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ARTICLES

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Review

Enzootic bovine leukosis and the risk to human health

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The bovine leukemia virus (BLV) was first reported as enzootic bovine lymphosarcoma in Eastern Europe in the late 19th century, highlighted by the presence of slightly yellow nodules in the enlarged spleen of cattle. It was believed to be an infectious disease because it spreaded through the herds. With changes observed in sensitivity of diagnostic techniques, the opinion that "BLV does not infect humans" starts to change after more than two decades. Several researches tried to link the BLV and human health in some studies in which BLV and human breast cancer have been shown. Studies about the possible routes of infection and to explain the genetic transformation processes in humans are raised. Multiple reports on this disease that link it to human health concluded on the need for a new mind set to understand relation between BLV and human health so as to improve the prevention, control and eradication of cattle herds.

Key words: Zoonotic disease, bovine leukemia virus, polymerase chain reaction (PCR), cancer.

INTRODUCTION

For years, the disease known as enzootic bovine leukemia (EBL) has been defined as a sickness limited to cattle, particularly dairy cattle. Researchers around the world have made significant efforts to demonstrate, without a doubt, that this disease is not related to any pathology in humans (Gilden et al., 1975; Donham et al., 1977; Burridge, 1981). With the emergence of new diagnostic technologies, the detection of a large diversity of pathogenic agents has improved, including the said disease. With the currently obtained results (Buehring et al., 2003; Buehring et al., 2007; Nikbakht et al., 2010; Mesa et al., 2013; Buehring et al., 2014; Buehring et al., 2015; Villalobos, 2016), doubts have once more emerged about whether this virus is capable of affecting human health. The objective of this review is to provide relevant

information on this disease from its discovery in 1871 to 2016, including the history, classification and epidemiology, mechanisms of action of the virus to cause harm, cancer and its association with the virus, entry routes and mechanisms of cellular transformation, all of which cast new light on the implication of bovine leukemia virus (BLV) in human health. The definitive response on this subject still lacks a categorical response.

HISTORY

Bovine leukemia virus was first reported as enzootic bovine lymphosarcoma in Eastern Europe in the late 19th

century, highlighted by the presence of slightly yellow modules in a splenomegaly observed in cattle; it was believed to be an infectious disease because it spread through herds (Leisering, 1871; Buehring et al., 2014). In 1917, its transmission through an infectious agent was demonstrated (Schwartz and Levy, 1994). Subsequently, Miller et al. (1969) used electron microscopy to demonstrate the presence of the viral particle within a lymphocyte in cows with lymphosarcoma; finally, using Koch's postulates, the causal association between the virus and enzootic bovine lymphosarcoma was successfully demonstrated (Olson et al., 1973; Olson, 1974). The first studies that showed evidence on the possible transmission of BLV in human cells and other species were conducted by Graves and Ferrer (1976), who obtained samples from cows infected with BLV in a state of persistent lymphocytosis that were cultivated in boundary cap (BC) cells; these samples proceeded to infect cell cultures of humans, simians (chimpanzees and rhesus), canines, sheep, goats and bats. In all of the cell cultures of the mentioned species, production of the complete virus was observed. The authors concluded that BLV can infect cells of the rhesus monkey, chimpanzees and humans, and it merits special attention in terms of its potential as a biohazard towards human beings.

Subsequently, Slavikova et al. (1987) isolated 60 clones of human myeloma B cells that were infected with BLV. Amongst the 60 clones evaluated, two harboured proviral sequences and the presence of virus proteins, confirming their expression ability in human cells *in vitro*. Altaner et al. (1989) obtained similar results using clones of foetal sheep cells (FLK) infected with virus to which human neuronal cells were exposed. The data showed that the neuronal cells could be infected by direct contact and the virus is capable of replicating itself; therefore, *in vitro*, these cells are susceptible and permissive to BLV. During the 1990s, Ursin et al. (1990) showed interest in the possibility that there is a risk to human health from BLV. These authors presented a prospective study in which a possible association between the consumption of cow's milk and cancer was investigated. The study followed 15,914 individuals for a period of eleven years, of whom 1,422 were diagnosed with cancer. The types of cancer evaluated were cancer of the lip, mouth, throat, oesophagus, stomach, colon, rectum, pancreas, larynx, respiratory tract, breast, cervical-uterine, ovarian, prostate, kidney, bladder, melanoma, skin cancer, thyroid, lymph organs, multiple myeloma and leukaemia. Risk factors considered were active tobacco consumption and ex-smokers and alcohol, meat, egg and coffee consumption. Although the investigation did not identify an association between milk consumption and total incidence of cancer, there was a strong association between milk consumption (two cups daily) and cancer in lymph organs, particularly lymphosarcoma, the same condition that is present in cattle. The study also found a slight positive association with kidney cancer and female reproductive organs, except for cervical-uterine cancer. In 1990, an increase in cancer was observed by Davis et al. (1990) in the agricultural population of several countries (Germany, Italy, United States, Japan, England and Wales), particularly in the lymphatic and haematopoietic system compared to the general population; thus, special attention was demanded on this topic (Blair et al., 1992). However, in other studies, such as those by Fritschi et al. (2003) in meat workers or reports by Sellers et al. (2008) on the risk of consuming unpasteurized milk, there have not been consistent results on the risks of contracting any type of cancer in particular.

CLASSIFICATION, HOSTS AND REPLICATION STRATEGY OF THE VIRUS

BLV (Retroviridae family; Orthoretrovirinae subfamily, *Deltaretrovirus* genus) is an exogenous retrovirus, responsible for EBL, which is the most common neoplastic disease in cattle worldwide (Schwartz and Levy, 1994; Dequiedt et al., 1999; Beyer et al., 2002; Moratorio et al., 2013). BLV is related to *human T-lymphotropic virus* types 1, 2 and 3 (HTLV 1, 2 and 3) and *primate T-lymphotropic virus* types PTLV 1, 2 and 3 (Gelmann et al., 1983; Tanaka et al., 1990; Heenemann et al., 2012). Infection is transmitted horizontally, through the transfer of infected cells by direct contact, ingestion of colostrum, milk and possibly through bloodsucking insects (Ferrer et al., 1978; Gillet et al., 2007). Vertical transmission (cow to calf) has also been demonstrated transplacentally (Ferrer, 1979; Van der Maaten et al., 1981; Romero et al., 1983; Lassauzet et al., 1991; Hübner et al., 1997). Although the virus has been demonstrated to have cattle as its main host, infection also occurs in buffalo and capybaras (Schwartz and Levy, 1994). Sheep and goats have been experimentally infected and have been routinely used in the investigation of BLV, with the particularity that goats develop cancer faster than cattle (Schwartz and Levy, 1994; Gillet et al., 2007; Merimi et al., 2007); therefore, it is proposed that the mechanisms of leukemogenesis in cattle and goats are likely different (Graves and Ferrer, 1976; Djilali and Parodi, 1989).

The propagation of BLV in the host occurs through two distinct processes. In the first process, the infectious cycle results from the virion coupling with the target lymphocyte, entry of the single-stranded viral RNA, reverse transcription and integration as a provirus into the host genome (also known as the infectious cycle). The second replication strategy depends on the management of cellular proliferation using viral regulatory proteins such as *Tax*. The two routes of viral replication produce a group of infected cell populations composed of distinct clones (Gillet et al., 2013). In the same study, Gillet et al. (2013) also demonstrated that BLV is initially directed to

transcribed regions of the genome for its integration; afterwards, a massive selection of clones is produced during primary infection, disfavouring proviruses located near genes; however, the abundance of long-term clones benefits transcriptional activity of the genomic region surrounding the provirus.

EPIDEMIOLOGY AND DIAGNOSIS OF THE EBL

The Guaymí and Guabalá are breeds reported in 2010 (Villalobos et al., 2010) and have been the subject of various studies in order to preserve them and use them for the production of milk and meat (Delgado et al., 2012; Martinez et al., 2012). With the emergence of an outbreak of EBL in native cattle in Panama in 2011, new diagnostic protocols were developed in order to replace the agar gel immunodiffusion (AGID) by better methods such as blocking enzyme-linked immunosorbent assay (ELISA) and nested polymerase chain reaction (PCR), and the development of genetic studies of disease resistance (Villalobos and Gonzalez, 2015). With the recent reports of the virus in human beings, a new line of research was created by the use of gene markers env, gag and *Tax* in human lymphocytes (Villalobos, 2016).

THE ZOONOTIC POTENTIAL OF BLV

In a study conducted by Buehring et al. (2001) under the premise that BLV is present in many of the meat products and milk on the market and because the incidence of breast cancer is higher in countries with a high consumption of food products from bovine species, the authors found that many humans possess antibodies against BLV, particularly the envelope glycoprotein (gp51) and capsule protein (p24), suggesting the possibility of infection with the virus. These same authors, using immunohistochemistry and PCR *in situ* in patients diagnosed with cancer and subject to surgical excision, showed that most mammary tissues studied presented evidence of a proviral genome of BLV, and four of the 27 samples were positive for the virus capsule protein.

With changes observed in low sensitivity techniques, such as complement fixation or agar gel immunodiffusion (AGID) in the 1970s and 1980s (Gilden et al., 1975; Donham et al., 1977; Burridge, 1981), compared to more modern techniques such as the enzyme linked immunosorbent assay (ELISA) and immunoblotting, the opinion that had prevailed for more than two decades that "BLV is not transmissible to humans and no disease in humans has been attributed to BLV" could be changing. In a study performed at the University of Berkeley, California, by Buehring et al. (2003) based on serum samples of 257 people, four isotypes of antibodies were used (IgG$_1$, IgM, IgA and IgG$_4$) to detect the capsule (p24) antigen of BLV. At least one reactive isotype to the protein was detected in 74% of the evaluated population.

Although the investigation did not conclude there was infection to humans, it again opens the debate on the possibilities of additional studies with techniques such as real time PCR because of the possibility that the reaction was in response to denatured antigens from heat action in ingested foods. Conversely, Lee et al. (2005) investigated the possible relationship between leukaemia, lung cancer and meat consumption in Korea, using new primer sets of the envelope gene; the results were negative, indicating that virus infection did not occur in any of the cases.

To provide evidence of possible zoonotic behaviour of BLV, Ochoa-Cruz et al. (2006) selected 56 cases diagnosed with ductal carcinoma, of which the immunoperoxidase assay was applied for the purpose of detecting glycoprotein gp51 of BLV in the cytoplasm of tumour cells. The technique showed that 7% of samples were positive for gp51, demonstrating the presence of BLV in humans with the ability to produce viral protein; the appearance of this molecule implies that the active provirus inserted in the genome is capable of producing structural viral proteins to assemble progeny. Subsequently, Buehring et al. (2007) used *in situ* PCR with primers of the *Tax* region of BLV to evaluate 213 samples of mammary gland tissue sections fixed with formalin distributed in 110 women with breast cancer and 103 controls (women without a history of breast cancer). The investigation showed positive reactions to BLV in 59% of the cases of women with breast cancer and in 29% of the control cases. Amongst the samples from women with breast cancer, 69% showed proviral BLV DNA in the accompanying non-malignant mammary epithelium, suggesting that the development of cancer may be an exceptional case, delayed within a population of cells infected with BLV in mammary tissue (field effect). These data provide a first promising step in the establishment of a causal role of BLV in human breast cancer.

Another investigation in which serological (ELISA) and genomic (PCR) techniques were used was conducted by Nikbakht et al. (2010) in the School of Veterinary Medicine of the University of Tehran; this study analysed 454 samples of human patients without any clinical symptoms in particular. Based on the serological test, 57 patients (12.5%) were diagnosed as positive. For the PCR test, 77 patients were evaluated (57 positive and 20 negative to the ELISA test). It was possible to isolate provirus in 12.3% of the 57 positive ELISA samples. Although the examination revealed the presence of provirus, the authors remained cautious in the conclusions because the provirus may not necessarily be integrated into the genome but rather be a non-integrated element (episome); in the latter case, people may not be actively infected. A study similar to that conducted by Buehring et al. (2007) was developed by Mesa et al. (2013) in Colombia with 106 mammary tissue samples from female patients (53 patients were positive for breast

cancer and 53 were negative). A PCR analysis was performed on these patients to detect the gag segment of BLV. Of the analysed samples, 35% of the patients positive for breast cancer were positive for gag amplification and 45% of the patients were negative. Given the discovery of antibodies against BLV in humans, Buerhing et al. (2014) used human mammary tissue for BLV infection tests using liquid phase PCR (L-PCR), sequencing, in situ PCR and immunohistochemistry (IHC). The studies focused on mammary tissue because in the original host, bovine cattle, BLV DNA and p24 protein are found in greater abundance than in lymphocytes. The findings of this investigation conclude that there is evidence showing that the BLV DNA and protein found present a high likelihood of constituting the *in vivo* presence of BLV in humans. Subsequently, Buehring et al. (2015) conducted a study on the cause-effect relationship between BLV and breast cancer in 239 patients with positive and negative records, using anatomopathological studies of mammary tissue. The presence of the virus was observed in 59% of malignant tissues, 38% of tissues with premalignant changes and 29% of tissues from normal controls. The study concluded that there is a highly significant cause-effect relationship. However, the authors also noted that one control study is not conclusive on its own, and validation from other investigators is required. In a randomized study conducted in a region in Panama by Villalobos (2016), 20 patients were sampled anonymously in order to detect the gag gene of enzootic bovine leukosis virus and 75% of them were positive. A larger project is currently being conducted with the aim of increasing the number of human patients and the virus markers like *Tax*, env and pol. Furthermore, a prospective study is required that demonstrates that viral infection precedes the development of cancer to support the idea of causality of the virus towards breast cancer. In light of the possible public health consequences of BLV in humans, future research should address how humans are infected by BLV, the frequency with which BLV infection is produced in different populations and whether the virus is associated with disease in humans.

ROUTES OF BLV ENTRY INTO THE HUMANS

As the presence of the virus within humans has been successfully demonstrated, whether by indirect methods such as ELISA and immunoblotting or direct methods such as PCR, several entry routes have now been proposed, such as direct contact with animals or animal products. The consumption of unpasteurized milk, artisanal cheeses and improperly cooked meat could be entry vehicles of the virus in populations likely to be exposed in rural areas such as Panama, Peru, Mexico and the United States, where a very common practice is ingesting milk from cows. For example, a study

conducted in the United States by Oliver et al. (2009) shows that between 35 and 60% of families and employees at farms ingest unpasteurized milk, and cattle herds infected with BLV are found throughout the world. In the United States, close to 38% of cattle herds, 84% of dairy herds and 100% of herds of large-scale dairy operations are infected (USDA, 1999, 2008). The detection of antibodies due to the consumption of foods derived from the bovine species had previously been reported by Barnes et al. (1988), particularly the reactivity from isotypes IgG2 and IgG4 towards antigens of bovine milk. Buehring et al. (2003) reported using the isotype IgG4 in the reactivity to BLV. Notably, viral particles from BLV denatured by pasteurization or heat can cause reactivity from the human immune system; however, it is not possible to differentiate them from un-denatured viral particles (Buehring et al., 2003). Another possible entry route could be injection of biological products contaminated by BLV, for example, with the development of anti-hemoparasite vaccines (Callow et al., 1997) and the incidence of many vaccines contaminated with BLV, as reported by Rogers et al. (1988), which forced the implementation of more rigorous diagnostic techniques against BLV in Australia. However, there are no reports of BLV contamination or production of the virus in *in vitro* cell lines for vaccines (Buehring et al., 2003). Furthermore, epithelial cells are identified as the entry route of the virus, through a genome integration process and the recognition of similar receptors to those of cattle (BVLRcp1) or through the interference of other unidentified molecules such as IgM, CD5+ and CD11b integrins, similar to experimental infection in studies on goats (Mesa et al., 2013). Importantly, once a zoonotic virus enters the human population, the majority are capable of dispersing amongst the population, a process that constitutes the most severe threat for human health (Christou et al., 2011). On this line of thought, BLV is known for crossing towards other species easily; the virus naturally infects capybara, Zebu cattle water buffalo, and it has experimentally infected sheep, goats, pigs, rabbits, rats and chickens (Schwartz and Levy, 1994). Moreover, human cells (fibroblasts) are susceptible to infection with BLV *in vitro* (Diglio and Ferrer, 1976).

MECHANISM OF LEUKEMOGENESIS BY BLV

Leukemogenesis mechanisms (induction of leukemia) through animal retroviruses that belong to the *Alpharetrovirus* and *Gammaretrovirus* genera induce tumour production by two mechanisms: activation of a viral oncogene or insertion of a gene from the cell, such as a proto-oncogene (Weiss et al., 1985). However, deltaretroviruses such as BLV lack a known oncogene (Sagata et al., 1984). Most of the studies on leukemogenesis induced by BLV have focused on the Tax protein because it is considered a potent

transcriptional activator of viral gene expression. In addition to its function as a transcriptional activator, the Tax protein induces the immortalization of fibroblasts of the rat embryo (Willems et al., 1990, 1998). This ability to induce immortalization may be the first step in the transformation process mediated by BLV. However, once the cattle are infected and during the latent period, the expression of BLV is blocked at the transcriptional level (Kettmann et al., 1982; Lagarias and Radke, 1989). Such repression appears to be very important for the escape of BLV from the immune surveillance system of the host, and subsequently only a small proportion of infected animals would rapidly develop the terminal stage of the disease (Gillet et al., 2007). In fact, transcription of the BLV genome in fresh tumour cells or in peripheral mononuclear cells (PBMCs) in fresh blood of infected individuals is almost undetectable by conventional techniques (Kettmann et al., 1982; Tajima et al., 2003; Tajima and Aida, 2005).

LEUKEMOGENESIS AND *PX* REGION

All retroviruses possess the genes gag, pro, pol and env, which encode the internal structural proteins, viral protease, reverse transcriptase and envelope glycoproteins of the virion, respectively, and are essential for the production of infectious viral particles. The genes are flanked by two identical long terminal repeats, LTRs (Alfaro et al., 2012). Although the genome sequences of BLV and HTLV-1 differ, they have a sequence in common called pX that is located between the env gene and the 3'LTR region that encodes a regulatory gene. In both viruses, the regulatory proteins Tax and Rex are encoded in the pX region. The R3 and G4 proteins are encoded in the pX region of BLV, whereas $p12^I$, $p13^{II}$ and $p30^{II}$ are encoded in the pX region of HTLV-1 (Sagata et al., 1984; Franchini et al., 2003). The pX sequences do not originate from the host cells and thus it is not an oncogene. In both BLV and HTLV-1, the Tax protein acts as an activator of transcription with oncogenic potential, and Rex interferes with the exportation of messenger RNA of both viruses from the nucleus (Derse, 1987; Willems et al., 1987; Felber et al., 1989; Katoh et al., 1989; Willems et al., 1990; Kashanchi and Brady, 2005; Matsuoka and Jeang, 2011). In cattle, the R3 and R4 proteins contribute to the maintenance of a high viral load (Willems et al., 1994; Florines et al., 2007). Furthermore, $p12^I$ and $p13^{II}$ proteins from HTLV-1 are similar in some functions to R3 and G4, respectively. $p12^I$ resembles R3 in that both are maintained in the nucleus and contribute to infectivity of the virus (Collins et al., 1998; Gillet et al., 2007); the $p13^{II}$ protein resembles the G4 protein because both bind to the farnesyl pyrophosphate synthetase, which farnesylates ras (Lefebvre et al., 2002) in addition to promoting ras dependent apoptosis (Hiraragi et al., 2005). Similarly, suppressions have been

observed in the sequences of gag, pol and env in all of the BLV-positive panel samples, and the presence of LTR sequences and Tax in these samples is consistent with the results reported for HTLV-1, which are associated with escape from immune surveillance (Kamihira et al., 2005; Buehring et al., 2014).

MECHANISMS OF CELLULAR TRANSFORMATION

Given the reports of the presence of BLV found integrated to the genome or as an episome in humans in the United States by Buehring et al. (2007), in Iran by Nikbakht et al. (2010) and in Colombia by Ochoa-Cruz et al. (2006), Mesa et al. (2013) and Buehring et al. (2014), possible mechanisms of transformation in mammary tissue must be proposed. These include involvement of the *Tax* gene in oncogenic processes *in vivo* and *in vitro*, such as viral transcription and increased expression in the proportion of the Bcl-2 protein (a proto-oncogene) over its protein homologue, Bax, which are related to resistance to apoptosis and the production of leukaemia in infected cows (Takahashi et al., 2005). A similar mechanism of resistance to apoptosis and chronic lymphocytic leukaemia occurs in humans (Pepper et al., 1997). Some authors mention that in the BLV genome, there is no preference for a particular site in the host genome (Murakami et al., 2011), and BLV could integrate itself in active sites associated with the control of cell division (Fulton et al., 2006; Klener et al., 2006). However, Gillet et al. (2013) demonstrated that BLV and HTLV-1 have surprisingly similar genomic regions where it is expected that the provirus of both viruses is inserted with a greater likelihood in their respective hosts. These insertion sites are the regions transcribing Pol II (polymerase) and Pol III, the regions close to the CpG islands, tRNA genes (transfer RNA) and tRNA pseudogenes. Similarly, in a study conducted by Elemans et al. (2014) on the mortality rate of cytotoxic T lymphocytes CD8+ (CTL) against infection of BLV and HTLV-1, both viruses are in the lowest range observed in the literature. This similarity could lead to finding similar mechanisms of action of BLV in humans.

Actions of the virus on the block of tumour suppressor and apoptosis genes could be a possible cause, as Melana et al. (2002) reports in breast cancer in the case of mouse mammary tumour virus (MMTV). Mutations of the p53 gene have been reported in 20% of women with breast cancer, and 30% of malignant processes in humans have been attributed to mutations of the oncogene ras (Javier and Butel, 2008).

Given the possibilities of action of the virus in humans, cases of benign, pre-malignant and malignant cell transformations and cases of latent virus presenting in apparently healthy patients could possibly be found without presenting changes in tissue, as shown in several previously mentioned reports and in the bovine species

or other retroviruses such as HIV and HTLV (Mesa et al., 2013). An additional finding has been made in recent years by Kinkaid et al. (2012), who demonstrated the presence of micro RNA (miRNA) in BLV. These miRNAs are small regulatory sequences encoded by most eukaryotic cells and some viruses that collectively have DNA type genomes. The miRNA type BLV-miR-B4 has been identified in BLV, which is transcribed by RNA polymerase III (pol III). The BLV-MiR-B4 shares partial identity of its sequences. It also shares target sequences with the miRNA of the bovine host (miR-29), and because the overexpression of miR-29 is associated with neoplasias of B lymphocytes that resemble tumours associated with BLV, a possible mechanism is suggested that contributes to the tumour genesis of BLV, similar to the participation of cell transformation in humans. Currently, six viruses are causally associated with cancer in human patients: The Epstein-Barr virus (EBV), human T-lymphotropic virus type 1 (HTLV 1), hepatitis B virus (HBV), hepatitis C virus (HCV), human papilloma virus (HPV) and human herpes virus 8 (HHV-8). However, given the growing research, using more advanced techniques such as ELISA, immunoblotting, nested PCR and RT-PCR, BLV is a new candidate to join the list of potentially hazardous viruses to human health and particularly cancer of the mammary gland (Rees, 2012). Other viruses together with BLV have also been proposed as potentially hazardous to human health, such as MMTV and cytomegalovirus (Lawson, 2006; Mason et al., 2011); thus, a change of thinking on the relationship between cancer and viruses is necessary. There is research in progress that could resolve various hypotheses that remain unsupported; however, as long as new lines are opened with methodologies that allow a better understanding of the action of the virus, particularly BLV and cancer in humans, new and better diagnostic, prevention and control methodologies of these diseases will continue to develop. Breast cancer occupies a significant position worldwide in terms of morbidity and mortality. Between 5 and 10% of all breast cancer cases are associated with hereditary factors. The rest are associated with other factors such as infections, of which 8% of the malignancy is reported in developed countries and an impressive 23% in developing countries.

CONCLUSION

Considering the scientific facts on the high prevalence of the EBL virus in countries such as the United States, the consumption of milk, meat and animal by-products positive for the disease, the constant exposure to the virus, the immune response against it and, ultimately, the demonstrated presence of the virus in the human genome, it is clear that there is a real potential risk. It is necessary to direct greater investment in research; rethink a new vision on the risks to human health; and develop programmes of diagnosis, prevention, control

and eradication of the virus, particularly in countries with high prevalence. These steps may result in less exposure to the virus and a consequent reduction of the risk that it becomes, from the effects of evolution, a true zoonosis.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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Full Length Research Paper

In vitro anti-tyrosinase and anti-elastase activity of collagen from sea cucumber (*Holothuria leucospilota*)

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Collagen is the main structural component of connective tissues in vertebral and invertebral animal tissues and organs. The collagen isolated from the body wall of sea cucumber (*Holothuria lecospilota*) can be used in the pharmaceutical field. The research aimed at isolating collagen from the sea cucumber body wall and testing the anti-tyrosinase and anti-elastase activity. This was done by isolation of collagen by soaking the body wall of sea cucumber using Tris-HCI-EDTA 0.1 M and dialyzing using 0.5 M acetic acid. The step was followed by characterization of collagen using FT-IR spectrophotometer and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resulting collagen was tested for anti-tyrosinase and anti-elastase *in vitro*. The research showed that the resulting collagen was of the amide group with molecular weight of 166.43 and 138.35 kDa, showing α1 and α2 chains. Anti-tyrosinase activity test showed an IC_{50} value of 1.20 mg/mL, while anti-elastase activity test showed an IC_{50} value of 125 μg/mL. The collagen isolated from the sea cucumber has weak anti-tyrosinase and moderate anti-elastase activity.

Key words: Crude collagen, anti-tyrosinase, anti-elastase, sea cucumber (*Holothuria leucospilota*).

INTRODUCTION

Sea cucumber has important economic values. Besides its use for diet purpose, sea cucumber has promising marine-derived medicinal potentials (Bordbar et al., 2001). In addition, it can be used for its anti- cancer, bacterial and malarial activities (Janakiram et al., 2005; Layson et al., 2014; Liu et al., 2007). Generally, major sources of collagen are the skin and bone of pigs and cows. However, the occurrence of mad cow disease has resulted in unease among cattle gelatin users. Additionally, collagen obtained from pig bones cannot be used by many, due to religious constraints (Sadowska et al., 2003). Thus, there is a strong need to develop alternative collagen sources. Marine organisms have been recognized as potential alternative sources, due to their availability, lack of dietary restriction, lack of disease risk and high collagen yields (Liu et al., 2007).

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Figure 1. *Holothuria leucospilota* (black sea cucumber).

Collagen is a component that forms the sea cucumber body and account for 70% of its total protein content. Few studies have been conducted on sea cucumber from Indonesia, while collagen is widely used in pharmaceutical and cosmetic industries. Pharmacological activity of collagen is to scaffold mesenchymal stem cell and extracellular biometrical matrices to enhance cellular growth and development (Sadowska et al., 2003). In Indonesian waters, there are 53 species of sea cucumber, including *Holothuria, Actinopyga, Bohadschia, Labiodemas, Thelonata* and *Stichopus*. Among the species of sea cucumber, only 29 are internationally traded (Darsono, 2011), all of which belong to *Holoturiidae* and *Stichopodidae* families. One of them is *Holothuria lecospilota*, which is widely found in Pramuka Island, Kepulauan Seribu, Jakarta Bay area. The study aimed at isolating collagen from the body wall of sea cucumber and conduct *in vitro* test for anti-tyrosinase and anti-elastase activity in sea cucumber.

MATERIALS AND METHODS

Isolation of crude collagen

Two fresh samples of Black sea cucumber, *H. lecospilota* were taken from Pramuka Island, Kepulauan Seribu, Jakarta Bay area weighing between 700 and 800 g and were handpicked by the divers. The body wall of *H. leucospilota* was dissected for free adherent tissues, cut into small pieces (about 2 × 2 cm), and stored in a small container filled with phosphate buffer saline (PBS). The body wall of sea cucumber was soaked in destilled water for 1 h. The dissected body wall of sea cucumber was soaked in 0.1 M NaOH solution for 6 h, then soaked in 0.1 M pH 8.0 Tris-HCl for 24 h, washed using pH 7.0 double destilled water, and then stirred using 0.5 M acetic acid for 48 and 72 h. The filtrates were centrifuged under 8000 rpm speed for 20 min, salted out using 0.9 M NaCl for overnight, and then centrifuged under 8000 rpm speed for 20 min for obtaining deposits. The deposits were dialyzed by

using a dialysis bag and 0.1 M acetic acid as medium under a temperature of 4°C for 12 h (Sadowska et al., 2003; Sionkowska et al., 2015; Zhong et al., 2015). Then, the medium was replaced with destilled water, and then dialyzed for a similar period. The resulting collaged was put in a freeze dryer.

