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Full Length Research Paper

Morphological and proteomic analyses of *Zea mays* in response to water stress

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Water stress affects plant growth and development, leading to agricultural crop losses in maize cultivation. It also threatens food security in economical crops such as maize, one of the major crops produced worldwide. Transcriptomic studies associated with morphological assessments have been widely conducted on the mechanisms of crop development and stress response; however, data on maize is still very much limited. Hence herein, we used both the morphological and proteomic analyses to investigate and establish physical features and proteins associated with maize in response to osmotic stress. In addition, proteomic analysis (1DE and 2DE techniques) was used to separate and enumerate water stress responsive proteins. Morphologically, a decrease in the overall growth of the maize plant as a result of water stress was observed, whereby features such as leaf colour and size, shoot height and stem diameter were negatively affected. Through proteomics analyses, a total of nine expressed proteins were revealed in response to water stress. Overall, this work, has successfully profiled the water stress responsive proteins and specifically indicating the efficiency of proteomic tools in the detection and analysis of qualitative proteins from maize.

Key words: *Zea mays*, water stress, induced proteins, proteomics, plant response, crop losses.

INTRODUCTION

Climate change and global warming accelerates the risk of drought, which has several detrimental effects on various organisms including humans, animals and plants (Dai, 2013). However, plants as the primary producers are constantly exposed to various abiotic stresses, which affect their essential roles in the general life systems of mankind (Jin et al., 2015). Due to climate change, some regions on earth are not receiving enough rainfall, thus such regions do experience drought. Soil water supply is an important environmental factor, controlling seed germination and seedling establishment (Kramer and Kozlowski, 1980; Bargali and Bargali, 2016). Hence, when water potential is reduced, seed germination will be delayed or halted depending on the extent of its reduction (Hegarty, 1977; Zobel et al., 1995). Seed germination and early seedling growth are considered the most critical

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phases for the establishment of any plant species (Bargali and Singh 2007; Pratap and Sharma, 2010; Vibhuti et al., 2015; Pantola et al., 2017).

Water deficiency is one of the major abiotic stresses that affect plant growth, development and productivity worldwide (Zhao et al., 2011; Shi et al., 2014). With such effects, it is estimated that by the end of the 21st century, drought terrestrial areas will increase and threatens food security (Zhao et al., 2011). Hence, it is imperative to determine and understand the mechanisms that are employed by plants when experiencing drought, in order to improve their tolerance to water stress.

To deal with water-deficit stress, plants have developed various mechanisms to regulate the balance of cells, through optimization of their morphology, physiology and metabolism at a cellular level (Boyer, 1982). Previous studies have shown various signal responses to drought, where a plant would undergo leaf abscission, while in other plants, the response may be deadly (Chaves et al., 2002). In some cases, slow water loss results in acclimation to water stress condition thus limiting the drastic effects of plant damage (Bray, 1997), whereas in rapid water loss, acclimatization is prevented because plants have limited time. In addition, water stress results as a physiological condition, where plants have less than full turgor pressure, due to the transpiration demand exceeding root water uptake (Dejonge et al., 2012). The physiological impact of water stress at both the tissue and cellular levels in plants have been shown to result in new metabolic and structural abilities mediated by the changed gene and protein expression that will assist in plant functioning (Bray, 1997; Kasuga et al., 1999; Hasegawa et al., 2000; Seki et al., 2003; Shinozaki et al., 2003). In addition, some of the physiological and biochemical modifications that are involved during water stress include growth inhibition as a result of stomatal closure, which affects photosynthesis and respiration (Fathi and Tari, 2016).

Maize (*Zea mays* L.) is one of the major cultivated crops in South Africa, which serves as a staple food to many homes. Maize being a thermophilous crop, requires temperatures that exceed 10°C for its proper growth as well as related physiological processes such as canopy photosynthesis and root system activities (Liu et al*.,* 2010). However, it needs to be produced under optimal conditions for maximum production and the coordination of its high sensitivity to harsh environmental conditions such as water deficit (Lobell et al., 2011). Maize exhibits varying physiological and biochemical effects during water stress such as development and growth inhibition during the early growth stages, structural damage, reduction in kernel number and ear size (Bassetti and Westgate, 1993; Farré and Faci, 2006). In addition, water stress induces stomatal closure, which results in decreased CO_2 absorption that reduces photosynthetic activity (Nayyar and Gupta, 2006).

Water deficiency intensely affects the agricultural sector, thus limiting total crop yield, which in turn, affects food security and pose a serious threat to the growing population. An increase in crop production is hindered by drought stress (Fathi and Tari, 2016). Various studies are being conducted on maize that includes the mechanisms of crop development and the environmental adaption of crops to stress, in order to improve quality and yield. This raises a need to better understand the mechanisms used by crop plants when they are exposed to drought stress. Thus, our study, reported herein aimed to profile water stress induced proteins from leaf extracts of a *Z. mays* cultivar (R450 w/uo2550 CML550).

Transcriptomic studies have previously been carried out to reveal the large-scale drought modulated gene expression in leaf meristem and reproductive tissues and seedling shoots of maize (Zheng et al., 2010; Kakumanu et al., 2012). Recently, proteomics analyses have been performed on various maize tissues to study water stress responsive protein expression in drought-tolerant and drought sensitive genotypes (Riccardi et al., 2004). Although, proteomics approaches have been studied in various plant species (Cui et al., 2005; Dani et al*.,* 2005; Ndimba et al., 2005), the published proteomic data on responses of maize to water stress is still limited (Yoshimura et al., 2008). Ultimately, information on such studied genes/proteins can then be possibly genetically transferred into other maize varieties and/or related crops that exhibit sensitivity to water stress.

MATERIALS AND METHODS

Plant material and treatment growth conditions

The R450w/uo2250w CML550 *Z. mays* seeds cultivar used in this study were obtained from Molelwane Farm, Department of Crop Science, North-West-University, RSA. The seeds were selected for size homogeneity in terms of size and physical appearance for each pot. The seeds were surface-sterilized with 70% (v/v) ethanol for a minute, followed by decontamination with 1.25% sodium hypochlorite solution (bleach) for 10 min. Immediately after sterilization, the seeds were rinsed three times with sterile distilled water. Three seeds were sown in each of the 12 plastic plant pots, filled with a 3:2 (v/v) mixture of sterilized organic soil (Levington F2, seed and modular compost) and vermiculite. The intended maize plants were grown in a randomized design to eliminate the effect of variations in environmental conditions at different positions. Thereafter, plants at the same developmental stage and of similar height, were selected for all experiments. The sown seeds were watered daily with 100 ml of sterile tap water up until germination begun on day 7. Germinated plants were grown under greenhouse conditions of 16-h days and 8-h nights, day/night air temperature of 26/22°C and relative humidity of 75%. Treatment of plants began when seedlings were 8 days old, whereby plants were divided into two groups: well-watered plants irrigated after every 2 days (control) with 100 ml sterile tap water while the water-stressed plants did not receive any water up until the recovery period (16 days) (treatment). On the 16th day, leaves of both the control and treatment plants were harvested, rinsed with sterile distilled water and immediately snap-frozen in liquid nitrogen. Each treatment group was conducted in three independent biological replicates.

Total protein extraction from maize leaf tissues

Maize leaf protein extracts were prepared from sixteen, 16-day old maize seedlings. The snap-frozen leaf material was ground into fine powder using pre-chilled sterile mortar and pestle. The powdered tissues were precipitated with 10% (w/v) trichloroacetic acid (TCA). The generated precipitate for each sample was individually washed 3 times with 1 ml of ice-cold 80% (v/v) acetone through centrifugation at 13 400 *g* for 10 min at room temperature. Immediately after washing, the pellet was air-dried for 5 min at room temperature. The air-dried pellet was solubilized in 1 ml of lysis buffer (9 M urea, 2 M thiourea and 4% (w/v) 3-cholamidopropyl dimethylammonio 1-propanesulfonate (CHAPS)) through vigorous vortexing at room temperature for an hour. After an hour of vortexing, the homogenate was centrifuged at 15 700 *g* at room temperature for 10 min. The supernatants for each sample were then transferred into fresh sterile Eppendorf tubes and stored at -20°C. Total protein concentration of the leaf extracts were quantified using a 2000 Nanodrop spectrophotometer (Thermo Scientific Inc., California, USA). One-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) or 1DE of about 10 µg protein on a 12% (w/v) was performed to evaluate the quality of the obtained protein extracts.

Two-dimensional electrophoresis (2-DE) of total soluble proteins

Prior to further analysis with 2-dimensional gel electrophoresis (2DE), the resolved total protein samples were further purified using the ReadyPrep™2-D Clean-up kit (catalog # 163-2130, Bio-Rad Laboratories Inc., California, USA) following the manufacturer's instructions to improve the quality of proteins. Good quality resolved proteins from the 1DE samples were then selected for further analysis through a 2DE. The protein samples were mixed with a rehydration buffer (8 M urea, 2% (w/v) CHAPS, 50% (w/v) dithiothreitol (DTT), 0.2% (v/v) ampholytes, 0.1% (w/v) of bromophenol blue (Bio-Rad Laboratories Inc., California, USA) to make a final volume of 125 µl. The immobilized pH gradient (IPG) strips (7 cm), pH 3 - 10 (Bio-Rad Laboratories Inc., California, USA) were passively rehydrated overnight in an equilibration tray with the rehydration solution containing equal amounts (150 µg) of protein samples at room temperature on a flat surface. Subsequently, the strips were then subjected to an isoelectric focusing (IEF) on a PROTEAN i12 IEF cell (Bio-Rad Laboratories Inc., California, USA) in a step-wise program. Focusing was carried out at 20°C and 50 µA current per IPG strip, following the set procedure of: 250 V for 20 min, followed by 4 000 V for 2 h and finally, 4,000 V until it reached 10,000 Vh.

The focused IPG strips were then equilibrated with 2.5 ml of SDS containing equilibration buffers as described by Ngara and Ndimba (2011). The equilibrated IPG strips were dipped into a 100 ml of SDS-PAGE running buffer and loaded onto the 10% (w/v) polyacrylamide resolving gels of 1-mm thickness. The strips were then overlaid with pre-warmed overlay agarose solution (100 ml 1 x SDS-PAGE running buffer; 0.5% (w/v) agarose; 0.002% (w/v) bromophenol blue), which was allowed to cool and solidify. The gels were electrophoresed on a Mini-PROTEAN Tetra hand cast system (Bio-Rad Laboratories Inc., California, USA) at a constant voltage of 150 V for 45 min or until the dye front had reached the bottom of the gel. Immediately, the electrophoresis was complete, gels were stained in a Coomassie staining buffer solution followed by de-staining (100% (v/v) ethanol, 100% (v/v) methanol, 100% (v/v) acetic acid) for 50 min, shaking on an ultra-rocker (Bio-Rad Laboratories., USA) until the protein spots were visualized. The de-stained gels were then image-captured on a Chemi DOC™ Imaging system (Bio-Rad Laboratories Inc., California, USA) using the Bio-Image Lab™ software.

RESULTS

Morphological responses of *Zea mays* to water stress

After the successful growth of the *Z. mays* cultivar (R450w/uo2250w CML550), morphological differences in the appearance of the control (water supplied plants) and experiment (water deprived plants) were documented. The resultant phenotypic changes between the two sets of plants were recorded for 16 days as illustrated in Figure 1. Water stress treatment resulted in noticeable phenotypic changes as shown by the gradual effects on the plants. Reduction in the overall plant growth was exhibited in treated plants as compared to the control treatment. In addition, leaf discoloration was also evident, whereby all leaves of the experimental plants had a dull green appearance while those of the control were somewhat bright green (Figure 1C and D). Control (well-watered) plants showed fully expanded leaves (Figure 1E) as compared to the experiment (water deficit) leaves that revealed a rolled morphology (Figure 1F). The width of the leaves showed a detectable difference, with the control leaves having a larger width than the experiment (Figure 1E and F). Number of leaves per plant was reduced, with the control having larger number of leaves than the experiment (Figure 1). Also, a decrease in shoot height and stem diameter (Figure 1) was evident in the experimental plants (Figure 1B, D and F), while shorter and thin in the controls (Figure 1A, C and E).

One-dimensional gel electrophoresis (1DE) expression profile of maize proteome

In order to investigate the changes in the maize leaf proteome in response to water stress, 1DE analysis of the total soluble proteins was undertaken. Maize total soluble leaf protein extracts were separated by 1DE to evaluate the quality of the extracts and visualized after staining with Coomassie (Figure 2). The total soluble protein expression profiles exhibited a mixture of numerous higher and lower abundant proteins (Figure 2). The protein extracts exhibited a relatively uniform protein expression, abundance and loading across biological replicates for both the control and water stressed treatment (Figure 2A). In addition, newly synthesized water stress proteins were observed in E1 and E2 (25, 27, 55 and 120 kDa), as compared to the control, where they were absent (Figure 2A). In order to eliminate contaminants, the protein extracts were further purified. No evident differences were detected in protein profiles (Figure 2B) between the control and water-stressed treatment.

