

Tomato variety AMIRAL F1-hybrid [variety of tropical, semi-tropical and Sahelian zones (produced/imported, packed and marketed by SAKATA SEED INDIA PVT., LTD)] was used. Tomato seeds were surface disinfected by soaking in 96% ethanol for 3 min, rinsed thoroughly with sterile distilled water and then disinfected in calcium hypochlorite solution ($CaCl_2O_2$ at 3.3%, w/v) for 3 min and finally rinsed thoroughly with sterile distilled water before sowing. These seeds were sown at a rate of 4 seeds per pot.

Fungal material was composed of two efficient local AMF isolated from the rhizosphere of cowpeas grown in Burkina Faso (Haro et al., 2012, 2017): Mycorrhizal complex [*Scutellospora* sp., *Gigaspora* sp., *Glomus* sp. (M1)] and *Glomus* sp. (M2). The inocula were obtained by multiplication of cowpea rhizosphere indigenous arbuscular mycorrhizal fungi (Haro et al., 2012). The inoculum constituted of spores, mycorrhizal root fragments and soil.

Culture substrate

The growing substrate was a sterilized soil of Ouagadougou and its physical and chemical characteristics are presented in Table 1. Culture substrate was homogenized, sieved with a 2 mm sieve and sterilized at 121°C for 1 h.

Test implementation

The test consisted of growing tomatoes in 2 L pots containing 2 kg of sterilized culture substrate (Table 1). The inoculation was carried out at the sowing time with 10 g of inocula [each inoculum constituted of spores, mycorrhizal root fragments and soil and was kept at room temperature (about 25°C)] (Haro et al., 2017) for each inoculated treatment. The inoculation consisted to place in the middle of each pot containing the culture substrate, 10 g of inoculum at 2 or 3 cm deep. Control pots were not inoculated. The experiment unit was composed of 3 treatments (two inoculated treatments (M1 and M2) and one control)] with 5 replicates per treatment. The tomato was sown at the rate of 4 seeds per pot and a wedge was carried out two weeks after the plants emergence to allow only one plant per pot. The experimental design used was a simple randomization complete block design. This experiment lasted 60 days (flowering stage).

Grow parameters measurement

To estimate the effect of mycorrhizal inoculation on the tomato, the height, the diameter at the collar, the rate of relative growth in height and the rate of relative growth of the diameter at the collar were calculated at the 30th and at the 60th day after sowing. The relative growth rate in height (RGRH) was calculated according to the following formula:

$$RGRH = (Hf - Hi)/Hi$$

With H: height, i: initial and f: final.
The collar diameter was measured using a caliper at the separation zone between the root system and the aerial part at the30th and at the 60th day after sowing. The relative growth rate of the collar diameter (TCRdc) was calculated by the following formula:

$$TCRdc = (Dcf - Dci)/Dci$$

With Dc = Diameter at the collar, I = initial and f = final.

Table 2. Tomato mycorrhizal frequency and intensity 60 days after sowing inoculated with 2 mycorrhizal inocula [mixed inocula (M1) and *Glomus* sp. (M2)].

Treatment	Mycorrhizal frequency (%)	Mycorrhizal intensity (%)
M1	86±4[a]	40.5±4.07[a]
M2	82±6.63[a]	35.3±5.35[a]
Control	0[b]	0[b]
Significance level	<0.000 1	<0.000 1

For the same parameter, data followed by the same letters are not significantly different according the Newman-Keuls test ($p < 0.05$). Standard error of the mean ($n = 5$).

Table 3. Plant height, the diameter at the collar, the rate of relative growth in height and the rate of relative growth of the diameter at the collar of tomato inoculated with 2 mycorrhizal inocula [mixed inocula (M1) and *Glomus* sp. (M2)].

Treatment	Height 1 (cm)	Height 2 (cm)	RGRH (%)	Diameter 1 (mm)	Diameter 2 (mm)	TCRdc (%)
M1	14.5±1.55[a]	35.7±2.67[a]	1.54±0.24[a]	2.53±0.32[a]	3.54±0.3[a]	0.45±0.16[a]
M2	12.6±1.95[a]	20±4.71[ab]	1.46±0.25[a]	2.2±0.28[a]	2.87±0.35[b]	0.21±0.14[a]
Control	10.2±1.06[a]	13.5±4.15[b]	0.74±0.36[a]	1.61±0.14[a]	2.02±0.28[b]	0.4±0.14[a]
Significance level	NS	0.004	NS	NS	0.01	NS

For the same parameter, data followed by the same letters are not significantly different according the Newman-Keuls test ($p < 0.05$). Standard error of the mean ($n = 5$). Height 1 and 2: Height measured respectively at 30 and 60 days after sowing. Diameter 1 and 2: diameter at the collar measured respectively at 30 and 60 days after sowing. NS: Not significant.

Shoot, root and total biomass measurement

At 60 days after sowing, each plant was carefully removed in order to recover the aerial part and all the roots of the plants. All these parts were dried in an oven at 70°C for 72 h for the measurement of shoot, root and total biomass. After the biomass measurement, the roots were used for the mycorrhizal infection study.

Staining for mycorrhizal colonization

About 10 g of roots from each treatment were thoroughly washed and placed in falcon tubes and then cleared using 10% KOH. They were heated in 90°C water bath for one hour. The roots were washed with tap water. Staining was then done by adding 0.05% trypan blue in lactic acid and heating in 90°C water bath for 30 min (Phillips and Hayman, 1970) and the observation was done under microscope (OLYMPUS BH-2) (magnification = 10x). The mycorrhizal frequency and intensity were estimated by Trouvelot et al. (1986) method.

Statistical analysis

Data were statistically analyzed using a one-way analysis of variance (ANOVA) with XLSTAT 2018 statistical software, and the means were compared using the Newman-Keuls test ($p < 5\%$).

RESULTS

Mycorrhization frequency and intensity evaluation

Table 2 shows the frequency and intensity of mycorrhization of the tomato. The frequency of mycorrhization was quite high (86% for M1 inoculum and 82% for M2 inoculum) while the intensity of mycorrhization remains low (40.5% for M1 inoculum and 35.3% for M2 inoculum) (Table 2). Statistical analyzes show significant differences between the different treatments. However, the roots of the control treatments are not mycorrhized (Table 2).

Effects of the tested AMF on the tomato growth

Table 3 presents the results of the measurements of the height, the relative growth rate in height, the collar diameter and the relative growth rate of the collar diameter of the tomato inoculated with native arbuscular mycorrhizal fungi. The results showed that the plant height varied according to the inoculum used and over time. At 30 days after sowing, the statistical analysis does not show any significant difference ($P > 0.05$) between the different treatments, neither for the height nor for the collar diameter (Table 3 and Figure 1). At 60 days after sowing, the differences appear between the different treatments for both the height and the collar diameter and the highest values were obtained for inoculation with M1 inoculum (35.7 cm for the height and 3.5 mm for the collar diameter). This inoculum improved the tomato height growth by 164.44% and that collar diameter by 75.25% compared to controls (Table 3). However, M2 inoculum did not improved tomato growth compared to the control (Table 3). Moreover, the statistical analysis did not show any significant difference ($P > 0.05$) between

Figure 1. Tomatoes plants 60 days after sowing and inoculated with 2 mycorrhizal inocula [mixed inocula (M1) and *Glomus* sp. (M2)].

the different treatments either for the relative growth rate in height, or for the relative growth rate in the collar diameter. In general, the M1 inoculum improved the tomato growth (Table 3 and Figure 1).

Assessment of shoot, root and total biomass

The results on shoot, root and total biomass are presented in Table 4. These results varied according to the treatments. Statistical analysis showed significant differences between the different treatments. The highest values were obtained with M1 inoculum (Table 4). This inoculum improved the shoot biomass by 540%, the root biomass by 1061.97% and the total biomass by 638.1% compared to the control (Table 4). However, M2 inoculum did not improved tomato biomass production compared to the control (Table 4).

DISCUSSION

The objective of this study was to assess the effects of mycorrhizal inoculation with native strains from Burkina Faso on tomato growth. The mycorrhizal results showed that the roots of the tomato were highly colonized by the mycorrhizal strains used. The absence of mycorrhizal infection on the controls roots showed that this treatment was free from any mycorrhizal contamination. The growth and biomass stimulation between the different treatments and the control could be attributed to the effect of arbuscular mycorrhizal fungi inoculation.

The results on tomato growth show that inoculation improved the growth of this plant. Statistical analysis of plants height measured at 30 days after sowing did not show any significant difference between inoculated plants and control. This can be explained by the fact that the substrate used contained the necessary nutrients which were directly accessible to the plant's roots. These results are in agreement with those of Haro et al. (2012) who showed that the plant will not find a need to form the mycorrhizal symbiosis if the nutrients are available in the environment. This also explains the absence of significant differences in the rate of relative growth in height and the rate of relative growth in the collar diameter of the tomato. However, the statistical analysis on the height and the collar diameter of the plants measured at 60 days after sowing show significant differences between the different treatments. This can be explained by the fact that the growth of the plants over time has led to the depletion of the mineral elements directly accessible by their roots. Thus, the mycorrhizal symbiosis was established and developed gradually with the exhaustion of the mineral elements in the soil; hence

Table 4. Plant shoot, root and total biomass 60 days after the sowing of tomato inoculated with 2 mycorrhizal inocula [mixed inocula (M1) and *Glomus* sp. (M2)].