Characterization of crude collagen

FT-IR spectrophotometer

FT-IR spectroscopy of freeze-dried sample of crude collagen was based on a Bio- Rad FT-IR –40, USA. Sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed to make a salt disc (10 mm in diameter) for reading the spectrum further. Spectra were collected between wave numbers of 4000 and 500 cm^{-1}, which was compared with standard collagen (Sigma) (Sambrook and Russel, 2001; Kong and Yu, 2007).

SDS-PAGE electrophoresis

The molecular weight of the collagen was determined by SDS-PAGE and was compared with molecular marker–Sigma (Kim and Uyama, 2005).

Anti-tyrosinase activity test

The anti-tyrosinase activity of crude collagen was measured spectrophotometry. The enzymatic reaction was initiated by addition of a known amount of the enzyme to substrate solution containing dimethyl formamide (DMF and MBTH). DMF was added to the reaction mixture in order to keep the resulting colored complex in soluble state during the course of investigations. The progress of the reaction was followed by measuring the intensity of the resulting pink color at 505 nm. A typical reaction mixture with a total volume of 1.0 ml contained 100 μl enzyme solution (a), 500 μl substrate solution (b) and 400 μl phosphate buffer (pH 6.8). The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as, the concentrations of each sample that inhibited 50% of tyrosinase activity. The resulting data were expressed as a percentage of inhibition of tyrosinase activity (Kothary et al., 1984).

Anti-elastase activity test

The method of Kothary et al. (1984) was adapted to detect the production of elastase among test crude collagen. These activities were determined by colorimetric assay of Sachar et al. (1955) with some modifications, employing elastin Congo red (Sigma) as a substrate. Briefly, 250 µl each of 50 mM sodium borate buffer (pH 8.5) and elastin Congo red (20 mg ml^{-1} in 50 mM sodium borate buffer, pH 8.5) was mixed and vortexed at room temperature for 5 min. Next, 250 µl of test sample was added and incubated at 37°C for 3 h at 180 rpm.

Test sample was replaced by 250 µl of buffer as control. After incubation, 750 µl of 10% trichloroacetic acid was added to stop the reaction and kept for 30 min on ice. Insoluble material in the assay mixture was removed by centrifugation at 5000 rpm, 30 min and the absorbance was read at 495 nm using a double beam UV-vis spectrophotometer (Sachar et al., 1955).

Statistical analysis

All the experiments were performed three times with three replicates per experiment and data are expressed as mean ± standard deviation. Statistical significance of the differences was determined by the one way ANOVA test using Minitab (V.11.0 for Windows). Percentage reduction in elastase and tyrosinase activity in test in the presence of collagen was compared with untreated control by one way ANOVA using Duncan's method. P-values of ≤ 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

H. leucospilota morphologically has a round body cross section, relatively flat ventral side, and a round anus. It has dark skin with smooth and thick surface (Figure 1). The spicules found on dorsal area were of table, rosette and slab types. It is mostly found at the area with coarse sand substrate and the body is covered with smooth sand (Conand et al., 2013). Most parts of the sea cucumber are made of collagen and have been identified as type 1 collagen. Type 1 collagen is found in human skin; therefore, the collagen can be used for cosmetic products. Collagen extraction was conducted using a method proposed by Trotter et al. (1995) and Saito et al. (2002). The extraction did not obtain crude collagen extract because of too long soaking process in distilled water. Consequently, the resulting pellets were much fewer. Another study that combined the method proposed by Trotter et al. (1995) and Saito et al. (2002) used acetic acid and Tris HCl-EDTA. The research found collagen yield of 7.92% after soaking in acetic acid for 72 h. The calculation was based on crude weight. The resulting yields were higher than those found in the research conducted by Saito et al. (2002), in which the resulting crude collagen of the sea cucumber, *Stichopus japonicus* was only 3.3%.

Characterization of the collagen was conducted by using FT-IR spectrophotometer and SDS-PAGE. IR spectroscopic test was conducted to find out the functional groups or collagen-chitosan compound. FT-IR spectroscopy is a tool to measure radiation absorbance at infra-red area at various wavelengths. Qualitatively, FT-IR spectroscopy can be used to identify functional groups in a molecular structure. The data resulting from FT-IR spectrum are the characteristic spectral peaks, which are represented as transmittance curve (%) and wavenumber (cm^{-1}) of the tested sample and are analyzed. To analyze the data resulting from the infrared spectroscopic measurement, international conversion table, namely Handbook IR is necessary (Kong and Yu, 2007). The result of characterization using FT-IR spectrophotometer showed IR spectrum as presented in Figure 2.

The figure of FT-IR spectrum of sea cucumber collagen shows that the collagen resulting from isolation using 0.5 M acetic acid was amide A and 1. Amide A in the sea cucumber collagen was found at wavenumber of 3438.84 and 3433.06 cm^{-1}, respectively. The N-H group of peptide was influenced by hydrogen bonding whose position was shifted to a lower frequency. Amide A is an area, in which there is paired overextended NH group that formed hydrogen bonding and OH from hydroxyproline. Hydroxyproline is a specific amino acid in collagen. The peak of amide 1 in sea cucumber collagen was found at wavenumber of 1631.67 and 1629.74 cm^{-1}, respectively. Amide 1 has wavenumber range of 1690 to 1600 cm^{-1} and represents C=O group, which is secondary structure of protein. Amide 1 is related to stretching vibration of carbonyl group. It is a group with specific function to form collagen. The functional groups in sea cucumber (*H. leucospilota*) are different from other collagens, such as those found in *S. variegatus, Oreochromis niloticus, Thunnus albacares*, and puffer fish, namely amide A, B, I, II and II (Sionkowska et al., 2015; Zhong et al., 2015).

FT-IR absorbance spectrum of the collaged showed amide A at wavenumbers of 3438.84 and 3433.06 cm^{-1}, respectively. Peak amide 1 was found at wavenumbers of 1631.67 and 1629.74 cm^{-1}, respectively. The collagen has a specific peak at wavenumber of 3452.64 cm^{-1}, showing that it belongs to hydroxyl group (-OH); meanwhile, at wavenumber of 1657.57 cm^{-1}, amide 1 was found. Amide 1 is a crucial factor for understanding the secondary structure of protein (Vidal, 2013). Amide II was shown at wavenumber of 1560.47 cm^{-1}. Amide II represents a helix structure. Observation of the isolated collagen using SDS-PAGE was conducted to determine the protein band pattern and molecular weight of the protein. The collagen of sea cucumber (*H. leucospilota*) has 3 protein bands with molecular weight after soaking with 0.5 M acetic acid for 72 h (Figure 3).

Protein band after 72 h of treatment showed α_1 and α_2 chain at molecular weights of 166.43 and 138.35, respectively; the α chain showed molecular range of 112 to 199 kDa and β chain was shown at molecular weight of above 200 kDa. Characterization was conducted by using SDS PAGE based on the difference in the migration rate and molecular weight on a single electric field. The protein band pattern of the *H. leucospilota* collagen
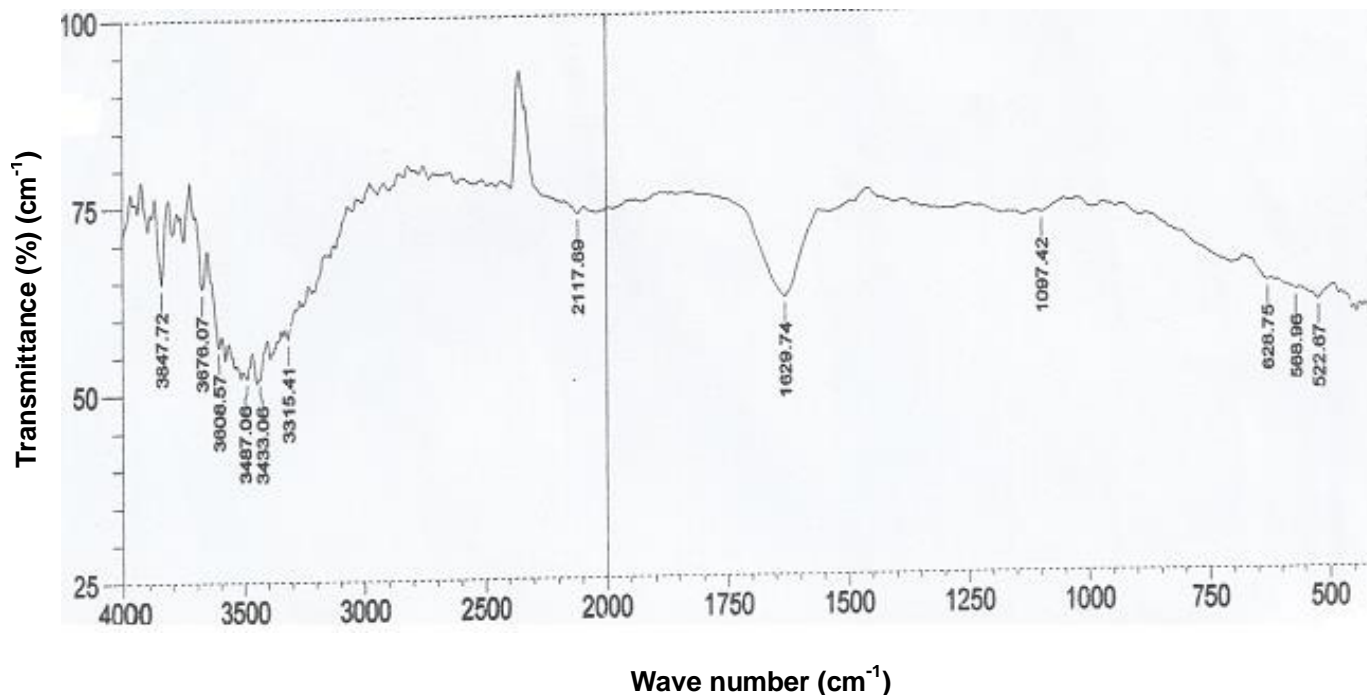
Figure 2. FT-IR spectrum of *H. Leucospilota* collagen.

consists of 3 protein bands, namely, 247.29 166.43 and 138.35 kDa. The protein bands showed α1 and α2 chains at molecular weights of 166.43 and 138.35 kDa, respectively, in which α chain was shown at molecular weight range of 112 to 199 kDa and β chain at molecular weight above 200 kDa. The research shows that protein band pattern of *S. Japonicus* collagen was 135 kdA as compared to 137 kDa in sea cucumber *Stichopus monotuberculatus* collagen (Cui et al., 2007). The primary structure of type 1 collagen consists of continuously repeated sequence Gly-X-Y (most of X is proline and Y is hydroxyproline). In a section that has more than a thousand of residues, every third residue is glycine. The repeated GI-X-Y sequence in α1 chain plays an important role in forming the secondary structure of triple helix. Therefore, the collagen is type 1, which is mostly found in connective tissues, including those of tendon, bone and skin (Saito et al., 2002). Collagen is considered to be one of the most useful biomaterials because it has a wide range of industrial applications (Lafarga and Hayes, 2014).

Skin aging (whether extrinsic or intrinsic type) causes wrinkling, sagging, laxity, dyspigmentation, and telangiectasia. As the skin ages, the collagen (a major component of skin) and elastin in the dermis lose elasticity resulting in wrinkles. To prevent the skin from aging or wrinkles, natural phytochemical source is desirable. Elastase are known to be a major enzyme responsible for dehydration and wrinkle formation on the skin surface (Lafarga and Hayes, 2014). The results of *in*
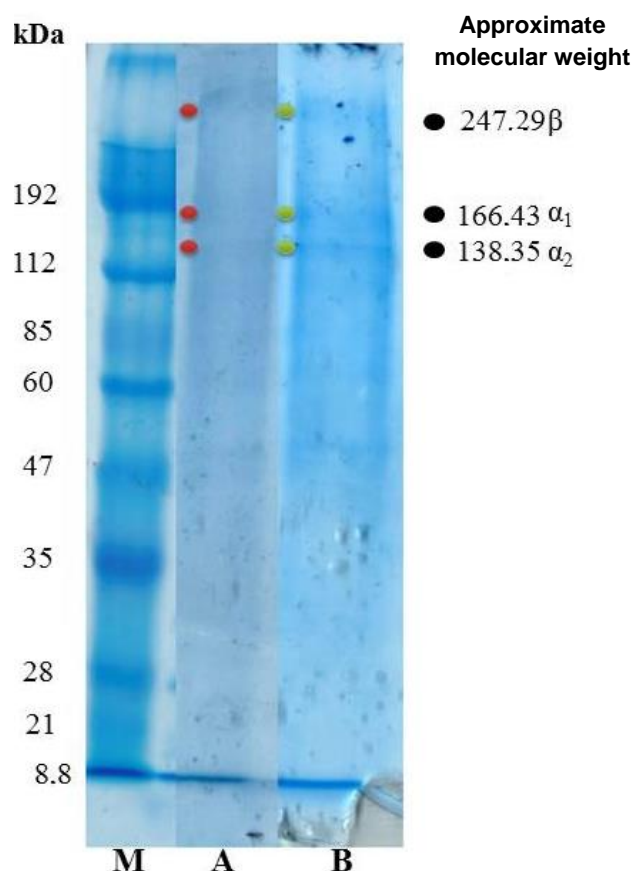
Figure 3. Protein band pattern of *H. leucospilota* collagen.
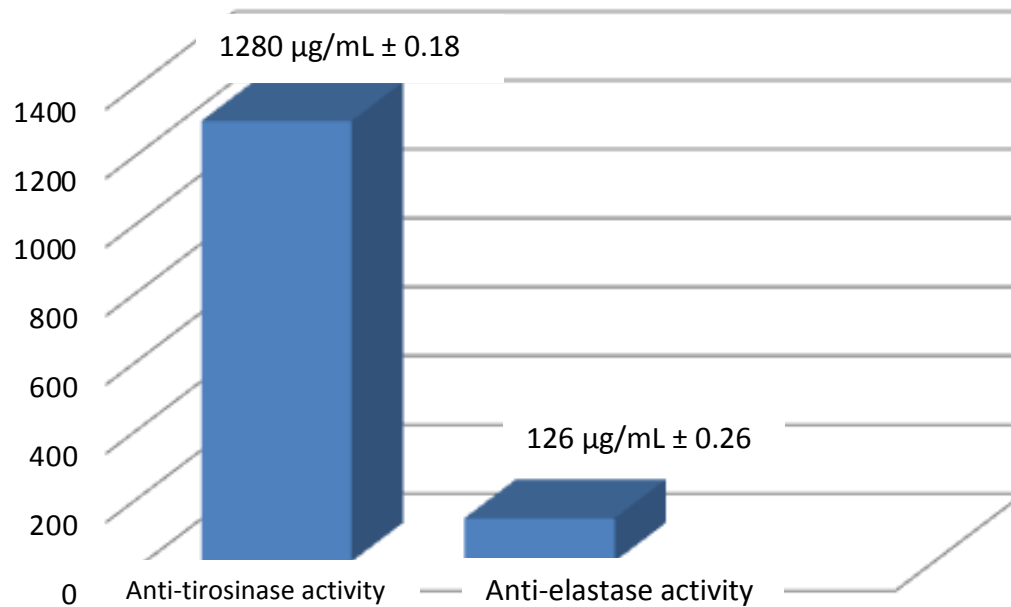
Figure 4. The results of measurement for anti- tyrosinase and elastase activity of the collagen.

vitro measurement for anti-tyrosinase and anti-elastase activity of the collagen are presented in Figure 4.

Measurement of anti-tyrosinase and anti-elastase activity of the collagen was conducted by varying the collagen concentrations from 50 to 2000 ppm. Then, the absorbance and percentage (%) inhibition were measured. The inhibitory effects of collagen on these two enzymes are shown in Figure 4. Measurement of anti-tyrosinase and anti-elastase activity of the collagen resulted in IC_{50} value of 1280 ± 0.98 and 126 ± 0.76 µg/mL. The study conducted by Choi et al. (2015) found that hydrolyzed collagen of *Todarodes pacificus* has anti-tyrosinase activity. On the other hand, this result corresponded to the results of Zhuang et al. (2009) that tyrosinase inhibitory activity of jellyfish (*Rhopilema esculentum*) collagen hydrolysate fraction was 47.2% at 5 mg/mL. Tyrosinase is a copper containing oxidase, widely distributed in micro-organisms, plants and animals, and a key enzyme in melanin biosynthesis.

Conclusion

In this study, the novel anti- tyrosinase and elastase activities of collagen from sea cucumber (*H. leucospilota*) showed an indication of the efficacy as potential anti-wrinkle. However, *in vivo* evaluations are needed for the development of natural source to formulate advanced skin care cosmetics.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Production of invertase from *Saccharomyces cerevisiae* Angel using date syrup as a cost effective carbon source

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Invertase (β-fructofuranosidase) finds major applications in food, cosmetics, and pharmaceutical industries. In the present study, fermentation conditions for invertase production from *Saccharomyces cerevisiae* Angel using date syrup in shake flask were investigated. The effect of time period (0-96 h), carbon sources (sucrose, date syrup, maltose, galactose, and glucose), nitrogen sources including yeast extract, peptone, urea, meat extract, and ammonium sulphate, pH (4 to 6.5), temperature (30 to 40°C), date syrup concentration (5 to 50 g/L), and inoculum size (1 to 10% v/v) were optimized. Optimum conditions for yeast growth and invertase yield were 20 g/L date syrup, 10 g/L yeast extract, temperature of 37°C, pH 5.5, and 60 h fermentation time. In addition, effect of date syrup concentration and size of inoculum were also optimized. Highest invertase titer of ~190 U/mL were obtained when 40 g/L of date syrup and 10% v/v inoculum were used. These results show great practical potential for invertase production by saving enzyme production cost.

Key words: *Saccharomyces cerevisiae* Angel, invertase, date syrup, β-fructofuranosidase.

INTRODUCTION

Invertase (β-fructofuranosidase, EC 3.2.1.26) breakdown sucrose and releases equimolar mixture of glucose and fructose (Alegre et al., 2009; Qureshi et al., 2012b). Invertase finds potential applications in several industries,

for instance in food (for confectionery products, infant food products, and beverages), in production of artificial honey, in cosmetics as plasticizing agent, in pharmaceutical products, in paper industry, and in manufacturing of sucrose biosensors (Alegre et al., 2009; Kotwal and Shankar, 2009; Uma et al., 2010a). Sucrose hydrolysis could be performed either by conventional method, for example, acid hydrolysis that produce invert sugar but, this method has certain limitations, including lower conversion efficiency, higher energy consumption, and high production cost. However, invertase applications show 100% conversion efficiency of sucrose into glucose and fructose (Kumar et al., 2008).

Invertase are produced from several microorganisms, including *Mucor geophillus* (Qureshi et al., 2012b), *Aspergillus caespitosus* (Alegre et al., 2009), *A. niveus* (Guimarães et al., 2009), *A. niger* (Rubio and Navarro, 2006), *Rhodotorula glutinis* (Rubio et al., 2002), *Saccharomyces cerevisiae* (Ali et al., 2008). However, *S. cerevisiae* is considered as best microorganism for invertase production, largely due to its high efficiency for sucrose consumption (Ali et al., 2008). Therefore, *S. cerevisiae* Angel baker yeast was purchased from local market and its invertase production capability was determined. Invertase production from Angel yeast using date syrup as a carbon source has never been investigated. So, this research work could provide new insight in fermentation processes.

Increasing invertase production demands, high nutrients cost, and environmental pollution problems has urged to utilize agro-industrial residues as cost effective nutrients for production of invertase and other value added products. Several studies have evaluated efficiencies of various agro-industrial residues as cost effective carbon sources, for example, date syrup for pectinase production (Qureshi et al., 2012a), oilcake for invertase production (Qureshi et al., 2012b), and protease production from molasses (Qureshi and Dahot, 2009). In the present study, efficiency of date syrup as a cost effective carbon source for invertase production from *Saccharomyces cerevisiae* Angel was investigated. Highest invertase titer of ~190 U/mL were obtained when 40 g/L of date syrup and 10% v/v inoculum were used. These results show great practical potential for invertase production from date syrup medium at industrial scale.

MATERIALS AND METHODS

Microorganism and cultivation conditions

S. cerevisiae Angel was purchased from local market, Shanghai, China. Strain was incubated in YPD medium containing 20 g/L of glucose, 20 g/L of peptone, and 10 g/L of yeast extract for 24 h at 37°C in shaking incubator. After 24 h, 10% v/v culture was transferred into seeds culture medium (sucrose 20 g/L, yeast extract 10 g/L, MgSO$_4$·7H$_2$O 1.0 g/L, KH$_2$PO$_4$ 2 g/L) and incubated at 37°C for 12 h in shaking incubator. Mature seeds culture (5.0% v/v) was inoculated in the fermentation medium (sucrose 20 g/L, yeast extract 10 g/L, MgSO$_4$·7H$_2$O 1.0 g/L, KH$_2$PO$_4$ 2 g/L), and culture

was incubated at 37°C for 96 h in shaking incubator. Samples were collected at regular interval of 12 h, and growth (OD) was spectrophotometrically measured. Culture broth was centrifuged at 12,100 g for 10 min to collect supernatant that was used for invertase activity. All media were sterilized at 115°C for 20 min prior inoculation.

Effect of carbon and nitrogen sources

Effect of different carbon sources (20 g/L) on microbial growth and invertase production was determined by replacing sucrose in the above mentioned fermentation medium with date syrup, maltose, galactose and glucose. Culture was incubated at 37°C for 60 h. For evaluating the effect of nitrogen source, various nitrogen sources, including yeast extract, peptone, urea, meat extract, and ammonium sulphate at a concentration of 10 g/L were supplemented in synthetic medium.

Influence of initial pH and temperature

Initial pH of the enzyme production medium was adjusted in the range of 4 to 6.5 pH with 2.0 M H$_2$SO$_4$/2.0 M NaOH. Effect of temperature on cell growth and invertase production was checked at various temperature ranges from 30 to 40°C. Culture was incubated for 60 h at different conditions.

Effect of inoculum size and date syrup concentration

Effect of inoculum size (1-10 % v/v) was determined on invertase production. For optimizing the date syrup concentration, separate set of experiments at different date syrup concentrations (5-50 g/L) were performed under optimized cultural conditions.

Invertase assay

Invertase activity was assayed from culture supernatant collected after centrifugation 12,100 g for 10 min, according to method adapted from (Qureshi et al., 2012b). 1 mL of crude sample was taken, then 1 mL of substrate (sucrose solution prepared in 20 mM acetate buffer pH 5.5) was added. Reaction contents were mixed thoroughly and incubated at 37°C for 15 min. Then, 2 mL of dinitrosalicylic acid (DNS) reagent was added for color formation and to stop the reaction, and then reaction mixture was boiled for 5 min. The absorbance of samples was read at 540 nm against blank, according to reducing sugar determination method (Miller, 1959). Enzyme and substrate blank were prepared by replacing substrate or enzyme with 1 mL of double distilled water. One unit of invertase activity was defined as, the amount of crude enzyme required for releasing 1 µg of reducing sugar under assay conditions. All experiments were performed in triplicate and average of results are shown in figures as well in text, standard deviation was calculated in MS excel shown as error bars in all figures.

RESULTS AND DISCUSSION

For obtaining highest possible invertase yield, cultural conditions must be optimized in terms of carbon and nitrogen sources, pH, temperature, and size and age of inoculum. There were two main reasons of this study, first was to optimize cultural conditions for obtaining the highest possible invertase titer. Second was the

Figure 1. Batch profile of invertase activity and yeast growth.
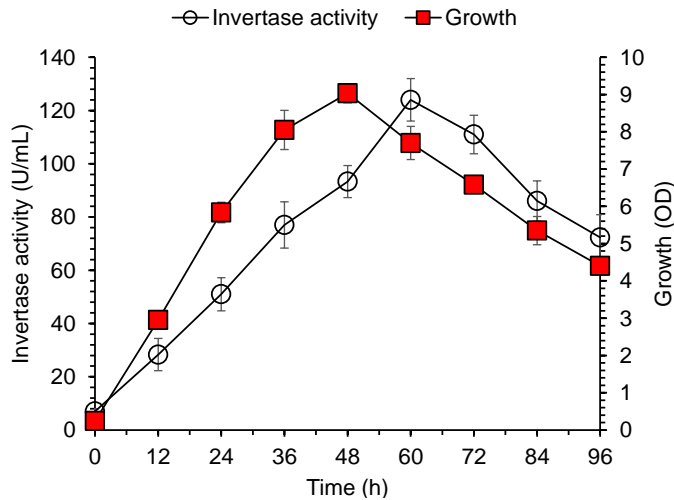
Figure 2. Effect of carbon source on invertase yield and *S. cerevisiae* Angel growth.
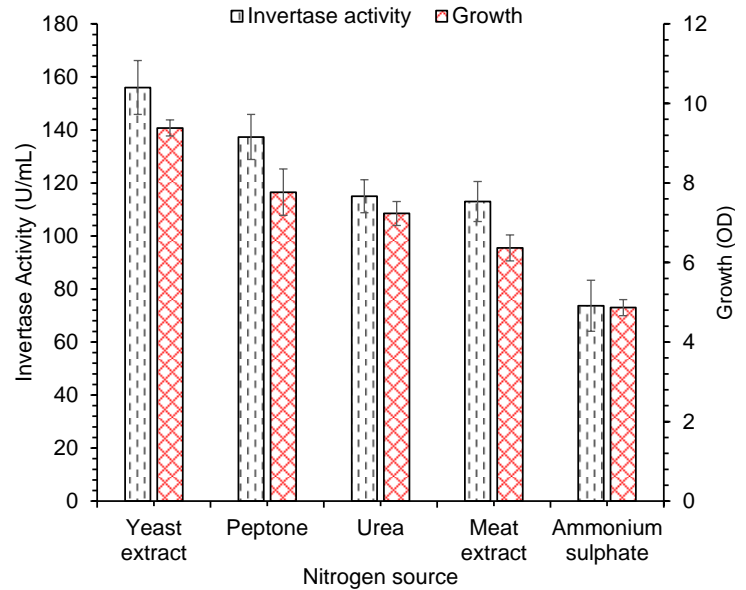
Figure 3. Effect of nitrogen source on enzyme production.

(Ali et al., 2008). Enzyme production decreased on further incubation perhaps due to decrease of nutrients, cell death, carbon catabolite repression, and inhibitors accumulation in the fermentation medium (Ali et al., 2008; Qureshi et al., 2012b).

The effect of different carbon sources (sucrose, date syrup, maltose, galactose, and glucose) on yeast growth and enzyme production was determined; results are shown in Figure 2. S. cerevisiae Angel growth and invertase yield were highest when 20 g/L of date syrup was used as carbon source (156 U/mL) followed by sucrose (124 U/mL) and glucose (109 U/mL). Most of researchers have performed invertase production from pure sugars, that is increasing the fermentation medium cost. However, agro-industrial residue could be attractive carbon source for production of enzymes and other valuable products to solve the pollution problem and save the fermentation medium cost (Pandey et al., 2000). In this study, date syrup medium secreted highest invertase yield probably due to growth promoting substances present in date syrup (Qureshi et al., 2012a).

Figure 3 shows the effect of nitrogen sources (10 g/L) on *S. cerevisiae* growth and invertase yield. Several nitrogen sources including yeast extract, peptone, urea, meat extract, and ammonium sulphate were tested. Highest invertase yield was attained when 10 g/L of yeast extract was used. Our results were similar to results presented in literature (Qureshi et al., 2012b) for invertase production. By contrast, other researchers obtained maximal invertase yield when urea was used as nitrogen source (Ali et al., 2008).