Two-dimensional (2D) gel electrophoresis expression profile of maize proteome

Purified total soluble proteins were separately (two

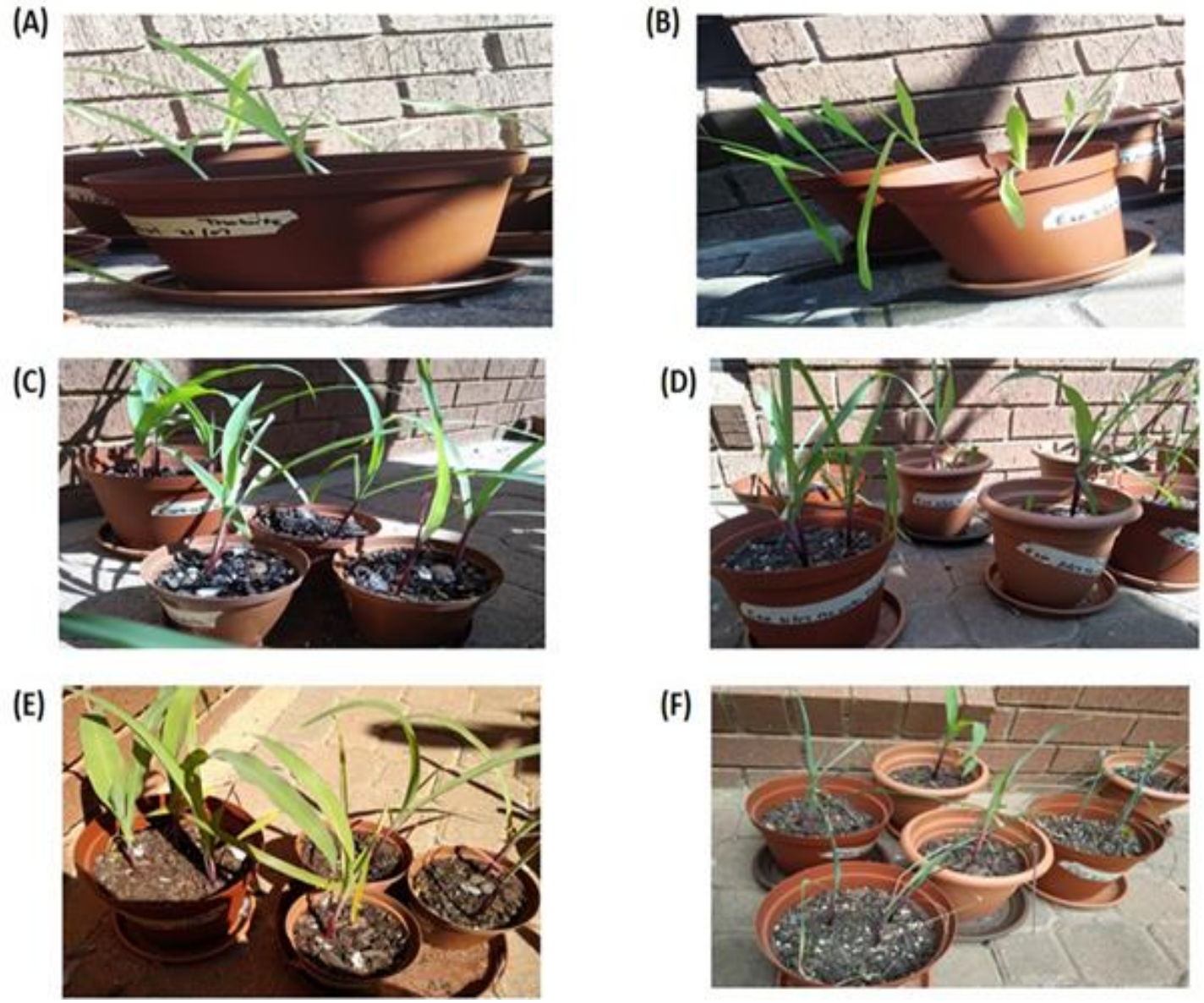

Figure 1. Morphological appearance of the *Zea mays* seedlings in withdrawing water conditions after 8, 13, 16 days. (A) represents the eight-day Z. mays control (water supplied) seedlings, while (B) represents the experimental (water-deprived) seedlings; (C) represents a 13-day control (well-watered), whereas (D) represents the treated (water-deprived) seedlings at the same duration, while (E) shows the last day of treatment (day 16) of the control well-watered seedlings and (F) represents the experimental (water deprived) seedlings.

treatment groups) subjected to 2D gel electrophoresis or 2DE analysis to evaluate the changes in 16-day old maize leaf proteome in response to water deficit using the 7 cm IPG strips, pH 3-10. The resolved control (well-watered) protein profile (Figure 3A) produced a minimal number of Coomassie stained spots, while the treated group (water deficit) exhibited an increased number of induced protein spots (Figure 3B), which indicate the effect of water deficit on the expression of most proteins. A total of nine differentially expressed protein spots were visualized through a comparison between the well-watered and water deficit leaf extracts (Figure 3).

DISCUSSION

Water deficit is one of the most serious abiotic factors that threaten the agricultural sector since it limits crop production especially in maize worldwide (Farooq et al., 2009; Raos et al., 2016). Many research groups have invested most of their time in attempting to discover the various complex mechanisms by which plants can cope with the different biotic and abiotic stress factors. Hence, in our study, we focused on the effects of abiotic stress on maize (*Z. mays*), specifically water deficit after day seven to the sixteenth-day of water-deficit exposure

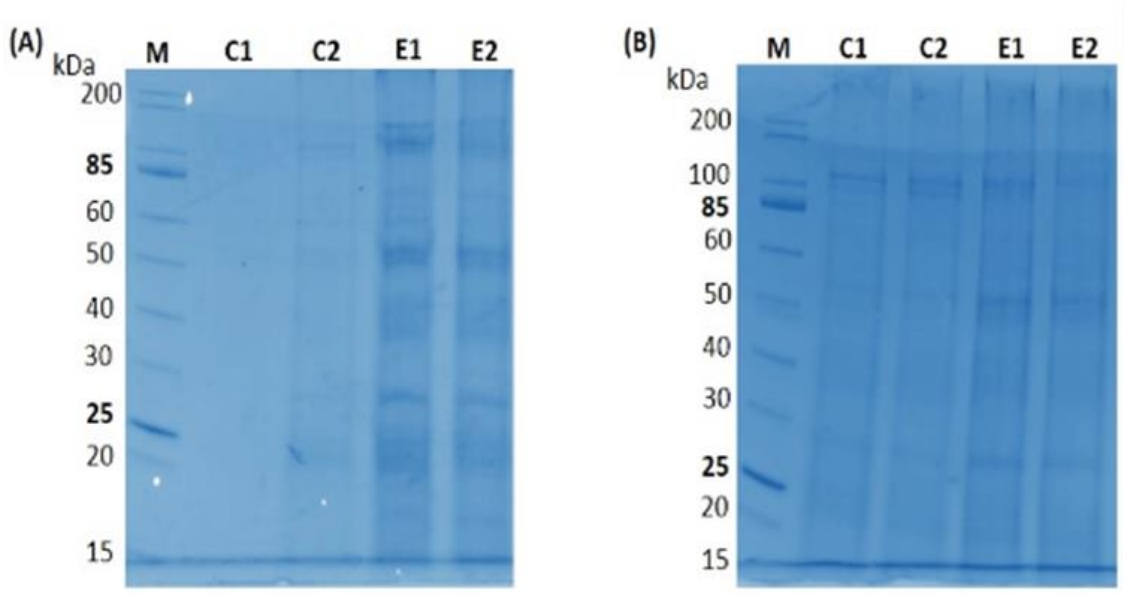

Figure 2. Comparative 1D SDS-PAGE profiles of *Zea mays* total soluble proteins. (A) An SDS-PAGE of the expressed non-purified protein fractions under water stress, where lane M is the molecular weight marker (Catalog# P7704S New England Biolabs Inc., Massachusetts, USA), lanes C1 and C2 represent the control (well-watered) protein samples, while lanes E1 and E2 represent the experimental (water-deprived) samples. (B) An SDS-PAGE of the expressed purified protein fractions under water stress, where lane M represents the unstained molecular weight marker (Catalog# P7704S New England Biolabs Inc., Massachusetts, USA), lanes C1 and C2 are control (well-watered) protein samples while lanes E1 and E2 represent the experiments (water-deprived).

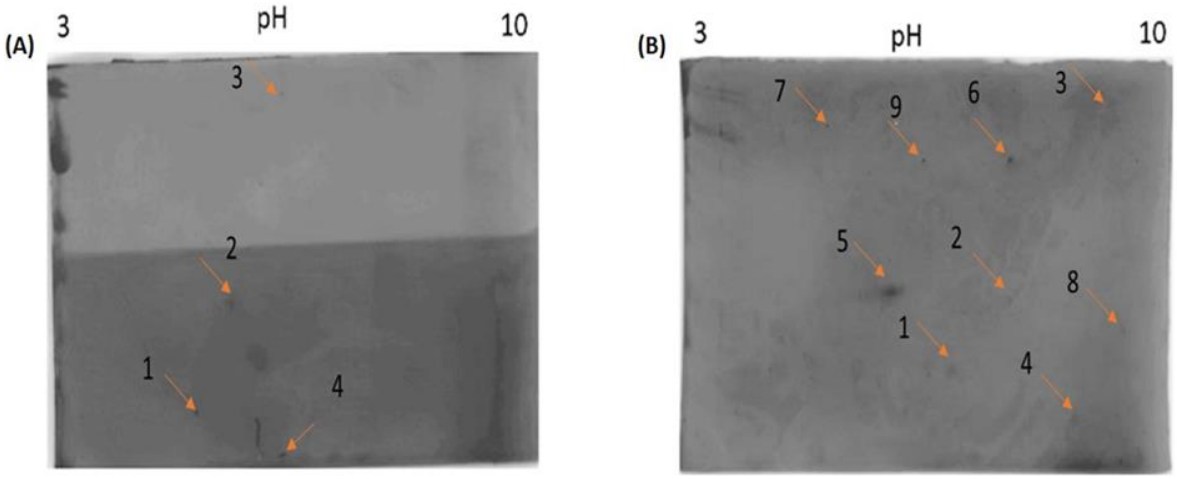

Figure 3. Coomassie blue stained 2D electrophoresis gels of *Zea mays* leaf proteins. A 10% (w/v) comparative acrylamide gel expression protein profiles of two treatments (A) control (well-watered) and (B) experiment (water stressed) showing total leaf proteome of Zea mays plants. Results indicated here are representatives of three independently carried out experiments.

period at the seedling stage. During water stress, plants are subjected to a multiplex of biochemical, physiological and molecular influences, which ultimately affect their growth, development and osmotic homeostasis (Zhu, 2002).

Combined morphological and proteomics approaches were used in our study to investigate the responses of water stress in *Z. mays*. Leaves are the most essential organ of a developing plant due to their role in photosynthesis; as they are the main indicators of the

plant's health. Various studies support the fact that water deficit stress causes a reduction in the overall plant growth as indicated in *Brassica species* (Hasanuzzaman et al*.,* 2014) and other plant species (Rizhsky et al*.,* 2002; Jaleel et al*.,* 2009). Our experimental findings concur with the previous investigations, where a decrease in the overall plant growth was evident under water deficiency (Figure 1). Water stress induces various morphological changes that includes modifications in leaf anatomy and ultrastructure, shrinkage in leaf size, decrease in stomata number; thickening of leaf cell walls, cutinization of leaf surface, and induction of early senescence (Seyed et al., 2012).

In our study, leaf rolling and reduced leaf area, were evident in the water deficient treated plants as compared to the well-watered control, which remained unrolled with expanded leaf area (Figure 1E and F) that clearly indicates the effect of water stress with longer exposure. Similarly, the rolling of leaf, reduction in leaf area and low rate of transpiration, were reported as coping mechanism employed by plants in arid areas against water loss (Clarke, 1986). The same leaf morphological changes were observed as a result of water loss (Kadioglu et al., 2012; Kim et al., 2014).

A notable difference was observed in the leaf width, with the control leaves having a larger width than the water deficit experiment (Figure 1E and F). Our findings concurred with previous experiments, which indicated inhibition of leaf expansion during water stress (Salazar et al., 2015; Fathi and Tari, 2016). In addition, water stress decreased the number of leaves per plant and shoot height, with the control plants having larger number of leaves than the experimental plants (Figure 1C and D). A decrease of shoot height in water stressed plants demonstrates that drought stress has an apparent effect on plant height (Hasanuzzaman et al., 2014). According to Fathi and Tari (2016), water deficit has a negative impact onto the development of shoot/root, thus affecting the height of the plant. The stem diameter was relatively thicker for control plants as compared to the experimental plants, which was thin but strong enough to support the whole plant. The obtained results firmly agree with other studies conducted on crop species such as tomato (Gallardo et al., 2004) and pepper (Cohen et al., 1998).

Maize total soluble leaf protein extracts were separated by 1DE to evaluate the quality and clear visualization with Coomassie staining (Figure 2). A relatively uniform protein expression, abundance and loading across the three biological replicates for both the control and water stressed treatments was observed (Figure 2A). In addition, water stress led to the observation of newly synthesised and/or more pronounced proteins that were clearly detected in E1 and E2 (25, 27, 55 and 120 kDa) as compared to the control, where the protein were absent (Figure 2A). In order to eliminate phenolic and ionic contaminants that normally associate with extracted protein samples, the protein extracts were further subjected to purification. Our findings showed no apparent difference in purified protein profiles (Figure 2B) between the control and water stressed treatment. The obtained results contrast those of a similar study conducted on plant seeds by Parchin and Shaban (2014), which found that there is always higher protein abundance in irrigated plants than those that are not irrigated. In general, our total soluble protein expression profiles exhibited a mixture of higher and lower abundant proteins (Figure 2).

In our study, the purified total soluble proteins from the stressed and unstressed groups were subjected to 2D gel analysis (2DE) on 7 cm IPG strips, pH 3-10, to profile water stressed proteins in maize. Notably, 2DE remains one of the highly recommended techniques for the identification of the total expressed proteins in both the stressed and unstressed treatment groups, due to its advantage in providing an overview of proteome separation in terms of their isoelectric point (*pI*) and molecular mass (Kim et al., 2015). In our case therefore, the separation on 2DE was carried out to determine the expression profiles of leaf protein extracts between the stressed (water deficit) and unstressed (well-watered) 16-day old maize plants, and depending on the nature, composition and complexity of the protein mixture.

The protein profile for the control (well-watered) (Figure 3A) produced a minimal number of Coomassie stained spots, while the experiment (water deficit) demonstrated an increase in the number of induced protein spots (Figure 3B), which indicates the effect of water deficit on the general expression of most proteins. A total of nine differentially expressed protein spots were visualized through a comparison between the well-watered and water deficit leaf extracts (Figure 3). From the control, four proteins were expressed (Figure 3A), while in the experiment, about five proteins were newly induced (spots 5, 6, 7, 8 and 9) under water deficit stress (Figure 3B). Our results indicate that water stress induced the abundance of several proteins in *Z. mays* leaves, and some of the affected proteins were either up-regulated (spots 1, 3) or down-regulated (spot 4) when water was withdrawn for days. Generally, most protein spots were however, confined between an experimental IEF pH restriction of 3-10. Nonetheless, our findings concur with previous studies carried out on drought stress in various plant species (Ngara et al., 2012; Kim et al., 2015; Cao et al., 2017).