Treatment	Shoot biomass (g)	Root biomass (g)	Total biomass (g)
M1	0.79±0.17[a]	0.33±0.07[a]	1.12±0.18[a]
M2	0.29±0.11[b]	0.22±0.1[ab]	0.41±0.11[a]
Control	0.12±0.07 [b]	0.03±0.02 [b]	0.15±0.07 [b]
Significance level	0.009	0.016	<0.001

For the same parameter, data followed by the same letters are not significantly different according the Newman-Keuls test ($p < 0.05$). Standard error of the mean ($n = 5$).

the effectiveness of the mycorrhizal strains in improving the growth in height and the collar diameter of the tomato plants at 60th day after sowing. Similar results have been found by Haro et al. (2012) on cowpeas. The highest values obtained with M1 inoculum, can be explained by the high mineral absorption power of the M1 inoculum.

For shoot, root and total biomass, M1 inoculum showed the best efficiency. As with the height and collar diameter measured at the 60th day after sowing, the effectiveness of the M1 inoculum on stimulating the production of biomass could be explained by the improvement of the tomato mineral nutrition by this inoculum. These results are in agreement with those of Ndoye et al. (2016) who showed that inoculation with *G. verriculosum*, *G. manihotis* and *R. irregularis* significantly improved the biomass of fonio plants. Similar results were found by Laminou Manzo et al., (2009) who showed the effectiveness of *Glomus intraradices* on the production of total biomass of *Acacia raddiana*, *Acacia nilotica*, *Acacia senegal* and *Prosopis chilensis*.

It appears that M1 was the most effective of all the inocula used in this study. This could be explained by the fact that M1 contains three AMF genera (*Scutellospora* sp., *Gigaspora* sp., *Glomus* sp.) whose effects can be added compared to M2 which has only one genus (*Glomus* sp.). Similar results were found by Haro et al. (2012) who showed that native strains containing at least 2 genera have effects that can add up compared to monospecific inocula.

The M2 inoculum did not improve either the tomato growth or the biomass production. This can be explained by the ineffectiveness of *Glomus* sp. in the tomato mineral nutrition improvement, which is justified by a host preference for endomycorrhizal fungi. Similar results were found by Haro et al. (2012) on cowpea.

Conclusion

From these results, it generally appears that mycorrhizal inoculation improves the growth and production of tomato biomass. This study showed that Burkina Faso native mycorrhizal strains can improve both the production of biomass and the growth in height and in the collar diameter. Although, it has shown promising results, this study deserves to be supplemented by *in situ* tests which would make it possible to assess the effect of this inoculation on the tomatoes production.

CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

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Development of starter cultures carrier for the production of high quality *soumbala*, a food condiment based on *Parkia biglobosa* seeds

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In this study, three *Bacillus subtilis* (B7, B9 and B3) and one *Bacillus amyloliquefaciens* (I8) strains previously selected as potential starter cultures were cultivated on boiled dehulled African locust bean seeds to produce ready to use ferments. These ferments were then used to produce *soumbala*. The microbial load changes during ferments production were evaluated. Biochemical and microbiological characteristics of the obtained *soumbala* were also determined using standard methods. Variable growth ability on the carrier material was observed for the tested starters with *Bacillus* loads ranging between 8.21 and 10.37 Log CFU/g in the ferments. The highest microbial counts were observed for ferments prepared with the starters B9 and B7. These ferments also demonstrated the strongest fermentation capacity of *soumbala*. The ferment-based dried *soumbala* had a pH and moisture ranging from 7.17 to 7.37 and 5.67 to 8.46%, respectively. On dry matter (DM) basis, it contained 1.77 to 2.11% of ashes, 41 to 43% of proteins, 37 to 40% of fat and 13 to 15% of carbohydrates. *Soumbala* prepared with the starter B7 showed the highest content in valine, isoleucine, leucine, phenylalanine, tyrosine and proline.

Key words: African locust bean, starter culture, *Bacillus*, fermentation, *soumbala*, carrier.

INTRODUCTION

Fermented food condiments obtained by the fermentation of proteagenous seeds, are well appreciated in Africa for their high nutritional value and organoleptic properties. In Burkina Faso, the well-known of these fermented food condiments is *soumbala*, obtained by spontaneous alkaline fermentation of African locust bean (*Parkia biglobosa*) seeds (Parkouda et al., 2009). *Soumbala* is also well known and used in Côte d'Ivoire, Guinea, and Mali. It is known under different names such as *dawadawa*/*iru* in Nigeria and Ghana (Onzo et al., 2014; Ajavi et al., 2015), *afitin*/*sonru*/*iru* in Benin (Azokpota et al., 2006) and *nététu* in Senegal (N'Dir et al., 2000).

Soumbala is an affordable source of proteins (34-42%), fat (21-37%), carbohydrates (15-17%), minerals (calcium, phosphorus and iron), vitamins (B1 or thiamine, B2 or riboflavin and PP or niacin) and essential amino acids (Ouoba et al., 2003b) for low income inhabitants. In addition, the production of *soumbala* constitutes an income generator for producers who are generally illiterate women.

The production of *soumbala* includes successive cleaning of the seeds, a first cooking which often lasts more than 24 h, a dehulling of the cooked seeds, a second cooking which lasts between 1 and 2 h and then a spontaneous fermentation of 48-72 h (Sawadogo-Lingani et al., 2003). *Bacillus subtilis* group species were identified as the dominant *Bacillus* involved in the spontaneous fermentation of *soumbala* (Ouoba et al., 2004). Despite increasing consumption today in Burkina Faso, *soumbala* still faces competition from imported seasonings. This is partly due to the use of unsuitable fermentation methods in the production of traditional *soumbala* leading to a product with poor organoleptic and sanitary quality resulting sometimes in the presence of pathogenic bacteria and biogenic amines (Parkouda et al., 2010).

Several studies carried out on *soumbala* and other fermented condiments of Burkina Faso provided evidence on how to isolate and characterize some *Bacillus* species with potential uses as starter cultures in controlled fermentation to improve its hygienic, nutritional and organoleptic quality (Ouoba, 2003; Kaboré, 2012; Compaoré, 2013). However, the form in which these potential starter cultures can be easily transferred to *soumbala* production units has not been proposed yet. As a consequence, *soumbala* processing units are still producing *soumbala* in a traditional way with uncontrolled fermentation. The objectives of this study were, therefore on one hand to assess the possibility of using the dehulled seeds of African locust bean as local carrier material for the transfer of starter cultures of *Bacillus* spp. to *soumbala* production units, and on the other hand to compare the biochemical and microbiological characteristics of *soumbala* prepared with starter cultures used in single or in combination.

MATERIALS AND METHODS

African locust bean seeds

African locust bean seeds were purchased with a *soumbala* producer in Ouagadougou, Burkina Faso, stored in polypropylene bags and kept in the pilot plant of Département Technologie Alimentaire (CNRST/IRSAT/DTA) at room temperature.

Microorganisms

The starter cultures used in this study included two strains of *B. subtilis* (B7 and B9) isolated from *soumbala*, one strain of *Bacillus amyloliquefaciens* (I8) isolated from *bikalga* (fermented seeds of *Hibiscus sabdariffa*) and one strain of *B. subtilis* (B3) originating from *maari* (fermented seeds of *Adansonia digitata*). These strains were identified based on molecular methods (Rep-PCR, ITS-PCR, M13-PCR, 16S rRNA and *gyrB* gene sequencing) and selected as starter cultures in previous studies based on their technological properties among other proteolytic, saccharolytic, lipolytic and antimicrobial activities (Ouoba et al., 2003a, 2003b, 2007; Kaboré et al., 2012; Compaoré et al., 2013b). All the strains were kindly provided by the laboratory of microbiology of CNRST/IRSAT/DTA where they were stored in a -80°C freezer.

METHODS

Preparation of the carrier material

African locust bean seeds were first dried and cleaned by winnowing to remove light impurities. They were then dehulled using a mechanical dehuller (prototype CNRST/IRSAT, Ouagadougou, Burkina Faso, 1997). Following dehulling, the cotyledons were separated from the hulls by winnowing and manual sorting. The cotyledons were then collected for use as carrier for the production of the ferments.

Preparation of the inocula

The stock cultures were sub-cultured in Brain Heart Infusion (BHI) agar (Liofilchem, 610007, Italy) and incubated for 24 h at 37°C. From BHI agar plates, the *Bacillus* strains were sub-cultured for 18 h at 37°C in 10 ml of BHI broth (Liofilchem, 610008, Italy). After incubation, the cultures were centrifuged at 5 000 g for 10 min and the pellet resuspended in 5 ml of sterile diluent containing 8.5 g/l NaCl and 1.5 g/l peptone (Difco 218971, Becton Dickinson & Co, Sparks, MD, USA). The number of cells was then estimated by microscopy using a counting chamber (Neubauer, Wertheim, Germany) and dilutions were made in sterile diluent to obtain a rate of inoculation of 10^5 - 10^6 cells/ml.