Figure 4 shows the influence of initial pH (4 to 6.5) on the invertase biosynthesis by *S. cerevisiae* using 20 g/L of date syrup and 10 g/L of yeast extract in mineral medium

evaluation of cost effective carbon source for invertase production from *S. cerevisiae* Angel. Date syrup, a liquid containing high nutrient has been evaluated as carbon source for pectinase production (Qureshi et al., 2012a), but never used as a carbon source for invertase production. Utilization of agro-industrial waste as substrate not only reduce the enzyme production cost but also reduce the pollution problems occurring due to accumulation of waste material.

Effect of time of incubation on synthesis of invertase by *S. cerevisiae* Angel is shown in Figure 1. Cell growth and enzyme concentration increased with time of incubation, and reached to maximum after 60 h (124 U/mL). Similar results for invertase production are reported in literature

Figure 4. Effect of pH on invertase production.
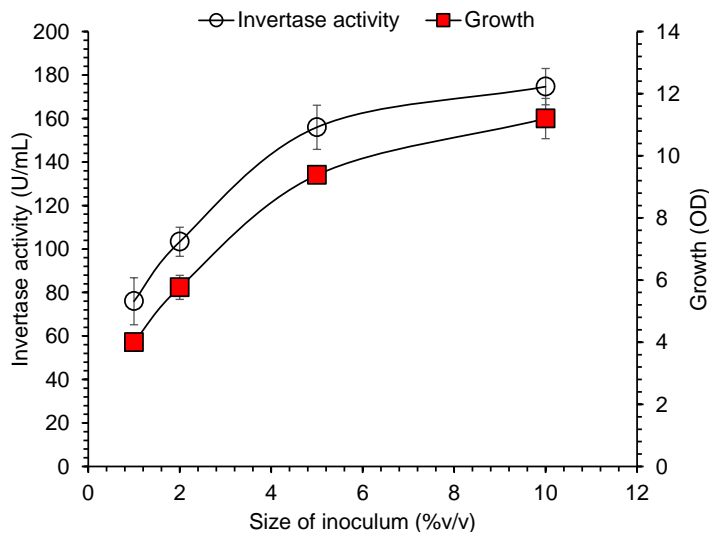
Figure 6. Effect of inoculum size on invertase titer.
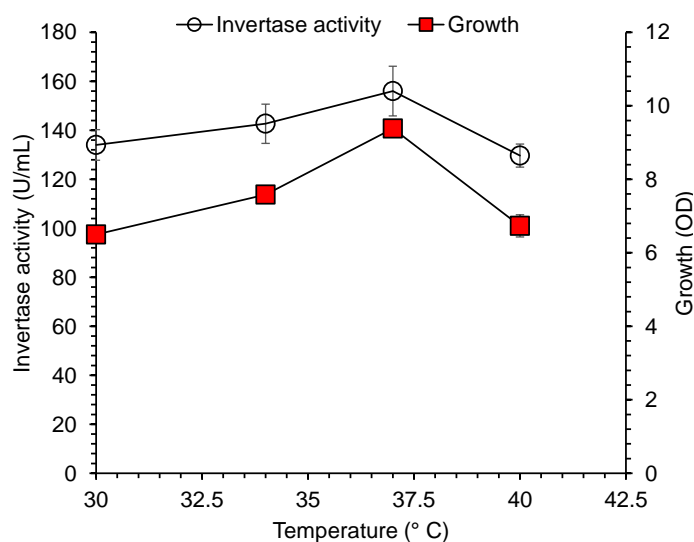
Figure 5. Effect of temperature on enzyme biosynthesis.

when incubated at 37°C for 60 h. Maximum growth was obtained at pH value of 6.0, whereas highest invertase yield was noted at   5.5. Yeast growth and enzyme secretion sharply decreased at pH 6.5. Although, there was no significant difference in the cell growth at the pH values of 5.5 and 6.0. Only 0.1 OD increase in cell growth was observed at pH 6 comparing to 5.5 pH. Therefore, pH 5.5 was selected for further experiments based on optimum enzyme activity and microbial growth. Our results are similar to results of (Uma et al., 2010) for invertase yield. Figure 5 shows the effect of incubation temperature on the invertase synthesis by yeast (30-40°C), when grown in fermentation medium containing 20 g/L of date syrup and 10 g/L of yeast extract, culture was

incubated for 60 h at the initial pH 5.5. The maximum yield of invertase was noted at 37°C, on further increase of temperature, invertase titer decreased. Generally speaking, data presented in figure show almost similar invertase activity in the range of 30-37°C temperature with 134 U/mL (30°C) and 156 U/mL (37C°). But, looking at industrial production unit minute increase save enzyme production cost at large extent. At per milliliter there is only 22 Units difference at both temperature ranges, but this difference increases to 22,000 U/L and if production fermenter has 100 L medium than 220,000,0 U enzyme titer could be increased at 37°C operation instead of 30°C. This minor change at laboratory scale is of significant importance at commercial scale.

Among several factors that affect enzyme production, age and size of inoculum are of significant importance. Figure 6 shows the effect of inoculum size (1-10% v/v) on invertase production. Maximum invertase titer of 174.6 U/mL was obtained when (10% v/v) inoculum was used. Enzyme yield increased by increasing size of inoculum, this occurred due to increase of yeast growth (Figure 6). There was significant difference in enzyme activity when inoculum size was increased from 1 to 5% v/v and enzyme titer increased from 76 to 156 U/mL. However, further increase in size of inoculum from 5 to 10% v/v enhanced enzyme titer from 156 to 174 U/mL. This increase could be important achievement at commercial scale compared to laboratory stage. Previous studies have reported best enzyme titer at different size of inoculum; Ali et al. (2008) found 2% v/v to be optimal for invertase production. Gancedo (1998) obtained maximum invertase titer when 10% v/v inoculum was used. In the present study, lower invertase titer was observed when less than 10% v/v inoculum was added in the fermentation medium. This might be due to lower concentration of yeast cell that was not sufficient for
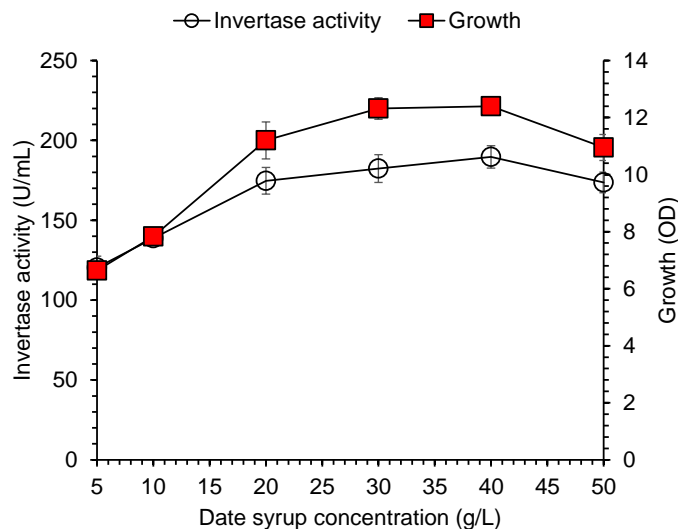
Figure 7. Effect of date syrup concentration on invertase production.

higher invertase yield.

Effect of date syrup concentration (5-50 g/L) on cell growth and enzyme production was checked, results areshown in Figure 7. Invertase concentration increased withdate syrup concentration upto 40 g/L (~190 U/mL), on further increase of date syrup concentration, enzyme yield and microbial growth decreased. There was no major difference in invertase activity either of the 20 g/L (174 U/mL) or 40 g/L (189 U/mL) date syrup used. From economical point of view, 20 g/L date syrup could be applied at commercial scale invertase production from *S. cerevisiae* Angel. This was probably due to accumulation of inverted sugars that was responsible for catabolite repression (Ali et al., 2008).

Conclusion

Increasing invertase demands, high nutrient cost, and environmental pollution has compelled the utilization of agro-industrial residues as inexpensive nutrients for enzyme and other valuable products.

In the present study, cultural conditions were optimized for invertase production from *S. cerevisiae* Angel using date syrup in shake flask under submerged fermentation conditions. Highest invertase titer of ~190 U/mL was obtained when 40 g/L of date syrup and 10% v/v inoculum were used. These results show great practical potential for invertase production from date syrup medium at industrial scale.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Assessment of genetic diversity among cultivated Pearl millet (*Pennisetum glaucum*, Poaceae) accessions from Benin, West Africa

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Simple sequence repeat (SSR) molecular markers were used for genetic diversity analysis and population structure of the cultivated Pearl millet in Benin, West Africa. In order to assess the level of genetic diversity, 14 polymorphic SSR markers were used to screen 114 accessions from different agro-ecological zones in Benin. SSR markers were found to reveal a total of 57 alleles with an average of 4.071 allele per locus. Genetic diversity index varied from 0.099 to 0.633 with an average of 0.405. The average observed heterozygosity was found to reach 0.425. The analysis of molecular variance showed no real differentiation between regions. Only 5% of genetic variation was observed between samples collected from north-eastern and north-western region. A high level of variation (95%) was observed among accessions. Moreover, both principal component analysis (PCA) and the dendrogram obtained from the genetic distance among accessions revealed the absence of any specific structuration of accessions from each region under study. Our results confirmed diversity among cultivated Pearl millet in Benin and such diversity is not clustering according to geographical patterns.

Key words: Cereal, simple sequence repeat (SSR) markers, genetic variability.

INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br., 2n = 2x = 14) is one of the most important food crops widely cultivated in the arid and semi-arid area. It is an important cereal food grain in sub-Saharan Africa and India, where it is mostly used for human consumption (Loumerem et al., 2008). Because of its good performances in low-fertility soils such as semi-arid regions of Africa and Southeast Asia, it is an important staple cereal cultivated

in regions severely affected by malnutrition. It has a high nutritional value compared to other cereals and presents therefore a high potential in contributing to food and nutrition security (Vanisha et al., 2011). In Benin, national production (27000 tonnes in 2013, USDA, 2013) ranks fourth among cereals after rice, maize and sorghum, although it ranks second in the northern part of the country. Being a drought-tolerant crop, pearl millet is cultivated in dry northern regions of Benin under increasingly short and marginal raining seasons. It is used in various local food preparations like local porridge, cakes and traditional beverage. Despite its importance in the daily food supply of the northern populations of the country, pearl millet is considered as a neglected and underutilized species (NUS) (Dansi, 2010). The combined effects of the decrease in cultivated areas dedicated to pearl millet versus other cereals in this part of the country and the reduction of the rainy season as a result of climate change (MAEP, 2007), may lead to either decrease or loss of genetic diversity on the long term. An international initiative aiming at securing plant genetic resources has recently been launched (FAO, 2010), in order to help farmers to respond to climate change and to mitigate the losses due to genetic erosion. This endeavour needs to be further supported through the implementation of adequate preservation measures at the local scale, starting with an inventory of the existing diversity.

At this time, there is no national collection of pearl millet landraces from Benin and moreover, very little information is available on the diversity of cultivated pearl millet accessions in the northern part of the country. Overall, 22 Beninese pearl millet accessions have been sampled as part of larger studies targeting landraces spanning Western and Central Africa (Haussmann et al., 2006; Stich et al., 2010), and stored in the international genebank. However, these population is insufficient to provide information of their genetic diversity. It therefore appears necessary to conduct a new sampling campaign in this region in order to close the gaps in the previous datasets and to prevent the loss of existing local pearl millet diversity. Pear millet has an anemophilous pollination, which contributes to the maintenance of genetic variability through gene flow among wild and cultivated population (Pesson, 1984; Tostain, 1993). The use of agromorphological parameters for the characterization of diversity is not sufficient and will not provide an accurate classification of the accessions, since morphological criteria are strongly influenced by the environment (Bahram et al., 2014). On the contrary, the molecular markers have been proven to be a powerful tool to assess the genetic varaition and to point up any relationship among accessions in various crop species such as *Gossypium* and Broomcorn millet (Wu et al., 2007; Hu et al., 2009).

Several molecular genetic tools based on either dominant or codominant markers can be used in the molecular characterization of genetic diversity in plants. Among these, Single Sequence Repeat (SSR) or microsatellite markers, which target tandem repeats of di- tri- or tetra-nucleotide DNA motifs, are both highly polymorphic within populations and randomly distributed throughout the genome in both transcribed and non-transcribed sequences (Saghai-Maroof et al., 1994; Manzelli et al., 2007). SSR markers have been successfully used to evaluate genetic diversity in several crop species such as wheat (Fufa et al., 2005), *Pennisetum purpureum* (Azevedo et al., 2012), *Capsicum* (Nicolai et al., 2013), Sorghum (Cuevas et al., 2014), barley (Shakhatreh et al., 2015), *Vigna subterranea* (Molosiwa et al., 2015), maize (Salami et al., 2016) and some underutilized species such as buckwheat and wild melon (Roy et al., 2012; Zhang and Zao, 2013). For pearl millet characterization, several SSR markers were developed (Mariac et al., 2006; Stich et al., 2010; Nepolean et al., 2012) and used to evaluate its genetic diversity at the regional scale in west Africa. In the Stich et al. (2010) study, it has been proposed that Western and Central Africa (WCA) are the primary center of origin and genetic diversity of pearl millet. Moreover, with respect to Beninese accessions, few of them (22 accessions) were included in the 2010 study since the country is regarded as a secondary centre for pearl millet production.

A more in-depth knowledge of genetic diversity is the key for both crop breeding programme and the development of *in situ* conservation strategies. The main objectives of the present study were: (1) to evaluate the level of genetic diversity of cultivated millet in Benin and (2) to evaluate genetic diversity structure in the cultivated areas, and to explore relationship between such structure and the local usages of the accessions.

MATERIALS AND METHODS

Plant collection and DNA extraction

Pearl millet accessions were collected in December 2013 to January 2014 from the northern part of Benin, which is divided between the northeast and the northwest covered by the Departments of Borgou-Alibori and Atacora-Donga, respectively. The northern region is located in arid and semi-arid agro-ecological zones characterized by unpredictable and irregular rainfall oscillating between 800 and 950 mm/year. The overall climate is Sudanian with alternating dry (November to April) and rainy (May to October) seasons (Jalloh et al., 2013). Additionally, the northwest region is characterized by a particular climate called Atacorian climate. The annual mean temperatures range from 26 to 28°C and may exceptionally reach 35 to 40°C in the far northern localities (Adomou, 2005; Akoegninou et al., 2006). Figure 1 presents distibution of the prospected villages across the northern part of the country. During these surveys, samples were collected from farmers providing panicles or seeds, whenever panicles were not available.

For genomic DNA extractions, five seeds from each accession were germinated in a polyethylene bag and leaves from 4 week-old plantlets were harvested. DNA was extracted through the grinding
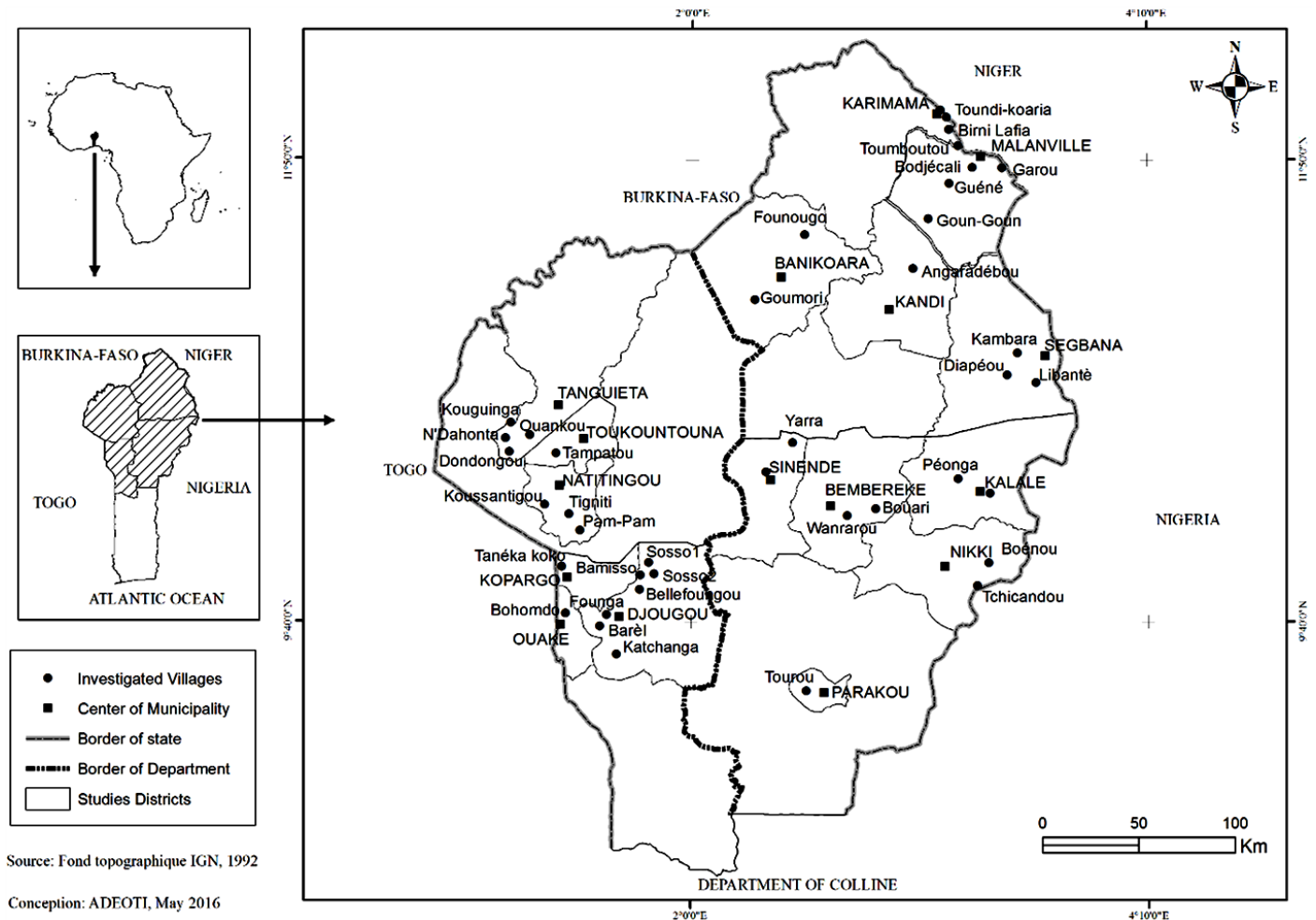
Figure 1. Geographical distribution of villages across study areas.

of 100 mg of leaf tissues sampled from 4 week-old plantlets in 750 µl of extraction buffer (100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG 6000, 0.5% sodium sulphite, and pH 8) pre-heated at 65°C. The lysate was then decanted into a 2 ml Eppendorf tube and incubated at 65°C for 20 min. DNA was purified through two successive steps of phase separation with chloroform-isoamyl alcohol (24:1, v/v) and precipitation from the supernatant with one volume of cold isopropyl alcohol, respectively, followed each by a centrifugation at 13,000 rpm for 10 min at 4°C temperature. The DNA pellet was then washed twice in 70% ethanol and dried at 40°C for 1 h, before re-suspension in 150 µl of TE 1X. RNAs were eliminated through incubation for 1 h at 37°C with 6 µl of RNase A (SIGMA). The integrity of the DNA extracts was assessed through agarose gel electrophoresis and their concentration was determined through fluorimetric quantitation using the Hoechst 33258 dye with a GENios Plus fluorimeter (Tecan Scientific Instruments).

DNA amplification and SSR markers analysis

A total of 14 SSR primers pairs, selected from previous analyses of pearl millet genetic diversity (Mariac et al., 2006; Azevedo et al., 2012), were used in the present study (Table 1). For each pair, the 5' end of the forward primer was labeled with a fluorescent dye

(yellow, blue, green or red). The amplification was performed on 25 ng of genomic DNA as two multiplex groups consisting of 7 primer pairs each, in a reaction mix including 2.5 mM dNTPs, 20 µM of each primer pair, Taq Hot start Q 5U/µl (QIAGEN), SolQ 5X in a final volume of 10 µl. The amplification procedure consisted of an initial denaturation step at 95°C for 15 min followed by 35 cycles of 94°C for 30 s, 55°C for 1.5 min and 72°C for 1 min, followed by a final extension cycle of 72°C for 30 min. Amplification products were then diluted and mixed with GS 500Liz (Applied Biosystems Inc.) internal size standard and denaturated through heating at 95°C for 5 min prior to capillary electrophoresis in an ABI Prism 3500 Genetic Analyzer sequencer (Applied Biosystems). Fragment analysis and allele scoring were performed using the GeneMapper Software version 4.1 and double-checked manually.

Data analysis

GenAlEx software version 6.502 (Peakall and Smouse, 2012) was used to perform the genetic data analysis. Total number of alleles, allele frequencies, number of alleles per locus for each locus and population, observed heterozygosity and gene diversity (He) (Nei, 1973) were calculated. In order to graphically represent the relationships between accessions, genetic similarity indices between genotypes were calculated using the DARwin 6 software

Table 1. List of microsatellites markers used for diversity study.

SSR marker	Forward 5' - 3'	Reverse 5' - 3'
Psmp2201	CCCGACGTTATGCGTTAAGTT	TCCATCCATCCATTAATCCACA
Psmp2227	ACACCAAACACCAACCATAAAG	TCGTCAGCAATCACTAATGACC
Psmp2237	TGGCCTTGGCCTTTCCACGCTT	CAATCAGTCCGTAGTCCACACCCCA
Psmp2247	CCAAACCGTAACCTGAAAAGCTACTG	GTGTCGGTTTGCTTCGTTCCTT
Psmp2249	CAGTCTCTAACAAACAAACACGGC	GACAGCAACCAACTCCAAACTCCA
IRD12	ACTCGTTCGGATGCACTTCT	CGGGGAAGAGACAGGCTACT
IRD46	GAACAATTGCTTCTGTAATATTGCTT	GCCGACCAAGAACTTCATACA
Psmp2202	CTGCCTGTTGAGAATAAATGAG	GTTCCGAATATAGAGCCCAAG
Psmp2206	AGAAGAAGAGGGGGTAAGAAGGAG	AGCAACATCCGTAGAGGTAGAAG
Psmp2214	CGCACAGTACGTGTGAGTGAAG	GATTGAGCAGCAAAAACCAGC
Psmp2219	ACTGATGGAATCTGCTGTGGAA	GCCCGAAGAAAAGAGAACATAGAA
Psmp2220	GCATCCTTCACCATTCAAGACA	TGGGAAACAGAATGGAGAAAGAG
Psmp2231	TTGCCTGAAGACGTGCAATCGTCC	CTTAATGCGTCTAGAGAGTTAAGTTG
IRD25	CGGAGCTCCTATCATTCCAA	GCAAGCCACAAGCCTATCTC

(Perrier and Jacquemoud-Collet, 2006). The neighbor-joining method was used to construct the dendrogram. A principal component analysis (PCA) was also performed in order to visualize the distribution of the accessions according to their microsatellite diversity. Finally, an Analysis of Molecular Variance (AMOVA) was also performed using the GenAlEx software in order to estimate the total molecular variance between and within populations.

RESULTS

Diversity of species and vernacular names among area of study

During these surveys, a total of 114 accessions corresponding to 27 different popular names were collected through villages. The number of varieties varied from one area to another and across ethnic groups. Venacular names recorded were related to local usages such as quality and quantity of flour obtained from seeds, development cycle of variety (late or early). In the east-northern part of the country, pearl millet were used mainly to prepare meal and porridge for children. Nevertheless in the west-northern, the second use of *P. glaucum* is related to the preparation of traditional beverage. These results point out the diversity of use of pearl millet in the Northern Benin. Indeed, traditional beverage were consumed daily by people and represents a part of identity of most of ethnic group live in the west-northern of the country and could justify the difference observed for local usages in east-northern and west-northern parts.

Microsatellite marker polymorphism and global diversity among population

With the fourteen SSR primer pair used, 57 alleles were detected among the 114 pearl millet accessions with an average of 4.071 alleles per locus. Table 2 shows a summary of genetic values obtained for each locus. The number of alleles per locus varied from 2 to 8. The lowest and the highest values were obtained respectively for the loci Psmp2202 and Psmp2231 (Table 2). The number of private allele varied from 1 (Psmp2201, Psmp2220, Psmp2237, Psmp2247, IRD46) to 4 (Psmp2231). Genetic diversity ranged from 0.099 to 0.633 with an average of 0.405. For the observed heterozygosity, the obtained values varied from 0.105 to 0.771 with an average of 0.425. These values were obtained for the loci Psmp2247 (for the lowest) and Psmp2220 (for the highest). Most of the loci presented an excess of heterozygosis. The fixation index obtained for all loci varied from 0.303 to 0.147 with an average of 0.050.

Genetic relationship among and within population

The genetic diversity information among geographic regions of Northern Benin was presented in the Table 3. The observed allelic diversity differed from one region to another. The average number of alleles ranged from 2.429 to 3.929. The department of Donga was found to show the lowest average number of alleles while the highest value was obtained for the Alibori. On the other hand, the genetic diversity varied from 0.311 for the Donga to 0.482 for Borgou Department.

The fixation index values F varied from 0.132 to 0.021 with an average of 0.050 corresponding to a deficiency of heterozygosis within the whole population. However, the values obtained for each department showed in their majority an excess of heterozygosis for regions Borgou, Atacora, and Donga. Only the region of Alibori presented a deficiency of heterozygosis.

The Fst data translate the existing differentiation among populations (Table 4). The values obtained for the

Table 2. Genetic diversity parameters of microsatellites loci used to evaluate the diversity of pearl millet (*Pennisetum glaucum*, Poaceae).

SSR marker	Number of different alleles	Mean of different alleles Na/locus	Ne	I	Ho	He	F
Psmp2201	3.000	2.500	1.450	0.501	0.284	0.287	0.022
Psmp2227	4.000	3.750	1.691	0.762	0.467	0.401	-0.157
Psmp2237	4.000	3.250	1.850	0.762	0.404	0.447	0.123
Psmp2247	3.000	2.250	1.114	0.211	0.105	0.099	-0.051
Psmp2249	3.000	3.000	1.909	0.789	0.391	0.465	0.140
IRD12	5.000	3.500	2.205	0.881	0.568	0.533	-0.093
IRD46	3.000	2.500	1.845	0.665	0.409	0.445	0.046
Psmp2202	2.000	2.000	1.872	0.655	0.607	0.463	-0.303
Psmp2206	5.000	3.500	1.868	0.740	0.402	0.411	-0.025
Psmp2214	5.000	3.000	1.288	0.406	0.201	0.209	0.010
Psmp2219	3.000	2.750	2.106	0.815	0.624	0.523	-0.202
Psmp2220	6.000	4.750	2.757	1.188	0.771	0.633	-0.218
Psmp2231	8.000	5.250	2.175	1.071	0.453	0.531	0.147
IRD25	3.000	2.500	1.335	0.414	0.266	0.224	-0.173
Mean	4.071	3.179	1.819	0.704	0.425	0.405	-0.050
SE	0.425	0.163	0.068	0.041	0.028	0.023	0.029

Ne: Number of effective alleles, I: Shannon' information index, Ho: observed heterozygousity, He: gene diversity, F: fixation index.

Table 3. Genetic diversity parameters among geographical group of Pearl millet (*Pennisetum glaucum*, Poaceae).

Population	Number of accessions	Average number of alleles per locus	Ho	He	F
ALIBORI	50	3.929	0.432	0.448	0.021
BORGOU	16	3.357	0.494	0.482	-0.036
ATACORA	29	3.000	0.412	0.380	-0.056
DONGA	19	2.429	0.362	0.311	-0.136
Mean	-	3.179	0.425	0.405	-0.050
standard error	-	0.163	0.028	0.023	0.029

Ho: Observed heterozygousity, He: gene diversity, F: fixation index.

Table 4. Estimates of Pairwise Fst values (below diagonal) among the different collection regions.