Conclusion

Water stress induced a number of morphological and molecular changes in the R450w/uo2250w CML550 *Z.mays* cultivar. Our study, has in this regard, established and profiled the total soluble stress responsive proteins in the maize leaf proteome using 2DE. A total of nine differentially expressed proteins were identified, indicating

that the proteomic tools used herein were able to separate and allow for the detection of qualitative proteins in *Z. mays*.

In addition, proteins profiled in this study with their probable associated biochemical pathways provide new information regarding the response of maize to water stress, since maize is known to be highly sensitive to water stress. Findings of this study aid insights regarding molecular pathway responses in an understanding of the morphological and molecular mechanisms used by maize cultivars in response to water deficit. Future work on further identification of the profiled water stress proteins by mass spectrometry, iTRAQ and bioinformatics analyses will strongly assist in confirming the response mechanisms employed by the R450w/uo2250w CML550 cultivar against water stress.

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CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Morphological and molecular characterisation of *Colletotrichum gloeosporioides* (Penz) isolates obtained from *Dioscorea rotundata* (Poir)

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Anthracnose disease is a major constraint to yam production in tropical West Africa and anywhere the crop is cultivated. This study determined the cultural characteristics and growth rates of mycelia and also characterised 6 isolates of *Colletotrichum gloeosporioides,* the causal agent of the yam anthracnose disease, obtained from *Dioscorea rotundata* leaves, vines and setts in the Tolon District of Ghana. The cultural characteristics and mycelial growth rates of the isolates were determined on Potato Dextrose Agar (PDA). The *C. gloeosporioides* isolates were characterised using polymerase chain reaction technique with the universal primer pairs ITS1/ITS4 and NS1/NS2, *C. gloeosporioides* species specific primer pairs CgInt/ITS4 and CgLac-f/CgLac-r, and *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1. Based on the PCR, six isolates of *C. gloeosporioides* with distinct cultural characteristics were obtained. There were no significant differences ($P \leq 0.05$) in mycelial growth rates among the isolates. The *C. gloeosporioides* isolates produced characteristic band sizes on ITS1/ITS4, NS1/NS2, CgInt/ITS4 and CgLac-f/CgLac-r. None of the isolates produced a band on CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1. The proper identification of *C. gloeosporioides,* the pathogen responsible for the *D. rotundata* anthracnose is important for the proper management of the disease.

Key words: Anthracnose disease, cultural characteristics, DNA band, morphological characteristics, Mycelial growth rate, polymerase chain reaction (PCR).

INTRODUCTION

Yam, a staple crop is mainly cultivated in the tropical and subtropical regions for its tubers (Agrios, 2005; Achar et al., 2013). Unfortunately, these regions also provide favourable conditions that support the growth and survival of the yam anthracnose disease pathogen, *Colletotrichum gloeosporioides* (Penz); a major threat to yam production worldwide (Agrios, 2005; Chaube and Pundhir, 2009; Lebot, 2009; Reddy, 2015). In West African yam growing countries, the most important commercial yam species such as *D. alata* and *D. rotundata* are susceptible to the anthracnose disease (Ayodele et al., 2000; Lebot, 2009). The majority of

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cultivated yams in Ghana are varieties of *D. rotundata* and *D. alata* (Demuyakor et al., 2013), with the former being the most preferred (Otoo et al., 2015). During cultivation, the disease affects the yam leaves and vines, and severe infection results in yam plant defoliation and vine dieback (Ayodele et al., 2000). *C. gloeosporioides* has also been reported to cause an orange-brown yam tuber rot known as "dead skin" (Green and Simons, 1994; Reddy, 2015).

Yams are mainly cultivated from yam setts, which if not obtained from certified sources could be infected with *C. gloeosporioides* which is capable of initiating anthracnose disease on yam crops (Aighewi et al., 2003; Asiedu and Sartie, 2010; Ayoola, 2012; Osei-Adu et al., 2016). *C. gloeosporioides* is not host specific and as such the presence of other susceptible crops in and around yam fields could also be a source of inoculum for the establishment of the disease on yam crops (Lebot, 2009). The spores of *C. gloeosporoides* require moisture, optimum temperature (20 - 30°C) and high relative humidity to germinate and establish on yam crops, with mature spores mainly disseminated by rain splash (Sharma and Kulshrestha, 2015).

To enhance yam production, there is the need to manage the anthracnose disease on the crop. The proper identification of the causative agent of the disease is crucial for appropriate disease management. Identification of *C. gloeosporioides* based on cultural, mycelial growth rate and morphological characteristics can be confused with other species within the genus, more especially *C. acutatum* (Chowdappa and Kumar, 2012; Reddy, 2015). According to Serra et al. (2011), different species of *Colletotrichum* are capable of infecting a single host. The foliage infection of *C. acutatum* and *C. gloeosporioides* are difficult to differentiate in terms of their symptoms and cultural morphology (Shi et al., 2008). This makes it difficult to differentiate between *C. acutatum* and *C. gloeosporioides;* hence the need to use molecular techniques for the proper identification of *Colletotrichum* isolates (Serra et al., 2011). The polymerase chain reaction (PCR) technique has been documented as one means of properly identifying *C. gloeosporioides* isolates (Shi et al., 2008; Serra et al., 2011; Raj et al., 2013; Chagas et al., 2017). The proper identification of the *D. rotundata* anthracnose disease pathogen is essential for selecting appropriate strategies in managing the disease to enhance the crop's productivity. This study sought to determine the cultural characteristics, mycelial growth rates and molecular characteristics of *C. gloeosporioides* isolates obtained from infected *D. rotundata* leaves, vines and setts in the Tolon district of Ghana.

MATERIALS AND METHODS

Study site

The study was conducted in the Spanish Laboratory at the Nyankpala Campus of the University for Development Studies, during the 2016 and 2017 main cropping seasons. The site which is located in the Tolon district of Ghana (latitudes 9° 15' and 10° 02' North and Longitudes 0° 53' and 1° 25' West), were the *D. rotundata* leaves, vines and setts with anthracnose symptoms were obtained for the study. The mean annual rainfall ranges from 950 to 1,200 mm and humidity between April and October can be as high as 95% in the night, falling to 70% in the day. The soil is generally of the sandy loam type and the vegetative cover is basically Guinea Savanna interspersed with short drought resistant trees and grassland.

Sampling of anthracnose infected *D. rotundata* plant parts

Ten anthracnose infected *D. rotundata* leaves were randomly obtained from each of 48 yam farms in the Tolon district. Infected *D. rotundata* vines (a total of 5) were obtained by a complete survey of each of the 48 farms for die-back symptoms. One hundred and fifty *D. rotundata* planting setts (5 per farmer) were randomly obtained from 30 yam farmers. These setts were carefully examined and those with "dead skin" symptoms selected for the study. A total of 21 symptomatic "dead skin" setts were obtained for the study. The samples were then conveyed in well labelled envelops to the laboratory.

Preparation of culture media

The medium was prepared as directed by the manufacturer's (Sigma-Aldrich Co., Spain) recommendation of 39 g of Potato Dextrose Agar (PDA) per litre of distilled water. Two hundred and fifty (250) mg of amoxicillin was added to suppress bacterial growth. The mixture was heated to completely dissolve the solutes and then autoclaved at 1.03 kg cm^{-2} pressure at 121°C for 15 min. About 20 ml each of the molten media were poured into Petri dishes (9 cm diameter) and allowed to solidify before use.

Isolation of *C. gloeosporioides*

Fragments (1 cm) of symptomatic tissues consisting of diseased and healthy parts were obtained from the anthracnose infected *D. rotundata* leaves, and vines affected by die-back. Similarly, 0.5 cm^3 fragments were obtained from portions of the yam setts with the "dead skin" symptoms. These tissues were each washed with tap water and surface sterilised in 70% alcohol for 3 min. The tissues were then rinsed with three changes of sterilised distilled water. Each tissue was inoculated on the PDA and incubated at ambient temperature (28±2°C) for 7 days. The mycelia that grew were sub-cultured onto fresh PDA, and further sub-cultured until pure cultures of *C. gloeosporioides* were obtained.

Identification of *C. gloeosporioides*

The cultural and microscopic view of the morphological characteristics of 7 days old pure culture of the isolates under a compound microscope (Leica DME, Leica Microsystems, Shanghai, China) were compared to those documented by Barnett and Hunter (2006).

Determination of mycelial growth rate

The method of Than et al. (2008) was employed with some modifications. For each *C. gloeosporioides* isolate, the mycelial growth on PDA plate was measured daily until the fifth day. The

Table 1. Primer pairs and their annealing temperatures.

Primer pair	Sequence (5´→3´)	Annealing temperature (°C)
ITS1/ITS4	Forward: TCC GTA GGT GAA CCT GCG G Reverse: TCC TCC GCT TAT TGA TAT GC	56
NS1/NS2	Forward: GTA GTC ATA TGC TTG TCT C Reverse: GGC TGC TGG CAC CAG ACT TG	53
CgInt/ITS4	Forward: GGC CTC CCG CCT CCG GGC GG Reverse: TCC TCC GCT TAT TGA TAT GC	65
Ca-f1/Ca-r1	Forward: TGA ACA TAC CTA ACC GTT GC Reverse: AGG GTC CGC CAC TAC CTT TA	55
CaGlu-f1/CaGlu-r1	Forward: CGT TCA CGA CAA ACA CCT TG Reverse: ATC GAG TCG TGA TCG AAT CC	55
CgLac-f/CgLac-r	Forward: GAA GAT CTC GGC ACC ATC AT Reverse: AAC AAC AGG GAC CAG GTC AG	56

mycelial growth rate was calculated as 5 days average of the mean daily growth. Each isolate was replicated four times.

DNA extraction using the CTAB protocol

Extraction of DNA from the fungal mycelia was done according to the CTAB protocol (Lodhi et al., 1994). The mycelia were grinded to a fine paste in 400 µl of extraction buffer in microfuge tubes using a pestle and then incubated in a re-circulating water bath at 65°C for 15 min, followed by centrifugation at 12000 rpm for 5 min. Four hundred (400) µl of supernatant was transferred into new Eppendorf tubes and 250 µl of Chloroform: Iso Amyl Alcohol (24:1) was added to each tube, mixed with the solution by inversion and centrifuged at 13,000 rpm for 1 min. The upper aqueous phase was transferred into a clean microfuge tube and 50 µl of 7.5 M Ammonium Acetate, followed by the addition of 400 µl of ice-cold ethanol to each tube to precipitate the DNA. This was then mixed by slow inverted movements that caused the DNA to precipitate at the bottom of the tubes. The tube containing the DNA was then centrifuged at 13,000 rpm for 5 min after which the propanol was decanted. This was washed twice with 0.5 ml of 70% ethanol and centrifuged at 15,000 rpm for 5 min. The DNA was then dried and 50 µl of TE buffer was added to dissolve it. It was then stored at -20°C until required.

PCR amplification of *C. gloeosporioides* strains

The reaction volume was 20 µl containing 2 µL of genomic DNA, 2X Master Mix with standard buffer (New England Biolab, UK) and 1 µL of each primer. PCR amplification was started at an initial denaturation step at 94ºC for 5 min followed by 35 cycles of denaturation at 93ºC for 1 min; for each primer pair, DNA annealing was done at a specific temperature (Table 1) for 1 min with extension at 72ºC for 2 min and a final extension at 72ºC for 5 min. The formation and size of the PCR products were checked by electrophoresis in 1.0% agarose gel and stained with ethidium bromide.

Pathogenicity test

This was determined by a modified detached leaf method described by Shivanna and Mallikarjunaswamy (2009) with some modifications. Fully expanded apparently healthy *D. rotundata* leaves were detached from their plants and washed with tap water to remove any dust particles on them. The leaves were then surface sterilized with 70% alcohol for 3 min and rinsed in three changes of sterile distilled water and left to air-dry in a microflow laminar flow workstation. A leaf was then placed on moistened blotter discs (filter paper) in a Petri dish, wounded by gentle pricking with a sterilized needle and inoculated with 1 ml spore suspension (2 x 10^6 spore/ml) of *C. gloeosporioides* and then incubated under light-dark cycle of 12/12 h at 23±2°C for 7 days. The leaves that served as control were prick inoculated with sterile distilled water. Fungal pathogens were re-isolated on PDA plates and isolates compared with the inoculants culture based on colony and morphological characteristics.

Data analysis

The mycelial growth rate data were subjected to one-way Analysis of Variance (ANOVA) with GenStat (12th edition) statistical software. Treatment means were separated with the least significant difference of Tukey's multiple-range test at 5% significance level.

RESULTS

Cultural characteristics and mycelial growth rate of the *C. gloeosporioides* isolates

Based on the cultural and morphological characteristics, the *C. gloeosporioides* isolates obtained from the anthracnose infected yam leaves, vines and planting setts were grouped into six (CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6) (Table 2). Each of the isolates had cylindrical conidia with both ends rounded. Setae were not recorded for the isolates CDr1, CDr2, CDr3, CDr4, and CDr6, except for CDr5. The mycelial growth rate of the *C. gloeosporioides* isolates ranged from 4.55±0.287 (CDr4) to 5.35±0.263 (CDr3) mm per day (Table 2). There were no significant differences ($P \leq 0.05$) in mycelial growth rate among the various isolates (Table 2).

Table 2. *Colletotrichum gloeosporioides* isolates obtained from *D. rotundata* crops, their cultural characteristics and mycelial growth rate.