Four different inocula were prepared: Inoculum of *B. subtilis* B7, inoculum of *B. subtilis* B9, inoculum of *B. subtilis* B3 and inoculum of *B. amyloliquefaciens* I8.

Cultivation of the starter cultures on the carrier material

The African locust bean cotyledons were weighed and washed before being boiled for 6 h. After cooking and draining, the cotyledons were distributed (500 g) in baskets and autoclaved at 121°C for 20 min. After cooling at 45 to 50 °C, each basket was inoculated with each inoculum (2% v/w) in single and left to ferment for 48 h at room temperature (35 - 38°C). One non-inoculated basket served as control.

The fermented cotyledons from each basket were dried in an oven at 60 to 65°C for 24 h before being aseptically ground using a

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blender of mark XPREP (Model MX1200XT11CE, USA). The resulting powder was aseptically packaged (5 g) in sterile plastic bags and stored at room temperature. Four ferments FB7, FB3, FI8 and FB9 in powder form were then prepared from inoculum of *B. subtilis* B7, *B. subtilis* B3, *B. amyloliquefaciens* I8 and *B. subtilis* B9, respectively. Samples were collected after autoclaving, after inoculation (0 h of fermentation), at the end of the fermentation (48 h) and after the grinding of dried product to determine pH and growth of *Bacillus* for each single starter culture fermentation batch. The experiment was performed on three separate occasions and 16 samples were taken at each trial. In total, 48 samples were collected for microbiological analyses.

Production of *soumbala* with the ferments

The production of *soumbala* was carried out with non-dehulled African locust bean seeds following the traditional processing described by Sawadogo-Lingani et al. (2003) with slight modifications as follows: the seeds were cleaned, cooked for 18 h and dehulled manually with mortar and pestle; the dehulled seeds were cooked again for 1 h, drained, distributed in baskets, autoclaved at 121°C for 20 min and cooled to 45 to 50°C. Seven parallel fermentation batches were prepared as follows: the batches 1, 2, 3 and 4 containing 1 kg of sterilized cotyledons each, were inoculated with 5 g of each single ferment (FB7, FB9, FB3 and FI8); the batches 5, 6 and 7 containing 2 kg of sterilized cotyledons each were inoculated with 10 g of mixed ferments (FB7+FB3, FB7+FI8 and FB7+FB9). The batches were then left to ferment for 48 h at room temperature (35 - 38°C). Traditional spontaneously fermented *soumbala* was prepared according to Sawadogo-Lingani et al. (2003) to serve as a control (batch 8). After the fermentation, fermented cotyledons were sun-dried and kept in a dry place. Samples were collected at 0 h, at the end of the fermentation (48 h) and after drying. The different types of *soumbala* produced were:

(1) *Soumbala* with single ferment: SB7, SB3, SI8, and SB9
(2) *Soumbala* with mixed ferments: SB7 + B3, SB7 + I8, SB7 + B9
(3) Spontaneously fermented *soumbala*: SN.

The experiment was conducted in triplicate and 24 samples were taken at each assay. In total 72 samples were collected for microbiological analyses. Physicochemical analyses were performed only on the final dried products (24 samples).

Microbiological analyses

For all samples, 10 g were aseptically homogenized with 90 mL of sterile diluent by using a stomacher (Stomacher 400 lab blender, England) at normal speed for 2 min to obtain 10^{-1} dilution (ISO 6887-1, 2017). Serial dilutions were made from the homogenate using 9 mL sterile diluent. From appropriate ten-fold dilutions, *Bacillus* strains were enumerated by pour plate technique using BHI Agar incubated aerobically at 37°C for 72 h. After incubation, plates with 15 to 300 colony forming units (CFU) were counted (ISO 4833, 2003) and results expressed as Log CFU/g.

Biochemical analyses

Ten grams of sample were homogenized with 20 mL of distilled water in a stomacher bag for 1 min at normal speed. The pH of the homogenate was determined using an electronic pH meter (Hanna, Romania) calibrated with standard buffer solutions pH 4.0 and 7.0.

Moisture content was determined by drying the sample at 105 ± 2°C for 12 h according to ISO 712 (2009); total ash content was determined by incineration in a muffle furnace (Nabertherm, Germany) at 550°C for 4 h, according to ISO 2171 (2007); crude protein content (N×6.25) was determined by the Kjeldahl method after acid digestion (AFNOR NF V03-050, 1970); crude fat content was determined with Soxhlet apparatus using n-hexane according to ISO 659 (1998). Total carbohydrates content was determined by spectrophotometric method at 510 nm using sulfuric orcinol as reagent (Montreuil and Spik, 1969).

For amino acids profile determination samples were first defatted using Soxhlet method (ISO 659. 1998). The amino acid profile was carried out by high performance liquid chromatography (HPLC) using Waters PICO-TAG method (Kristofferson, 2011) which consists of three steps: hydrolysis of samples, sample derivatization pre-column and HPLC-reverse phase analysis. The identification and determination of the concentrations of the different amino acids were done from the Empower software by comparing retention times obtained with retention times of the standards.

Statistical analysis

All the data were submitted to Analysis of Variance (ANOVA) with the statistical software XLSTAT-Pro 7.5.2 and the means were compared using a Fischer test for post-hoc comparisons with a probability level $p < 0.05$. The curves and the standard deviation were obtained using Microsoft Excel 2007.

RESULTS

Microbial growth during the production of ferments

No microbial growth was observed after autoclaving of African locust bean cotyledons for all samples (results not shown). Figure 1 shows the growth ability of the different starter cultures inoculated in single in the cooked dehulled African locust bean seeds. At the onset of the fermentation (t = 0 h), *Bacillus* counts ranged between 4.33 and 4.87 Log CFU/g. At the end of the fermentation (t = 48 h), a significant increase of the *Bacillus* load was observed in all samples with values ranging between 7.11 and 9.70 Log CFU/g. However, the starter cultures showed variable ability to grow in the cooked dehulled seeds of *P. biglobosa*. *Bacillus amyloliquefaciens* I8 (originating from *bikalga*) counts were the lowest, followed by *B. subtilis* B3 (originating from *maari*). On the contrary, *B. subtilis* B7 and *B. subtilis* B9 (originating from *soumbala*) yielded the highest counts. In the final dried and ground products (ferments), the *Bacillus* counts had increased to between 8.21 and 10.37 Log CFU/g and the highest microbial counts were observed for ferments prepared with *B. subtilis* B9 and B7.

Microbial growth during controlled fermentation of *soumbala* using single and mixed ferments

The growth of *Bacillus* ferments inoculated in single during the production of *soumbala* is as shown in

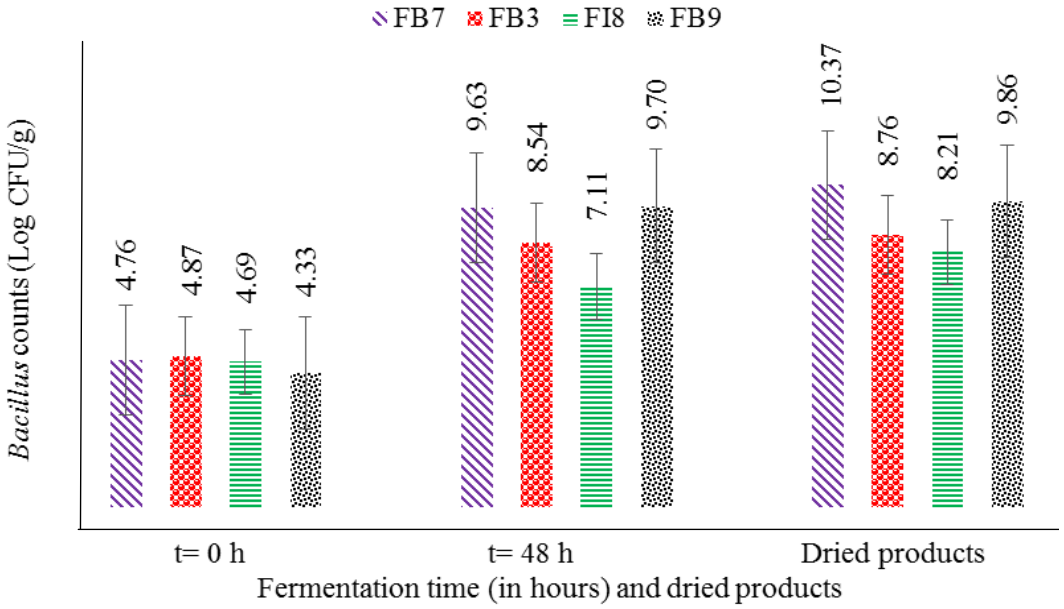

Figure 1. Growth of *Bacillus* starter population during the production of ferments. FB7: Ferment produced with starter culture *B. subtilis* B7; FB3: Ferment produced with starter culture *B. subtilis* B3; FI8: Ferment produced with starter culture *B. amyloliquefaciens* I8; FB9: Ferment produced with starter culture *B. subtilis* B9.