Region	Alibori	Borgou	Atacora	Donga
ALIBORI	0.000			
BORGOU	0.000	0.000		
ATACORA	0.035	0.043	0.000	
DONGA	0.060	0.079	0.008	0.000

index of differentiation Fst among pairs of geographical groups varied from 0 (between Borgou and Alibori) to 0.079 (between Borgou and Donga). The obtained values showed a very low level of differentiation among the various geographical groups. No differentiation could be observed between Borgou and Alibori on one hand and a very low level between Atacora and Donga on the other hand. This result could be justified by the close

geographical proximity of such regions. Indeed, Borgou and Alibori represent the north eastern part of Benin, while Atacora and Donga represent the north western one. It is then understandable that seeds exchange could have happened between Borgou and Alibori on one hand and between Atacora and Donga on the other hand. The only and moderate differentiation observed was found to occur between Alibori and Donga and Borgou and Donga. This situation could be probably explained by the relative geographical isolation of these regions.

An AMOVA was also performed among regions, populations, accessions and within accessions (Table 5). The results of AMOVA showed that variations could be observed at two levels: firstly within accessions and then among regions. This analysis revealed that the widest variation (95%) was observed within accessions and not between accessions. No variation was captured among populations and among accessions. However, little variation (5%) was observed between geographical

Table 5. Results from analysis of molecular variance (AMOVA) using two regions and 4 groups of populations.

Parameter	df	SS	MS	Estimated variance	Variation (%)
Among regions	1	19.509	19.509	0.150	5
Among populations	2	5.687	2.844	0.000	0
Among accessions	110	321.366	2.922	0.000	0
Within accessions	114	338.623	2.970	2.970	95
Total	227	685.185	-	3.121	100

df: Degrees of freedom; SS: sum of squares; MS: mean square.
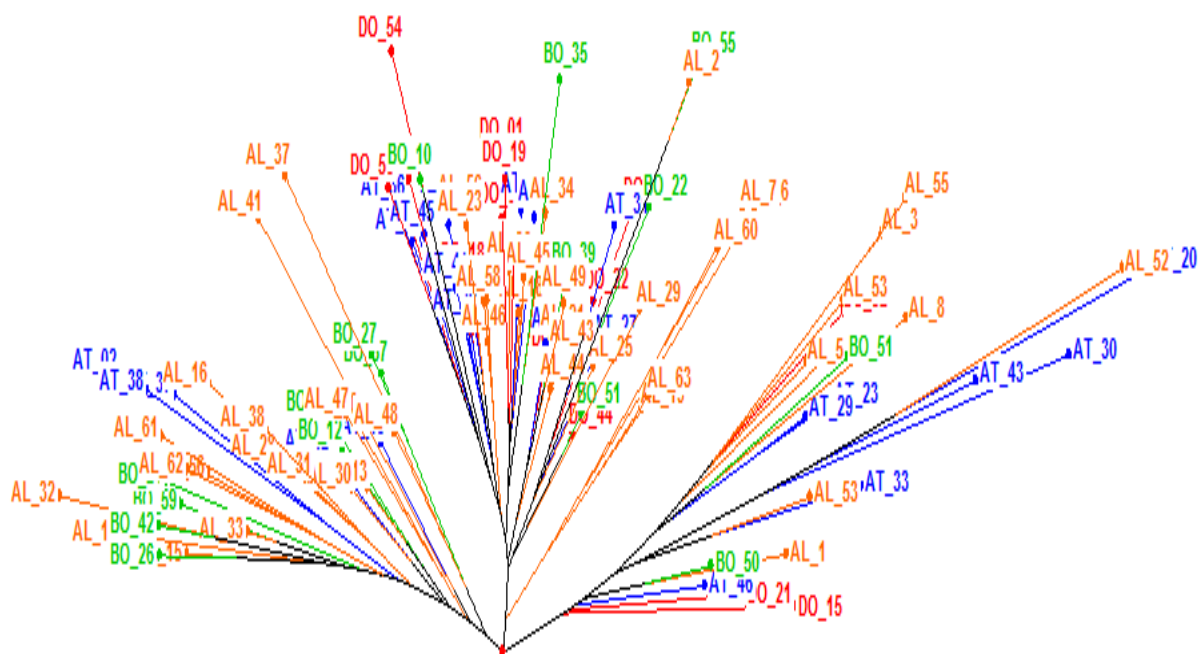
Figure 2. Neighbour joining dendrogram from 114 pearl millet accessions obtained from the UPGMA method. Accessions from Alibori, Borgou, Atacora and Donga are respectively shown in orange, green, blue and red.

regions.

The structure of accessions under study and PCA

The graphical representation of genetic relationships among accessions was drawn with a dendrogram taking into account the various genetic distances of Nei. The analysis of this dendrogram revealed that there is no particular structuration of the diversity of accessions according to their geographical origin. Even if three clusters were obtained from the dendrogram (Figure 2), each of them was found to gather accessions originating from each of the four regions.

In order to better visualize the distribution of variability and to point out any genetic information related to the 114 accessions under study from the previous

dendrogram, a PCA was also performed (Figure 3). The first two axes of PCA explained respectively 22.19 and 7.69% with a cumulative variation of 29.88%. However, neither a clear pattern nor a geographical separation could be evidenced. A compact homogeneity was observed from the PCA representation. The PCA showed a similar result when an analysis of the dendrogram based on the genetic similarity among accession was performed; also, it indeed revealed the absence of any geographical structuration among accessions.

DISCUSSION

The northern where Pearl millet was cultivated, its consumption differed from one region to another. Indeed, in the part of the north east, it is used mainly to prepare
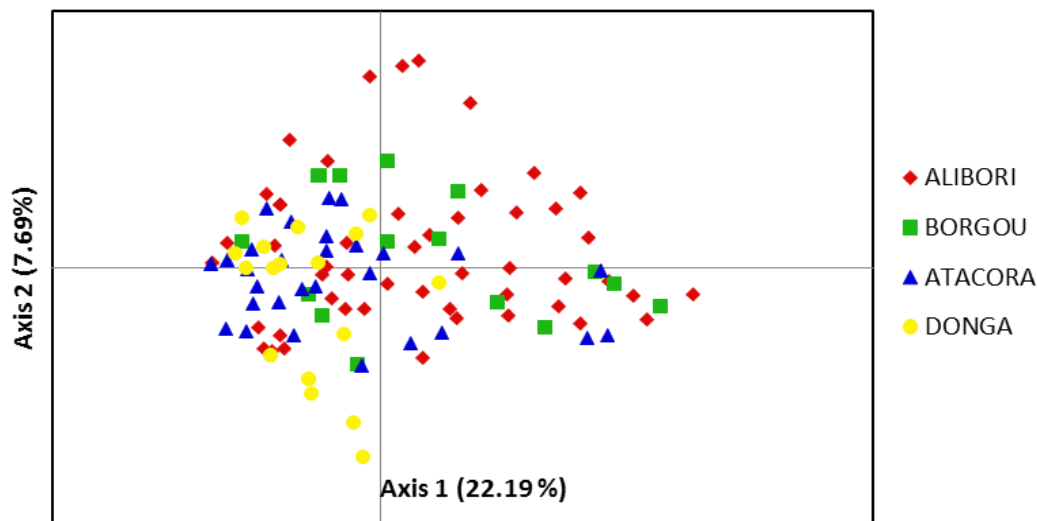
Figure 3. Principal component analysis performed on the 114 pearl millet accessions under study.

local beverage and meal with its flour. In the north west, it is used principally for beverage. This difference in usage could also be observed in the choice of the varieties cultivated throughout the various regions.

Our study aimed to assess the level of genetic diversity among cultivated pearl millet accessions collected in Northern Benin using SSR molecular markers. Using this approach, no significant variations were found among pearl millet accessions for most of the studied agromophological parameters (Bahir et al., 2014). These variations showed some limitations such as phenotype modifications due to environmental influence, when compared with molecular markers (Ferreira, 2006). The SSR molecular markers are known to be efficient for genetic diversity studies of pearl millet (Mariac et al., 2006; Bashir et al., 2015; Shakhatreh et al., 2015). The current study showed a lower average (4.071) number of alleles per locus compared to that reported by Mariac et al. (2006) which was 6.2 for 421 cultivated accessions from Niger, and 16.4 for accessions from a wide geographic range in West and Central Africa (Stich et al., 2010). Other authors reported the analysis of 214 Sudanese pearl millet accessions in which they found a high level of alleles was reported with an average of 13.3 per locus (Bashir et al., 2015). The low number of alleles observed in the present study could be explained by (1) the limited number of markers involved and (2) a smaller sample size when compared to previous studies.

Genetic variability contributes to a long term selection (Allard, 1960). According to our results, there is a moderate genetic diversity level among accessions. The average gene diversity (He = 0.405) obtained for the 114 cultivated accessions was lower than those reported in others pearl millet genetic diversity studies based on SSR molecular maker. Indeed Stich et al. (2010) reported on a He = 0.74 across west and central pearl millet inbreds,

Mariac et al. (2006) 0.49 among landraces from Niger and 0.77 for Sudanese cultivated accessions (Bashir et al., 2015). Although the number of samples and markers used did vary from one study to another, the estimated genetic diversity cannot be a function of the size of our sample. This explanation had been also underlined by Stich et al. (2010). The obtained value could be explained by a relatively weak polymorphism in our pearl millet samples when compared with others' studies. This remark was also supported by the number of alleles obtained per marker. However, our results revealed a level of diversity among local pearl millet landraces that could be considered as useful for improving existing landraces and the development of local conservations strategies for biodiversity. As mentioned by Wang et al. (2012), high genetic diversity is favourable for genetic marker development, construction of segregating populations and provides enriched gene resources for gene mining in the grass family.

However, the AMOVA and Fst pairwise comparison revealed that no differentiation was observed within geographical regions. The lack of differentiation among populations could be explained by the closeness of populations of the northeast and the northwest which contributed to the high rate of pollen-mediated gene flow and by a high frequency of seeds exchange among farmers. Moreover, the possibility of alleles sharing and exchange contributed also to that situation. Similar results were reported by Bashir et al. (2015). According to Stich et al. (2010), the lack of regional differentiation could be explained by the fact that pearl millet is a highly allogamous plant with an out-crossing rate exceeding 85%. The little variation observed among the regions could be attributed probably to the climatic conditions existing in the north-eastern and the north-western part of Benin. Indeed, the northwest is characterized by a high

mountainous landscape which probably influenced the genetic adaptability of landraces. Most of the differentiation (95%) was present within accessions. This could be due to the natural selection and gene flow as it had been reported in many studies (Turpeinen et al., 2003; Nevo et al., 2004; Shakhatreh et al., 2015). According to Nevo et al. (1998), natural selection appears to be a major differentiating and orienting force of regional evolutionary change, maintaining genetic polymorphisms under conditions of environmental heterogeneity and stress.

The PCA showed no clear regional structuration of landraces. Moreover, the dendrogram obtained from a neighbour joining revealed also a similar result. These observations confirmed the previous results reported earlier and suggested that there is a great genetic similarity among cultivated regions of pearl millet in the Benin northern. It could be related to seeds exchange among regions which highly contributed to intermixture of accessions as mentioned earlier. Similar results were reported among the cultivated landraces in Niger (Mariac et al., 2006) and Sudanese (Bashir et al., 2015). Our results will contribute highly in the development of the pearl millet conservation strategies in the Northern Benin.

Conclusion

Conclusively, the present study revealed the genetic relationship and diversity among cultivated pearl millet accession in Northern Benin. Our results revealed a moderate genetic diversity and the weak extent of differentiation between regions. Given the number of alleles obtained per locus, the local plant material could be useful for the genetic improvement of existing varieties. However, an agromorphological characterization of accessions must be conducted and combined with genetic information in order to identify the best accessions which could be integrated into future plant breeding programs.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

Abbreviations

AMOVA, Analysis of molecular variance; **MATAB,** mixed alkyltrimethylammonium bromide; **SSR,** simple sequence repeat.

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Full Length Research Paper

Enhancing the production of syringomycin E in *Pseudomonas syringae* pv *syringae* by random mutagenesis and molecular characterization of the *SyrB1* gene

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Syringomycin E (SRE) is a phytotoxin produced by *Pseudomonas syringae pv syringae* with high potential as a safe and effective therapy for the control of human fungal infections and as a preservative for the food industry. In this study, 27 strains of *P. syringae* pv *syringae* were isolated from plums, potatoes, apricots and peaches, of which *P. syringae* pv *syringae* 120 (PS120) showed the highest SRE production levels. Furthermore, random mutagenesis induced by ultraviolet (UV) radiation resulted in the generation of a *P. syringae pv syringae* mutant that produced 30% more SRE than the parental strain PS120. To elucidate molecular mechanism underlying the higher SRE production ability of the mutant strain, *syrB1* and *syrB2* genes, which are known to be involved in SRE production, were cloned and sequenced. The nucleotide and amino acid sequences analysis showed that UV radiation induced numerous mutations at the AMP binding site on adenylation domain of *syrB1*, while no mutation was detected in *syrB2* gene. Real-time polymerase chain reaction (PCR) results showed that expression of *syrB1* gene in mutant strain was six-fold higher than that of PS120 strain. Taken together, these results suggest that the mutations at AMP binding site and overexpression of *syrB1* were responsible for increased biosynthesis of SRE in *P. syringae pv syringae*.

Key words: Antifungal protein, syringomycin E, UV mutagenesis, *Pseudomonas syringae* pv *syringae, syrB1*.

INTRODUCTION

The widespread resistance to commonly used antimicrobials and an increasing public health concern about the presence of chemicals in the food-chain have stimulated search for novel, effective and safe

antimicrobial compounds. As a result, new resources and approaches are being explored to identify/develop the next generation of antimicrobials. Syringomycin has emerged as a new class of peptide presenting strong antimicrobial activity. For example, numerous studies have demonstrated the effectiveness of syringomycin E (SRE) against multiple filamentous fungal and yeast pathogens (Mazu et al., 2016; Im et al., 2003; Bender et al., 1999; De Lucca and Walsh, 1999; Fukuchi et al., 1992). The SRE can kill 95 to 99% of *Aspergillus* strains (*Aspergillus flavus, Aspergillus niger,* and *Aspergillus fumigatus*) and *Fusarium* (*Fusarium moniliforme* and *Fusarium oxysporum*) in concentrations from 1.9 to 7.8 µg/ml, but is non-toxic to mammalians and plant cells (De Lucca and Walsh, 1999; Takemoto et al., 2010). The SRE is also more effective at killing germinated spores of *Aspergillus* and *Fusarium* species than other peptides such as cecropin A, cecropin B and dermaseptin (De Lucca and Walsh, 1999). Kawasaki et al. (2016) showed that SRE inhibits 50 and 90% of *P. ultimum* oospore germination at 31.3 and 250 µg/ml, respectively.

In vitro growth inhibition experiments have further shown that SRE is toxic to a number of fungi such as *A. fumigatus* (opportunistic fungal pathogen) and *Mucor* species, *Trichophyton* species (parasitic skin fungus), responsible for causing fungal infections in humans (Muedi et al., 2011). In addition, the potential of SRE as an effective preservative for agricultural products has also been demonstrated; SRE was reported to be effective against a range of fungi involved in food spoilage including *Penecillium digitatum* in lychee, and *Aspergillus, Rhizopus* and *Fusarium* in orange, lychee, mango and dragon.

Syringomycin E, a natural phytotoxic, is a small cyclic lipodepsinonapeptide (ca. 1,200 Da) produced by the plant bacterium *P. syringae pv. syringae* (Guenzi et al., 1998; Raaijmakers et al., 2006). *P. syringae pv. syringae* also produces the large cyclic lipodepsipeptide, syringopeptins (Bensaci et al., 2011). The syringomycin gene cluster in *P. syringae pv. syringae* contains four genes involved in the biosynthesis of syringomycin E, including *SyrB1, SyrB2, SyrC* and *SyrE* (Guenzi et al., 1998); of which expression levels are regulated by *salA, syrG,* and *syrF* (Wang et al., 2006; Vaughn and Gross, 2016). *SyrB1* and *syrB2* function in generating one of the unusual amino acids of SRE scaffold, 4-Cl-$_L$-Thr residues. *SyrB1* gene with approximately 2 kb in length encodes for a 66 kDa enzyme consisting of an adenylation (A) domain which is responsible for amino acid selection and activation, and a thiolation (T) domain which is responsible for thioesterification of the activated substrate (Bender et al., 1999). The A domain catalyzes the activation and binding of L-Thr to phosphopantetheninyl

in T domain, where it undergoes halogenation catalyzed by SyrB2 to create 4-Cl- thr-S- SyrB1 (Vaillancourt et al., 2005). Next, SyrC enzyme transfers the 4-Cl-Thr group from SyrBs1 to SyrE to create the complete SRE (Singh et al., 2007). Syringomycin is a cyclic lipodepsinonapeptide composed of a 3-hydroxy fatty acid tail. SRE, which contains a 3-hydroxy dodecanoic acid tail, is the major form produced by *P. syringae pv.syringae* strain B301D (Bender et al., 1999). Syringomycin is synthesized via a nonribosomal peptide synthetase system (Bender et al., 1999; Zhang et al., 1995). The syringomycin gene cluster is located in a 55 kb DNA region in the genome of B301D strain containing genes that are responsible for biosynthesis, regulation, and secretion of syringomycin (Lu et al., 2002; Quigley et al., 1993; Zhang et al., 1995, 1997).

The lower production efficiency of SRE by *P. syringae pv. Syringae* has been the major barrier to its cost-effective production and industrial application. Currently, much effort is focused on finding new ways to improve SRE production efficiency and develop SRE-based fungicides. Hence, the key objectives of this study was to determine if exposure to UV could be used to generate *P. syringae pv. syringae* mutants with enhanced SRE production efficiency and to examine molecular mechanisms, especially the role of *syrB1* and *syrB2* genes.

MATERIALS AND METHODS

Bacteria isolation

P. syringae pv. *syringae* was isolated from stone fruit trees such as plums, apricots or peaches, and potatoes in Hanoi, Vietnam according to the method of Mohammadi et al., (2010). Briefly, biological samples were surface-sterilized by 70% ethanol for 2 min before incubation in distilled water with shaking for 2 h. 100 µl of suspension were plated on CMB agar supplemented with 5% sucrose. Colonies that produced fluorescence under UV light were picked and grown on King's B media; gram stained and identified by standard biochemical assays for *Pseudomonas* species. Identification methods include catalase, oxidase, arginine dihydrolase, gelatin hydrolase and nitrate reductase assays together with levan production. Strains with correct biochemical activity were finally confirmed as *P. syringae* by 16S rDNA analysis.

Syringomycin production bioassay

The ability to produce SRE of *P. syringae pv syringae* was determined as described previously (DeVay and Gross, 1977). In brief, bacterial cells were grown overnight in nutrient broth yeast (NBY) medium at 25°C. The cells were harvested by centrifugation, washed with sterile deionized water, and adjusted to a final cell density of ~2 × 10^8 CFU/ml. Five microliters of the bacterial suspension was inoculated to the previously prepared PDA plates

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Table 1. PCR primer sequences.

Gene	Primer	Sequence	Product size (kb)
16SrRNA	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	1.4
	1492R	5'-CGGTTACCTTGTTACGACTT-3'	
SyrB1	Forward	5'-GTTAGTCGGTGCTGAAATGC-3'	2.2
	Reverse	5'-CATTTCTTCTGGCGAGTAAGC-3'	
SyrB2	Forward	5'-GAGCAAGCTCAAGACACATTAC-3'	1.3
	Reverse	5'-GCATCGGAGGAAATAGTCATGG-3'	

and incubated at 25°C for 4 days. The inoculated plates were overlaid with *Geotrichum candidum* VTCC-Y-0483 as an indicator organism and were further incubated for 24 h at 25°C in the dark. Clear zones of fungi growth inhibition around bacterial colonies were considered as an indicator of syringomycin production. One unit of antifungal activity was defined as the amount of the final dilution of cell-free extract in 1 mM HCl required to completely inhibit the growth of *G. candidum* in the area where a 10 µl droplet was applied on PDA plate (Sinden et al., 1971). Each assay was repeated independently three times with three plates per replicate.

The presence of syringomycin in *P. syringae* fermentation broth was confirmed by HPLC and LC-MS as previously described (Singh et al., 2007). Briefly, cells from 24-h cultures in NBY media at 25°C were pelleted by centrifugation, and supernatant extracted with water-saturated *n*- butanol at pH 2 before purification by cation-exchange chromotography and analysis by HPLC. HPLC was carried out with 20 µl of sample loaded onto a octadecylsilane bounded silica (5 µm) column (25 cm × 4.6 mm). The mobile phase, a mixture of 50 volume of 0.2% (w/v) acetic acid in water, 25 volume of acetonitrilc and 25 volume of methanol, was filtered and de-gased. HPLC was performed at a flow rate of 1 ml/min and observed at 240 nm. LC-MS analysis was performed on an LCMS-QP8000 a spectrometer (Shimadzu, Japan) with a Vydac C18 LC-MS column (Thermo Fisher Scientific, USA).

Random mutagenesis

P. syringae pv syringae parental strain was cultured at 180 rpm at 26°C on nutrient broth (NB) medium for 16 h. Biomass was collected by centrifugation; pellet was re-suspended in sterile distilled water to an OD of 0.5 to 0.6 and subjected to a UV light treatment with power of 40 W at various time doses. After 48 h culturing at 26°C, cells were plated to count the surviving colonies.

DNA preparation

P. syringae pv *syringae* mutant and parental strains were grown for 48 h at 28°C in SRM medium and collected by centrifugation at 16128 rpm for 2 min. Total DNA extraction was performed using PureLink[TM] Genomic DNA Mini Kit (Invitrogen, USA) according to the manufacturer's instructions. DNA was stored at -20°C until used.

Cloning and sequencing of 16S rRNA, syrB1 and syrB2 genes

The 16S rDNA, syrB1 and syrB2 genes were amplified by PCR with the specific primer pairs (Table 1) using Thermo cycler engine (AB system). The reaction mixture (25 µl) containing 12.5 µl of master mix (Thermo Fisher Scientific, USA) 10 pmol of each forward and reverse primers and 2 µl total DNA. The PCR conditions were as follows: 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 56°C for 40 s, 72°C for 1 min 30 s, and a final extension at 72°C for 7 min samples were then stored at 4°C until used. The PCR products were purified using the GENCLEAN® II Kit (Q-BIO gene, Carlsbad, CA, USA), and directly cloned into a TOPO TA cloning vector (Invitrogen, USA) according to the manufacturer's instructions and sequenced with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA) using M13 forward primer and M13 reverse primer.

Real-time RT-PCR assay

Total cellular RNA was extracted using the TRIzol reagent (Invitrogen, USA) as per the manufacturer's instructions. The extracted RNA was treated with DNase I at 37°C for 3 h to remove DNA. Reverse transcription was carried out with the Superscript TM III First-Strand Synthesis System (Invitrogen, USA) according to the manufacturer's instructions. In detail, 500 ng mRNA was used as template for reverse transcription reaction. The cDNA was then used as a template for qualitative real-time PCR analysis using specific primers (Table 2). As a control for the input RNA, level of a housekeeping gene 16S rRNA was also assayed. The data was analyzed by LightCycler 2.0 software (Roche, USA).

RESULTS

Screening of *P. syringae* strains for SRE production

In this study, a number of bacterial strains were isolated from leaf tissues of plums, peaches, apricots, tomatoes and screened for production of syringomycin as described earlier. Among them, 120 strains were *P. syringae pv syringae* strains as indicated by biochemical assays and 16S rDNA analysis (data not shown). 14 strains produced antifungal metabolites characteristics of syringomycin and PS120 strain showed the highest amount of antifungal activity (36 U/ml) (Table 3).

Characterization of antifungal compounds produced by strain PS120

The antifungal compounds of PS120 were extracted and purified and analyzed by SDS-PAGE; similarly extracted metabolites from a non-syringomycin producing strain

Table 2. Real-time PCR primer sequences.

Gene	Primer	Sequence	Product size (bp)
SyrB1(PS120)	Forward	5'-GAGCACATCGTGCGTGACAG-3'	154
	Reverse	5'-TCGATACGGGTGTCTTCAGC-3'	
SyrB1(PS120-15)	Forward	5'-TGGTTATTACACAGGAGCCTTGG-3'	260
	Reverse	5'-CGACAATTTCCTGAGCGCAC-3'	
SyrB2(PS120)	Forward	5'-TTAACTGCGGAACAGCGTGC-3'	179
	Reverse	5'-CATAGTTGGCGATGTTGGTAC-3'	
SyrB2(PS120-15)	Forward	5'-TTAACTGCGGAACAGCGTGC-3'	189
	Reverse	5'-CGGTCATAGTTGGCGATGTTG-3'	
16SRNA	Forward	5'-ACACATGCAAGTCGAACGAG-3'	100
	Reverse	5'-CGTCCGTTTCCAGACGTTAT-3'	

Table 3. Syringomycin production levels of the isolated *P. syringae* strains.

S/N	Strains	Antifungal activity (U/ml)	No.	Strains	Antifungal activity (U/ml)
1	PS13	17	8	PS57	12
2	PS41	13	9	PS17	15
3	PS28	10	10	PS89	27
4	PS53	20	11	PS52	34
5	PS120	36	12	PS15	15
6	PS54	17	13	PS25	25
7	PS32	26	14	*P. syringae* ATCC55389	31

were used as a negative control. Fifteen micrograms of the purified compound and an equal amount of standard SRE (Sigma, USA) were separated on 12% polyacrylamide gel, and then stained with Coomassie-blue. The results showed that the purified compound was a protein with MW similar to that of the standard SRE (Figure 1). To determine whether this compound was syringomycin, its antifungal activity was examined against *G. candidum* strain VTCC-Y-0483 using a standard plate bioassay. It was observed that this compound produced large zone of inhibition against *G. candidum*. In contrast, no zone of inhibition was observed with the compound extracted from non-syringomycin producing strain (Figure 2).