Isolate	Yam part of isolation			Cultural characteristics	Mycelial growth rate per day (mm)
	Leaves	Vines	Setts		
CDr1	+	-	-	Mycelial growth with a pinkish centre and whitish towards the margins.	5.30 ± 0.208[a]
CDr2	+	+	+	Whitish mycelial growth with concentric rings and having abundant orange conidia masses at the centre.	5.20 ± 0.141[a]
CDr3	+	+	-	White cottony sparse mycelial growth.	5.35 ± 0.263[a]
CDr4	+	-	-	White cottony dense mycelial growth.	4.55 ± 0.287[a]
CDr5	+	-	+	Whitish cottony mycelial with dark conidia masses occurring in concentric masses.	5.05 ± 0.171[a]
CDr6	+	+	-	White mycelial growth with concentric rings.	4.95 ± 0.377[a]
F (pr)					**0.291**

Means ± standard errors in the same column followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Tukey's multiple-range test. Key: (+) = isolated, (-) = not isolated.

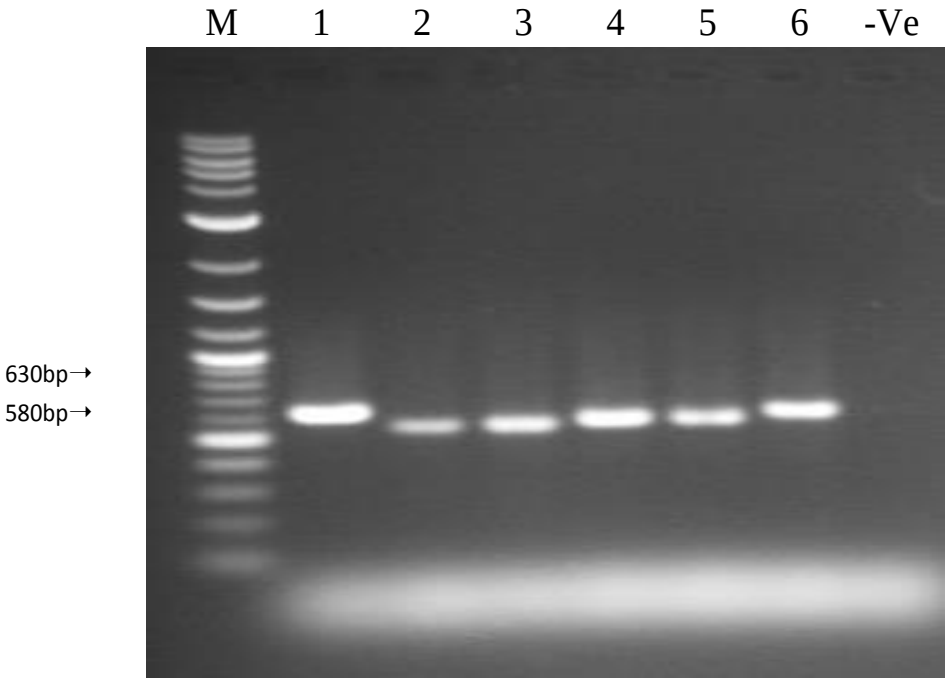

Figure 1. Amplified band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using the universal primer pair ITS1/ITS4. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb); 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

Polymerase chain reaction

The PCR analysis carried out for the detection of the ITS region of the *C. gloeosporioides* isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 produced characteristic band sizes of approximately 580 bp when run on the universal primer pair ITS1/ITS4 (Figure 1). A band size of 560 bp was amplified when the DNA of each of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 were run with the 18S rRNA universal primer pair NS1/NS2 (Figure 2). The species-specific primer pair for *C. gloeosprioides,* CgInt/ITS4 produced a band size of 463 bp for each of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 (Figure 3). Also, CgLac-f/CgLac-r primer pair which is specific to *C. gloeosporioides* produced band sizes which ranged from 200 bp to 1300 bp for the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 (Figure 4). The *C. acutatum* species specific primer pairs

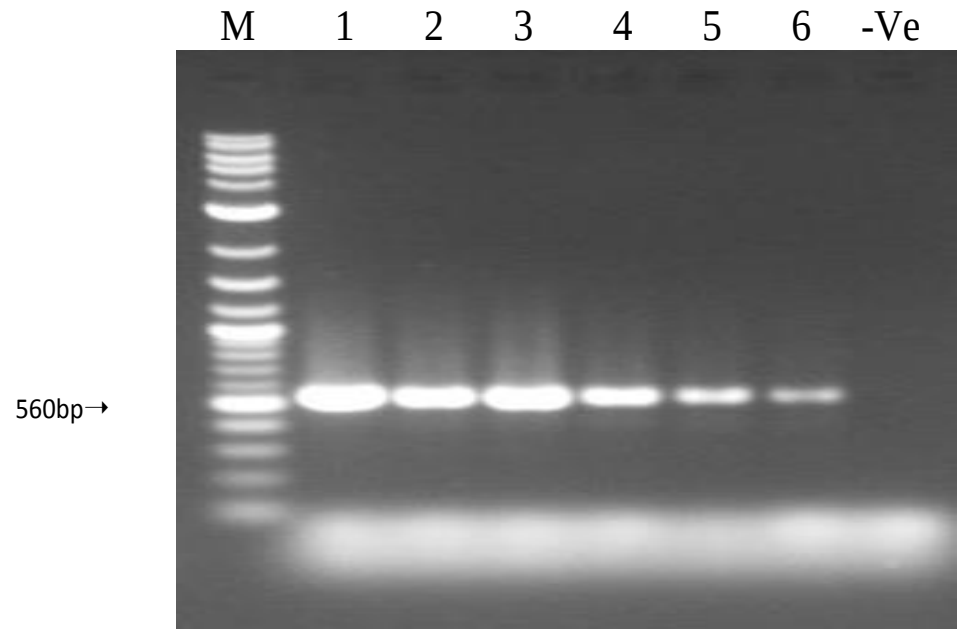

Figure 2. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using the universal primer pair NS1/NS2. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb; 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

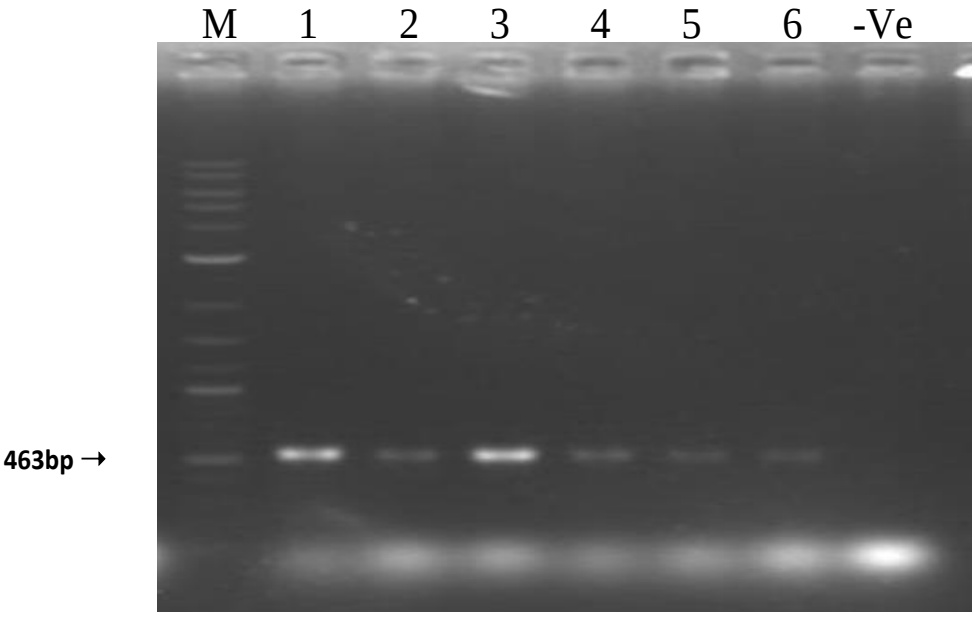

Figure 3. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using *C. gloeosporioides* species specific primer pair CgInt/ITS4. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb; 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1 each did not produce bands for all the isolates of *C. gloeosporioides* (Figures 5 and 6). There was no PCR amplification for the negative control (nuclease-free PCR water) on the various primer

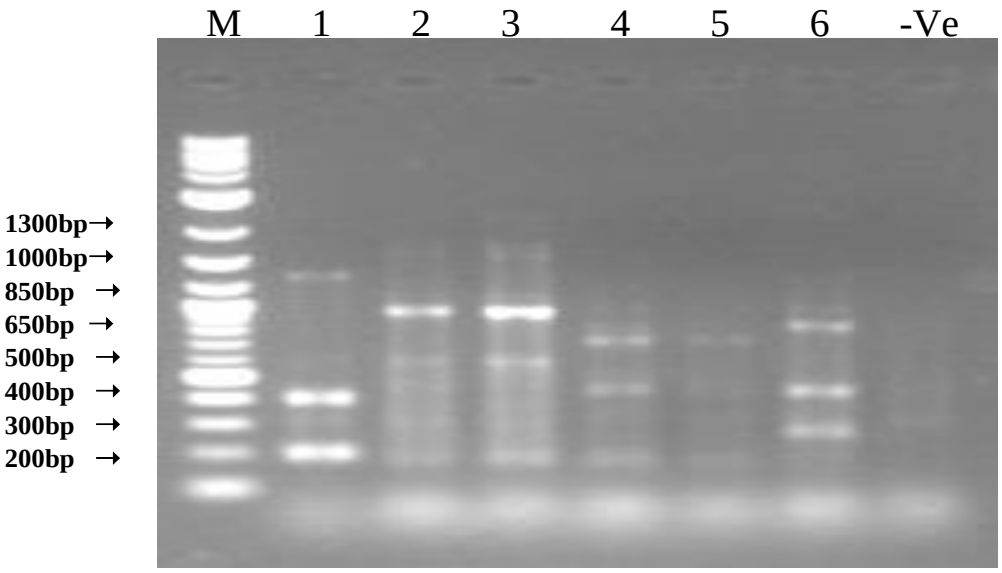

Figure 4. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using C. *gloeosporioides* species specific primer pair CgLac-f/CgLac-r. M = Molecular size marker, Quick-Load Purple 2-Log DNA Ladder (0.1-10.0 kb); 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

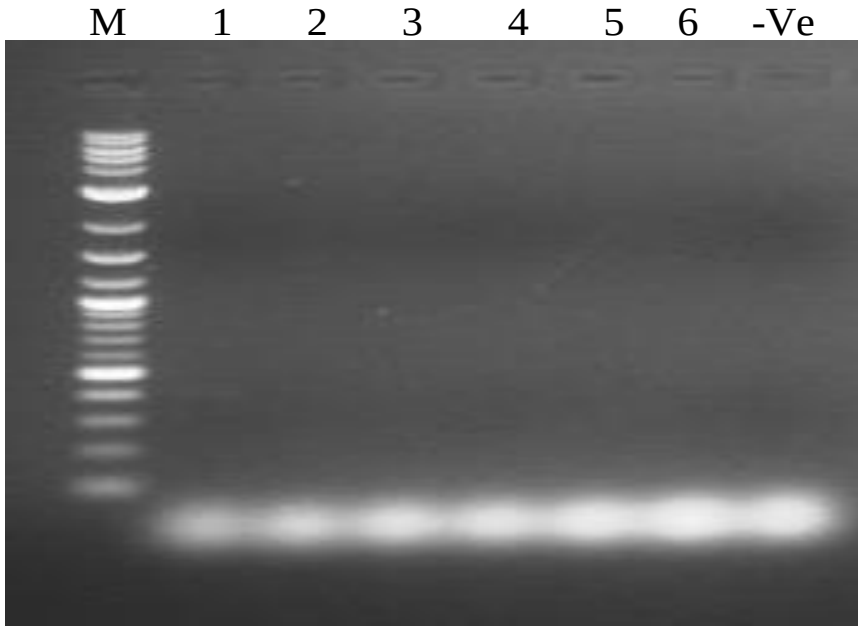

Figure 5. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using *C. acutatum* species specific primer pair CaGlu-f1/CaGlu-r1. M = Molecular size marker (Quick-Load Purple 2-Log DNA Ladder, 0.1-10.0 kb; 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

pairs ITS1/ITS4, NS1/NS2, CgInt/ITS4, CgLac-f/CgLac-r, CaGlu-f1/CaGlu-r1 and Ca-f1/ Ca-r1 (Figures 1, 2, 3, 4, 5 and 6).

Pathogenicity test

The yam leaves inoculated with the *C. gloeosporioides*

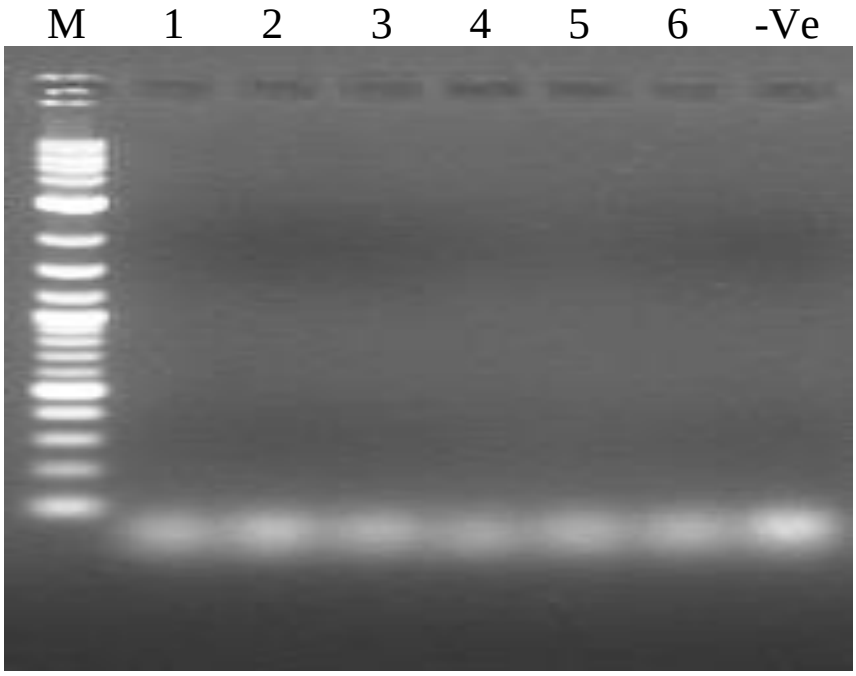

Figure 6. Amplified DNA band sizes of *Colletotrichum* strains (lanes – 1, 2, 3, 4, 5, 6) using *C. acutatum* species specific primer pair Ca-f1/Ca-r1. M = Molecular size marker, Quick-Load Purple 2-Log DNA Ladder (0.1-10.0 kb); 1 = CDr1, 2 = CDr2, 3 = CDr3, 4 = CDr4, 5 = CDr5, 6 = CDr6, -Ve = Negative controls (nuclease-free PCR water).

conidia suspension developed symptoms of anthracnose disease. The re-isolation of the pathogen from these diseased leaves on PDA produced similar cultural and morphological characteristics as the mother culture. Anthracnose disease symptoms were not developed on the negative control leaves. These outcomes satisfied Koch's postulate.