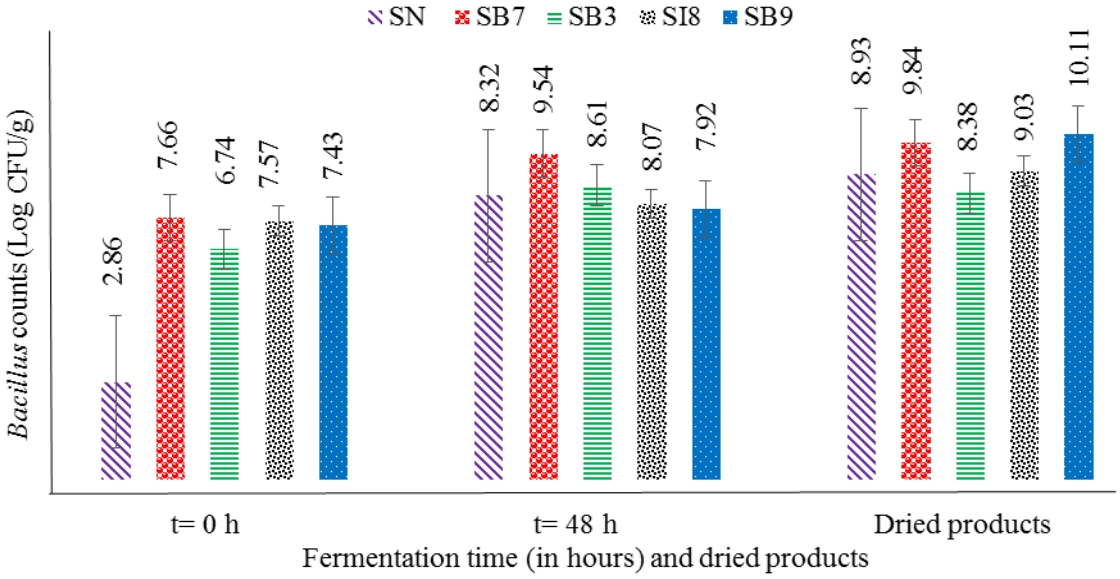

Figure 2. Growth of *Bacillus* starter population during the production of *soumbala* using single ferments. SN: spontaneous *soumbala*; SB7: *soumbala* produced with ferment B7; SB3: *soumbala* produced with ferment B3; SI8: *soumbala* produced with ferment I8; SB9: *soumbala* produced with ferment B9.

Figure 2. At the beginning of the fermentation, *soumbala* produced with single ferment (SB7, SI8, SB3 and SB9) had a *Bacillus* load between 6.74 and 7.66 Log CFU/g whereas spontaneous *soumbala* had a load of 2.86 Log CFU/g. At the end of the fermentation (48 h), the *Bacillus* loads increased in all types of *soumbala* and ranged

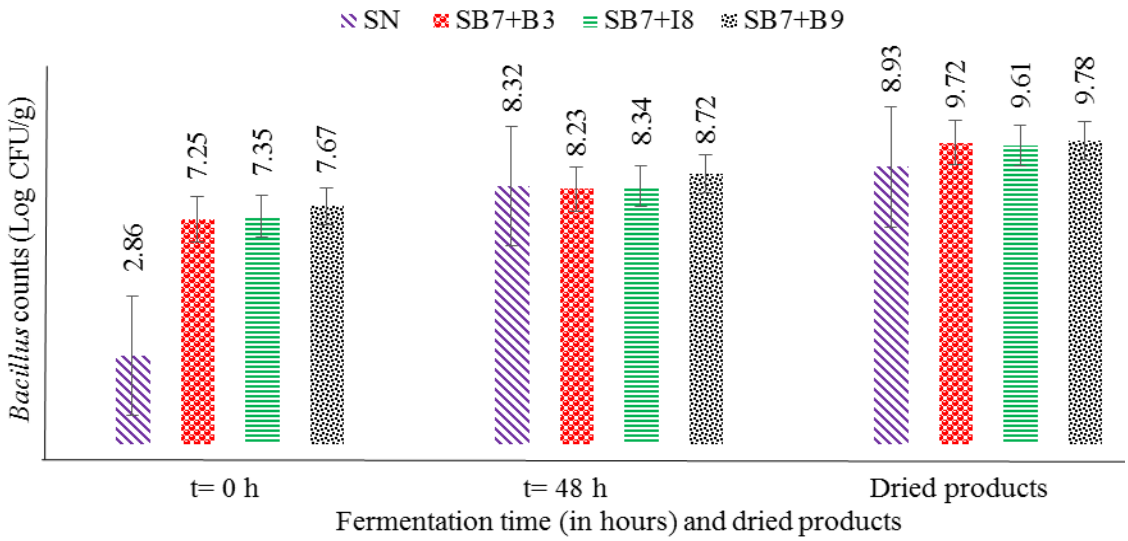

Figure 3. Growth of *Bacillus* starter during the production of *soumbala* using mixed ferments. SN: spontaneous *soumbala*; SB7+B3: *soumbala* produced with ferment B7 in association with ferment B3; SB7+I8: *soumbala* produced with ferment B7 in association with ferment I8; SB7+B9: *soumbala* produced with ferment B7 in association with ferment B9.

between 7.92 and 9.54 Log CFU/g. The increase of *Bacillus* count was also observed in final dried products between 8.38 and 10.11 Log CFU/g. The highest *Bacillus* counts were obtained with ferments of *B. subtilis* B9 (10.11 Log CFU/g) and *B. subtilis* B7 (9.84 Log CFU/g). Regarding the *soumbala* obtained by spontaneous fermentation (SN), its *Bacillus* load after drying was 8.93 Log CFU/g.

Figure 3 shows the growth capacity of the *Bacillus* ferments used in mixture during the controlled fermentation of *soumbala*. Used in mixture, the starter cultures' loads increased from 7 Log CFU/g at the onset of the fermentation to 8 Log CFU/g at the end of the fermentation, while for spontaneous fermentation, microbial population increased from 2 to 8 Log CFU/g. After drying, the *Bacillus* counts were between 9.61 and 9.78 Log CFU/g for *soumbala* produced with ferments and 8.93 Log CFU/g for spontaneous *soumbala*.

Proximate composition of *soumbala* produced with ferments of starter cultures

Results from Table 1 show the proximate composition of the different samples of *soumbala*. The pH of *soumbala* produced with ferments of starter cultures varied from 7.17 ± 0.01 to 7.25 ± 0.11 for single culture *soumbala* and from 7.31 ± 0.04 to 7.37 ± 0.02 for mixed cultures *soumbala*. However, *soumbala* from spontaneous fermentation had a pH of 7.21 ± 0.01. The lowest pH value (7.15 ± 0.01) was obtained with single starter culture *B. amyloliquefaciens* I8 (originating from *bikalga*) while the highest pH (7.37 ± 0.02) was recorded with the combination of *B. subtilis* B7 and B9 (originating from *soumbala*). There was no significant difference ($p > 0.05$) between the pH of the different *soumbala* produced using combined starter cultures. The moisture content of controlled fermented dried *soumbala* ranged between 5.67 ± 0.51 and 8.46 ± 0.67% whereas the spontaneous dried *soumbala* showed a moisture content of 6.19 ± 0.39%. Dried *soumbala* obtained with mixed starter culture SB7+I8 showed the highest moisture content (8.46 ± 0.67%). Single culture inoculated *soumbala* and mixed culture inoculated *soumbala* ash content ranged from 1.95 ± 0.16 to 2.11 ± 0.07%/DM and 1.77 ± 0.05 to 1.90 ± 0.04%/DM, respectively. Meanwhile, ash content of spontaneous *soumbala* was 2.11 ± 0.02%/DM. *Soumbala* obtained with a mixed culture of *B. subtilis* B7 and *B. amyloliquefaciens* I8 showed the lowest ash content (1.77 ± 0.05%/DM). Analyses showed that there was no significant difference ($p > 0.05$) between ash content of spontaneous *soumbala* and *soumbala* from starter B7 (SB7), I8 (SI8) and B9 (SB9). Protein content varied between 41 and 43%/DM with spontaneous *soumbala* giving the weakest rate (41.19 ± 0.89%/DM) compared to mixed culture *soumbala* (SB7+I8) which gave the highest rate (43.78 ± 0.13%/DM). There was significant difference ($p < 0.05$) between protein contents of all samples of *soumbala*. Regarding the crude fat content, it ranged from 37.46 ± 0.30%/DM (obtained with SB7+B3) to 40.67 ± 0.17%/DM (obtained with SB3) for *soumbala* produced with starter cultures and was

Table 1. Proximate composition of spontaneous *soumbala* and *soumbala* produced using ferments in single or mixed culture.