Further analysis of the purified compound by HPLC revealed that it has the same retention time as the standard SRE (Sigma, USA). The standard SRE appeared at 4.283 min, while that from *P. syringae pv syringae* PS120 was at 4.281 min (Figure 3), indicating the purified compound was SRE. The identity of the purified compound was further analysed using ESI mass spectroscopic analyses. It was found that the purified compound generated MH+ species with molecular weights of 1224 to 1226 and major fragmentation species with molecular weight of 613 and 614 (Figure 4) that are
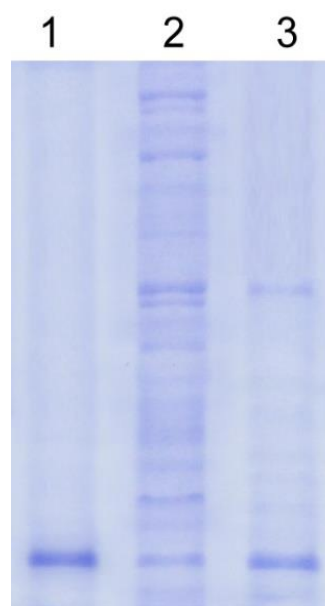
Figure 1. SDS-PAGE gel electrophoresis of SRE product. 1, standard SRE; 2, fermentation supernatant; 3, purified SRE of PS120 strain.
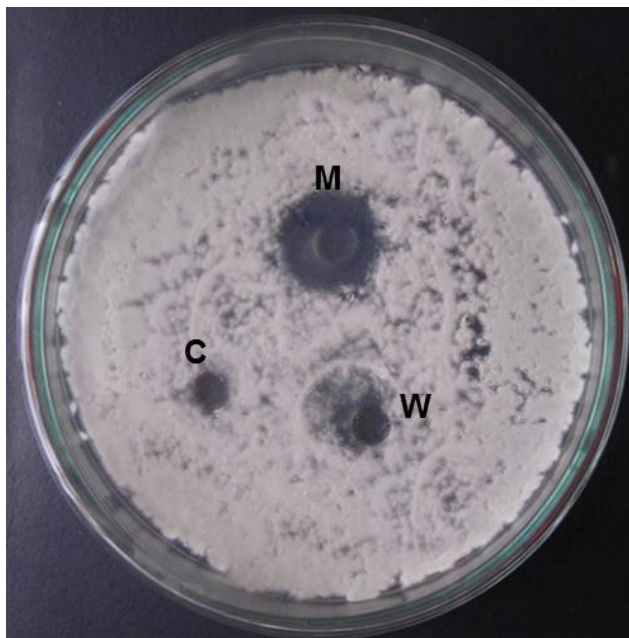
Figure 2. Plate bioassays for syringomycin production by *P. syringae pv. syringae* parental strain PS120 and mutant strain PS120-15. Indicator strain *G. candidum* VTCC-Y-0483 was overlaid on pre-inoculated bacterial suspension and zones of growth inhibition were observed after 24h at 25°C. C, the compound extracted from non-syringomycin production strain as negative control; W, the compound extracted from PS120; M, the compound extracted from PS120-15 mutant.
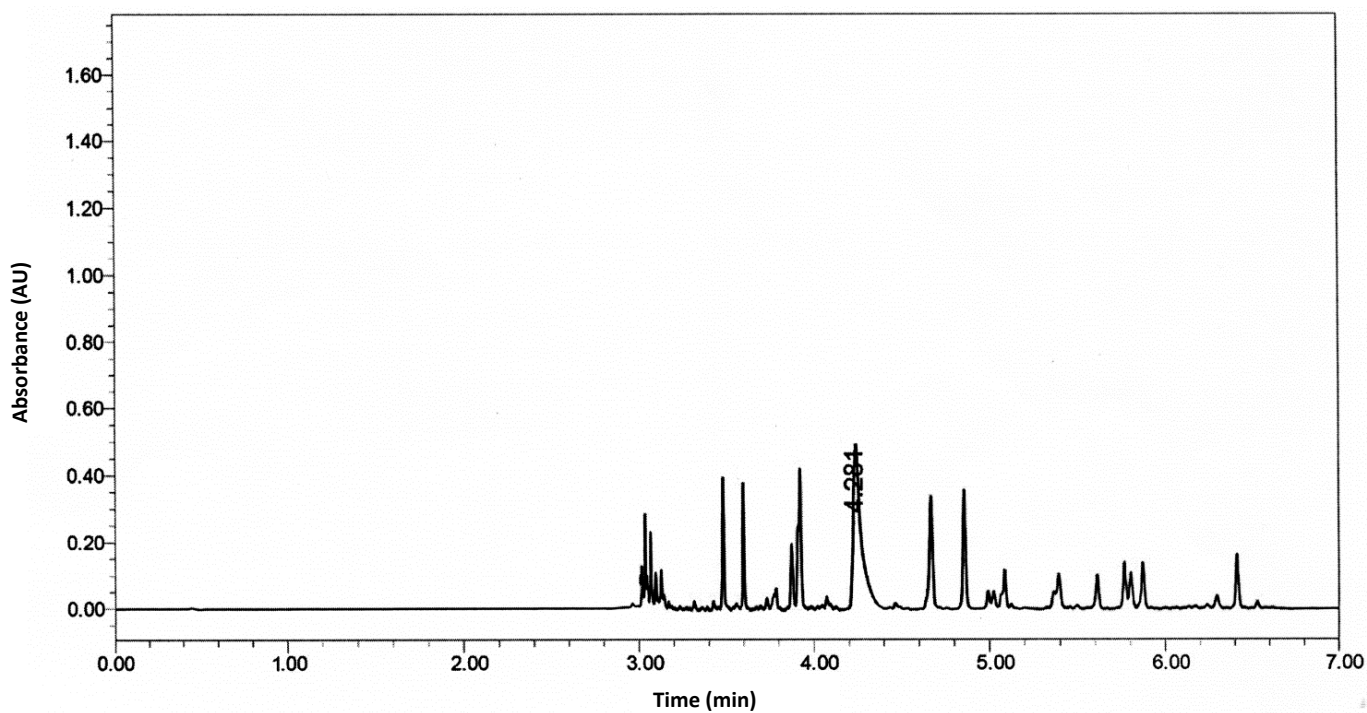
Figure 3. HPLC analysis of purified syringomycin. Purified fermentation broth of *P. syringae pv syringae* PS120 was analyzed using HPLC and showed a major peak among others at retention time of 4281 min, matched retention time of standard SRE at 4283 min, indicating the presence of syringomycin in fermentation broth.
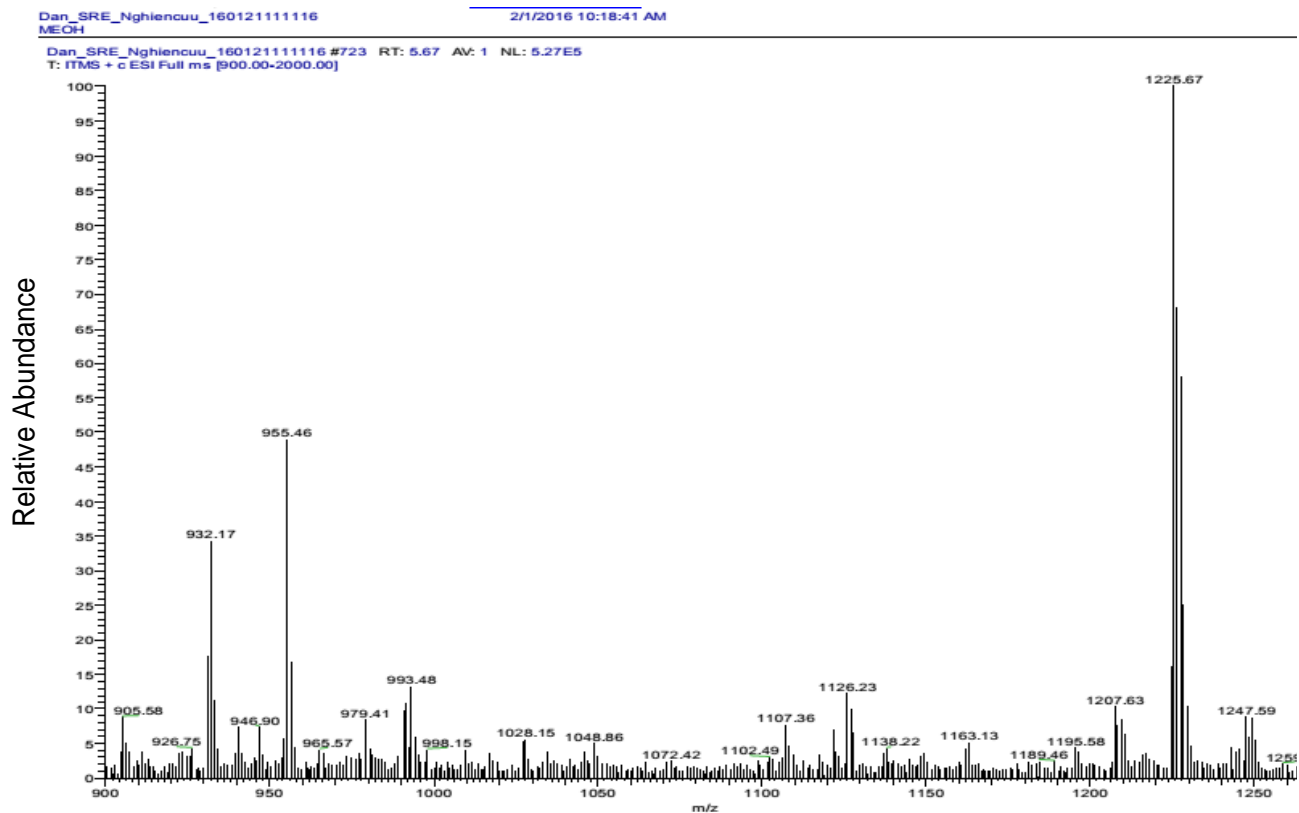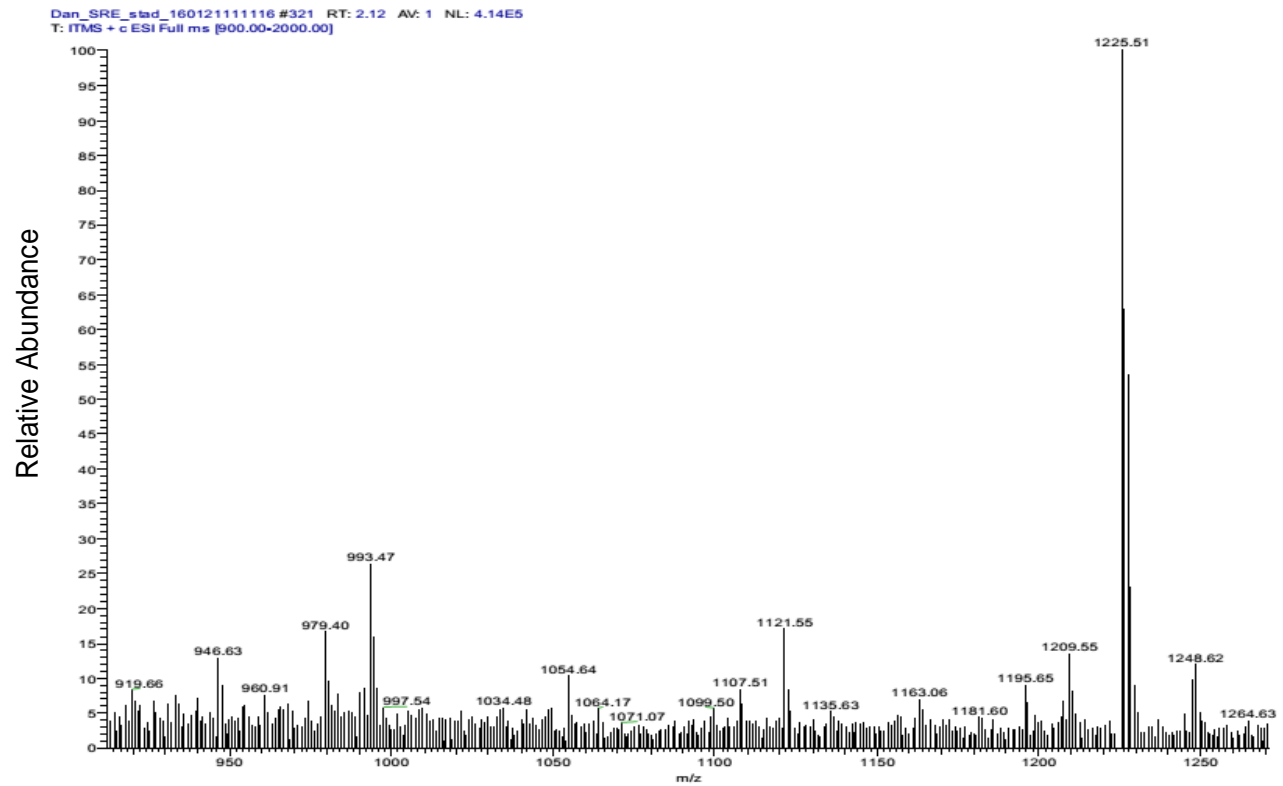
Figure 4. ESI mass spectroscopic analysis of the purified syringomycin. Purified compounds gave MH+ species with molecular weights of 1224-1226 and major fragmentation species with molecular weight of 613 and 614, compatible with the proportion of 2 isoforms of chlorine (^{35}Cl and ^{37}Cl), which are characteristics of SRE.
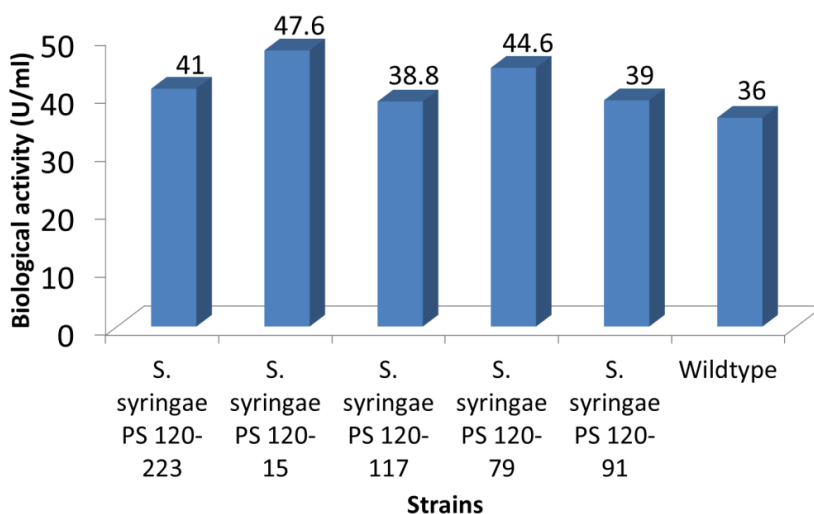
Figure 5. Biological activity of SRE from top five mutants. Among all mutants, five trains PS120-223, -15, -117, -79 and -91 showed the highest increase in SRE production, with 20 to 32.3% increase comparing to parental strain.

consistent with the proportion of 2 isoforms of chlorine (^{35}Cl and ^{37}Cl), the key characteristics of SRE.

Enhancing the production level of SRE

PS120 strain was exposed to various doses of UV to create mutant strains with enhanced SRE production. Following initial screening, 334 mutants were selected that survived after exposure to UV light for 180 s. The SRE production capacity of these mutants was assessed from their ability to inhibit the growth of *G. candidum.* The mutants with increased SRE production were selected following the method described by DeVay and Gross (1977). Of the 334 mutants, five were found to produce significantly higher levels of SRE (20 to 32.2%) compared with the parental PS120 strain (Figure 5). *P. syringae* mutant (PS120-15) showed the highest SRE production (47.6 U/ml), that is 32.2% higher than the parental strain (36 U/ml). This result was further supported by a larger inhibitory zone to *G. candidum* induced by PS120-15 compared to PS120 (Figure 2). PS120-15 mutant was further studied to understand the molecular mechanism responsible for the increased SRE production.

Analysis of syrB1 and syrB2 genes and their expression

SyrB1 and *syrB2* genes from both wild type (PS120) and mutant (PS120-15) strains were PCR amplified using specific primers as described earlier. The PCR products were then cloned, sequenced and analyzed using Bioedit

(Tom Hall, Ibis Biosciences). While no change in *syrB2* sequence was detected following UV treatment, an additional nucleotide fragment was found inserted in *syrB1* sequence in PS120-15 mutant that led to the insertion of an amino acid fragment in syrB1 protein (Figure 6). Further analysis revealed that the inserted fragment was located at the AMP binding site (between the position of 304-408 of syrB1), an important element in the biosynthesis of SRE (Kleinkauf and Von Dohren, 1996).

The expression of *syrB1* and *syrB2* in wildtype and mutant was then measured by real-time PCR assay. The data showed that expression level of syrB1 in mutant strain was six-fold higher than that in the wild type strain (Figure 7). On the other hand, the expression level of *syrB2* remained unaffected (data not shown). This suggests that the insertion of amino acid fragment in syrB1 was associated with increased expression of *syrB1* and that this may be responsible for increased production of SRE in the mutant strains.

DISCUSSION

Because of the SRE antimicrobial activities and due to the fact that it has no impacts on both human health and environment, SRE has been the focus of much research with ultimate aim to develop new generation of antimicrobials. SRE has been shown to be an effective fruit preservative agent against a range of fungal species including *G. candidum, Rhodotorula pilimance, Botrytis cinerea, Fusarium, Pythium ultimum, Rhizoctonia, Greeneria uvicola, Aspergillus japonicus, Mucor* species,

A

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PS120      GGCGAAGCGTTGCCCGCCAGCGTGCTGCGTCCCTGGGTCGAGCGCCATGGCGA-------
           ||||||||||||||||||||||||||||||||||||||||||||||||||||
PS120-15   GGCGAAGCGTTGCCCGCCAGCGTGCTGCGTCCCTGGGTCGAGCGCCATGGCGACCAGAAG

PS120      ------------------------------------------------------------
PS120-15   CCTGCGCTGATCAACATGTACGGCATCACCGAGGCCACCGTTCACACCACCTTCAAGCGA

PS120      ------------------------------------------------------------
PS120-15   GTGTTGGCGCAGGATCTGGAAACAGCAGCCATGGTGTCTCTCGGCAAGCCGCTGGACGGC

PS120      -----------------------------------------------------------G
                                                                      |
PS120-15   TGGCGCCTGCACCTGCTTGACGCGAGTCAGACTCCGGTCGCGGCAGGCACTACTGGCGAG

PS120      TTGTACATCGAAGGCGCGGGCGTGGCTCAGGGCTACCTGAACCGTGAGGCGCTCAACGTC
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PS120-15   TTGTACATCGAAGGCGCGGGCGTGGCTCAGGGCTACCTGAACCGTGAGGCGCTCAACGTC
PS120      GAGCGCCTTCATCGAGTTG
           |||||||||||||||||||
PS120-15   GAGCGCCTTCATCGAGTTG
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B

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PS120      287  GEALPASVLRPWVERHG----------------------------------------------- 338
PS120-15   287  GEALPASVLRPWVERHGDQKPALINMYGITEATVHTTFKRVLAQDLETAAMV 338
```

Adenylation domain →

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PS120      339  -------------------------------------------ELYIEGAGVAQGYLNREALNVERFIEL 392
PS120-15   339  SLGKPLDGWRLHLLDASQTPVAAGTTGELYIEGAGVAQGYLNREALNVERFIEL 392
```

Adenylation domain →

Figure 6. Comparison of nucleotide sequence (A) and amino acid sequence (B) of SyrB1 of the Ps120-15 with PS120. Sequence analysis using Bioedit showed an insertion in SyrB1 sequence in PS120-15 mutant strain leading to the insertion of an amino acid fragment located at the AMP binding domain of SyrB1 protein.
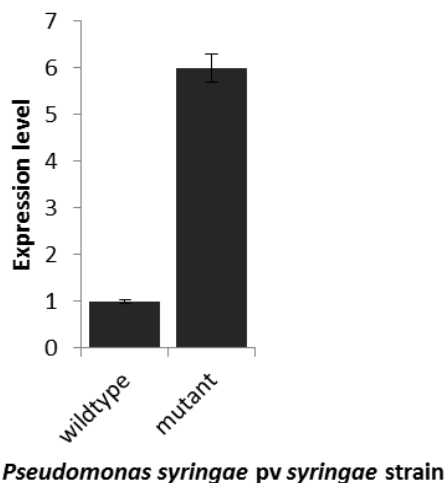
Figure 7. Comparing expression of SyrB1 in mutant and wildtype strains at transcriptional level. Expression level of SyrB1 in both strains was measured by realtime PCR using specific primers and normalized against 16S rRNA. Mutant strain expressed SyrB1 six times higher than that of parental strain.

Trichophyton species*, Curvularia brachyspora, Nigrospora sphaerica, Penicillium thomii* and *Penicillium sclerotiorum* (Breen et al., 2015; D'aes et al., 2010; Bull et al., 1998; Janisiewicz and Bors, 1995). In addition, it exhibits toxicity against Hela cells at 20-times higher concentrations than its fungicidal concentration that kills 95% of pathogenic fungi (De Lucca et al., 1999).

A number of studies have been conducted to isolate *P. syringae* pv. *Syringae* strains which produce SRE (Janisiewicz and Bors, 1995; Cirvilleri et al., 2005; Singh et al., 2007). In this study, a total of 260 *P. syringae pv syringae* strains was isolated and 27 strains produced antifungal metabolites characteristics of syringomycin was selected. Among these, PS120 showed the highest amount (36 U/ml). However, the production efficiency of SRE of PS120 is still low and has been the major barrier to its cost-effective production and industrial application. With the aim to create *P. syringae* pv. syringae mutants with enhanced ability to produce SRE, random mutagenesis approach was applied using UV light treatment. The effectiveness of UV light induced mutagenesis has

previously been reported (Ikehata and Ono, 2011), such as to increase production of clavulanic acid by *Streptomyces clavuligeru*s (Lee et al., 2002) and bio-insecticides production in *Bacillus thuringenesis* (Ghribi et al., 2004). Interestingly, five of the 334 mutants, resulting from random mutagenesis induced by exposure of PS120 strain to UV light, produced significantly higher levels (20 to 32%) of SRE than the parental strain PS120. Out of the 334 mutants screened, a mutant strain (PS120-15) producing the highest level of SRE was selected that synthesized SRE up to 30% higher than its parental strain. These findings are consistent with the results of earlier studies that used UV light-induced mutagenesis to enhance production of bioinsecticides by *B. thuringiensis* (Ghribi et al., 2004) and clavulanic acid by *S. clavuligerus* (Muedi et al., 2011).

Although significant efforts have been made toward understanding the molecular mechanisms that trigger and control the biosynthesis of SRE, much is still unknown about this process (Roongsawang et al., 2011). Transposon mutagenesis performed by Xu and Gross (1988) and Zhang and Gross (1995) has proven that a chromosomal region larger than 25 kb is involved in the biosynthesis of SRE. Within this region, four genes, namely, *syrB, syrC, syrD* and *syrP* have been sequenced and partially characterized (Martinie et al., 2015; Singh et al., 2007; Gross, 1991; Zhang and Gross, 1997; Quigley et al., 1993). *SyrD* has similar sequence to the ATP binding cassette transporter superfamily, thus, it is hypothesized that *syrD* product is involved in the transportation of SRE across the cytoplasmic membrane (Quigley et al., 1993). Further sequencing investigation by Guenzi et al. (1998) showed that *syrB* is actually organized in two ORFs, *syrB1* and *syrB2* encoding a 67 kD protein and a 36 kD protein, respectively. The 67 kD protein encoded by *syrB1* carries all known conserved regions of the amino acid binding modules of peptide synthetases.

It is well documented that *syrB1* and *syrB2* are involved in the biosynthesis of SRE of *P. syringae* pv *syringae*. It was hypothesized that UV radiation is likely to induce mutations and/or enhance the expression of these two enzymes to influence the production of SRE directly or indirectly. To test this hypothesis, *syrB1* and *syrB2* genes was cloned and sequenced. Previous studies have shown while *syrB1* carries all consensus sequences of amino acid binding module of peptide synthetases, *syrB2* sequence shows little to no consensus to other known peptide synthetase (Guenzi et al., 1998). In this study, the sequence analysis showed that an insertion occurred at AMP binding site in *syrB1* gene at nucleotide position 304 to 408, which belongs to adenylation domain responsible for recognizing and activating a specific amino acid (Kleinkauf and Von Dohren, 1996; Marahiel et al., 1997). Previous study has indicated that SRE is synthesized by nonribosomal mechanism (Singh et al., 2007) with the participation of *syrB1, syrB2, syrC* and

syrE. Nonribosomal peptide synthetases, such as *syrB1* and *syrB2*, are multimodular enzymes that produce peptide via thiotemplate mechanism independent of ribosomes. Well-known products of nonribosomal peptide synthetases are antibiotics (such as penicillin, erythromycin, vancomycin), immune suppressants (such as cyclosporine and rapamycin) and antitumor agents (Lee et al., 2005). Minimal nonribosomal peptide synthetase module consists of an AMP-binding adenylation domain (A) for substrate recognition and activation; and a thiolation (T, also called peptidyl carrier protein) domain, downstream of A domain, functioning as cofactor binding site (Schwarzer and Marahiel, 2001). This A domain is highly conserved; therefore, mutations in AMP-binding site might lead to the improvements in SRE production ability of the mutant strain.

To test whether the UV treatment induced the expression of syrB1, a realtime PCR assay was conducted. The real-time PCR result showed that expression level of *syrB1* in mutant strain was six-times higher compared with the wildtype strain. The increased expression level of *syrB1* might be due to some acquired mutations in its promoter region and responsible for the increased biosynthesis of SRE. It is likely that insertion of new fragment of amino acid into the adenylation domain of SyrB1 increases its amino acid activation rate, indirectly boosting SRE biosynthesis. At present, the molecular mechanism(s) underlying the higher SRE production ability of the mutant strain was not fully understood. This study showed a successful enhance-ment of SRE production in *P. syringae pv syringae* using UV induced mutagenesis, proving involvement of at least one gene, *syrB1*. These results shed more lights into the biosynthesis pathway of SRE in *P. syringae*, giving directions for further characterization of the pathway in the future.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ACKNOWLEDGMENTS

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Full Length Research Paper

The impact of salinity stress on morphological and anatomical aspect of water hyssop *Bacopa monnieri* (L.) Wettst grown *in vitro*

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This study was conducted at Marine Science Center, Basrah University, Basrah, Iraq during the period of 2015 to 2016. In the present work, we used NaCl and polyethylene glycol (PEG) to induced abiotic stress *in vitro*. Results show that high shoot numbers (24) were produced on MS (solid) medium enriched with 0.8 g/L NaCl in addition to cytokinins while, inclusion of drought agent PEG to proliferation medium (solid and liquid) produced a minimum shoots of 5 and 11 respectively. Root growth and development were also investigated in this study. It was found that high root development was observed on MS (solid or liquid) medium free of hormone (control) and MS (solid or liquid) medium containing 0.8 g/L NaCl 26.67, 25, 21.67 and 18 respectively whereas, less root development was obtained on MS medium (solid or liquid) enriched with NaCl and 26 g/L PEG. In this study it was found that inclusion of the amino acid proline to the stressed media did not improve the growth of shoots or roots. Histological analysis of multiplied shoots under abiotic stress agents showed accumulation of insoluble starch granules in the parenchyma cells of the cortex layer and this may be a part of protective tolerate mechanism used by medicinal hyssop (*Bacopa monnieri*) against abiotic stress.

Key words: *Bacopa monnieri*, medicinal hyssop, Brbin, micropropagation, salt stress, polyethylene glycol (PEG), proline.

INTRODUCTION

Bacopa monnieri (L.) Wettst a water hyssop, known as Brahmi, or Brbin belonging to the family Plantaginaceae is widely distributed in warmer parts of Asia, Australia, and America. It is an important medicinal plant used in the Ayurvedic system for centuries. This creeping herb with a light purple flower is used for various ailment but is best known as a brain tonic, a nerve tonic for enhancing memory, improves intellectual and cognitive functions,

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anti-inflammatory, analgesic, antipyretic, sedative and antiepileptic agent. In India and Pakistan this plant has been used as a cardiac tonic, digestive aid and improves the respiratory system (Bammidi et al., 2011; Charoenphon et al., 2016). It also used in medical therapy for insomnia, asthma, hoarseness, snake bite, rheumatism, leprosy, eczema, water retention, blood cleaning and insanity (Banerjee and Modi, 2010).

Environmental stress and in particular salinity is one of the important factor that limits the distribution, crop productivity, morphogenesis and the formation of secondary metabolites of the glycophytic plant (Liu and Cheng, 2008; Charu et al., 2015; Vibhuti et al., 2015; Awasthi et al., 2016). However, plants have developed complex mechanisms to adapt to osmotic, ionic and oxidative stresses induced by salt stress; for instance, the accumulation of proline (Debnath, 2008; Bargali and Bargali, 2016). Tissue culture is used to evaluate the impact of abiotic stress on cell metabolism morphologically, anatomically and physiologically; it also opens a new path for the production of secondary metabolites (Debnath, 2008; Naik and Al-Khayri, 2015). Tissue culture is a novel technology used to investigate plants' tolerance to different abiotic stresses factors. So, the present study aimed to investigate the effect of salinity (NaCl) and drought agent *polyethyleneglycol* (PEG) on morphogenetic potential and histological aspect of *in vitro* grown shoots of Bacopa monnieri. (L.) Wettst.

MATERIALS AND METHODS

Source of the plant materials and surface sterilization

This project was conducted at Marine Science Center, Basrah University, Basrah, Iraq during the period of 2015 to 2016. A plant was collected from different parts of Shatt- Al-Arab coast. Young and healthy shoots were selected as a material for *in vitro* culture use. Shoots were treated with 10% (v/v) liquid detergent solution for 5 to 10 min, followed by rinsing under running tap water for 15 to 20 min. Shoot tips of 1 to 2 cm were detached from the young shoots and immersed directly in antioxidant solution consisting of 100 mg/L ascorbic acid and 150 mg/L citric acid until the time of sterilization. The clean shoot tip explants were treated with 20% (v/v) bleach solution containing two drops of Tween-20 per 100 ml as a surfactant for 10 min under aseptic conditions (Laminar flow air cabinet). Shoots were rinsed three times with sterile distilled water to remove the traces of bleach solution.