DISCUSSION

The cylindrical shaped conidia with rounded ends recorded for each of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 were similar to the observations made by Abera et al. (2015) who also worked on *C. gloeosporioides.* The mycelial growth rate per day for the various isolates which ranged from 4.55±0.287 to 5.35±0.263 mm were within the growth rate range of 3.6 to 11.2 mm recorded for *C. gloeosporioides* (Than et al., 2008; Abera et al., 2015). The insignificant differences ($P \leq 0.05$) in growth rate among the *C. gloeosporioides* isolates confirmed Than et al. (2008) report. Interestingly, Abera et al. (2015) recorded significant differences in mycelial growth rate among *C. gloeosporioides* isolates. These contradictory reports on significant differences in mycelial growth rate among *C. gloeosporioides* isolates attested that, it is not reliable for distinguishing among *Colletotrichum* isolates.

The observed variation in colour and pattern of growth among the pure cultures of CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 agreed with the findings of Than et al. (2008), Gautam (2014), Abera et al. (2015) and Appiah-Kubi et al. (2016) who also documented differences in cultural characteristics among isolates of *C. gloeosporioides*. The differences in cultural characteristics among the *C. gloeosporioides* isolates could be attributed to environmental rather than genetic factors. This is because according to Than et al. (2008), the use of morphological and phenotypic characteristics in distinguishing among C*olletotrichum* species can be deceptive, since different environmental conditions can cause variation among these traits. This makes it unreliable to depend solely on the cultural and morphological characteristics of *Colletotrichum* species for their identification. According to Cannon et al. (2000), the DNA traits of an organism are not directly influenced by environmental conditions and hence the most reliable method of distinguishing among *Colletotrichum* species will be the use of molecular techniques such as PCR.

When the DNA extracts of the C*. gloeosporioides* isolates were subjected to PCR run with the universal primer pairs ITS1/ITS4, the band size amplification of approximately 580 bp was yielded for all isolates. This agreed with the findings of Raj et al. (2013) who also

observed band amplifications of 580 bp when *C. gloeosporioides* isolates were subjected to PCR using the universal ITS1/ITS4 primer pair. Also, the various isolates were not separated when run on NS1/NS2 primer pair, because each of them produced a band size of 560 bp. This agreed with the findings of Shi et al. (2008) who also observed a 560 bp band size for *C. gloeosporioides* isolates after subjecting them to PCR using the universal primer pair NS1/NS2. The 463 bp detected with the C. gloeosporioides specific primer pair CgInt/ITS4 for isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 also confirmed all the isolates as the same strain of *C. gloeosporioides.* A similar observation was made by Shi et al. (2008). Also, Serra et al. (2011) reported that *Colletotrichum* isolates that produced band sizes on the primer pair CgInt/ITS4 was a confirmation of their identity as *C. gloeosporioides.*

The PCR analysis of the isolates CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 on the *C. gloeosporioides* species specific primer pair CgLac-f/CgLac-r produced band sizes for the various isolates; also revealing them as *C. gloeosporioides.* This agreed with the observation of band size production by *C. gloeosporioides* on the primer pair CgLac-f/CgLac-r documented by Shi et al. (2008) and Chagas et al. (2017).

The difficulty in distinguishing between *C. gloeosporioides* and *C. acutatum* using cultural and morphological characteristics necessitated the PCR run of all isolates on the *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/ Ca-r1 to further clarify their identity as *C. gloeosporioides*. The lack of band production by the various *C. gloeosporioides* isolates when they were subjected to PCR on each of the *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1 further confirmed their identification as *C. gloeosprioides*. A similar observation was made by Shi et al. (2008) who also recorded no DNA amplification for the PCR analysis of *C. gloeosporioides* on each of the *C. acutatum* species specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1. The pathogenicity test which showed that the *C. gloeosporioides* isolates produced anthracnose disease symptoms on the yam leaves confirmed the isolates as the causative agents of the disease.

The isolates of this study were identified as *C. gloeosprioides* based on their cultural and morphological characteristics, as well as mycelial growth rate. The isolates were confirmed as *C. gloeosprioides* as they produced characteristic bands on the universal primer pairs ITS1/ITS4 and NS1/NS2, and *C. gloeosprioides* species specific primer pairs CgInt/ITS4 and CgLac-f/CgLac-r. Also, the lack of bands amplification by CDr1, CDr2, CDr3, CDr4, CDr5 and CDr6 on the *C. acutatum* specific primer pairs CaGlu-f1/CaGlu-r1 and Ca-f1/Ca-r1 further confirmed all the isolates as *C. gloeosporioides*. The proper identification of *C. gloeosporioides* as the pathogen causing the *D. rotundata* anthracnose disease would aid in the development of appropriate strategies and tactics in the management of the disease in the Tolon district.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Evaluation of different methods for total RNA extraction from *Thaumastocoris peregrinus*

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Thaumastocoris peregrinus **is a pest that damages eucalyptus plantations. Recently in Brazil, there are few studies related to its control. With the advancement of molecular biology, several techniques can assist in the discovery of an effective and sustainable control. The different techniques of analysis of gene expression start with the extraction of the total RNA from the material for genomic analysis, hence it is essential that the RNA be intact. Numerous insect RNA extraction protocols are available, but none of them are widely effective, therefore, several factors and intrinsic characteristics of the tissue to be analyzed can affect the quality of the extracted material. Thus, it is necessary to modify the protocols in order to optimize the extraction of RNA. This study aimed to analyze the efficiency of three methods for RNA extraction from *T. peregrinus*. The results obtained demonstrated that the RNA extracted with only one method was more efficient due to less contamination with DNA and greater integrity of the samples. It should be noted that the determination of an efficient protocol for the extraction of RNA from *T. peregrines* will assist in future research, which will assist in the discovery of genes present in the extracted RNA sample.**

Key words: Ribonucleic acid, bronze bug, *Eucalyptus.*

INTRODUCTION

Eucalyptus production is becoming increasingly prominent in the Brazilian economy. Countless advances have been achieved in terms of productivity and the total planted area has been growing. However, since their introduction in the country for commercial purposes, eucalyptus species have suffered from the attack of pests and diseases. Among these, the bronze bug, *Thaumastocoris peregrinus* (*T. peregrinus*) Carpintero and Dellapé, 2006 (Hemiptera: Thaumastocoridae), was detected in the year. 2008. The bronze bug is an exotic pest and has since attacked eucalyptus plantations, reducing its production. This insect pest has adapted and spread throughout the Brazilian territory, starting to cause direct damage to eucalyptus plants, a fact that caused a considerable increase in production costs and generated serious commercial losses (Wilcken et al., 2010).

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As a phytophagous species, when feeding, the tan bug perforates the leaves and fine branches to suck the sap, causing a silvering of the leaves and, later, the leaves look tan and suffer abscission, with consequent reduction of photosynthetic area of the plant. In case of severe attacks, it can lead to death (Jacobs and Neser, 2005).

When it sucks the leaf sap, it causes severe damage due to the possible release of enzymes, which leaves the leaf with a tanned appearance, reducing the photosynthetic area of the plant. The identification and characterization of the molecular mechanisms of the insect responsible for leaf damage are important to support population control strategies for *T. peregrinus*, mainly through the resistance of plants. Molecular biology techniques favor this approach and in this way, this work aims to identify and characterize the expression of important genes that are related to leaf damage caused by the insect.

However, the use of these molecular techniques depends directly on obtaining good quality nucleic acids including RNA in adequate quantities. Studies in the field of molecular biology have evolved rapidly and new techniques have been shown to be useful in the quantification of expression patterns. However, the numerous techniques of analysis of gene expression currently available directly require obtaining good quality and adequate quantities of the extraction of pure and intact RNA (Aras et al., 2003). These steps are essential for a comparative quantitative analysis.

Extraction procedures are not absolutely reproducible for all species, since they possess different types of tissues and cells (Gouveia and Regitano, 2007). In addition, it is essential to accumulate a significant amount of RNA for some procedures, which can become complicated to small insects, such as the bronze bug (*T. peregrinus*).

In practice, the procedures are empirical, as it is essential to making adaptations and modifications to the protocols (Chiari et al., 2009); also, it is essential to use particular methodologies in order to optimize the extraction of RNA from a good quality sample (Waldschmidt et al., 1997). Furthermore, the protocol in use must be appropriate to the objective that will be given to the extraction of the nucleic acid, such as quantitative real-time PCR (qRT-PCR), sequencing, cloning, gene expression, etc., to find results conducives and acceptable to the application (Bartlett and Strirling, 2003).

RNA extraction and isolation consists of the initial lysis and cell denaturation phase in which cell membranes are disrupted and total nucleic acids extracted. The predominant difficulty in RNA extraction is its degradation by the present stable and active ribonucleases (RNases) in the tissues (Bitencourt et al., 2011). Therefore, initial extraction buffers must have RNase inhibition to prevent the action of RNases (Romano and Brasileiro, 1999). The second stage consists of the separation of RNA from other unwanted cellular components, such as membrane debris, proteins and DNA and the third and last stage, involves precipitation of RNA, usually with the use of alcohols (ethanol or isopropanol) (Gouveia and Regitano, 2007).

With the premise of ensuring high quality RNA extraction from small amounts of biological material, numerous commercial kits are manufactured, and for the most part, they are in fact efficient; however, it is essential to observe the viability for the species and tissue you want to work on (Bitencourt et al., 2011). As an alternative, different protocols and reagents for the extraction of RNA from arthropods have been described in several studies; however, for the extraction of *T. peregrinus* specifically, they are not efficient. We were able to extract the RNA, but for use after extraction it is not satisfactory.

Considering that it is not only in places where *T. peregrinus* has been introduced that there is a need to generate and make available information that helps in the management of this insect pest, in a bid to reduce economic losses and guarantee the productive and environmental sustainability of the stands of Eucalyptus, as well as the the difficulty in sources of resistance for genetic improvement of the eucalyptus for the pest in focus and the limitations in the efficiency and durability of chemical treatments and biological control, a promising alternative method of control such as gene silencing by interfering RNA (RNAi) can be developed. The genomic and transcriptomic sequences of *T. peregrinus* necessary for the selection of target genes are still unknown. With focus on sequencing the transcriptome of the bronze bug, the present study aimed to establish protocols for obtaining the extraction of total RNA from *T. peregrinus* samples.

MATERIALS AND METHODS

The adult insects of *T. peregrinus* were collected in the field at the Campus II of the Federal University of the Jequitinhonha and Mucuri Valleys, located in the city of Diamantina-MG. Subsequently, 5 insects were released in 5 Petri dish for each protocol (15 Petri dish) with 5 repetitions for each protocol (Figure 1), where they were fasted for 2 h, then insects from Petri dish were transferred to an Eppendorf tube and immediately frozen in liquid nitrogen, in order to maintain the integrity of the samples, facilitate the extraction processes and keep the metabolic conditions related to the attack process of *T. peregrinus* stable; thereafter, we proceeded to extract the RNA immediately.

Three protocols used to extract total RNA from insects were used in the experiment and are described below.

All procedures were adopted with the following cleaning / sterilization measures to ensure that the site was free of any contaminants:

1) The solutions used were made with DEPC water (diethylporocarbonate) properly autoclaved;
2) All glassware and other utensils were previously autoclaved at 121°C for a period of 30 min and then dried in an oven;
3) Polypropylene tips and microtubes are new and free of RNAs;
4) The bench used is exclusively for the handling of RNA and was