Type of formulation	Type of *soumbala*	Content					
		pH	Moisture (%)	Ashes (g/100 g DM)	Proteins (g/100 g DM)	Fat (g/100 g DM)	Carbohydrates (g/100 g DM)
Without ferment	SN	7.21 ± 0.01^{cd}	6.19 ± 0.39^{bc}	2.11 ± 0.02^{a}	41.19 ± 0.89^{d}	39.66 ± 0.05^{bc}	14.57 ± 0.04^{bcd}
Single culture	S_{B7}	7.17 ± 0.01^{cd}	5.67 ± 0.51^{c}	2.11 ± 0.07^{a}	42.51 ± 0.43^{bc}	39.13 ± 0.25^{c}	14.77 ± 0.26^{abc}
	S_{B3}	7.25 ± 0.11^{bc}	6.23 ± 0.74^{bc}	1.95 ± 0.16^{bc}	41.58 ± 0.22^{cd}	40.67 ± 0.17^{a}	15.11 ± 0.47^{ab}
	S_{I8}	7.15 ± 0.01^{d}	6.06 ± 0.20^{bc}	2.05 ± 0.10^{ab}	41.87 ± 0.12^{bcd}	40.28 ± 1.03^{ab}	14.22 ± 0.09^{cde}
	S_{B9}	7.21 ± 0.04^{cd}	6.30 ± 0.82^{bc}	2.09 ± 0.06^{ab}	42.75 ± 0.42^{b}	39.68 ± 0.22^{bc}	15.33 ± 0.57^{a}
Mixed culture	S_{B7+B3}	7.31 ± 0.04^{ab}	7.15 ± 0.90^{ab}	1.85 ± 0.01^{cd}	42.02 ± 0.23^{bcd}	37.46 ± 0.30^{d}	13.81 ± 0.18^{e}
	S_{B7+I8}	7.35 ± 0.01^{a}	8.46 ± 0.67^{a}	1.77 ± 0.05^{d}	43.78 ± 0.13^{a}	38.76 ± 1.03^{c}	14.13 ± 0.51^{de}
	S_{B7+B9}	7.37 ± 0.02^{a}	6.74 ± 1.47^{bc}	1.90 ± 0.04^{cd}	41.47 ± 1.18^{d}	38.86 ± 0.38^{c}	14.16 ± 0.03^{de}
P value		< 0.0001	0.0162	0.0003	0.0011	0.0001	0.0005
F value		9.67	3.59	8.20	6.36	9.63	7.31

SN: Spontaneous *soumbala*; S_{B7}: *soumbala* with ferment of *B. subtilis* B7; S_{B3}: *Soumbala* with ferment of *B. subtilis* B3; S_{I8}: *Soumbala* with ferment of *B. amyloliquefaciens* I8; S_{B9}: *Soumbala* with ferment of *B. subtilis* B9; S_{B7+B3}: *Soumbala* with association of ferment of *B. subtilis* B7 + *B. subtilis* B3; S_{B7+I8}: *Soumbala* with association of ferment of *B. subtilis* B7 + *B. amyloliquefaciens* I8; S_{B7+B9}: *Soumbala* with association of ferment of *B. subtilis* B7 + *B. subtilis* B9. For each column, the values with a common letter are not significantly different according to Fisher test at the 5% threshold.

39.66 ± 0.05%/DM for spontaneously fermented *soumbala* (SN). Fat content of SB7+B9, SB7+I8 and SB7 was not significantly different ($p > 0.05$). Likewise, there was no significant difference ($p > 0.05$) between fat content of SN and SB9. The *soumbala* that was fermented using the ferment B9 had the highest total carbohydrates value (15.33 ± 0.57 %/DM), while the combination of ferments B7 and B3 gave the lowest content (13.81 ± 0.18%/DM). SN had a content of total carbohydrates of 14.57 ± 0.04%/DM.

Amino acid profiles of *soumbala* produced with ferments of starter cultures

Amino acid profiles (in g/100 g DM) of *soumbala* produced with ferments of *Bacillus* starter as well as *soumbala* from spontaneous fermentation are presented in Table 2. The different *soumbala* presented variable content in essential amino acids. The highest contents in valine (1.038), leucine (1.138), isoleucine (0.772), phenylalanine (0.722), tyrosine (1.064) and proline (0.641) were obtained with *soumbala* fermented using the ferment produced with the starter B7. However, these amino acids were observed in low concentrations in *soumbala* produced with the combination of starter cultures B7 and B9. The *soumbala* SI8 presented the lowest content in histidine (0.102) while the highest content was observed for *soumbala* SB9 (0.208). Threonine, methionine and alanine were found in highest concentrations in *soumbala* SB7+I8 with respective values of 0.109, 0.077 and 0.506. Regarding lysine, the highest content was obtained with SB7 (0.791) while the lowest content was found in SI8 (0.520).

DISCUSSION

The increase of *Bacillus* loads during the production of ferments indicates that the starter cultures used in the study are able to use African locust bean cooked cotyledons as substrate for their growth. However, the fermentation capacity varied among the strains. The highest loads observed with the starter B7 and B9 are probably due to the fact that these strains were previously isolated from the fermentation of the same substrate and are therefore more able to use this substrate for their growth. Indeed, the autochthonous character of these starters gives them a better implantation during the fermentation process (Fessard, 2017). The low concentrations of *Bacillus* in the ferments prepared with starter cultures B3 and I8 may be explained by their non-autochthonous character. Therefore, African locust bean seeds cotyledons may not be a favorite substrate for their growth.

The *Bacillus* loads (9.63 - 9.70 log CFU/g) found in the ferments prepared with starter cultures B7 and B9 were close to those of Agbobatinkpo et al. (2012) who also

Table 2. Amino acid profiles of spontaneous *soumbala* and *soumbala* produced using ferments in single or mixed culture (g/100 g DM).

Amino acid	Different type of *soumbala*								P value	F value
	SB7	SB3	SI8	SB9	SB7+B3	SB7+I8	SB7+B9	SN		
Aspartic acid	0.128^{c}	0.351^{a}	0.140^{c}	0.214^{bc}	0.248^{ab}	0.211^{bc}	0.143^{bc}	0.141^{c}	0.0056	4.57
Glutamic acid	0.077^{cd}	0.089^{bc}	0.062^{d}	0.112^{a}	0.073^{cd}	0.076^{cd}	0.086^{c}	0.110^{ab}	0.0011	6.38
Serine	0.069^{ab}	0.054^{ab}	0.035^{b}	0.093^{a}	0.062^{ab}	0.095^{a}	0.052^{ab}	0.060^{ab}	0.1475	1.84
Glycine	0.276^{a}	0.260^{a}	0.165^{b}	0.227^{a}	0.231^{ab}	0.238^{ab}	0.177^{b}	0.204^{ab}	0.0673	2.43
Histidine	0.161^{bc}	0.192^{ab}	0.102^{d}	0.208^{a}	0.181^{abc}	0.176^{abc}	0.146^{c}	0.169^{abc}	0.0025	5.42
Arginine	0.075^{b}	0.075^{b}	0.071^{b}	0.075^{b}	0.096^{ab}	0.137^{a}	0.079^{b}	0.102^{ab}	0.1651	1.76
Threonine	0.083^{b}	0.057^{c}	0.049^{c}	0.053^{c}	0.074^{bc}	0.109^{a}	0.054^{c}	0.062^{bc}	0.0022	5.57
Alanine	0.399^{ab}	0.425^{a}	0.275^{b}	0.465^{a}	0.453^{a}	0.506^{a}	0.376^{ab}	0.482^{a}	0.0631	2.48
Proline	0.641^{a}	0.355^{cd}	0.484^{bcd}	0.560^{ab}	0.344^{d}	0.488^{bc}	0.428^{bcd}	0.528^{ab}	0.0058	4.55
Tyrosine	1.064^{a}	0.992^{ab}	0.786^{bc}	0.876^{ab}	0.815^{bc}	0.919^{ab}	0.655^{c}	0.794^{bc}	0.0252	3.21
Valine	1.038^{a}	0.682^{bcd}	0.603^{cd}	0.677^{bcd}	0.728^{bc}	0.877^{ab}	0.493^{d}	0.708^{bcd}	0.0042	4.87
Methionine	0.076^{a}	0.066^{ab}	0.065^{ab}	0.063^{ab}	0.069^{ab}	0.077^{a}	0.032^{b}	0.062^{ab}	0.3236	1.27
Cysteine	0.012^{c}	0.030^{ab}	0.015^{bc}	0.044^{ac}	0.020^{b}	0.04^{a}	0.040^{a}	0.036^{a}	0.0012	6.21
Isoleucine	0.772^{a}	0.392^{c}	0.374^{c}	0.370^{c}	0.439^{c}	0.566^{b}	0.330^{c}	0.436^{c}	<0.0001	12.03
Leucine	1.138^{a}	0.685^{cd}	0.617^{cd}	0.711^{cd}	0.794^{bc}	0.950^{ab}	0.594^{d}	0.790^{bc}	0.0003	8.09
Phenylalanine	0.722^{a}	0.595^{ab}	0.432^{c}	0.526^{bc}	0.556^{bc}	0.651^{ab}	0.427^{c}	0.555^{bc}	0.0052	4.66
Lysine	0.707^{ab}	0.791^{a}	0.520^{c}	0.751^{a}	0.670^{abc}	0.715^{ab}	0.565^{bc}	0.673^{abc}	0.0300	3.07