In vitro culture establishment

Surface disinfectant apical buds were inoculated onto Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) having 3% sucrose and supplemented with 0.5 mg/L benzyl adenine (BA) + 0.5 mg/L kinetin (AL-Aradi et al., 2016), and MS vitamins. pH of the medium was adjusted to 5.8. Then, 0.6% agar was added. The medium was then autoclaved at 121ºC for 20 min. *In vitro* generated healthy shoots were maintained by regular subculturing of propagules of four shoots each after every 8 weeks of culture. Shoots explants obtained from the *in vitro* cultures were used as inoculum and subjected to different abiotic stress treatments.

In vitro abiotic stress treatments

To determine the abiotic stress tolerance of medicinal plant *B. monnirri* by examining morphological and histological changes following water stress, the multiplied shoots were transferred to same proliferating MS (liquid and solid) medium containing different abiotic stress as follow: NaCl (0.8 g/L) and NaCl 0.8 g/L with 26 g/L polyethyleneglycol or 50 mg/L proline. Cultures were grown in a growth chamber at 27 ± 2°C with photoperiod of 16 h day/dark of 10 µmol m^{-2}s^{-1}.

To study the effect of abiotic stress on root development, multiplied shoot mass was transferred to hormone free MS (solid and liquid) medium fortified with different abiotic stress as follow: NaCl (0.8 g/L) and NaCl 0.8 g/L with 26 g/L polyethyleneglycol or 50 mg/L proline medium. Cultures were grown in a growth chamber at 27 ± 2°C with photoperiod 16 h day/dark of 10 µmol m^{-2} s^{-1}.

Regeneration potential development

Shoots and plantlets from all stressed media were transferred to medium free of abiotic stress agents for shoot and root regeneration. Cultures were maintained at temperature 27 ± 2°C and 16 h photoperiod of 10 µmol m^{-2} s^{-1}.

Data recording and statistical analysis

Experiments for shoots multiplication and rooting stress under normal and abiotic stress condition *in vitro* were repeated thrice. For each treatment, five tests tubes or jars were used. The data with respect to shoot and root numbers were recorded for each treatment after one and two months. Same date recording used for shoot and root regeneration after stress removed. All data were subjected to Analysis of Variance (ANOVA) for a completely randomized design (CRD). The differences among the treatment means were tested by Duncan's new multiple range test (DMRT) (Gomez and Gomez, 1984).

Microscopic study

A comparative histological study was conducted on the shoot for different treatments mentioned above in addition to control. Specimens were killed and fixed in (formalin: acetic acid: alcohol 90:5:5) (FAA) for 24 h, then dehydrated in ethanol series using 50, 70, 80, 90% and absolute 100% concentrations (Johanson, 1940). Specimens were then embedded in paraffin (solidification point about 57 to 60°C) and sectioned to thickness 10-micron sections. The sections were double-stained with safranin and fast green. Sections cleared with xylene and mounted in Canada balsam (Yilun et al., 1992). Slides were microscopy examined.

RESULTS AND DISCUSSION

Bud induction and shoot proliferation

It was observed that the addition of cytokinins to MS medium was essential for bud development and proliferation. Apical bud grown on hormone free medium showed less response and bud later died (data not shown in this paper). Proliferated shoots were transferred to same fresh medium after every eight weeks. Similar results were obtained by Binita et al. (2005), Sharma et al. (2010), Sundriyal et al. (2013) and Jain et al. (2014).

Table 1. Typical response for shoot numbers of *Bacopa monnieri* on liquid and solid medium, supplemented with 0.5 mg/L BA + 0.5 mg/L free of salinity and drought agents and subjected to salinity (NaCl), proline and drought (PEG) stress after one and two months.

S/N	Treatments	Rate of shoots number			Rate of roots numbers		
		After one Month	After two months	Regeneration potential (re-growth after stress relieved)	After one Month	After two months	Regeneration potential (re-growth after stress relieved)
1	MS solid medium (control)	25[b]	28.7[b]	29.33[a]	18[a]	26.67[a]	21[a]
2	MS liquid medium (control)	33.33[a]	42.3[a]	24[ab]	24[a]	25[ab]	20.7[a]
3	MS + 0.8 g/LNaCl (solid)	5[c]	24[b]	22[bc]	5[b]	21.67[ab]	19.3[a]
4	MS + 0.8 g/LNaCl (liquid)	6[c]	15.7[bcd]	19.33[bcd]	5[b]	18[bc]	19[a]
5	MS + 0.8 g/L NaCl + 50 mg/L proline (solid)	3.33[c]	13.7[cd]	15.33[cde]	4.67[b]	12.67[cd]	16.3[a]
6	MS + 0.8 g/L NaCl + 50 mg/L proline (liquid)	7.33[c]	12[cd]	12.33[de]	5.67[b]	11.67[cd]	14[a]
7	MS + 0.8 g/L NaCl + 26 mg/L polyethelenglygol(solid)	3.33[c]	5[d]	12[de]	6[b]	10[cd]	12.3[a]
8	MS + 0.8 g/L NaCl+ 26 mg/L polyethelenglygol (liquid)	7.67[c]	11[cd]	9.33[e]	7.33[b]	7.67[d]	11[a]
L.S.D.		6.96	13.07	7.309	8.443	8.406	11.29

Table 1 shows that the highest shoot numbers were obtained on liquid MS medium supplemented with 0.5 mg/L BA + 0.5 mg/L kinetin and free of salinity and drought agents after one and two months; 33.33 and 42.2 respectively (Figure 1A and B). These results are in line with those of Tiwari et al. (2000), who proposed an efficient and rapid method for *B. monnieri* by using a liquid medium. Moreover, the MS medium fortified with 0.5 mg/L BA + 0.5 mg/L kinetin liquid or solid medium showed no rosette clump; so it has been used for this study. *In vitro* shoots explants from control cultures were used as inoculums and subjected to salinity (NaCl) and drought (PEG) stress.

In vitro stress treatments

Concerning the number of shoots, the results illustrated in Table 1 shows that maximum shoot numbers rate (24 culture) were produced on MS (solid) medium supplemented with 0.8 g/L NaCl after two months. The most harmful effects of multiplied shoots were recorded on MS solid medium containing 0.8 g/L NaCl +26 g/L PEG, where, the less average shoot numbers (5 shoot/ culture) were recorded in this medium after two months respectively (Table 1). Results also recorded some yellowing on multiplied shoots grown on MS media containing stress agents and no necrosis was observed (Figure 2A and C). But, in spite of a long-term exposure to salinity and drought stress for two months, shoot

cultures were still viable. These findings indicated that *B. monnirri* can tolerate both NaCl and other water stress agents (Figure 2A to C). Table 1 also shows that cultures resumed their growth when stress relieved, and the shoots number increased distinctly compared with cultures subjected to stress. An identical observation was reported in same species *B. monnirri*, where plants are able to tolerate water stress for 45 days (Debnath, 2008).

Growth reduction in tissues grown in stressful media *in vitro* is the usual phenomenon, but stress metabolism resistance is really interrupted (Misra et al., 1997, Debnath, 2008). So, the response of shoots multiplication or roots formation in salinity and drought stress were different (Table 1).

NaCl stress involves both osmotic and ionic stress agents but the PEG is a high molecular weight osmotic agents. However, salinity and drought-induced production of active oxygen species (AOS) often cause oxidative stress (Sgherri et al., 2000; Khan and Panda, 2008). However, plants have a specific protective mechanism in various degree for defending themselves against activated oxygen (Sudhakar et al., 2001). In the present study, a significant statistically difference between shoots numbers of control and others of water stress treatments were recorded (Table 1 and Figure 2C) but, all the stressed plants showed the non-significant decrease in shoot numbers after one and two months. In the presence of both salinity and drought agent (PEG) in a liquid medium, a less shoots number was obtained; 3 and 5 shoots after 1 to 2 months because the (PEG) restrict
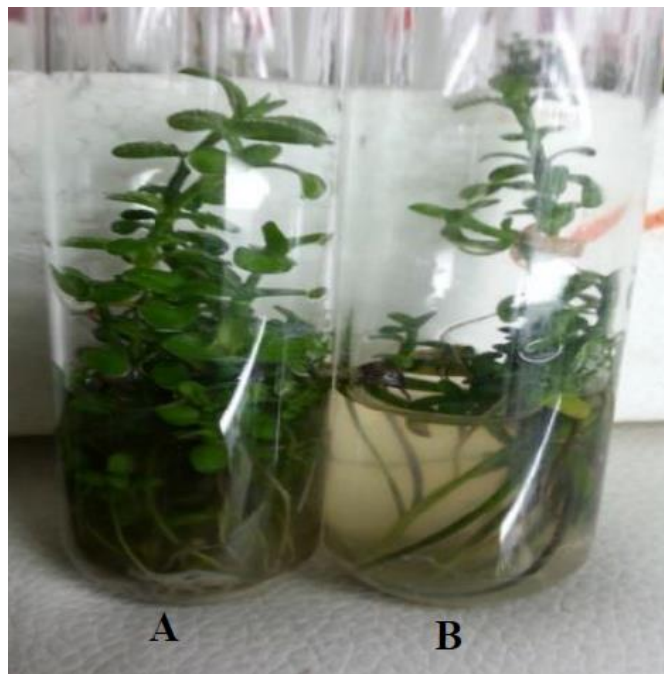
Figure 1. Initiation of shoots from apical bud on MS medium containing 0.5 mg/L BA + 0.5 mg/L kinetin. **A,** Shoot proliferation on liquid medium. **B,** solid medium.
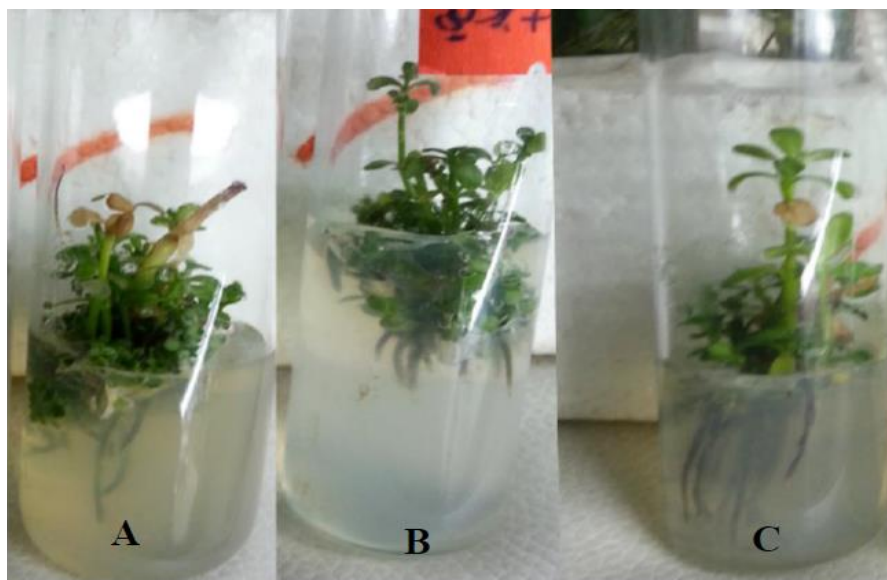
Figure 2. Shoot proliferation on MS solid medium containing abiotic stress agents. **A,** 0.8 g/L NaCl. **B,** 0.8 g/L NaCl + proline. **C,** 0.8 g/L NaCl + PEG.

the water translocation through plant inhibiting leaf expansion and plant growth (Yeo and Flowers, 1994). This restriction may affect shoot growth and proliferation by reducing the media uptake by shoot explants.

Concerning root growth and development, the impact of different abiotic stress represented in Table 1 shows that

the best root numbers were obtained on MS solid and liquid medium free of hormone and abiotic stress agents after one and two months were 18, 24, 26.67 and 25, respectively. The result also revealed that addition of salinity agent (NaCl, 0.8 g/L) to the medium did not decrease the root numbers. Instead, a high root number
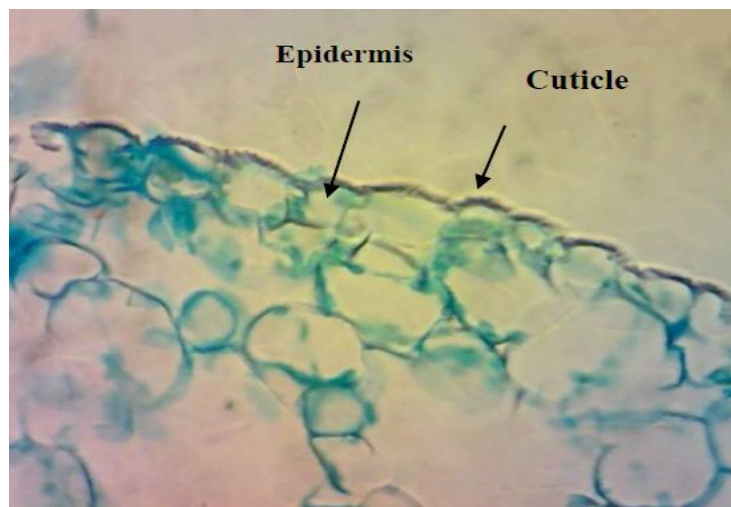
Figure 3. Cross section of stem: epidermis cells of stem cultured on MS medium free of abiotic stress and MS medium enriched with salinity, proline (anti abiotic stress) and drought (PEG) stress. No variation in cuticle thickness has been detected (200 times magnification).

(21.67 and 18) was recorded from shoots grown on MS (solid and liquid) medium containing salinity agent (NaCl, 0.8 g/L) after two months. However, on the rooting medium, the multiplied shoot on various stress media showed no significant increase in shoot growth after 1 to 2 months, and instead of that, it morphologically shifted from shoot proliferation to root formation (Table 1). This result is in accordance with that of Debnath (2008) who observed same phenomena on the *B. monnieri* hyssop cultured *in vitr*o under stress agents while, other researchers (Ehsanpour and Amini, 2003; Misra et al., 1990) suggested that increasing the root length and branching is one way of tolerance mechanism against drought and salinity stress.

Results also revealed that combined PEG to the culture media (solid and liquid) decreased the roots numbers to 10 and 7.67 respectively. This finding is in agreement with those of Said et al. (2015) who found that root growth parameters markedly decreased in Banana plantlet cultured *in vitro* when PEG was included in the media. Results also shows that adding the proline to the MS medium containing salinity agent alone or in combination with drought agent (PEG) did not improve the shoot multiplication or root development (Table 1).

After stress, relieved numbers of roots showed insignificance difference between control and other stress treatments (Table 1).

Histological study

Anatomical studies of plants under stress are very important because they show adaptive features of plants under these conditions (Younis et al., 2013). Histological study of *B. monnieri* stem subjected to various stress

treatments was conducted to describe its response to different abiotic stress *in vitro*. It was noticed that the epidermis of stems cultured in various salinity and drought stress agents, as well as control media, consist of one layer with thin- walled cells, no variation in cuticle thickness has been recognized (Figure 3). Stems grown in different abiotic stress agents' media showed high insoluble starch granules accumulation, in parenchyma cells of the cortex (parenchyma) (Figure 4B, C and D); whereas, no starch granules were recognized in stem grown in control medium (stress-free medium) (Figure 4A). The vascular area (phloem and xylem) of stems subjected to various abiotic stresses is wide, distinct and contained high numbers of vascular bundles in compared with control (Figure 5B). For the control, the vascular area is very small; fewer numbers of mature vascular bundles was detected (Figure 5A). So, anatomical results indicated that starch accumulation, as well as wide vascular area, are a possible productive tolerate mechanism use for salinity or drought stress in *glycophytic hyssop B. monnieri*. However, no phenolics or damaged parenchyma cells have been recognized in all sections stems subjected to drought or salinity stress treatments. These findings are paralleled to those of Theerawitaya et al. (2015) study, who demonstrated that the accumulation of soluble and insoluble starch granules was detected in rice seedling cultivars IR29 and Pokkalid, depending on the salts stress exposure times and plant organs.

Conclusion

Salinity and drought stress are the most important environmental factors that restrict glycophytic plant
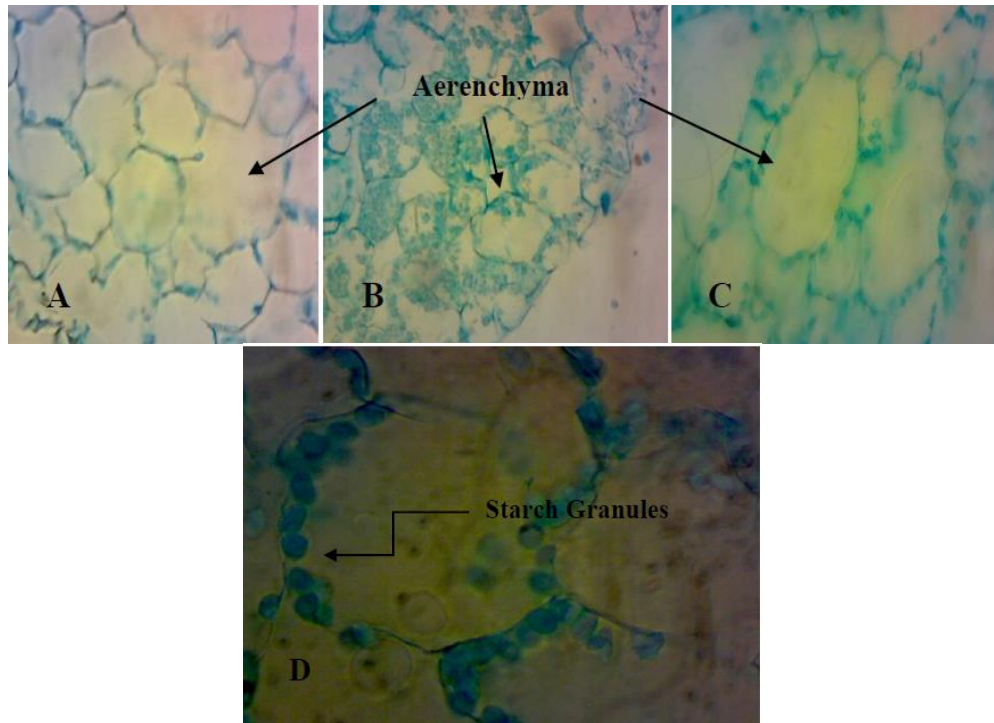
Figure 4. Cross section of stem. **(A)** Parenchyma cells of stem cultured in control medium (free of a biotic agents), no starch granules accumulation has been recognized (100 times magnification). **(B)** Parenchyma cells of stem cortex grown on MS medium enriched with salinity, proline (anti a biotic stress) and drought (PEG) stress, agents, parenchyma cells seems filled with insoluble starch granules (100 times magnification). **(C)** Parenchyma cells of stressed stem (200 times magnification). **(D)** Parenchyma cells of stressed stem under high power of magnification (400 times).
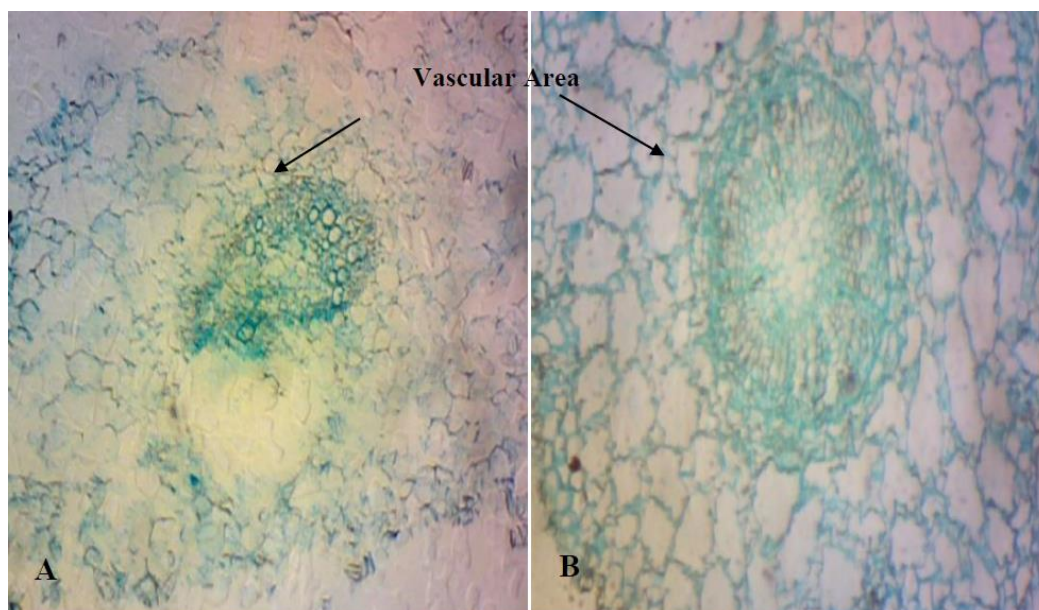
Figure 5. Cross section of stem: vascular of stem cultured in MS medium free of abiotic stress (A) and MS medium enriched with salinity, proline (anti a biotic stress) and drought (PEG) stress (B). (A) For stem grown in control medium, vascular area is very small with less numbers of mature vascular bundles. (B) For stems cultured under stress conditions, vascular area were more distanced and matured (40 times magnification).

growth. In this study we conclude that the deleterious effect on shoot multiplication and root development comes when PEG-containing medium was used. No adverse effects were recognized on shoot or plantlets grown on MS medium containing 0.8 g/L NaCl. Histological study revealed that the shoots grown under abiotic stress had a mature vascular area compared with the control. These preliminary results open the door to use a low salinity agent (0.8 g/L NaCl) instead of PEG and paclobutrazole to promote *in vitro* hardening.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Determination of proximate and mineral compositions of *Moringa oleifera* and *Moringa stenopetala* leaves cultivated in Arbaminch Zuria and Konso, Ethiopia

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This study presents the proximate and mineral composition analysis of *Moringa oleifera* and *Moringa stenopetala* leaves collected from two different agro-ecological zones namely Arbaminch Zuria and Konso in Southern Nation Nationalities and Peoples Region (SNNPR), Ethiopia. *M. oleifera* and *M. stenopetala* leaves were collected using random sampling method from four sampling sites namely Secha, Karat, Sikela, Gato. Before the samples were analyzed, parameters such as temperature, volume of reagents and time until complete digestion were systematically optimized. Analysis of variance was used to compare the proximate and elemental composition of leaf samples as well as mineral composition of soil. The results of this study revealed that, *M. oleifera* leaf samples from the Secha and Karat sampling sub-sites have shown the average moisture, ash, crude fiber, crude lipid and protein contents of 6.88±0.82, 15.3±1.02, 5.51±0.08, 7.11±0.52, 24.8±1.80% and 6.60±0.79, 15.6±1.11, 6.13±0.22, 7.35±0.48, 24.3±1.73%, respectively. The average moisture, ash, crude fiber, crude lipid and protein contents of 7.92±0.85, 14.9±0.93, 5.03±0.12, 3.65±0.11, 27.3±2.10% and 7.73±0.96, 14.1±0.87, 5.87±0.21, 4.29±0.12, 30.2±2.61% were determined for *M. stenopetala* leaf samples from the Sikela and Gato, respectively. The contents of the nutrients found in Moringa leaves have shown no significant differences from the two agro-ecological zones except some nutrients (ash, lipid, and protein). The K, Na, Mg, Ca, Mn, Zn, Fe, and Cu concentration determined from Moringa leaf samples from the two agro-ecological zones was ranged between 9570-14047, 1287-1298, 4273-4550, 18230-19026, 72.4-87.5, 21.3-57.6, 80.1-82.3 and 3.43-5.10 mg/Kg, respectively. The comparison of mean values of macro and micro nutrient of Moringa leaves and soils showed no significant difference between the two agro-ecological zones except some metals such as K, Zn and Cu. The present analysis revealed that Moringa leaves contained considerable amount of nutrients and hence used as food supplement for the community.

Key words: Nutritional compositions, elemental composition, Moringa species, agro-ecological zones, proximate analysis.

INTRODUCTION

In Africa, many studies have indicated that a vast number of indigenous wild plants play a significant role in the diet of the population (Muhammad et al., 2011). Vegetables are the cheapest and the most available sources of important nutrients, supplying the body with minerals, salts, vitamins and certain hormone precursors, protein,

energy and essential amino acids (Amaechi, 2009).

M. oleifera and *M. stenopetala* are the two most common species among the 13 species of the Moringa family. *M. oleifera* originates from the Himalaya and *M. stenopetala* is endemic to East Africa (Bosch, 2004). All parts of the tree except the wood are edible, providing a highly nutritious food for both humans and animals. *M. stenopetala* is one of the most frequently cultivated indigenous species for its palatable leaves in the semiarid areas of Konso, Derashe and Arbaminch Zuria districts of the Southern Rift Valley of Ethiopia and locally called as "Haleko" or "Shiferaw". It is also cultivated from the lower Omo Valley to the North and in the neighboring regions of South Omo, Gamo-Gofa and Borena (Grubben and Denton, 2004).

Moringa is a multi-purpose miracle tree with tremendous potential uses such as food for human beings, feed for livestock, dye, perfume, skin lotion, lubricant and water purification (Agena, 2009). The Moringa leaves are nutritionally rich and an excellent source of concentrated proteins, vitamins and minerals (Armelle and Melanie, 2010). It has also potential medicinal uses such as: the root bark is being used to kill different kinds of intestinal worms, increases food appetite, protect abdominal constipation, cure for different kinds of respiratory diseases such as bronchitis and influenza and the stem bark is being used to treat eye diseases, intestinal worms, and to decrease or neutralize the venom power of snake, bee and scorpion (Grubben and Denton, 2004).

A recent study conducted by Melesse et al. (2009) indicated that the leaves of *Moringa stenopetala* are rich in protein (28.2%) and contain reasonable amounts of essential amino acids of which some are comparable with those found in soybean meal. It is a multipurpose tree that is cultivated both for human food and animal feed in Southern Ethiopia. Moringa leaves have been consumed by Asian people for millennia as a healthy food product. Studies from other countries indicate that the leaves have immense nutritional value such as phytochemicals, vitamins, minerals, and amino acids (Busani et al., 2011). As such, the leaves have been used to combat malnutrition, especially among infants and nursing mothers. The Romans, Greeks, and Egyptians extracted edible oil from the seeds and used it for perfume and as a skin lotion. People in the Indian subcontinent have long used Moringa pods for food. The edible leaves are eaten throughout East Africa and parts of Asia. Moringa leaves are edible and are of high nutritive value (Tetteh, 2008). It has been reported that *M. oleifera* leaves product, especially leaf powder, is becoming increasingly popular in Ethiopia because of its outstanding indigenous nutritive and medicinal value. The leaves are also free of anti-nutritive factors such as phenols, tannins, and saponins (Ijarotimi et al., 2013).

In many developing countries, the supply of minerals is inadequate to meet the mineral requirements of farm animals and rapidly growing population. Mineral composition of a plant plays significant role its nutritional, medicinal and therapeutic values (Al-Kharusi et al., 2009).

M. oleifera contains several elements which are the basic building block of matter. Some of the elements are calcium, magnesium, potassium, sodium and the minor elements are iron, zinc, copper and manganese (Melesse and Berihun, 2013). However, complete information on the nutritional compositions of main edible parts of both Moringa species, that is, leaves cultivated in different agro-ecological zones of southern Ethiopia is still scarce. Therefore, the objectives of this study are: (1) to investigate the effect of agro-ecological variations in nutritional levels of leaves in Moringa species (2) to assess the nutritional levels of leaves in the two Moringa species in comparison with similar studies (3) to compare the proximate compositions in the leaves powder of *M. oleifera* and *M. stenopetala* grown in two different agro-ecological zones (4) to optimize the sample digestion method for both leaf and soil samples; and (5) to investigate and compare the elemental compositions of leaves powder of *M. oleifera* and *M. stenopetala* and the supporting soils in two different agro-ecologic zones..

MATERIALS AND METHODS

Description of the study area

The study was conducted in Arbaminch Zuria and Konso districts of SNNPR, Ethiopia. Arbaminch Zuria district is located at 6° 01'59" N and 37° 32'59" E, altitude of 1269 m.a.s.l and 505 km away from the capital city, Addis Ababa. Konso district is located at 5°15'00" N and 37°28'59" E and altitude of 1031 m.a.s.l. It is 536 Km far from Addis Ababa. Four sampling sub-sites (that is, two from each district) were selected, namely Secha and Sikela from Arbaminch Zuria and Karat and Gato from Konso district.