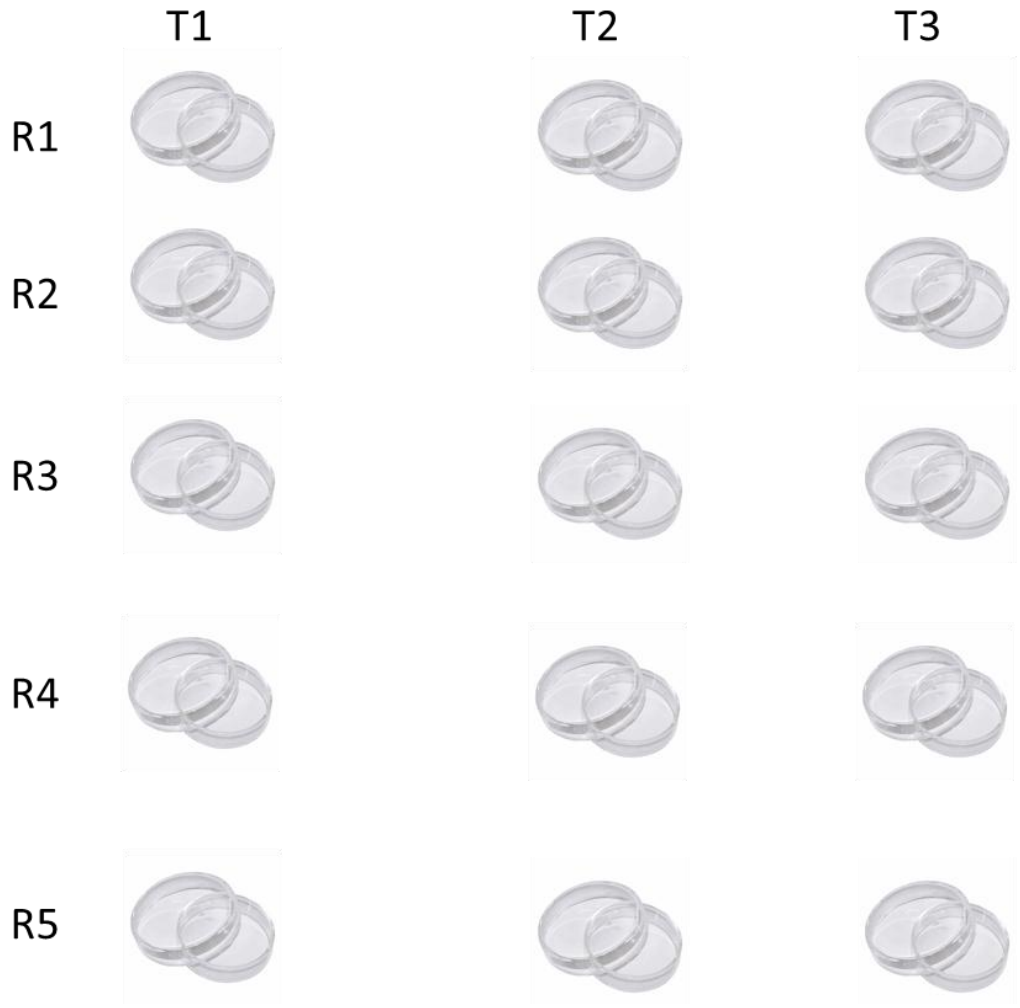

Figure 1. Design of the experimental scheme. T1: Samples extracted using (Protocol 01: TRI Reagent ® - SIGMA with modifications described. T2: Samples extracted using (Protocol 02: D-Sorbitol - SIGMA). T3: Samples extracted using (Protocol 03: RNeasy Mini-kit - Qiagen) R1, R2, R3, R4 and R5 are the repetitions.

constantly cleaned with SDS 10% and with 70% ethanol, as were all the equipment used in the experiment;
5) In addition, the necessary care was taken to keep the gloves used free of RNAs;
6) The experiments were carried out in a controlled environment at a temperature of 23°C.

Protocol 01: TRI Reagent ® - SIGMA with modifications described

In centrifugation microtubes (Eppendorf) of 1.5 ml, 5 insects were deposited along with 1000 µl of trizol® buffer (Sigma); thereafter 3 microspheres (beads) were added to the tube. With the aid of a mechanical disruptor (Mini-Beadbeater ™), the total RNA was extracted. This done, the samples were incubated for 5 min on ice, then 200 µl of chloroform were added and the microtubes were shaken for 15 s via manual inversion, and promptly incubated for another 15 min on ice. Subsequently, the samples were centrifuged for 15 min at 4°C and 10351 xg. The next stage consisted of washing the material and for this, the supernatant was transferred to a new tube, 500 µl isopropanol was added, the solution was mixed via inversion and incubated for 10 min on ice. Further, the sample was subjected to another centrifugation for another 10 min at 4°C and 10351 xg. In this step, the supernatant was removed so that only the precipitated material (Pellet) remained and immediately 1000 µl of 75% ethanol was added, mixed via inversion for approximately 15 s and again centrifuged, this time for 5 min at 4°C and 8000 RPM.

The supernatant was again discarded and the precipitated material dried for 10 min in a laminar flow chamber. To the dry material, 35 µl of water treated with the diethyl pyrocarbonate reagent (DEPC) was added, as it is a strong inhibitor of RNase activity.

Protocol 02: D-Sorbitol – SIGMA

In centrifugation microtubes (Eppendorf) of 1.5 ml, 3 microspheres (beads) were added together with up to 5 insects and stirred in the Disruptor (Mini-Beadbeater ™). Thereafter, 250 µl of the D-Sorbitol wash buffer and 15 µl of 2-βmercaptaethanol (β-ME) per sample

Table 1. RLT buffer volumes for sample rupture and homogenization.

Sample	Amount	Dish	Buffer RLT	Disruption and homogenization
Animal cells	$<5 \times 10^6$	< 6 cm	350 µl	Add Buffer RLT, Vortex ($\leq 1 \times 10^5$ cells); or use QIAshredder, TissueRuptor®, or needle and syringe.
	$\leq 1 \times 10^7$	< 6 - 10 cm	600 µl	
Animal tissues	< 20 mg	-	350 µl*	TissueLyser LT; TissueLyser II; TissueRuptor, or mortar and pestle followed by QIAshredder or needle and syringe.
	≤ 30 mg	-	600 µl	

Source: Manufacturer's protocol.

were added and mixed via stirring in the Disruptor (Mini-Beadbeater ™) for 10 s. Subsequently, the tubes were centrifuged for a period of 5 min at 5000 xg.

Further, the supernatant was discarded and the washing process repeated for at least one more time, or until the buffer shows no suspension. In this step, 120 µl of cetyltrimethylammonium bromide (CTAB), preheated to 65°C, together with 7 µl of β-ME per sample were added, the solution was homogenized by stirring with the aid of the Disruptor (Mini-Beadbeater™) for 10 s and then incubated in a water bath at 65°C for 60 min, with homogenization by manual inversion every 10 min.

Subsequently, the material was cooled on ice for 5 min, and after that, 700 µl of chloroformizo: isoamyl alcohol (CIA 24: 1) was added, followed by stirring for 10 s in the Disruptor (Mini-Beadbeater ™) and centrifugation at 5,000 xg for 10 min at 4°C. About 600 µl of the supernatant was promptly transferred to a new microtube, 700 µl of CIA was added, the solution was stirred again for 10 s in the Disruptor (Mini-Beadbeater ™) and subjected to centrifugation for 10 min at 4°C and 13000 xg.

After this step, the supernatant was transferred to another microtube, in addition to 1/10 of the volume of 3 M sodium acetate, pH 5.2 and 1/3 of the volume of cold isopropanol (isopropyl alcohol), the solution was mixed via inversion and the tubes stored at -20°C for a minimum period of 1 h.

Centrifugation was carried out at 13,000 xg for 10 min at 4°C. The supernatant was removed so that only the precipitated material was left (Pellet) and immediately 1000 µl of 70% ethanol was added. Again, centrifugation was carried out at 13,000 xg for 10 min at 4°C. Supernatant was again discarded and the precipitated material dried for 10 minutes in a laminar flow chamber.

Protocol 03: RNeasy Mini-kit – Qiagen

Before starting the extraction itself, the following was performed:

1. Addition of 10 µl of β-mercaptoethanol (β-ME) or 20 µl of 2 M dithiothreitol (DTT) to 1 ml of RLT buffer.
2. Addition of 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution.

A maximum of 30 mg of tissue (indicated by the manufacturer) was macerated with BUFFER RLT and then the appropriate volume of Buffer RLT was added, as described in Table 1. The tubes were centrifuged for a period of 3 min at a speed of 13,000 xg.

Furthermore, the supernatant was carefully removed with the aid of a pipette, 1 volume of 70% ethanol was added to the lysate and again the solution was homogenized with the aid of the pipette; also, the manufacturer did not recommend centrifugation. For this step, it was necessary to transfer 700 µl of the sample, including any precipitate, to an RNeasy Mini column spin previously placed in a 2-ml collection tube (provided in the kit) and the material was centrifuged for 15 s at ≥ 8000 xg. At the end, the tube was discarded, 350 µl of RW1 buffer was added to the RNeasy column and the samples were centrifuged for 15 s at ≥ 8000 xg. The residual solution was discarded and the column returned to the tube.

Concomitantly, 10 µl of DNase I stock solution (obtained by adding 550 µl of RNase-free water and homogenizing by manual inversion) was added to 70 µl of RDD buffer. The solution was gently homogenized by inversion and spun briefly. That done, 80 µl of the DNase incubation mixture was added directly to the RNeasy column membrane and the samples were incubated at room temperature for 15 min.

Subsequently, 350 µl of RW1 buffer was added to the RNeasy column, the solution was centrifuged for 15 s at ≥ 8000 xg and the flow was discarded. The RNeasy column was returned to the tube and 700 µl of RW1 buffer was added, with subsequent centrifugation for 15 s at ≥ 8000 xg and the flow discarded.

At the end of this period, 500 µl of RPE buffer was added to the RNeasy rotation column, the lid was closed and the solution was centrifuged for 15 s at ≥ 8000 xg; thereafter the flow was discarded. Further, 500 µl of Buffer RPE was added to the RNeasy rotation column, followed by decentrifugation for 2 min at ≥ 8000 xg.

In order to dry the column, centrifugation was performed at 13,000 xg for 1 min. Then, the RNeasy centrifuge column was transferred to a new 1.5 ml collection tube and 50 µl of RNAse-free water was added directly to the column membrane and centrifuged for 1 min at ≥ 8000 xg, to dilute the precipitated RNA.

At the end of each extraction process, the total RNA samples obtained were subjected to integrity analysis using the analytical method of electrophoretic visualization in 1.5% agarose gel, stained with ethidium bromide and visualized under ultraviolet light, in transilluminator, and photo-documented in a photo-documenter (LoccusBiotecnologiaTransluminator L. Pix) coupled to a microcomputer.

RESULTS

A difference was observed in the efficiency of the three protocols tested regarding the quality and quantity of the extracted RNA. The analysis by electrophoresis allowed verification such that the separation of the bands of the different types of RNAs was easily observed for the three protocols tested in this study (Figures 2 to 4).

However, the extraction performed with the commercial kit “RNeasy Mini-kit” (Qiagen) showed clearer and more intense bands, for all samples analyzed (Figure 4). Although with lower band intensity, extraction with D-Sorbitol (SIGMA) also allowed an invariable standard of total RNA quality for all samples (Figure 3).

Among the tested protocols, only Protocol 01, using the

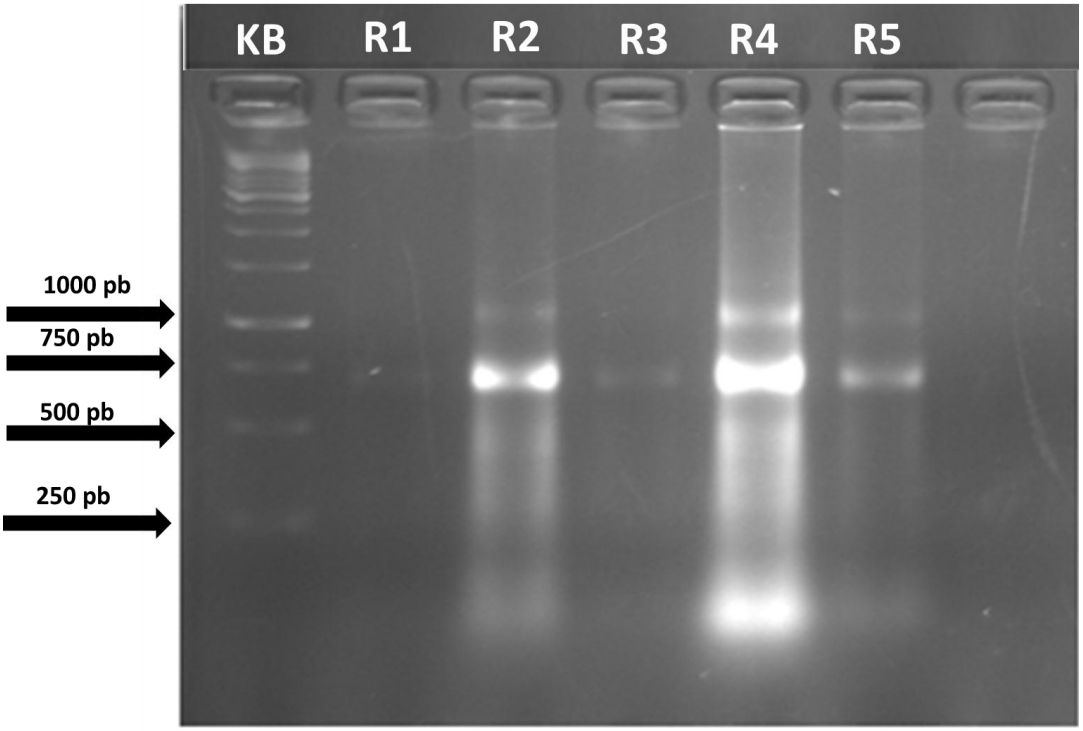

Figure 2. *T. peregrinus* RNA electrophoresis gel with Protocol 01: TRI Reagent ®. - SIGMA. Legend: KB Size marker 1 Kb DNA Ladder (Scientific Term), R1, R2, R3, R4, R5 is the repetitions. pb: Base pairs.