SN: Spontaneous *soumbala*; S_{B7}: *soumbala* with ferment of *B. subtilis* B7; S_{B3}: *soumbala* with ferment of *B. subtilis* B3; S_{I8}: *soumbala* with ferment of *B. amyloliquefaciens* I8; S_{B9}: *soumbala* with ferment of *B. subtilis* B9; S_{B7+B3}: *soumbala* with association of ferment of *B. subtilis* B7 + *B. subtilis* B3; S_{B7+I8} : *soumbala* with association of ferment of *B. subtilis* B7 + *B. amyloliquefaciens* I8; S_{B7+B9}: *soumbala* with association of ferment of *B. subtilis* B7 + *B. subtilis* B9. For each line, the values with a common letter are not significantly different according to Fisher test at the 5% threshold.

found *Bacillus* loads of 9.7 log CFU/g in *yanyanku* and *ikpiru*, two food additives (obtained by the fermentation of *H. sabdarifa* seeds) used in Benin for the fermentation of African locust bean seeds into *sonru* and *iru*. In addition, during the controlled fermentation of *Afitin* with *B. subtilis* starter cultures, the maximum load of *Bacillus* after fermentation was 9.5 log CFU/g (Ahonoukoun, 2014).

The increase in *Bacillus* load during the controlled fermentation of *soumbala* with the ferments demonstrates the fermentation capacity of these ferments. However, ferments produced with starter cultures B7 and B9 demonstrated the strongest fermentation capacity, with the highest loads when used in monoculture (9.84-10.11 Log CFU/g) and in mixed culture (9.79 Log CFU/g). Similar range of bacterial counts in *soumbala* or similar products have been reported previously (Sawadogo/ lingani et al., 2003; Parkouda et al., 2009; Amoa-Awua et al., 2014; Ajavi et al., 2015; Guissou et al., 2020).

In this study, the pH of *soumbala* produced with ferments of starter cultures was alkaline, like that of traditional spontaneous *soumbala*. This result is in agreement with those recorded for similar African fermented condiments by other authors (Azokpota et al., 2006; Akabanda et al., 2018; Mohammadou et al., 2018; Ibrahim et al., 2018). This alkaline pH is due to the proteolytic activity of the fermenting microorganisms, which degrade proteins and release ammonia in the medium (Mohammadou et al., 2018). The results reported here corroborate those of Agbobatinkpo et al. (2012) in Benin, during the study of the fermentation ability of *yanyanku* and *ikpiru,* who found an average pH ranging from between 7.1 and 7.3 for African locust bean cotyledons fermented with or without additives. However, Sawadogo et al. (2003) and Guissou et al. (2020) during spontaneous fermentation of *P. biglobosa* seeds to produce *soumbala* found lower pH values in the dried products. The low water content observed in the various *soumbala* would promote their conservation (Ajavi et al., 2015).

The content of ashes obtained for the different *soumbala* (1.77 - 2.11%) was lower than those found by Agbobatinkpo et al. (2012) which were 2.6 to 3.2%. This difference could be explained by the addition of ash solution during the preparation of the additives *yanyanku* and *ikpiru* used for the fermentation of *P. biglobosa* seed-based condiments in Benin, or by the difference in ash content of the seeds used in each country. The spontaneous *soumbala* as well as *soumbala* produced with ferments of starter cultures also presented lower concentrations of ashes compared to the results recently

presented by Guissou et al. (2020).

The protein levels obtained in this study were higher than those obtained for *sonru* and *iru* fermented with *yanyanku* and *ikpiru* additives, which average was 35% (Agbobatinkpo et al., 2012). The variation of the protein contents may be due to the proteolytic activity of the fermenting strains (Mohammadou et al., 2018) and also to the difference in the physicochemical composition of African locust bean seeds according to the localities. Results demonstrated that controlled fermented *soumbala* as well as spontaneous fermented *soumbala* were rich in proteins (> 40%). Therefore, *soumbala* could be a source of protein that could help poor population to meet their requirement for this nutrient, particularly in developing countries. High amount of protein was also noted for other alkaline fermented products and was related to *Bacillus* counts (Terlabie et al., 2006; Mohammadou et al., 2018).

The different *soumbala* prepared with the ferments of *Bacillus* spp. presented interesting fat contents (37 - 40%). The fat content of *soumbala* prepared using single ferment is comparable to that reported by Guissou et al. (2020), which was 40.47%. The carbohydrate contents found are also in agreement with that reported by Guissou et al. (2020).

Results showed that *soumbala* produced with ferments of *Bacillus* spp. contained more essential amino acids than the traditional spontaneous *soumbala*. *Soumbala* produced using the starter culture B7 had the highest levels of valine, leucine, isoleucine and phenylalanine. Similar results were previously obtained by Ouoba et al. (2003b) in *soumbala* produced by controlled fermentation using the same *B. subtilis* as starter culture. As reported by the same authors (Ouoba et al., 2003b), it was also found that *soumbala* produced with starter culture B9 contained high content of histidine compared to *soumbala* produced with starter culture B7.

The presence of high amounts of lysine is particularly interesting because lysine is a limiting amino acid in cereals and seeds that constitute the staple diet of the majority of African populations (Diawara et al., 2004). The *soumbala* produced with this starter culture could then be used to fortify foods. The presence of non-essential amino acids such as tyrosine, proline and glycine at significant content in certain samples is also of interest since these amino acids could be essential in some human physiological circumstances (Ouoba et al., 2003b). Variable concentrations of amino acids in African fermented condiments have been reported in other studies (Parkouda et al., 2015; Akabanda et al., 2018; Ibrahim et al., 2018).

Conclusion

In the present study, four *Bacillus* strains (*B. subtilis* B7, *B. subtilis* B9, *B. subtilis* B3 and *B. amyloliquefaciens* I8) previously isolated from spontaneous fermentation of three different condiments and selected as starter cultures were successfully developed on dehulled African locust bean seeds used as carrier material to produce ferments. These ferments have been used separately or in combination to control the fermentation of African locust bean seeds into *soumbala*, which present interesting microbiological and nutritional characteristics. The obtained results indicate that dehulled African locust bean seeds are a promising carrier material for the transfer of *Bacillus* starter cultures to *soumbala* production units. These results may help to standardize the *soumbala* production process as well as its quality. However, further investigations need to be performed to evaluate the performance of these ferments in real environment and assess their stability during storage.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Isolation and molecular characteristics of extended spectrum beta-lactamase-producing uropathogenic *Escherichia coli* isolated from hospital attendees in Ebonyi State, Abakaliki

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This present study was designed to determine the occurrence frequency of blaCTX-M and blaTEM genes in extended-spectrum beta-lactamase (ESBL)-producing uropathogenic *Escherichia coli*, and their antibiotic resistance patterns among hospital attendees in Abakaliki, Nigeria. Out of the 73 uropathogenic *E.coli* isolates analyzed, 52 were identified to be ESBL producers using double disk synergy technique. The frequency of beta-lactamse (bla) genes (CTX-M and TEM) among *E. coli* was molecularly determined by polymerase chain reaction (PCR). Out of 52 (71.2%) ESBL-producing uropathogenic *E. coli*, 17 (32.7%) were positive for blaTEM, 35 (67.3%) were positive for blaCTX-M while 8 (15.3%) harboured both blaTEM and blaCTX-M genes. CTX-M gene was the most prevalent gene. Isolates evaluated in our study were resistant to cefotaxime (83.6%), ceftazidime (79.5%), amoxycillin (72.6%), cefpodoxime (68.5%), aztreonam (61.6%), ceftriaxone (57.5%), and cefepime (37%). This study demonstrated the occurrence frequency of CTX-M and TEM genes in uropathogenic *E. coli* strains in Abakaliki. Even though molecular techniques are more reliable in the detection of ESBL production, routine clinical screening for ESBL-producing uropathogens using phenotypic method should be introduced and encouraged in clinical settings as they are less expensive. This will go a long way in checkmating drug resistance.

Key words: *Escherichia coli*, uropathogens, extended-spectrum beta-lactamase (ESBL), resistance genes, antibiotics.