Chemicals and reagents

All chemicals and reagents were of analytical reagent grade and obtained from Ambo University Chemistry Laboratory. Acid washed glassware and deionized water were used throughout the analysis.

Sampling protocol

Leaf samples

Samples of fresh Moringa leaves were collected from matured *M. oleifera* from Secha and Karat and *M. stenopetala* from Sikela and

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Gato sub-sites. Each sample was collected randomly from ten trees in each sampling sub-sites. About 500 g of the samples were collected from each agro-ecological zone and was placed in polyethylene plastic bags, labeled and transported to the laboratory for further treatment.

Soil samples

Soil samples were collected from the surface soil layers with a depth of about 0 to 15 cm at the point where Moringa leaves plugged. About 500 g of soil samples were collected from each sub-site into polyethylene plastic bags, labeled and transported to the laboratory for further treatment.

Sample preparation

Leaf samples

The leaves were washed with deionized water to remove dust materials and air dried in shade for two weeks to ensure their greenish coloration and maintain nutritional values. The dried leaves were milled and sieved with 0.5 mm sieve. The powders were packaged into polyethylene bags, labeled and stored at room temperature ($24 \pm 3\ ^{o}C$) prior to analysis.

Soil samples

The samples were air dried to constant weight for two weeks, grinded using mortar and pestle, sieved through a 0.5 mm stainless steel sieve and labeled. Finally, 0.5 g aliquot was taken from each sample for digestion.

Proximate composition analysis

Moisture content, total ash content, crude lipid content, and crude protein content were determined using AOAC methods as determined from (AOAC, 1990) and (AOAC, 2000) using AOAC 900.02A and AOAC 978.04 methods.

Moisture content

Moisture content was determined on dry basis. From each site, 2 g of Moringa leaf samples were weighed in triplicates and placed in an oven (Tianjin Taisite instrument, 101-0, China) at $105^{o}C$ for 3 h. The samples were removed and cooled in desiccators. Then the dried samples were reweighed. The percentage moisture was calculated as follows:

$$\text{Moisture content percentage (\%)} = \frac{(w_1 - w_2)}{w_1} \times 100 \qquad 1$$

W_1 = Weight of air dried leaves; W_2 = weight of oven dried leaves.

Total ash content

Total ash content was determined using AOAC 900.02A method as described in (AOAC, 1990). About 2.0 g of leaf samples from each site were weighed in triplicates. Dried and powdered samples were incinerated in a muffle furnace at 550°C for 4 h, cooled in desiccators and weighed until the weight is constant.

$$\text{Total ash (\%)} = \frac{\text{Weight of ash}}{\text{Initial weight of sample}} \times 100 \qquad 2$$

Crude fiber content

After introducing 2.0 g sample into the extraction unit, 150 mL of hot 0.2N H_2SO_4 was added and digested for 30 min. Then, the acid was drained and the sample was washed with hot deionized water for 1 h. The crucible was removed and oven dried overnight at 105°C, cooled, weighed, and heated at 550°C in a muffle furnace overnight and reweighed after cooling. Percentage extracted fiber was calculated as:

$$\text{Crude fiber (\%)} = \frac{\text{Weight of digested sample} - \text{Weight of ashed sample}}{\text{Weight of samples}} \times 100$$
$$3$$

Crude lipids content

The lipid content was determined as the weight change recorded after exhaustively extracting a food substance with a non-polar solvent using Soxhlet method and petroleum ether according to AOAC 945.16 method as described in (AOAC, 1990). The solute was separated from the solvent by evaporation of the solvent leaving the extracted lipid. Weight of the solvent flask with lipid was taken. In the present study, 2.0 g of leaf samples from each site were weighed in triplicates. The Soxhlet apparatus was set-up and the samples were extracted with 200 mL of petroleum ether for 6 h. The solvent free fat in the flux was dried in an oven for an hour at 105°C, cooled in desiccators and reweighed until the weight is constant.

$$\text{Crude lipid (\%)} = \frac{(\text{Weight of extract} + \text{Weight of flux} - \text{Weight of flux})}{\text{Initial weight of sample}} \times 100$$
$$4$$

Crude protein content

Crude protein was determined using AOAC 978.04 method as described in (AOAC, 2000). It is a micro Kjeldahl method. In the present study, about 2.0 g of leaf samples from each site were weighed in triplicates. Then, 10 mL of concentrated H_2SO_4, a mixture of 2.5 g of $CuSO_4$, K_2SO_4 and TiO_2 were added into each sample and digested in a Kjeldahl digestion flask (KDN-20C, China) at 380°C for 6 h until the mixture was clear. The digest was filtered into 500 mL volumetric flask and made up to mark with 100 mL deionized water and connected for distillation. Ammonia was steam distilled for an hour to which had been added 20 mL of 40% NaOH solution. Two hundred milliliters of the distillate was collected in 250 mL conical flask containing 20 mL of 0.2N H_2SO_4 and methyl red indicator.

The ammonia that distilled into the receiving conical flask was reacted with 0.2 N H_2SO_4 and the excess acid in the flask was estimated by back titration against 20 mL of 0.1 N NaOH with color change from red to yellow. A blank distilled was collected in 250 mL conical flask containing 20 mL of 0.2N H_2SO_4 and methyl red indicator. The distillate was titrated against 20 mL of 0.1 N NaOH. Total nitrogen in the sample was calculated using colorimetric methods (Nielson, 2010). Crude protein content was obtained by multiplying the nitrogen content using factor 6.25, that is, N × 6.25 (James, 1995).

Carbohydrate

The carbohydrate content was determined by difference, that is, addition of all the percentages of moisture, ash, crude lipid, crude protein, and crude fiber was subtracted from 100%. This gave the amount of nitrogen-free extract otherwise known as carbohydrate (Mahan et al., 2016).

Table 1. Proximate compositions of *Moringa oleifera* and *Moringa stenopetala* leaf powder (% Dry weight basis).

Sub-site	Sample type	Moisture (%)	Ash (%)	Crude fiber (%)	Crude lipid (%)	Crude protein (%)
Secha	*M. oleifera*	6.88±0.82	15.3±1.02	5.51±0.08	7.11±0.52	24.8±1.80
Sikela	*M. stenopetala*	7.92±0.85	14.9±0.93	5.03±0.12	3.65±0.11	27.3±2.10
Karat	*M. oleifera*	6.60±0.79	15.6±1.11	6.13±0.22	7.35±0.48	24.3±1.73
Gato	*M. stenopetala*	7.73±0.96	14.1±0.87	5.87±0.21	4.29±0.12	30.2±2.61

$$\%Carbohydrate = 100 - \begin{pmatrix} \%\text{Moisture} + \%\text{Ash} + \%\text{Crude lipid} + \\ \%\text{Crude protein} + \%\text{Crude fiber} \end{pmatrix}$$

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Energy value

The sample energy value was estimated (in kcal/g) by multiplying the percentages of crude protein, crude lipid, and carbohydrate with the recommended factors (2.44, 8.37, and 3.57 respectively) as proposed by Martin and Coolidge (1978).

Determination of soil pH

A pH glass electrode (Elmetron® EPSI-1, Poland) was used to determine the soil pH. Soil pH was measured in soil-water slurry. The determination of the soil pH is a three-step procedure: First the soil-water slurry in the ratio 1:5 was prepared. Air dried soil of 10 g was taken in a beaker and 50 mL of water was added. The mixture was stirred with glass rod for 15 min, and allowed to stand for 30 min. Secondly, the pH meter was calibrated with a buffer solutions of pH of 4.0, 7.0 and 9.0. Finally, the electrode was immersed into soil-water slurry and the pH was recorded.

Optimization of sample digestion

Sample digestion was carried out using wet digestion with a reflux condenser for both leaf and soil samples. The reagents used were mixture of nitric acid, perchloric acid and hydrogen peroxide. Before the samples were digested for analysis the parameters such as temperature, volume of reagents and time for complete digestion were carefully optimized. The optimum condition is one that meets: Minimum reagent volume, temperature, and digestion time to produce a clear and colorless digested solution.

Wet digestion method

Approximately 0.5 g of each of sample leaves was weighed in triplicate. Two milliliters of concentrated HNO_3, 1 mL of concentrated $HClO_4$ and 1 mL of H_2O_2, added to leaf samples and digested at a temperature of 210°C for 2 h. Similarly for soil samples, 6 mL of concentrated HNO_3, 1 mL of concentrated $HClO_4$ and 0.5 mL of H_2O_2 were added and digested at a temperature of 230°C for 2.5 h.

Determination of minerals

Flame atomic emission spectrophotometer (FAES, ELICO-CL-378, India) was used for analysis of K and Na. Working standard solutions of Mn, Fe, Zn and Cu were prepared from stock standard solution (1000 ppm) and absorbance was noted from standard solution of each element using flame atomic absorption spectrophotometer (FAAS, Model 210-VGP, USA). The concentrations of Ca and Mg were determined by back titration against $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$.

Statistical analysis

Each experimental analysis was done in triplicate. Data obtained from experiments were analyzed by one way ANOVA (Analysis of Variance) using SPSS version 17. Significance was accepted at 0.05 level of probability ($p < 0.05$). The analysis was used to compare the proximate and elemental composition of leaf samples of Moringa species as well as mineral composition of soil collected from two different agro-ecological zones.

RESULTS AND DISCUSSION

Proximate composition

M. oleifera leaves powder from Secha and Karat

The proximate compositions of *M. oleifera* and *M. stenopetala* leaves powder are presented in Table 1. As indicated in Table 1, the average moisture content range between 6.88 and 6.60% in Moringa oleifera leaves powder. The higher moisture content was recorded for the Secha *M. oleifera* leaf samples (6.88%) and the lower moisture content for Karat (6.60%). There was no significant difference in moisture contents of *Moringa oleifera* leaf samples (P > 0.05) from the two agro-ecological zones. The moisture content (6.88%) which was recorded from Secha *M. oleifera* was slightly higher than that of similar researches with moisture content values of 3.21% (Anthonia, 2012). Other studies have reported variable moisture contents ranging between 9.53 and 11.76% which was relatively higher than this study (Ogbe and John, 2011; Busani et al., 2011).

The total ash content ranged between 15.30 and 15.60%. The higher ash content was recorded for the Karat *M. oleifera* leaves powder (15.60%) and the lower ash content for the Secha *M. oleifera* leaves (15.30%). There was no significant difference in ash contents of *M. oleifera* leaf samples (P > 0.05) from the two agro-ecological zones. The ash content (15.60%), which was recorded from Karat *M. oleifera* leaves powder, was comparatively higher when compared to values obtained from similar research with ash content values of 7.13 and

10.6%, respectively (Onu and Aniebo, 2011; Busani et al., 2011). Thus, the result indicated that *M. oleifera* leaves collected from the two agro-ecological zones were rich in mineral elements.

Crude fiber of the *M. oleifera* leaves powder ranged from 5.51 to 6.13%. Higher crude fiber (6.13%) was recorded from Karat site and lower 5.51% was recorded from Secha site. There was no significant difference in crude fiber content of *M. oleifera* leaf samples (P > 0.05) from the two agro-ecological zones. The fiber content of *M. oleifera* leaves powder was quite low when compared with similar data reported by Ijarotimi et al. (2013). Dietary fiber, the indigestible cell wall component of plant materials, plays an important role in human health (Wani et al., 2014). Epidemiological studies have shown that high dietary fiber intake helps to prevent or treat hyperlipidemia, cardiovascular disease, hypertension, obesity, certain cancers, gastrointestinal disorders and diabetes (Ijarotimi et al., 2013).

The crude lipid content of *M. oleifera* leaves powder from Secha and Karat was 7.11 and 7.35%, respectively. The higher lipid content was recorded for the Karat *M. oleifera* leaf samples (7.35%) and the lower lipid content for the Secha leaf samples (7.11%). There was no significant difference in crude lipid contents of *M. oleifera* leaf samples (P > 0.05) from the two agro-ecological zones. The crude lipid (7.35%), which was recorded from Karat *M. oleifera* leaf samples, was comparatively higher compared to values obtained in similar research with crude lipid content values of 6.73% (Melesse et al., 2011).

The crude protein contents from the Secha and Karat *M. oleifera* leaves powder were 24.80 and 24.30%, respectively. The higher crude protein content was recorded for the Secha *M. oleifera* leaf samples (24.80%) and the lower crude protein content for the Karat leaf samples (24.30%). There was no significant difference in crude protein contents of *M. oleifera* leaf samples (P > 0.05) from the two agro-ecological zones. The results indicated that *M. oleifera* leaves from the Secha contained appreciable amount of crude protein content (24.80%) and was comparatively higher than that of similar studies (Sanchez et al., 2006) for the same Moringa species (22.8%). Other studies have reported variable protein contents ranging between 16 and 40% (Shad et al., 2016; Sanchez-Machado et al., 2010). This level of crude protein content is of particular nutritional significance as it may meet animal's protein and energy requirements and boost the immune system against disease (Melesse et al., 2012).

M. stenopetala leaves powder from Sikela and Gato

As indicated in Table1, the moisture contents of *M. stenopetala* leaves powder ranges between 7.92 and 7.73%. The result of the study indicated that dried *Moringa stenopetala* leaves from the Sikela contained higher moisture content (7.92%) and the lower moisture content was recorded from Gato leaf samples (7.73%). There was no significant difference in moisture contents of *M. stenopetala* leave samples (P > 0.05) from the two agro-ecological zones.

The total ash contents of *M. stenopetala* leaves powder ranges between 14.90 and 14.10%. The higher ash content was recorded from Sikela *M. stenopetala* leaf samples (14.90%) and the lower ash content from Gato leaf samples (14.10%). There was no significant difference in ash contents of *M. stenopetala* leaf samples (P > 0.05) from the two ecological zones. High ash content of (14.90%) from the Sikela *M. stenopetala* leaves powder in this study was comparable with that of similar study (Melesse et al., 2011) for the same Moringa species was found to be 14.80%.

Crude fiber of the *M. stenopetala* leaves powder ranged from 5.03 to 5.87%. Higher crude fiber (5.87%) was recorded from Gato site and lower 5.03% was recorded from Sikela site. There was no significant difference in ash contents of *M. stenopetala* leaf samples (P > 0.05) from the two agro-ecological zones. The fiber content of *M. stenopetala* leaves powder was quite low when compared with similar data reported by Ijarotimi et al. (2013).

The crude lipid contents for *M. stenopetala* leaves powder ranges between 3.65 and 4.29%. The higher crude lipid content was recorded for the Gato *M. stenopetala* leaf samples (4.29%) and the lower crude lipid content for the Sikela leaf samples (3.65%). There was no significant difference in crude lipid contents of *M. stenopetala* leaf samples (P > 0.05) from the two ecological zones. The average crude lipid content in *M. stenopetala* leaf samples from the Gato (4.29%) was comparatively higher than that of related study (Melesse et al., 2011) for the same Moringa species found (3.36%).

The crude protein content of *M. stenopetala* leaves powder ranges between 27.30 and 30.20%. The higher crude protein content was recorded for the Gato *M. stenopetala* leaf samples (30.20%) and the lower crude protein content for the Sikela leaf samples (27.30%). There was no significant difference in crude protein contents of *M. stenopetala* leaf samples (P < 0.05) from the two agro-ecological zones. The average crude protein content in *M. stenopetala* leaf samples from the Gato (30.20%) was comparatively higher than that of similar study (Melesse et al., 2011) for the same Moringa species found 26.6%.

All in all, there was no as such significant difference in nutritional composition of the two Moringa species grown in the two agro-ecological zones; this might be due to the lesser effect of variation in agro-ecological zones on compositions of nutrition of Moringa species. Therefore, other factors such as age, maturity of Moringa trees, soil conditions and so on might plays a major role in variation of nutritional as well as mineral composition of the species.

In addition, the proximate compositions of the two species have shown increment in their levels as compared with similar studies; in particular, the moisture content of the leaves was relatively lower and depicted higher values for the rest of proximate compositions (ash content, crude lipid, and crude fiber) in an indirect relationship as it decreases.

M. oleifera and *M. stenopetala* leaves powder from Secha and Sikela

As indicated in Table 1, the moisture contents of *M. oleifera* leaves powder from Secha was 6.88% and *M. stenopetala* leaves from the Sikela contained moisture content of 7.92%. Higher moisture content was recorded for the Sikela *M. stenopetala* leaf samples (7.92%). There was no significant difference in moisture contents of the two species (P > 0.05) with in the same agro-ecological zones.

The total ash contents of *M. oleifera* leave powder from Secha 15.30%. The lower ash content was recorded for the Sikela *M. stenopetala* leaf sample (14.90%). There was no significant difference in ash contents of the two leaf samples (P > 0.05) with in the same agro-ecological zones. High ash content of (15.30%) from the Secha *M. oleifera* leaves powder in this study was comparatively higher when compared to related study for the same Moringa species (13.20%). However, the ash content of *M. stenopetala* leaves from the Sikela was comparable for the same Moringa species which was found to be 14.60% (Melesse et al., 2012).

The crude fiber contents of *M. oleifera* leave powder from Secha 5.51%. The crude fiber content was recorded for the Sikela *M. stenopetala* leaf samples (5.03%). There was no significant difference in crude fiber contents of the two leaf samples (P > 0.05) with in the same agro-ecological zones.

The crude lipid contents for *M. oleifera* leaves powder from the Secha was 7.11% and the lower crude lipid content was recorded for the Sikela *M. stenopetala* leaf samples (3.65%). There was significant difference in crude lipid contents of the two species (P < 0.05) with in the same agro-ecological zones. The average crude lipid content in *M. oleifera* leaf samples from the Secha (7.11%) was comparatively lower compared to similar study for the same Moringa species found (10.40%). However, the crude lipid content of *M. stenopetala* leaves from the Sikela (3.65%) was comparatively lower than that of similar study for the same Moringa species was found to be 10.70% (Melesse et al., 2012).

The crude protein content of *M. oleifera* leaves powder from the Secha was 24.80%. The higher crude protein content was recorded for the Sikela *M. stenopetala* leaves sample (27.30%). There was no significant difference in crude protein contents of the two species (P > 0.05) with in the same agro-ecological zones. The average crude protein content in *M. oleifera* leaf samples

from the Secha (24.80%) was comparatively lower than the related study for the same Moringa species found 29.0% (Melesse et al., 2012).

M. oleifera and *M. stenopetala* leaves powder from Karat and Gato

As indicated in Table 1, the moisture content for the Karat *M. oleifera* leaf samples were (6.60%) and *M. stenopetala* leaves from the Gato contained moisture content of (7.73%). Higher moisture content was recorded from Gato *M. stenopetala* leaf samples (7.73%). There was no significant difference in moisture contents of the two species (P > 0.05) with in the same agro-ecological zones.

The total ash contents of *M. oleifera* leaves powder from the Karat was 15.60%. The lower ash content was recorded from Gato *M. stenopetala* leaf samples (14.10%). There was significant difference in ash contents of the two species (P < 0.05) with in the same agro-ecological zones. High ash content of (15.60%) from the Karat *M. oleifera* leaves powder in this study was comparatively higher than that of for the same Moringa species was found to be 13.20%. However, the ash content of *M. stenopetala* leaves from the Gato (14.10%) was comparable from similar study for the same Moringa species was found to be 14.60% (Melesse et al., 2012).

The crude fiber contents of *M. oleifera* leaves powder from Karat was 6.13%. The lower crude fiber content was recorded from Gato *M. stenopetala* leaf samples (5.87%). There was no significant difference in crude fiber contents of the two leaf samples (P > 0.05) with in the same agro-ecological zones.

The crude lipid contents for *M. oleifera* leaves powder from the Karat was 7.35%. Lower crude lipid content was recorded for the Gato *M. stenopetala* leaf samples (4.29%). There was significant difference in crude lipid contents of the two species (P < 0.05) with in the same agro-ecological zones. The average crude lipid content in *M. oleifera* leaf samples from the Karat (7.35%) was comparatively lower than that of related study for the same Moringa species found (10.4%). However, the crude lipid content of *M. stenopetala* leaves from the Gato (14.10%) was comparable to similar study for the same Moringa species was found to be 14.60% (Melesse et al., 2012).

The crude protein content of *M. oleifera* leaves powder from the Karat was 24.30%. Higher crude protein content was recorded for the Gato *M. stenopetala* leaf samples (30.20%). There was significant difference in crude protein contents of the two species (P < 0.05) with in the same agro-ecological zones. The average crude protein content in *M. oleifera* leaf samples from the Karat (24.30%) was comparatively lower than related study for the same Moringa species found (29.0%). However, the crude protein content of *M. stenopetala* leaves from the Gato (30.20%) was comparatively higher than that of

Table 2. Calculated carbohydrate and energy value of *Moringa oleifera* and *Moringa stenopetala* leafves powder from two different agro-ecological zones.

Sub-site	Sample type	Carbohydrate (%)	Energy value (kcal/g)
Secha	*M. oleifera*	40.4±3.21	264.3±5.23
Sikela	*M. stenopetala*	41.2±2.89	244.2±4.41
Karat	*M. oleifera*	40.0±2.76	263.7±6.01
Gato	*M. stenopetala*	37.8±2.53	244.6±3.96

Table 3. Concentration (mean ± SD, n=3, mg/kg air dry weight) of metals in *M. oliefera* and *M. stenopetala* leaf samples.

Metals	Metal concentration (mg/kg)				WHO permissible limit (mg/kg)
	Secha	Karat	Sikela	Gato	
	M. oleifera	*M. oleifera*	*M. stenopetala*	*M. stenopetala*	
K	12160±779.70	9570±21.0	12247±434.90	14047± 97.14	Not specified
Na	1296±101.5	1287±108.1	1293± 99.02	1298±99.50	Not specified
Mg	4370±120.0	4273.3±215.0	4550±310.5	4500±242.7	Not specified
Ca	19026±648.30	18803±114.0	18342±1010.0	18230±990.2	Not specified
Mn	72.37±3.37	76.77± 0.85	86.13±2.19	87.53±1.40	2.0[b]
Zn	31.87±2.11	21.30±2.09	57.63±4.45	27.90±1.59	27.4[b]
Fe	81.60±1.14	81.37±2.11	80.03±2.50	82.30±2.65	20.0[b]
Cu	5.10±0.20	3.90±0.53	3.43±0.15	4.13±0.15	3.0[b]

[b]source: FAO/WHO (2001).

related study for the same Moringa species was found to be 28.0% (Melesse et al., 2012).

The variations in ash, crude lipid and protein contents of the reported values may be due to different ages of trees, and possibly due to different stages of maturity (Yang et al., 2006).

Carbohydrate and energy values

The calculated carbohydrate and energy values for *M. oleifera* and *M. stenopetala* leaves powder collected from the four sub-sites were reported in Table 2. The %carbohydrate of Moringa species from Sikela, Secha, Karat and Gato were calculated using Equation 5 and found to be 40.4, 41.2, 40.0 and 37.8, respectively. There was no significant difference in %carbohydrate of *M. stenopetala* and *M. oleifera* leave samples (P > 0.05) from the two agro-ecological zones. The energy value (kcal/g) of Moringa leaves were also calculated as 264.3 (Secha), 244.2 (Sikela), 263.7 (Karat) and 244.6 (Gato). The result of energy value calculated for both Moringa species were found to be lower than related studies (Melesse et al., 2011).

Elemental compositions of *Moringa* species

Metal levels in the M. oliefera leaf samples from Secha and Karat

As shown in Table 3, the mean potassium concentration

in the *M. oliefera* leaves samples were 12160 and 9570 mg/kg for Secha and Karat, respectively. There were significant differences at ($P < 0.05$) among the mean concentrations of K metal in the leaf samples. These differences could probably be the result of plant nutrition, climate and soil conditions (Hamurcu et al., 2010).

The sodium concentration of the *M. oliefera* leaf samples analyzed (Table 3) were 1296 and 1287 mg /kg for Secha and Karat, respectively. F-test showed that there were no significant differences ($P > 0.05$) among the mean concentrations of Na metal in the leaf samples.

Magnesium concentrations were found to be 4370 and 4273.3 mg/kg in the Secha and Karat leaf samples, respectively (Table 3), which was greater than the leaf samples of the Karat site. The concentrations of Mg were not significantly different ($P > 0.05$) in the leaf samples.

The average concentrations of calcium in the *M. oliefera* leaf samples were found to be 19026 and 18803.30 mg/kg in the Secha and Karat leaf samples, respectively (Table 3). Ca concentration was greater in the leaf samples of the Secha. From the statistical F-test, there was no significant differences ($P > 0.05$) in Ca concentration were observed among the leaf samples analyzed.

Concentration of manganese was found to be 72.37 and 76.77 mg/kg in the Secha and Karat leaf samples, respectively (Table 3). Mn concentration was greater in the leaf samples of the Karat. There was no Significant difference ($P > 0.05$) in concentrations of Mn were found between the leaf samples from the results of statistical F-

test.

Both Mn concentrations were below the tolerable limit of 500 mg/kg, dry weight (Council of Europe, 2002). The critical concentration of Mn is 300 to 500 ppm dry weight and the estimated safe and adequate daily dietary intake in adults is 11 mg/day (Khan et al., 2008).

Zinc, analyzed in the *M. oliefera* leaf samples was 31.87 and 21.3 mg /kg in the case of Secha and Karat, respectively (Table 3). The statistical F-test showed no significant variation (p > 0.05) between the tested leaf samples. The amounts of Zn were below the US Recommended Daily Allowance (RDA) which is 15 mg/day for males and 12 mg/day for females and estimated maximum intake of 77 mg/day.

Iron was determined in the *M. oliefera* leaf samples and was found to be 81.60 and 81.37 mg/kg in leaf samples of Secha and Karat, respectively (Table 3). The statistical F-test showed no significant variation (p>0.05) between the tested leaf samples. Iron is an essential micronutrient required for human growth and development. The results showed that the Moringa leaves can be a good source of iron. The values are in accordance to recommended daily allowance of iron 100 to 130 mg/kg for children; 70 mg/kg for men and 120 to 160 mg/kg for women and feeding mothers (Ijarotimi et al., 2013).

Concentrations of copper in *M. oliefera* leaf samples were found to be 5.10 and 3.90 mg/kg in the Secha and karat sites, respectively (Table 3). The greater concentration of Cu was found in leaf samples of Secha site. Both concentrations were below the maximum permissible limit of 40 mg/kg dry weight (FAO/WHO, 1995). Therefore, the plant has no risk. From the statistical F-test, significant differences (P<0.05) in Cu concentration were observed among the leaf samples analyzed.

Metals levels in the M. stenopetala leaf samples from Sikela and Gato

The mean potassium concentration in the *M. stenopetala* leaves samples were found to be 12247 and 14047 mg/kg for Sikela and Gato, respectively (Table 3).There were significant differences at (P<0.05) among the mean concentrations of K metal in the leaf samples. These differences could probably be the result of plant nutrition, climate and soil conditions (Hamurcu et al., 2010).

The sodium concentration of the *M. stenopetala* leaf samples analyzed were 1293 and 1298 mg/kg for Sikela and Gato, respectively (Table 3). F-test showed that there were no significant differences at (P>0.05) among the mean concentrations of Na metal in the leaf samples.

Magnesium concentrations in the leaf samples were found to be 4550 and 4500 mg/kg in Sikela and Gato, respectively (Table 3). This was greater in the leaf samples of the Sikela site. There were no significant difference (P >0.05) in concentration of Mg between the

leaf samples.

The average concentrations of calcium in the *M. stenopetala* leaf samples were found to be 18341.70 and 18230 mg/kg in the Sikela and Gato, respectively (Table 3). Ca concentration was greater in the leaf samples of the Sikela site. From the statistical F-test, there were no significant differences (P > 0.05) in Ca concentration were observed among the leaf samples analyzed. High concentration of Ca is important because of its role in bones, teeth, muscles system and heart functions. The present study show satisfactory level of Ca accumulation as in earlier reports Moringa leaf samples (Abdul et al., 2012).