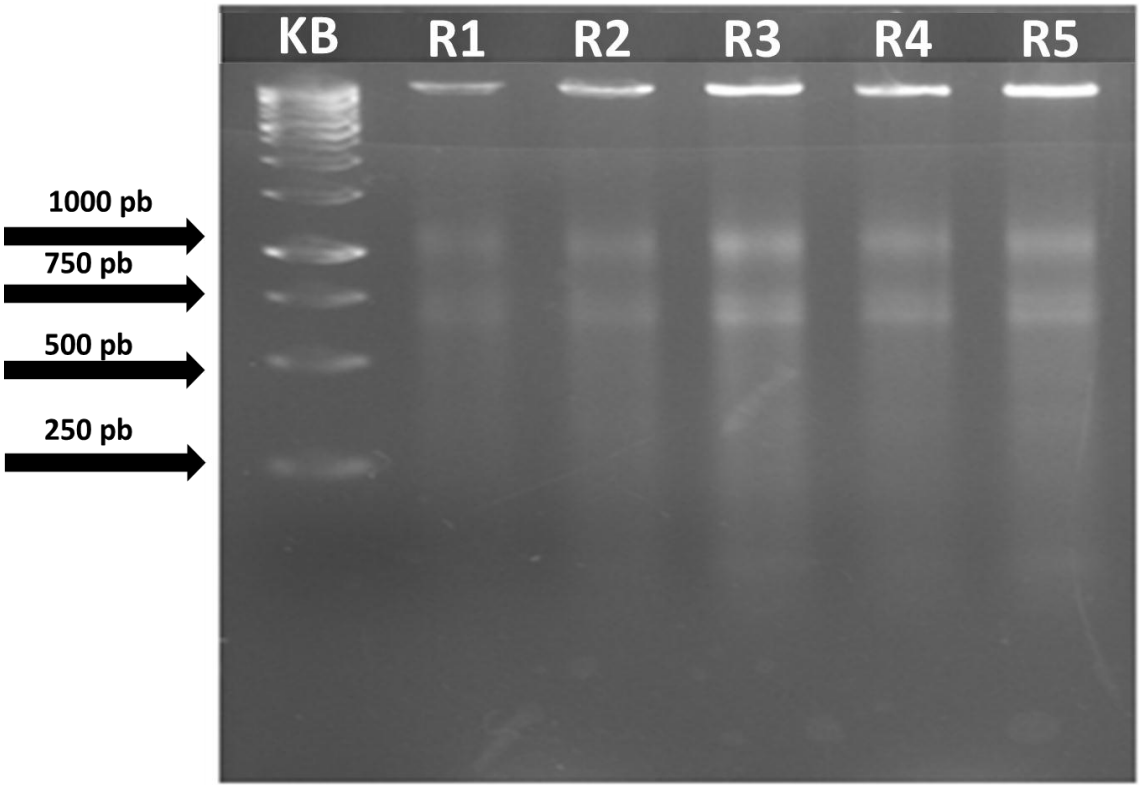

Figure 3. *T. peregrinus* RNA electrophoresis gel with Protocol 02: D-Sorbitol - SIGMA. KB Size marker 1 Kb DNA Ladder (Scientific Term), R1, R2, R3, R4, R5 are the repetitions. pb: Base pairs.

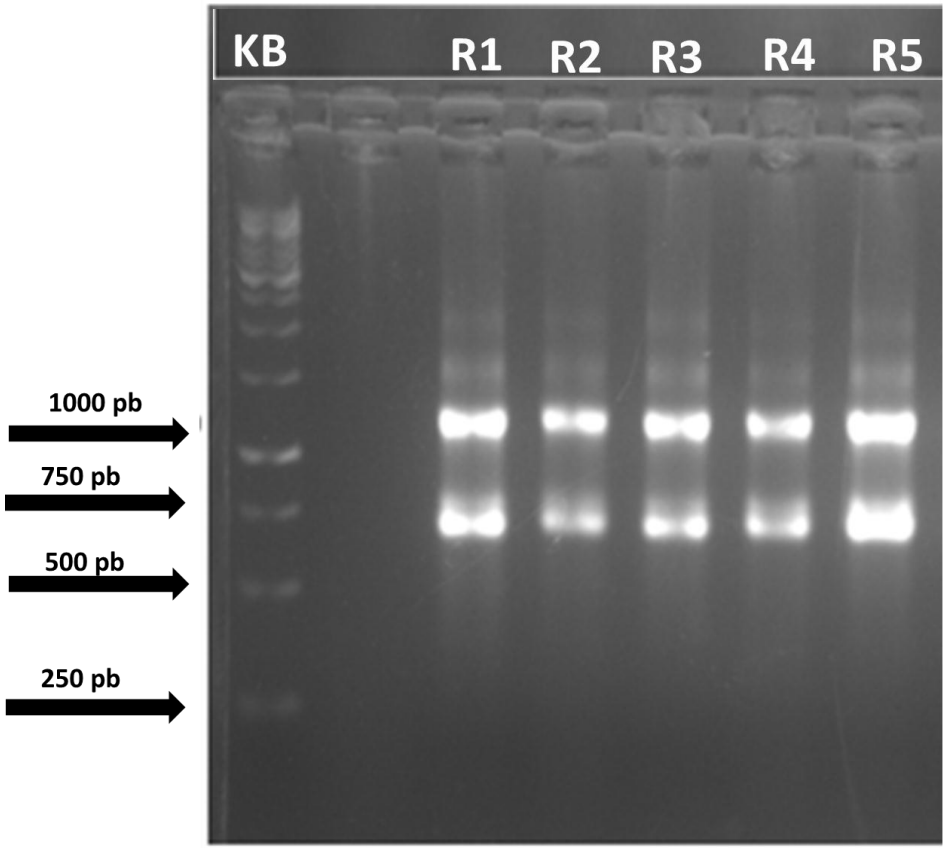

Figure 4. *T. peregrinus* RNA electrophoresis gel with Protocol 03: RNeasy Mini-kit – QIAGEN. KB Size marker 1 Kb DNA Ladder (Scientific Term), R1, R2, R3, R4, R5 are the repetitions. pb: Base pairs.

reagent TRIS (SIGMA), showed band intensity variability as a function of the sample used for extraction. No clear bands were observed for samples 1 and 6 (Figure 2). It is also noteworthy that, apart from the gel referring to Protocol 03 (Figure 4), it was not possible to verify the presence of DNA bands being the most suitable for possible future reactions. The results of the electrophoresis reveal that the best protocols for the extraction of total RNA, based on the estimated amount of RNA and the success of the extraction from the different materials, were Protocols 02 and 03 in which all presented RNA with pattern of desired band in the Protocol 03 agarose gel showed visible band and without drag indicating greater integrity of the obtained RNA. A difference was observed in the efficiency of the three protocols regarding the quantity and quality of the RNA extracted for *T. peregrinus*.

DISCUSSION

To carry out studies of gene expression and characterization of transcripts, it is necessary that RNA be extracted and purified efficiently, in such a way that its integrity and quality are maintained. Also, the extraction process needs to be safe, easy to perform, low cost, and allows its repeatability (Deng et al., 2005; Ibelli et al., 2007).

The techniques available for RNA extraction are numerous and despite their variations, the basic principle is the lysis of lipid membranes by a detergent solution, followed by purification, precipitation and RNA elution. The exposure of the genetic material to extraction buffers, as well as the initial stage of the process, helps in the precipitation of RNA and in the maintenance of its integrity in the subsequent stages of extraction, through the degradation of endogenous RNases (activities that are possible due to the presence of compounds such as lithium chloride and guanidine thiocyanate) (Sambrook and Russel, 2001).

The various commercially available reagents have combined substances in their compositions, such as guanidine isothiocyanate and phenol, which enable RNA extraction faster than that of conventional protocols and guarantee the integrity of the material. However, it is necessary to use other reagents in the subsequent steps

to assist in the sample purification process, such as the chloroform that solubilizes the lipids and allows their removal from the RNA molecules.

An ideal extraction technique should allow obtaining a large amount of pure and intact RNA, be easy and quick to perform, and also reproducible; in addition to being low cost and capable of extracting a large number of samples simultaneously. However, not all available techniques combine all of these characteristics together. Each technique has advantages and disadvantages that must be reviewed before a laboratory decides which one is most suitable for it, which makes works that perform protocol tests according to the tissue and/or species studied, of fundamental importance.

In the present study, the three protocols tested have different extraction buffers, with peculiar characteristics that seek to maintain the integrity of RNA throughout the process of extracting the molecule. The advantage of these reagents is that they are marketed ready for use, the procedure is quick and direct, and they promote rapid denaturation of nucleases and stabilization of RNA.

Among the tested processes, the extraction of RNA with TRIS (SIGMA) is considered one of the simplest and most used ways to extract RNA from cells or tissues. Based on the technique of Chomczynski and Sacchi (1987) and commercial availability, TRI Reagente® offers the possibility of simultaneous extraction of DNA and proteins present in the sample. It is a monophasic solution of phenol and guanidine isothiocyanide that disrupts cells and dissolves cellular components.

Despite presenting the advantages of being a ready-to-use reagent and a fast and direct procedure, capable of promoting rapid RNase denaturation and RNA stabilization, this protocol (Protocol 01) did not present satisfactory results for the extraction of *T. peregerinus* RNA. The inefficiency in extracting the RNA from the different samples of the insect used in the process was evidenced by the absence of ribosomal RNA bands observed in the image of the agarose gel, for samples 1 and 6, as well as, by the low intensity of the bands for samples 3 and 5 (Figure 2). The presence of sharp and shiny ribosomal RNA bands (28 S and 18 S) in the agarose gel is an indication of the good quality and integrity of the extracted RNA.

The analysis by agarose gel electrophoresis helped to verify also, that the only two samples that presented intense bands (samples 2 and 6), also, presented intense “traces”, that enabled us infer the existence of contamination and possible degradation of the samples. In addition, the presence of the DNA band was found, although not very evident, a fact that is directly linked to Trizol's inability to remove DNA from plasmids and DNA fragments, being able to efficiently remove only the large molecules of this acid nucleic.

In short, despite the homogenization of *T. peregrinus* samples obtained using the Mechanical Disruptor (Mini-Beadbeater™), equipment that causes cellular disturbances through constant and high-speed impacts and makes the process of homogenizing and extracting RNA extremely efficient in ensuring greater contact with the Trizol reagent, and which, in theory, should guarantee better denaturation of tissue proteins should be used. Also, despite the use of low temperatures in order to interrupt the enzymatic activity of RNases, it was not possible to obtain high-quality RNA samples with the use of the reagent TRI, which proved to be a poorly reproducible technique by allowing the extraction of RNA from only two samples, among the six analyzed.

Extractions performed with reagent IRT, following the manufacturer's recommendations, depending on the tissue and/or species, may have low yield and, consequently, high contamination due to the presence of proteins and phenolic compounds (Jaakola et al., 2001; Martins et al., 2018). As it is a reagent composed of phenol, when used in inadequate reagent/tissue proportions, it can also cause damage to the poly-A tail of the extracted mRNA (Azevedo et al., 2003), which can hinder further analysis. In addition, DNA fragments that are not removed in the extraction process with the reagent TRI may compromise procedures that depend on a high RNA yield such as the polymerase chain reaction via reverse transcriptase (RT-PCR).

In contrast, as with the application of Protocol 02 (D-Sorbitol - SIGMA) in this study, many protocols have been based on modifications of the classic extraction protocol with cetyltrimethylammonium bromide (CTAB) (Doyle and Doyle, 1987) to improve the purity and yield of DNA and RNA extraction. Among these modifications, the use of a sorbitol-based solution to pre-wash the tissue to be analyzed has allowed a reduction in the amount of extracellular contaminants and the obtaining of high quality and integrity RNA samples (Chang et al., 1993) with samples of different tissues and species.

Although, the use of D-Sorbitol in the present study promoted obtaining integral RNA samples and, apparently, free of polyphenols and polysaccharides, it can be inferred based on the absence of “traces” and the sharpness of the bands in the image of the agarose gel (Figure 3). The low intensity of the observed bands also shows a low amount of extracted RNA, which may be undesirable depending on the technique of analysis of gene expression subsequently employed. Another point to be highlighted refers to the presence of intense DNA bands observed in all samples processed with Protocol 02.

Thus, the electrophoresis analysis of the agarose gel showed that the use of D-Sorbitol together with 2-ßmercaptoethanol, which helps to reduce the oxidation of RNA pellets, constitutes a reproducible technique and provides RNA samples with high integrity. However, adjustments are necessary to obtain a higher concentration of RNA at the end of the process, alongside a subsequent treatment with the enzyme DNase I in order to remove fragments of genomic DNA. In comparison, Protocol 03 (RNeasy Mini-kit - Qiagen)

was the only procedure that managed to combine the integrity, quantity and quality of the extracted RNA with the repeatability of the extraction technique used. This allows the use of these samples in subsequent methods of analysis that involve, for example, enzymatic digestion, amplification and sequencing.

The use of extraction kits is based on RNA adsorption. They use the property of this nucleic acid to bind on surfaces such as magnetic spheres, silica, polystyrene latex materials, cellulose matrix or glass fibers, in the presence of certain salts or chaotropic agents that have the property of disrupting the three-dimensional structure of nucleic acids and proteins, by denaturing these macromolecules which allows high yield from small amounts of biological tissue.

RNA samples with these qualities, so desirable, are possible due to the use of the column that accelerates, facilitates, guarantees the extraction profit and reduces the risk of contamination during the process (Fanson et al., 2000; Siddappa et al., 2007). This works together with the use of the stabilizing solution RNAlater® that quickly permeates most tissues to stabilize and protect the RNA and slow down its degradation process, which is essential in the extraction process, as well as the addition of the enzymatic treatment with DNase I, which allows, as seen in the image of the agarose gel (Figure 4), the complete removal of the DNA fragments.

In contrast, the use of column kits for RNA extraction has the main disadvantage of the high price. In addition to not being a suitable technique for the extraction of small RNAs, some researchers also describe that it can result in the extraction of less pure RNA compared to organic extraction (Fanson et al., 2000; Siddappa et al., 2007).

Accordingly, it is understood that there is no nucleic acid extraction protocol (DNA or RNA) that can be considered standard. The presence of polysaccharides, polyphenols and a wide range of secondary metabolites makes it difficult to standardize between different tissues and obtain high-quality RNA (Campos et al., 2010; Cardillo et al., 2006) having all protocol advantages and disadvantages that must be carefully analyzed after the use of tests directed to the material under study. Each biological tissue or species of organism has characteristics intrinsic to its chemical and structural composition that can interfere with the success of the different stages of the extraction process. At the same time, each reagent used has its specificity and importance in this process, aimed at contributing to the isolation, purification and/or integrity of the RNA molecule. This makes it possible and important to test and manipulate the different protocols available for extraction, when starting a molecular study with a species such as *T. peregrinus*.

Conclusion

The commercial kit “RNeasy Mini-kit” (Qiagen) was the most efficient method for extracting total RNA from *T. peregrinus*. However, the extraction protocol with the regent D-Sorbitol (Sigma) has also shown satisfactory results and can actually replace the Kit, provided new tests are carried out that allow a greater yield in the extraction process with this reagent. On the other hand, extraction with the use of reagent Tris is not recommended due to its low effectiveness.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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HPTLC fingerprint profiles and UPLC-MS identification of potential antioxidant fractions and compounds from *Ambrosia maritima* L. and *Ammi majus* L.