INTRODUCTION

Microbial resistance by pathogenic *Escherichia coli* is a major worldwide concern. Antibacterial agents, especially beta-lactams are becoming less useful against Enterobacteriaceae (Dia et al., 2015). Urinary tract infections (UTIs), a common nosocomial and community-acquired bacterial infection, occur in all genders and age groups (Abdulaziz et al., 2018). Antibiotic resistance by *E. coli* to numerous antibiotics is now developing and evolving (Raju et al., 2019). *E. coli* implicated in UTIs are becoming multidrug-resistant as a result of their extended-spectrum beta-lactamase (ESBL)-producing ability. Beta-lactam resistance is mediated by ESBL genes that are mostly encoded by plasmid (Topaloglu et al., 2010). ESBLs are a branch of beta-lactamases that have the ability of hydrolyzing the β-lactam ring of penicillins, aztreonam, and cephalosporins. However, they often remain susceptible to cephamycins and carbapenems (imipenem and ertapenems) (Shehani and Sui, 2013). *E. coli* could acquire some resistance factors from environmental bacteria of surroundings; this could transmit its resistance genes to other bacterial pathogens in arrays of habitats. Diagnosing of UTIs accurately and proper usage of antimicrobials for treatment and prevention are paramount in reducing drug resistance (Roshan et al., 2020). CTX-M, TEM, SHV, and AmpC beta-lactamase genes have been identified in *E. coli* isolates from UTI patients over the years (Koshesh et al., 2017). TEM (Temoneira), SHV (sulfhydryl variable), and CTX-M (cefotaximase) belong to class A ESBLs. Shahid et al. (2011) and Iroha et al. (2010) observed different frequencies of ESBLs among Gram-negative bacteria to range between 6 and 88% in different health institution settings. Sima et al. (2016) also reported CTX-M (74%), SHV (45%), and TEM (67%) genes in *E. coli* isolates. CTX-M have been increasingly recorded in various clinical specimens and *E. coli* remains the major organism implicated (Mohammed et al., 2011). This study was therefore designed to molecularly characterize ESBL-producing uropathogenic *Escherichia coli* from hospital attendees in Ebonyi State, Nigeria.

METHODOLOGY

Collection of samples

Seventy three (73) *E. coli* were obtained from mid-stream urine samples of 133 patients attending Federal Teaching Hospital, Abakaliki metropolis, Nigeria between February, 2018 and November, 2018. Sterile universal container was used to collect urine samples from patients suspected of UTIs. Every patient was properly instructed on self-collection of urine samples. The samples were immediately transported to the laboratory of Applied Microbiology Department, Faculty of Sciences, Ebonyi State University, Abakaliki for bacteriological analysis.

Culturing of samples, isolation, and biochemical characterization of bacterial isolates

Mid-stream urine samples were streaked on MacConkey agar aseptically and then incubated for 24 h at exactly 37°C. Plates were then observed for *E. coli* growth (red or pink colonies) on MacConkey agar. These suspected bacterial isolates were thereafter characterized by standard microbiology techniques such as motility test, Gram-stain, and other biochemical tests such as methyl red, indole, urease test, Voges-Proskauer, and citrate (Cheesbrough, 2010, Moses et al., 2018). Pure colonies of isolates were then inoculated on nutrient agar slants, incubated for 24 h at 37°C and kept in a refrigerator for future use at 4°C (Moses et al., 2018).

Ethical clearance

Ethical approval was given by the Federal Teaching Hospital, Abakaliki (FETHA) research and ethical committee

Antimicrobial susceptibility test

Isolates were tested to evaluate their antimicrobial sensitivity patterns by the Kirby-Bauer disk diffusion method. Test organisms were adjusted to McFarland equivalent standards and inoculated using sterile swab stick on Mueller-Hinton (MH) agar plates. Cefpodoxime (10 µg), amoxicillin (20 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefepime (30 µg), cefotaxime (30 µg), and aztreonam (30 µg) antibiotic discs were carefully placed on the MH agar using sterile forceps. Antibiotics were allowed to properly diffuse for 10 min and plates were then incubated for 18 h at 37°C. The inhibition zone diameters (IZDs) were measured and results were interpreted as resistant or susceptible as per the CLSI guidelines after incubation (CLSI, 2018; Moses et al., 2020). The confirmed uropathogenic *E. coli* were stored in agar slant at -70°C and were subjected to further ESBL phenotypic and molecular identification.

Phenotypic test for ESBL detection

The turbidity of suspected potential ESBL-producers were properly adjusted to 0.5 McFarland standards. Sterile swab sticks were then used to make lawn culture of the standardized isolates on the surface of MH agar plates (CLSI, 2018). Cefotaxime and ceftazidime antibiotic discs were placed at a distance of 15 mm centre-to-centre from the central disc containing amoxicillin/clavulanic acid (20 µg/10 µg). Plate was incubated overnight at 37°C. An increase of ≥5 mm in the IZD for either of the cephalosporins (cefotaxime and ceftazidime) tested in combination with amoxicillin/clavulanic acid versus its zone when tested alone confirms ESBL production (Sima et al., 2016). *E. coli* ATCC 25922 was used as quality control.

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Table 1. PCR primers of 16S rRNA and ESBL gene.

Primer name	Targeted gene	Primer sequence (5'-3')[1]	Amplicon product size (bp)
27 805	16S rRNA	F-AGT TTG ATC MTG GCT CAG R-GGA CTA CHA GGG TAT CTA AT	797, Gudjónsdóttir (2015)
TEM	blaTEM	F-ATGAGTATTCAACATTTCCGTGT R-TTACCAATGCTTAATCAGTGAGG	861, Azam et al. (2016)
CTX-M	blaCTX-M	F-SCS ATG TGC AGY ACC AGT AA R-ACC AGA AYV AGC GGB GC	585, Gudjónsdóttir (2015)

DNA extraction and polymerase chain reaction (PCR) detection of blaCTX-M and blaTEM genes

Genomic DNA was extracted from pure colonies of an overnight growth of *E. coli* on Luria-Bertani agar using QIAamp DNA isolation columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purity and concentration of extracted DNA was determined using NanoDrop-Spectrophotometer at an absorbance of A260/280. Quality control of extracted DNA was done by testing all extracted genomic DNA for 16S rRNA gene. Table 1 shows the oligonucleotide primer sequences and the targeted genes (blaCTX-M and blaTEM) sizes. The PCR reactions for detection of blaCTX-M and blaTEM genes were done in a total reaction volume of 25 µl which contains 12.5 µl of Master Mix Red, 10 µl of Sigma water, 0.25 µl of forward primer, 0.25 µl of reverse primer, and 2 µl of the isolated genomic DNA. PCR was done with a C1000 Touch™ Thermo Cycler (Bio-Red) (Azam et al., 2016; Gudjónsdóttir, 2015).

Gel Electrophoresis

Exactly 1.0 g of agarose and 100 ml of 1X Tris-acetate-ethylenediaminetetraacetate (TAE; pH 8.0) buffer (Bio-Rad) was used to prepare 1% (w/v) agarose gel. The mixture was then heated up for about 3 min in a microwave for total dissolution of agarose. It was then cooled to about 50°C and ethidium bromide (1 µl/ml) was added to stain the prepared agarose gel. The molten agarose gel was then cast into a gel casting tray containing combs and allowed to solidify. After about 30 min of agarose gel solidification, gel combs were carefully removed and gel casting tray containing the gel was placed into a gel electrophoresis chamber filled with TAE buffer (40 mM Tris, 20 mM acetic acid, and 100 mM EDTA pH 8.0). For each run, 5 µl of Extend Quick-Load DNA Ladder (1 kb; New England, Bio Labs) was added to one of the wells to estimate the band sizes and 5 µl of negative control, comprising Sigma water, was added to another well. Exactly 5 µl of each PCR product was carefully and properly loaded in the remaining wells. Electrophoresis was run at 80 V and 400 mA (mini Ampere) for exactly 1 h. Gels were then visualized and photographed by a gel documentation system (Bio-Rad) (Gudjónsdóttir, 2015).

RESULTS AND DISCUSSION

This work provided insights into the antibiotic resistance profiles and molecular characteristics of uropathogenic *E. coli* with the ability to produce ESBLs. Out of the 130 samples analyzed between February and November, 2018, 73 uropathogenic *E. coli* isolates were obtained and fifty 52 isolates were ESBL producers as investigated phenotypically using double disk diffusion techniques (Tables 2 and 3). The isolates that demonstrated resistance or reduced susceptibility to ceftazidime, cefotaxime, and cefpodoxime were subjected to ESBL phenotypic detection. The keyhole pattern exhibited by uropathogenic *E. coli* isolates expressing ESBL production is as shown in Plate 1. This property is an important characteristic of ESBL-producing bacteria as a result of the synergistic effect between amoxicillin-clavulanic acid (a beta-lactamase inhibitor) and third generation cephalosporins (ceftazidime and cefotaxime). Three different sets of primers were used to amplify 16S rRNA, blaCTX-M, and blaTEM genes (Table 1). Agarose gel showed PCR product of the amplified 16S rRNA gene among the isolates to be 797 bp (Figure 1). Out of the 52 ESBL positive uropathogenic *E. coli* isolates, 17 (32.7%) harboured blaTEM gene, 35 (67.3%) harboured blaCTX-M gene, while 8 (15.3%) harboured both blaTEM and blaCTX-M genes (Table 4). PCR product band sizes of blaCTX-M and blaTEM genes were estimated to be 861 and 585 bp, respectively (Figure 2). Uropathogenic *E. coli* evaluated in this study exhibited varying frequencies of resistance to antibiotics tested. Isolates exhibited resistance to cefotaxime (83.6%), ceftazidime (79.5%), amoxycillin (72.6%), cefpodoxime (68.5%), aztreonam (61.6%), ceftriaxone (57.5%), and cefepime (37%) (Table 2). Indiscriminate use and abuse of beta-lactam antibiotics by individuals have caused problems in the treatment of microbial infections and diseases caused by these antibiotic-resistant organisms as a result of ESBL production. Some ESBL-producing bacteria failed to be detected using disk diffusion technique, thus resulting in serious treatment failures among infected patients/ individuals (Umadevi et al., 2011). The lack of routine screening and detection of bacteria among clinical isolates in hospitals with the ability to produce ESBL was evident in this present study. These types of discrepancies between susceptibility data and disc diffusion call for improved ESBL detection and incorporation into routine susceptibility techniques in

Table 2. Susceptibility patterns of uropathogenic *E. coli*.