Concentrations of manganese in the *M. stenopetala* leaf samples of were found to be 86.13 and 87.53 mg /kg in the Sikela and Gato, respectively (Table 3). Mn concentration was greater in the leaf samples of the Gato site. There was no significant difference (P> 0.05) concentrations of Mn were found between the leaf samples from the results of statistical F-test. For medicinal plants the WHO (FAO/WHO, 1984) limit has not yet been established for Mn. The range of Mn concentration in selective medicinal plants of Egypt was between 44.6 to 339 ppm (Jabeen et al., 2010). The concentration of *M. stenopetala* under the present study is in the range of 10 to 1800 ppm, indicating that all the concentration of Mn in the plants studied is around the normal range.

Zinc, analyzed in the *M. stenopetala* leaf samples were found to be 57.63 and 27.90 mg/kg in the case of Sikela and Gato, respectively (Table 3). Zn concentration was greater in the leaf samples of the Sikela site. The statistical F-test showed significant variation (p < 0.05) between the tested leaf samples. The permissible limit set by FAO/WHO in edible plants was 27.4 ppm.

According to Jabeen et al. (2010), the range of Zn in agricultural products should be between 15 to 200 mg/kg. Iron was determined in the *M. stenopetala* leaf samples and were found to be 80.03 and 82.30 mg/kg in leaf samples of Sikela and Gato, respectively (Table 3). Zn concentration was greater in the leaf samples of the Gato site. The statistical F-test showed no significant variation (p > 0.05) between the tested leaf samples. The permissible limit set by FAO/WHO (FAO/WHO, 1984) in edible plants was 20 ppm. For medicinal plants the WHO (FAO/WHO, 1984) limit has not yet been established for iron. The range of iron in selective medicinal herbs of Egypt in the study carried out was between 261 to 1239 ppm (Jabeen et al., 2010). The concentration of Fe in the *M. Stenopetala* is around the normal range for the element when compared with the medicinal herbs of Egypt.

Concentrations of Copper in *M. stenopetala* leaf samples were found to be 3.43 and 4.13 mg/kg in the Sikela and Gato, respectively (Table 3). The greater concentration of Cu was found in leaf samples of Gato site. Both concentrations were below the maximum

Table 4. pH of the soils (Mean ± SD, n=3).

Sample site	pH (H$_2$O)
Secha	7.86±0.10
Sikela	8.11±0.11
Karat	8.16±0.17
Gato	8.29±0.07

Table 5. Concentration (mean ± SD, n = 3, mg/kg air dry weight) of metals in soil samples.

Metals	Metal concentration (mg/kg)				WHO permissible limit (mg/kg)
	Secha	Karat	Sikela	Gato	
	Soil	Soil	Soil	Soil	
K	3523±205.5	1540±134.50	3343.30±236.90	2930±91.65	NA
Na	1980±87.18	1623±94.52	2776.70±76.38	1773.30± 80.02	NA
Mg	3606.7±66.58	3533±45.09	4253.30±197.60	4463.3± 204.29	NA
Ca	17210±2025.0	17633±985.3	17513.30±1746	17340±374.03	NA
Mn	84.53±3.24	11.0±0.10	5.33±0.25	29.67±3.79	2000[b][c]
Zn	36.57±2.40	68.27±8.44	74.23±9.82	77.87±26.23	300[a]
Fe	20.27±2.05	14.40±1.22	21.0±1.87	65.67±8.39	50000[b][c]
Cu	1.27±0.15	0.83±0.12	1.03±0.15	10.23±0.68	100[a]

[a]Source: Ewers (1991); [b]source: FAO/WHO, (2001); [c]Source: Pendias and Pendias, (1992); NA = Not available.

permissible limit of 40 mg/kg dry weight (FAO/WHO, 1995). From the statistical F-test, significant differences (P < 0.05) in Cu concentration were observed among the leaf samples analyzed. The permissible limit set by FAO /WHO for edible plants was 3.00 ppm (Jabeen et al., 2010). For medicinal herbs the WHO (FAO/WHO, 1984) limit has not yet been established for copper. In medicinal plants the permissible limit of copper set by China and Singapore were 20 and 150 ppm, respectively. The metal concentration of *M. stenopetala* was found to have below permissible limits of copper set by China and Singapore.

pH of soil samples

The mean pH values of soils from Secha and Karat

The mean pH values of the soils where *M. oliefera* was planted ranges between 7.86±0.10 and 8.16±0.17 (Table 4). Higher pH was recorded for the Karat soil sample (8.16±0.17) and lower pH for Secha soil sample (7.86±0.10). The analysis of variance showed that there was no significant difference in pH (P>0.05). The pH of the soil from both sites, where *M. oliefera* planted was within the slightly alkaline range of the pH scale. This was in line with the findings of Anjorin et al. (2010) on the soil survey of Abuja.

The mean pH values of soils from Secha and Karat

The mean pH values of the soils where *M. stenopetala*

was planted ranges between 8.11±0.11 and 8.29±0.07 (Table 4). Higher pH was recorded for the Gato soil sample (8.29±0.07) and lower pH for the Sikela soil sample (8.11±0.11). The analysis of variance showed that there was no significant difference in pH (P>0.05). The pH of the soils from both sites, where *M. stenopetala* planted was within the slightly alkaline range of the pH scale. This was in line with the findings of Anjorin et al. (2010) on the soil survey of Abuja.

Elemental compositions of soils

Metals levels in the soil samples from Secha and Karat

As shown in the Table 5, the mean concentrations of K, Na, Mg, Ca, Mn, Zn, Fe and Cu were 3523, 1980, 3606.70, 17210, 84.53, 36.57, 20.27 and 1.27 mg/kg, respectively in the Secha soil samples; and 1540, 1623.30, 3533.30, 17633.30, 11.0, 68.27, 14.40 and 0.83 mg/kg, respectively in the Karat soil samples. The concentrations of all metals were greater in the Secha soil samples except that of Ca and Zn which was greater in the Karat soil samples.

F-test showed significant differences (P<0.05) among the analyzed soil samples for the mean concentrations of all metals in except Mg, Ca and Zn. These differences could probably be the result of climate and soil conditions (Hamurcu et al., 2010).

Metals levels in the soil samples from Sikela and Gato

As shown in the Table 5, the mean concentrations of K, Na, Mg, Ca, Mn, Zn, Fe and Cu were 3343.30, 2776.70, 4253.30, 17513.30, 5.33, 74.23, 21.0 and 1.03 mg/kg, respectively in the Sikela soil samples; and 2930, 1773.30, 4463.30, 17340, 29.67, 77.87, 65.67 and 10.23 mg/kg respectively in the Gato soil samples. The concentrations of all metals were lower in the Sikela soil samples except that of K, Na and Ca which was lower in the Gato soil samples. F-test showed no significant differences (p >0.05) among the analyzed soil samples for the mean concentrations of all metals except Na, Mn and Fe. These differences could probably be the result of climate and soil conditions (Hamurcu et al., 2010).

Conclusion

The data derived from proximate and mineral composition of *M. oleifera* and *M. stenopetala* leaves collected from two different agro-ecological zones indicated that the plant leaves are rich in nutrients and has potential to be used as a feed additive for human and animal. These include serving as a protein, fats, fiber, carbohydrate and mineral resource feed formulations. There was no as such significant difference in nutritional composition of the two Moringa species grown in the two agro-ecological zones. It is recommended that further investigations on vitamin, amino acid profiles and anti-nutritional factors of fresh Moringa leaves be carried.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Effects of activated charcoal on medium-term conservation of yam (*Dioscorea* spp.) cultivated in Benin

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The effects of activated charcoal were tested on medium term conservation of yams cultivated through tissue culture techniques. Galzy glutamine basic medium and that supplemented with 3 g/l activated charcoal were used. Growth parameters such as number of leaves, height of stem, number of nodes and length of the main root are evaluated on plantlets after 20 months. The test T of Student Newman and Keuls (SNK) with two independent variables was used for the comparison of means with Minitab 16 software. Plantlets obtained in the control medium faded and presented a high number. The length of internodes obtained from the medium treated with activated charcoal had a length relatively greater than the control medium with increase of 33%. Probabilities (P = 0.001) associated with the T-test of equality of means of leaves, the height of stem and the length of internodes are lower than 0.05 but there was no significant difference between the number of nodes, length of internodes, number of roots, and length of the main root of plantlets obtained in both media at 5%. In addition, tests of correlation (Pearson) in the control medium revealed the existence of a highly significant link (P <0.001) between the number of nodes and number of leaves. In treated medium, there was a highly significant link (P <0.004) between the length of the stem and number of leaves; the height of the stem and length of the main root (P <0.001). Activated charcoal facilitates conservation and constitutes an alternative of conservation of yam. These results may be used to realize *in vitro* collection of different genotypes of yam cultivated in Benin.

Key words: *In vitro* conservation, plantlets, yam, activated charcoal.

INTRODUCTION

Yam (*Dioscorea spp.*) has a large morpho-ecological, physiological adaptation and is found in all continents (Degras, 1993). It is the second root and plants tuber after cassava with a global annual production estimated at 50 million tons (FAOSAT, 2012). This crop is a major source of food for more than 50 million people in west and central Africa (Asiedu and Sartie, 2010). In West Africa, it occupies an important place in food security and accounts for 95% of production. This tuber is an important source of income for farmers. In the savannas

extending from Cameroon to Ivory Coast, it is always considered as sacred food whose first harvest must be offered to gods and ancestors before yam consumption. So the royal powers associate with their diets, a tradition that is profoundly registered in the cultural and religious heritage of the populations (Degras, 1998). Nowadays, this older and secular plant which is promising crop for Africans (Degras, 1998) remains a major basic food in Africa. Benin is the fourth country producing yam in Western Africa after Nigeria, Ivory Coast and Ghana (FAOSTAT 2014). Despite the importance of this crop in the alimentary, cultural, religious and socioeconomic plan, it has not sufficiently drawn the attention of scientists and institutions, especially its conservation. Indeed, yams produced by vegetative propagation are submitted to viral and other pathologic infections that cause enormous damage for its conservation and generate genetic erosion for different varieties and species. More than 30% of yield tubers are lost (Adeniji, 1970; Okigbo and Ogbonnay, 2006). Due to erosion risks, conservation of genetic resources of cultivated and wild yams becomes more necessary. It is essential to implement appropriate conservation strategies to promote the accessibility of these species and varieties of yam. *In vitro* tissue culture of yams proves to be effective for the conservation of genetic resources (Ovono et al., 2010; Ahanhanzo et al., 2010). Malaurie et al. (1993) specified techniques to be applied in *in vitro* conservation of cultivated yam and the use of plantlets as planting materials. Several techniques used for conservation such as low temperature (Grout, 1995), low level of mineral nutrients, vitamins and carbohydrates, growth regulators as abscissic (Jarret and Gawell, 1991; Engelmann and Engelmann, 2014), activated charcoal (Agbidinoukoun et al., 2013; Polzin et al., 2014) and long term conservation techniques such as cryogenic conservation can be used to achieve high regeneration rates of the different species of *Dioscorea in vitro* (Das et al., 2013). In 2000, Central Laboratory of Plant Biotechnologies and Breeding, Faculty of Science and Technology (FAST), University of Abomey-Calavi applied some of these techniques in *in vitro* morphogenesis (Ahanhanzo et al., 2010) or in *in vitro* conservation of certain accessions of yam. The present work was a continuation of the previous studies and aimed to contribute to the genetic resources management through the verification of the influence of activated charcoal on *Dioscorea cayenensis-rotundata* in *in vitro* conservation.

MATERIALS AND METHODS

The plant materials are composed of healthy plantlets of yam of

complex *Dioscorea-Cayenensis – rotundata.* They were obtained from Central Laboratory of Plant Biotechnologies and Breeding, Faculty of Science and Technology (FAST), University of Abomey-Calavi (UAC) located approximately 16 km North of Cotonou in the municipality of Abomey – Calavi, Republic of Benin. The plantlets were twenty-months old**.**

The plantlets are cultivated in two different media: The medium of Galzy glutamine (2GG) (Doukoure, 2000) and the medium (2GG) supplemented with 3 g.l^{-1} activated charcoal. Cultures were placed in an air conditioned chamber at 28 ± 1°C, according to the standard proposed by Hunter. The relative humidity of the culture chamber is maintained at 80%. The culture chamber is equipped with lamp of types Philips TLD18W and Sibalec FL18W that ensure illumination of about 5000 luxes with a photoperiod of 12 h. 30 (15 × 2) healthy plantlets of yam of complex *D.-Cayenensis – rotundata* are used for each experiment repeated two times**.**

The following growth parameters were studied in order to evaluate the effect of activated charcoal on the conservation of yam plantlets concerned: number of leaves, number of roots, number of nodes, height of stem, the mean length of internodes and the mean length of main roots. Height of the stem and length of the main root were measured using a caliper with a precision of ± 0.1 mm. As for the length of internodes, it was taken with a ruler. The test T of Student Newman and Keuls (SNK) was used for the comparison of means with Minitab 16 software. The means were compared by using least significant difference method at 5% threshold. Pearson correlation test was performed to evaluate the relationship between the different growths parameters studied.

RESULTS

Effects of activated charcoal on the growth parameters

The main results indicated the influence of activated charcoal on plantlets of yam. Plantlets obtained in control medium present a high number of leaves (28 against 13) more than those presented in the medium containing activated charcoal (Figure 1).

The P value of 0.001 for the T-test of equality of means indicated that the leaves development was highly significant in the presence of activated charcoal at 5%.

Plantlets obtained in the control medium had height greater than those obtained in medium containing activated charcoal (Figure 2).

The P value of 0.022 for the T-test of equality of the average height of stem was lower than 0.05. There was a significant difference between the means length of stems obtained in both media on the threshold of 5%.

Figure 3 shows the number of nodes obtained in the two media: plantlets placed in the activated charcoal medium had eight nodes while those placed in control medium had seven nodes.

The P value of 0.582 for the T-test of equality of the two media showed that their nodes were more than 0.05; so there was no significant difference between the number

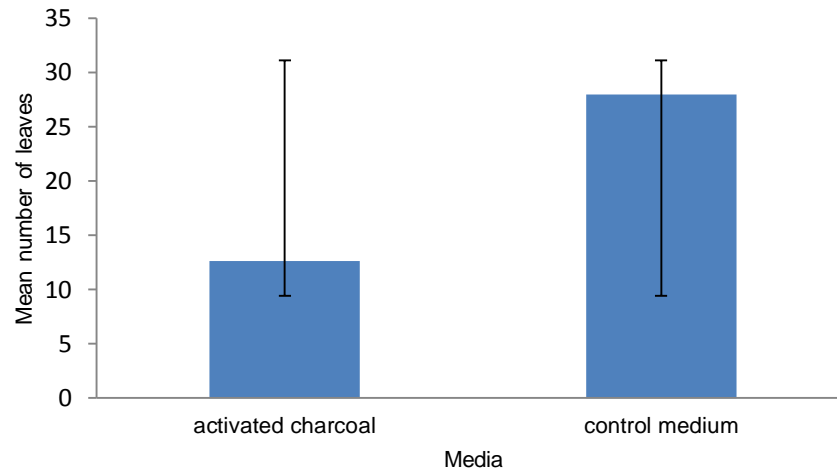*Corresponding author. E-mail: sedajas@yahoo.fr. Tel: 00229 97021533.

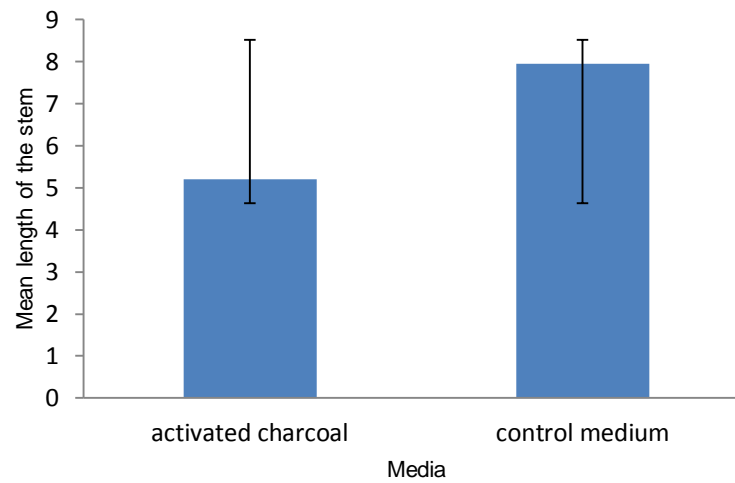**Figure 1.** Effect of activated charcoal on leaves of yam.

Figure 2. Effect of activated carbon on the length of the stem of yam.
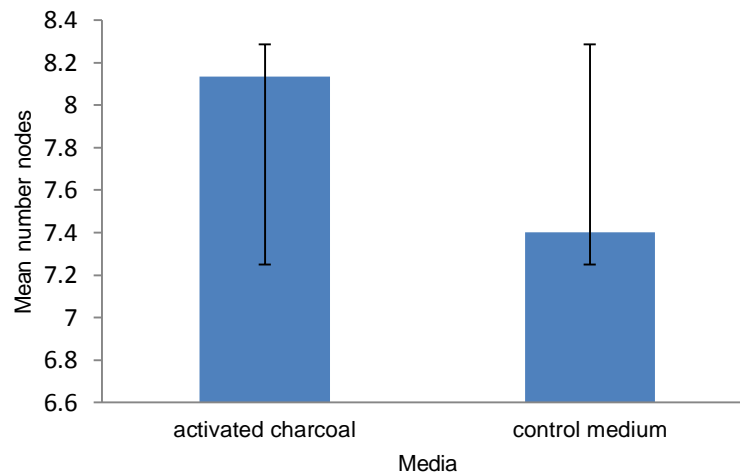

Figure 3. Effect of activated charcoal on the number of nodes of the yam.
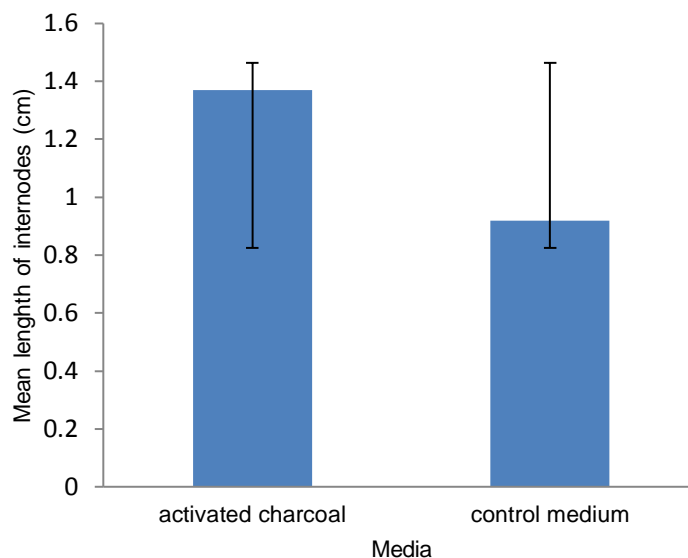
Figure 4. Effect of the activated charcoal on the length of internodes yam.
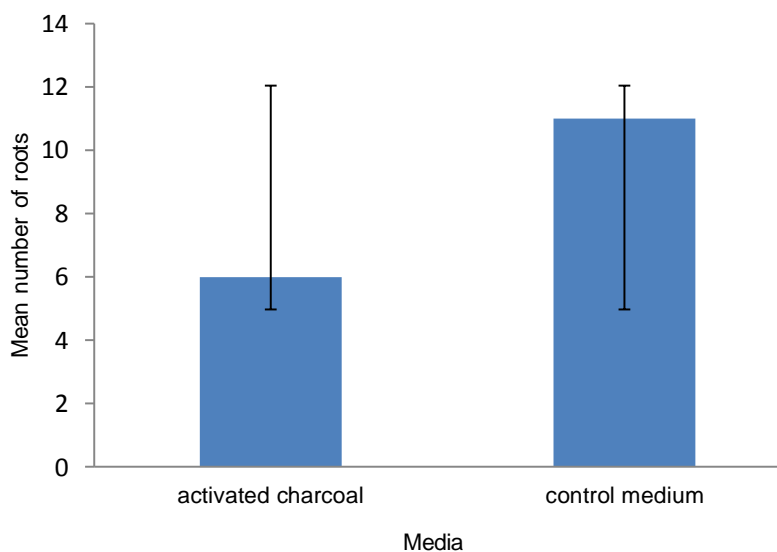

Figure 5. Effect of activated charcoal on the number of roots of yam.

of nodes obtained in both media at 5%.

The length of the internodes of the plantlets obtained from the medium treated with activated charcoal had a length relatively greater than those in control medium with an increase of 33% (Figure 4).

The P value of 0.001 for the T-test of equality of the mean of the lengths of the internodes of both media was lower than 0.05. There was a highly significant difference between the mean length of the internodes obtained in both media at 5%.

Plantlets of the control medium had a relatively high number of roots than those issued in the second medium (Figure 5).

The P value of   0.085 for n the T-test of equality of the two means of roots was greater than 0.05. At the threshold of 5%, there was no a significant difference between the mean number of roots obtained in both media.

In the same way, height of the main root of plantlets obtained in the medium without activated charcoal was
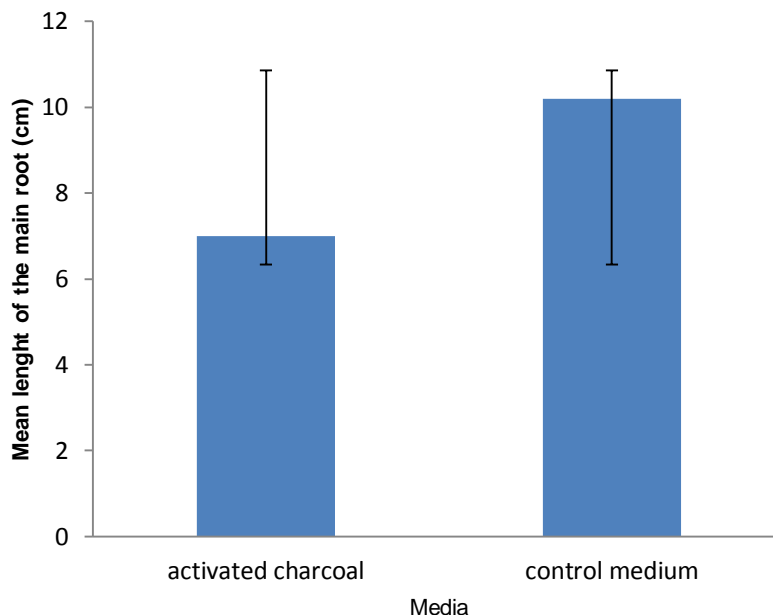
Figure 6. Effect of activated charcoal on the length of the main root of the yam.

Table 1. Pearson correlation and value of P established between the various parameters of the yam plantlets from the medium without activated charcoal.

Parameter	NL CG	NR CG	NN CG	LS CG	LEN CG	LR CG
NL CG	1					
NR CG	0.239[NS]	1				
NN CG	0.001**	0.422[NS]	1			
LS CG	0.406[NS]	0.228[NS]	0.285[NS]	1		
LEN CG	0.743[NS]	0.371[NS]	0.117[NS]	0.128[NS]	1	
LR CG	0.690[NS]	0.689	0.410[NS]	0.668[NS]	0.148[NS]	1

[NS]Not Significant; *significant; **highly significant; GG,Galzy glutamine medium; NL, number of leaves; NR, number of roots; NN, number of nodes; LS, length of the stalk; LR-P, length of main root; LEN, length internodes;a highly significant link (P <0.004) between the length of the stalk and the number of leaves; the length of the stalk and that of the main root (P <0.001).

relatively greater than those of the treated medium with an increase of 14% (Figure 6).

The P value of 0.521 for the T-test of equality of the average length of the main root was more than 0.05. There was no significant difference between the mean lengths of the main root of plantlets issued in the two media on the threshold of 5%.

Relations between the parameters studied on the medium with/ without activated charcoal

Analysis of Table 1 revealed a highly significant link (P <0.001) between the number of nodes and the number of leaves in the control medium.

In the treated medium, there was a highly significant link (P <0.004) between the height of stem and number of leaves, on one hand; and the height of the stem and length of the main root (P <0.001), on the other hand (Table 2). We noted also a significant relationship (P <0.043) between the length of the stem and length of internodes in this medium.

DISCUSSION

Plantlets obtained in the medium control presented faded leaves contrary to those in activated charcoal medium, which had quite green leaves. This state of fading plant may be explained by a temporary availability of nutrients

Table 2. Pearson correlation and P value established between the different parameters in vitro plantlets from the medium supplemented with activated charcoal.

Parameter	NL CG+CA	NR CG+CA	NN CG+CA	HT CG+CA	LEN CG+CA	LR CG+CA
NL CG+CA	1					
NR CG+CA	0.446[NS]	1				
NN CG+CA	0.065[NS]	0.918[NS]	1			
HT CG+CA	0.004**	0.584[NS]	0.163[NS]	1		
LEN CG+CA	0.673[NS]	0.968[NS]	0.379[NS]	0.043*	1	
LR CG+CA	0.042[NS]	0.634[NS]	0.476[NS]	0.001**	0.120[NS]	1

[NS]Not significant; *significant; **highly significant; CG + AC, Galzy glutamine medium supplemented with activated charcoal NL, number of leaves; NR, number of roots; NN, number of nodes; LS, length of the stalk; LR-P, length of main root; LEN, length internodes; a highly significant link (P <0.004) between the length of the stalk and the number of leaves; the length of the stalk and that of the main root (P <0.001).

for plantlets' rapid growth. Long term conservation made the medium to become impoverished in nutrients, which deficiency is pronounced on the plantlets. The activated charcoal seems to trap nutrients which were slowly absorbed by plantlets thus allowing continuous presence of those nutrients in the medium. A similar observation was made by Borges et al. (2004) who reported that the activated charcoal used in the preservation favors healthy tissue viability resulting in a high rate of shoots unlike the medium devoid of activated charcoal. Activated charcoal slowed the growth of plantlets through the mean number of leaves; the mean height of stem, the mean length of internodes except the number of node, the number of roots and the length of roots. These results were similar to those obtained by Agbidinoukoun et al. (2013) on yam. It was found that activated charcoal slowed down the formation of the shoots and leaves, but it led to a better rooting in all the accessions tested. Plantlets' growth can be slowed down in medium - term conservation of genetic resources. Our results confirmed those of Gomes and Canhoto (2009) in *Arbutus unedo* L., where the addition of charcoal did not improve root formation. In addition, the addition of activated charcoal had a positive effect on the growth of the internodes (Polzin et al., 2014). On the other hand, those results had been obtained (Etse et al., 2011) on *Zanthoxylum zanthoxyloides* Lam. Gübbük and Pekmezci (2004), working on Musa, revealed that activated charcoal had a favorable effect on roots of plantlets. Activated charcoal influenced significantly the increase in fresh matter (Agbidinoukoun et al., 2013). The same authors also found that from the sixth months, shoots of *Dioscorea* spp*.* cultivated on the control medium or with a low concentration (1 g.l^{-1}) of activated charcoal began a phase of fading while those cultivated on the medium rich in activated charcoal (2 and 3 g.l^{-1}) had good vigor. Damiano (1980) observed that the addition of 1 to 2 g. l^{-1} of activated charcoal in the medium reduces necessarily its transmission in strawberry roots. So activated charcoal action depends on its concentration in the medium and crop species. It sometimes has positive effects on the slowing growth of plantlets and is used *in vitro* for his antioxidant role. It

results in a considerable reduction in the browning of the medium caused by phenolic compounds, and reduces the effect of these phenolic compounds present in the medium (Shukla and Shukla, 2014).

On one hand, this study indicated significant relationship between the height of stem and length of root and on the other hand between the number of leaves and height of the stem on medium supplemented with activated charcoal. In addition, the plantlets on the medium with activated charcoal have more and longer nodes and they had shorter height. This antioxidant reduced the height in order to facilitate the conservation of plantlets with more potential nodes. It appeared that activated charcoal has an inhibitory action on the morphogenesis of the variety of yam tested. However, the age and physiological state of explants have an important role in the possibilities of conserving the material.

Conclusion

At the end of this study, it is clear that activated charcoal in the medium increases the conservation of the yam cultivars through *in vitro* tissue culture. The addition of this antioxidant has been favorable to plantlets in slowing growth. With this method of medium - term conservation, it is possible to proceed to massive production of this plant tuber culture at low -cost.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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