Antibiotics (concentrations in µg)	*Escherichia coli*, No. (%)	
	S	R
Cefepime (30)	46 (63.0)	27 (37.0)
Ceftazidime (30)	15 (20.5)	58 (79.5)
Ceftriaxone (30)	31 (42.5)	42 (57.5)
Cefpodoxime (10)	23 (31.5)	50 (68.5)
Aztreonam (30)	28 (38.4)	45 (61.6)
Cefotaxime (30)	12 (16.4)	61 (83.6)
Amoxycillin (20)	20 (27.4)	53 (72.6)

S-Susceptible; R-Resistant.

Table 3. Prevalence of ESBL-producing uropathogenic *Escherichia coli*.

Sample source	Sample size	Uropathogenic *E. coli*, No. (%)	ESBL-producing *E. coli*, No. (%)
Urine	130	73 (56.2)	52 (71.2)

Plate 1. Picture of Double Disc Synergy test for ESBL-producing Uropathogenic *Escherichia coli*.

hospitals. In the study done by other researchers, it was found that *E. coli* is usually implicated in urinary tract infections (UTIs) and frequently identified as ESBL-producers (Abhilash et al., 2010; Shanthi and Sekar, 2010; Umadevi et al., 2011). Phenotypic screening tests for ESBL detection only confirm whether ESBL is

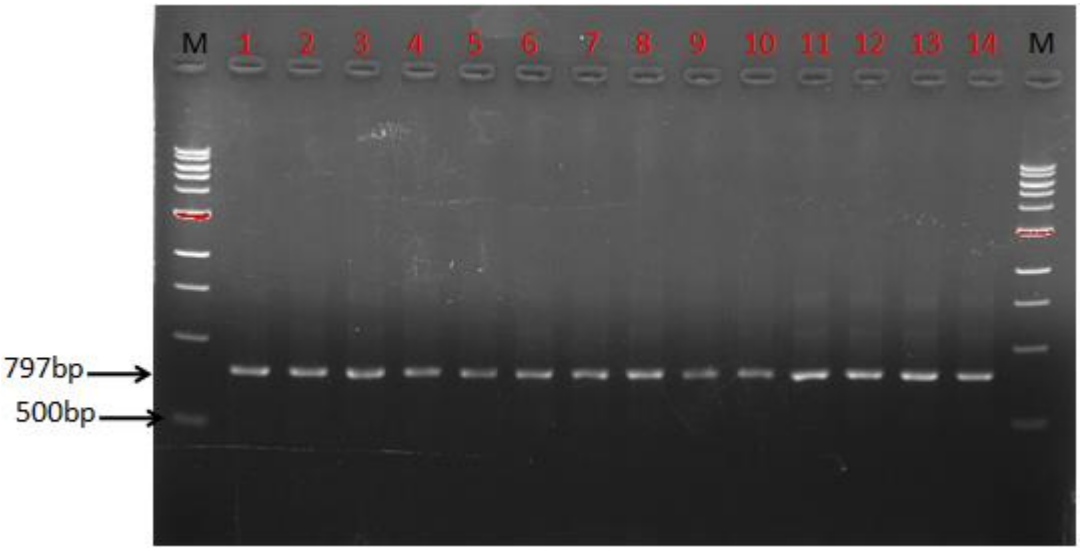

Figure 1. Gel picture of PCR amplification of 16S rRNA gene of bacterial isolates. Lane M = 1 kb Marker, Lane 1 - 14 = Uropathogenic *E. coli.*

Table 4. Distribution of ESBL genes among uropathogenic *Escherichia coli*.

Beta-lactamase (bla) genes	No. of positive isolates [n (%)]
TEM	17 (32.7)
TEM and CTX-M	8 (15.3)
CTX-M	35 (67.3)

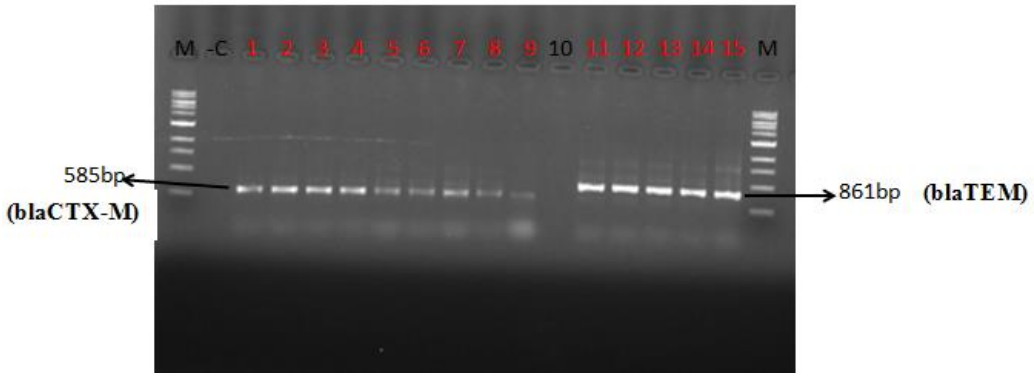

Figure 2. Gel picture of PCR products of amplified blaCTX-M and blaTEM genes among the bacteria isolates. Lane M= 1 kb Marker, Lane 1- 9 = blaCTX-M, Lane 11-15 = blaTEM, and -C= Negative control.

produced by the isolate but does not detect the presence of ESBL subtype. Currently, many researchers in the world have stated that although molecular methods appear more sensitive, but require specialized equipment and expertise, time consuming, and expensive (Sima et al., 2016; Varun and Parijath, 2014). A definitive identification is achievable only through molecular techniques. However, molecular methods such as PCR are not readily available in the hospital setting and can only be seen in research facilities. A study by Dia et al. (2015) identified 32 *E. coli* isolates to be ESBLs producers and blaCTX-M gene was reported to be the most frequent ESBL gene with 90.63% frequency. Similarly, Ugbo et al. (2020) and Sharma et al. (2010)

reported 55 and 56% blaTEM frequency, respectively among *E. coli* of clinical origin; and this is in agreement with this present study where we reported 32.7 and 67.3% frequencies for TEM and CTX-M genes, respectively. Roshan et al. (2020) reported 40.3% multidrug-resistant ESBL-producing isolates of *E. coli*; of which blaTEM (83.8%) and blaCTX (66.1%) were the common ESBL genotypes detected. In a study by Abdulaziz et al. (2018), blaCTX-M gene (93.9%) was the most prevalent ESBL genotype. Multiple ESBL gene carriage was also identified to be 45.5% among the uropathogenic *E. coli* (Abdulaziz et al., 2018). These observations by Roshan et al. (2020) and Abdulaziz et al. (2018) are in agreement with our present study, where blaCTX-M genotype was the most prevalent. A study done by Zongo et al. (2015) in Burkina Faso showed that high frequency (75.5%) of ESBL-producers was observed among *E*. *coli* isolates. They also reported that among the isolates with ESBL-producing ability, CTX-M (65, 49%) was the most prevalent, followed by TEM (25, 73%), and SHV (18, 71%).

Conclusion

This study has shown the presence of uropathogenic *E*. *coli* with ESBL-producing ability in the urine samples of hospital attendees in Abakaliki, Nigeria. Our study has also demonstrated the presence of ESBL genes, CTX-M and TEM, in the identified ESBL-producing uropathogenic *E*. *coli* isolates. Interestingly, blaCTX-M gene was the most predominant ESBL gene among the isolates in our study area. This research work showed that genotypic methods via PCR technique is more reliable for ESBL detection among bacterial isolates as PCR technique detected the presence of blaCTX-M and blaTEM in uropathogenic *E. coli* isolates. Thus, routine clinical detection of ESBL using phenotypic method should be introduced in the clinical setting since molecular methods are expensive to checkmate drug resistance due to ESBL production by bacteria. It is imperative that future studies should incorporate sequencing of isolates and resistance genes amplicons, together with bioinformatics so as to decipher the clonal relatedness/diversity and epidemiological identities of bacterial pathogens. This will greatly help in tracking disease occurrence, origins and sources of bacterial pathogens, and curtailing the spread of multidrug-resistant bacterial pathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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