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This study aimed to develop HPTLC fingerprint profile and identification of antioxidant molecules from active extract and fractions of *Ambrosia maritima* and *Ammi majus* using ultra performance liquid chromatography-mass spectroscopy (UPLC-MS). The antioxidant activity evaluated by using DPPH (1,1-diphenyl-2-picryl hydrazyl) method and HPTLC fingerprinting were carried out using CAMAG HPTLC system equipped with Linomat IV applicator, TLC scanner III, Reprostar 3, Camag twin through glass tank for development, and Wincasts1.2.3. The methanolic extract of *A. maritima* and *A. majus* shows highest antioxidant radical scavenging activity (87 and 58%, respectively). The ethyl acetate, aqueous fractions of *A. maritima* and aqueous fraction of *A. majus* shows the highest antioxidant activity (86, 82 and 81% respectively). The HPTLC profile of *A. maritima* ethyl acetate fraction indicates presence of nineteen compounds, ellagic (0.61%) and gallic phenolic acids (0.54%) content, respectively and ten compounds have been detected. The HPTLC profile of *A. majus* aqueous fraction indicate presence of twelve compounds, ellagic acid content (0.79%) and six compounds were detected. The HPTLC profile of *A. maritime* aqueous fraction indicate presence of nine compounds, ellagic acid content (2.54%) and eight compounds have been detected by UPLC-MS analysis.

Key words: *Ambrosia maritima*, *Ammi majus,* Antioxidant, HPTLC fingerprint, UPLC-MS analysis.

INTRODUCTION

Oxidative stress was initially defined as a serious imbalance between oxidation and antioxidants leading to potential damage to nucleic acid bases, lipids, and proteins that ultimately leads to cell death by necrosis or apoptosis (Halliwell and Poulsen, 2006; Mariusz and Sławomir, 2013). Cellular damage, due to free radical

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causes serious derangements such as gastric cancer (Vasavidevi et al., 2006), osteoarthritis (Yudoh et al., 2005), diabetes (Haydent and Tyagi, 2002), aortic valve stenosis (Peña-Silva et al., 2009), diabetic nephropathy (Taibur et al., 2012), high altitude pulmonary edema, high altitude cerebral edema (Bailey and Davies, 2001; Chao, 1999), neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and Huntington's disease (Chaitanya et al., 2010; Zhang et al., 1999).

Naturally occurring phytochemical antioxidants especially polyphenols (gallic and ellagic acids) (Indira et al., 2002) act as free-radical scavengers, oxidative stress relievers, and lipoperoxidation inhibitors (Velderrain-Rodríguez et al., 2018). Over the years, research on antioxidants and medicinal plants has gained enormous popularity and emerged as a potential therapeutic (Chandra et al., 2013).

*Ambrosia maritima (*Asteraceae) is a widely available weed in the Mediterranean region and African countries, particularly Egypt and Sudan, where it is locally known as Demsissa and grows abundantly near water catchments and on the banks of the Nile River (Tarig et al., 2018). It is widely used in Sudanese traditional medicine for the treatment of urinary tract infections, gastrointestinal disturbance, kidney stones, diabetes, hypertension, asthma, rheumatic pain, bilharziasis, and cancer. It is also used as appetizer, assisting digestion and tonic (Eman et al., 2014; Tarig et al., 2018).

Ammi majus (Apiaceae) is a glabrous annual plant used traditionally for the treatment of skin disorders, psoriasis and vitiligo. It was used as emmenagogue to regulate menstruation, as diuretic, and for treatment of leprosy, kidney stones, urinary tract infections, anti-asthmatic, antihyperglycemic, antispasmodic, carminative, digestive problems, preservative and against snakebites (Boulos, 2009; Corleto, 1993; Selim, 2012).

In recent years, HPTLC has become a conventional analytical approach for the standardization of herbal drugs due to its need for minimum sample clean up (Kaul et al., 2005; Alqasoumi et al., 2011), and many samples can be run simultaneously using a little volume of mobile phase, thus reducing the time and cost per analysis (Faisal et al., 2009; Alam et al., 2011). It can be used for the identification, assay and testing for purity, stability, dissolution or content uniformity of raw materials (Biringanine et al., 2006).

This study aimed to develop HPTLC fingerprint profile of antioxidant active extract fractions of *A. martima* and *A. majus* to quantify the content of gallic and ellagic acids in active fractions.

MATERIALS AND METHODS

Plant materials

The plant materials were collected from different regions in Sudan, authenticated by a taxonomist (Dr. Yahya Suliman) and voucher specimen were kept in the Medicinal and Aromatic Plant Research Institute (MAPRI) Herbarium (Sudan).

Preparation of plants extracts and fractions

Dry plant materials were powdered. 130 g of *A. majus* (seeds) and 50 g of *A. maritima* (leaves) have been taken for successive extraction two times using dichloromethane and methanol 80% as solvents by maceration for 48 h at room temperature. The extracts were filtered using cotton and Whatman filter papers; thereafter, the filtrates were concentrated under reduced pressure using Rotary evaporator and allowed to dry. The dry methanolic extracts of the plants with the higher antioxidant activity were re-dissolved in methanol (50%), and then fractionated three times using 15 ml of chloroform, ethyl acetate, and petroleum ether respectively. All fractions were concentrated under reduced pressure while the aqueous fractions were freeze dried.

Quantitative antioxidant activity using DPPH radical scavenging assay

The DPPH radical scavenging was determined according to the method of Brand et al. (1995) with some modification. In 96-wells plate, the test samples were allowed to react with 2,2-Di(4-tert-octylphenyl)-1-picrylhydrazyl stable free radical (DPPH) for half an hour at 37ºC. The concentration of DPPH was kept as 300 µM. The test samples were dissolved in DMSO while DPPH was prepared in ethanol. After incubation, decrease in absorbance was measured at 517 nm using multiplate reader spectrophotometer. Percentage radical scavenging activity by samples was determined in comparison with a DMSO treated control group. All tests and analysis were run in triplicate.

Sample preparation for chromatographic analysis

Accurately weighed 20 mg of sample was dissolved in 1 mL methanol. It was further vortexed, filtered through 0.45 µ membrane filter and used for analysis.

Selection of solvent system and HPTLC fingerprinting

After trying number of TLC in different solvent system, maximum number of spot was confirmed by TLC in a specific solvent system; thereafter that solvent system was used for analysis. Prepared samples were filtered and 8 µL of each of the solutions were separately applied on Silica gel 60 F_{254} precoated TLC plates, 5x10 cm (Merck, Germany) with the help of CamagLinomat-V (CAMAG, Switzerland) applicator and the plate was eluted to a distance of 8.5 cm at room temperature (25°C) in specific developed solvent system. The sample solution was applied to 6-mm wide band using CamagLinomat-V automated TLC applicator with the nitrogen flow providing a delivery speed of 150 nL/s from the syringe. Plates were developed in a Camag twin through glass tank pre-saturated with the mobile phase for 40 min. The plate was developed horizontally in Camag horizontal developing chamber (10 × 10 cm) at the room temperature. The scanning was carried out at 254 nm and 366 nm with a Camag TLC scanner III using the Wincats1.2.3 software.

UPLC-MS analysis of the active fraction

The 5 mg/mL solutions of each sample, filtered through 0.2 µM PTFE membrane filter as prepared previously were used for UPLC-MS analysis. In the present study, UPLC was performed on a Water's ACQUITY UPLC (TM) system (Waters Corp., MA, USA) equipped with a binary solvent delivery system, an auto-sampler, column manager and a tunable MS detector (Waters, Manchester, UK).

Table 1. List of the plant extracts and their radical scavenging activity.

Sample no.	Source	Solvent	%RSA ±SD (DPPH)
1	*Ambrosia maritima L*	Methanol	87± 0.08
2	*Ammi majus*	Methanol	58± 0.03
3	*Ambrosia maritima L*	Dichloromethane	13± 0.02
4	*Ammi majus*	Dichloromethane	Inactive
Standard	Propyl Gallate		90± 0.01

RSD = Radical scavenging activity, SD = Standard deviation.

Table 2. The antioxidant activity of the active extracts fractions.

Sample no.	Source	Solvent	%RSA ±SD (DPPH)
1	*Ambrosia maritima*	Ethyl acetate	86± 0.00
2	*Ambrosia maritima*	Aqueous	82± 0.01
3	*Ammi majus*	Aqueous	81± 0.02
4	*Ammi majus*	Ethyl acetate	43± 0.04
5	*Ambrosia maritima*	Chloroform	36± 0.04
6	*Ammi majus*	Petroleum ether	Inactive
7	*Ammi majus*	Chloroform	Inactive
Standard	Propyl Gallate		91± 0.01

The system was operated under the Empower software (Waters, USA). Data acquisition has been done in positive modes. Chromatography was performed using acetonitrile (A) and 0.5% v/v formic acid in water (B) as the mobile phases on monolithic capillary silica based C18 column (ACQUITY UPLC(R) BEH C18 1.7 µm, 2.1 x 100 mm), with the pre-column split ratio 1:5, flow rate 10 µL/min at ambient temperature. Separation was achieved by stepwise gradients from 5% B to 100% B for 20 min. The flow rate of the nebulizer gas was set to 500 L/h; for cone gas it was set to 50 L/h and the source temperature was fixed to 100°C. The capillary voltages and cone voltage were set to 3.0 and 40 KV respectively. For collision, argon was employed at a pressure of 5.3 x 10-5 Torr. The accurate mass and composition for the precursor ions and for the fragment ions were calculated using the Mass Lynx V 4.1 software incorporated in the instrument. Data obtained from UPLC-MS was processed by Mass Lynx V4.1 (Waters, USA). Separated metabolites present in different samples were tentatively identified based on their m/z ration and on literature.

RESULTS

Antioxidant activity of the extracts and fractions

The methanolic extract of *A. maritima* and *A. majus* shows variable antioxidant radical scavenging activity (87 and 58%) respectively, while dichloromethane extract of the two plants having either weak or no reactivity, is as shown in Table 1.

The ethyl acetate, aqueous fractions of *A. maritima* and the aqueous fraction of *A. majus* shows the highest antioxidant activity (86, 82 and 81% respectively); the chloroform fraction of *A. maritima* and the ethyl acetate fraction of *A. majus* shows moderate activity (36 and 43%); while the petroleum ether and chloroform fraction of *A. majus* are inactive as shown in the Table 2. All antioxidant activity results are estimated in comparison with the result of propyl gallate as standard.

Fingerprinting of active fractions using HPTLC

The HPTLC profile of the *A. majus* aqueous fraction indicate the presence of 12 compounds with retention factors (R_f) ranges (0.01-0.87) as shown in Table 3.

The HPTLC profile of the *A. maritima* ethyl acetate fraction indicates the presence of 19 compounds having retention factors (R_f) ranges from (0.01-0.68), while the aqueous fraction of same plant indicate the presence of 9 compounds with retention factors (R_f) ranges (0.03-0.84) as shown in Table 3.

All detected compounds from active fractions of the two plants had different area under the peaks (AU) calculated from the chromatograms obtained under 254 nm and 366 nm UV wavelengths (Figure 1). The purity of the sample was confirmed by comparing the absorption spectra at start, middle and end position of the band.

Quantification of gallic and ellagic acid content in the fractions

From the HPTLC profile using gallic and ellagic acid as referencing compounds, it is clear that the ethyl acetate

Table 3. R_f values of different constituents of *A. majus* and *A. martima* active fractions.

S/N	R_f	*A. majus* aqueous		*A. maritima* aqueous		*A. maritima* ethyl acetate	
		254 nm	366 nm	254 nm	366 nm	254 nm	366 nm
1	0.01	75.2	196.7			2865.6	
2	0.03			771.7	790.6	1940.7	
3	0.04					2117.7	
4	0.06					1686.6	8441.3
5	0.08					3484.3	7533.2
6	0.10	428.5	616.9				
7	0.12					5953.9	15113.3
8	0.13	761.3	910.2	1464.8	3612.7		
9	0.15			810.6	1743.5		
10	0.18					1163.9	5555.5
11	0.23					3682.4	7580.2
12	0.25			1074.5	3237.7		
13	0.30					1467.9	4085.7
14	0.32			1290.9	4859.1		
15	0.34	1578.8	1827.5			4200.7	3482.2
16	0.38						8106.2
17	0.40					3073.9	2879.4
18	0.43	1914.8				1610.6	3438.4
19	0.49					17095.3	23145.4
20	0.50			8468.2	19850.6		
21	0.52	7873.1	19543.7			3580.7	4878.2
22	0.56					11343.3	11801.6
23	0.58	9528.9	6726				
24	0.60						725.9
25	0.62						1113.9
26	0.63	16942.8		5314.9	8118.6		
27	0.66		13669.4				
28	0.68			3647.3	5078	1301.8	1730.3
29	0.74	23579.8	21055.8				
30	0.81	5225.9					
31	0.84			4484.6	13335		
32	0.87	9006.6					

fraction of *A. maritima* contain the two acids with different percentage (0.54 and 0.61% respectively), while the aqueous fractions of *A. maritima* and *A. majus* contain only ellagic acid with different percentage (0.79 and 2.54% respectively). Those have been calculated from the AU of each peak from the fingerprint compared to AU of the standard two acids (Table 4 and Figures 2 and 3).

UPLC-MS analysis of the active plant fractions

A. maritima active ethyl acetate fraction analysis

As shown in Table 5 and Figure 4, ten compounds have been detected from ethyl acetate fraction with different R_f values ranging from (0.82 - 11.24) with different peak intensities. 3 compounds (2',5-Dimethoxyflavone, (*R*)-3-Amino-4-hydroxybutyric acid and Psilostachyin A) were found having the highest peaks indicating their presence in high concentrations.

A. maritima active aqueous extract

Six compounds have been detected from aqueous extract of *A. maritima* with different R_f values ranging from (1.34 -11.24) with different intensities (Table 6 and Figure 5).

Ammi majus aqueous fraction

Eight compounds have been detected from aqueous extract of *A. majus* with different R_f values ranging (0.08 - 6.55) with different intensities (Table 7 and Figure 6).

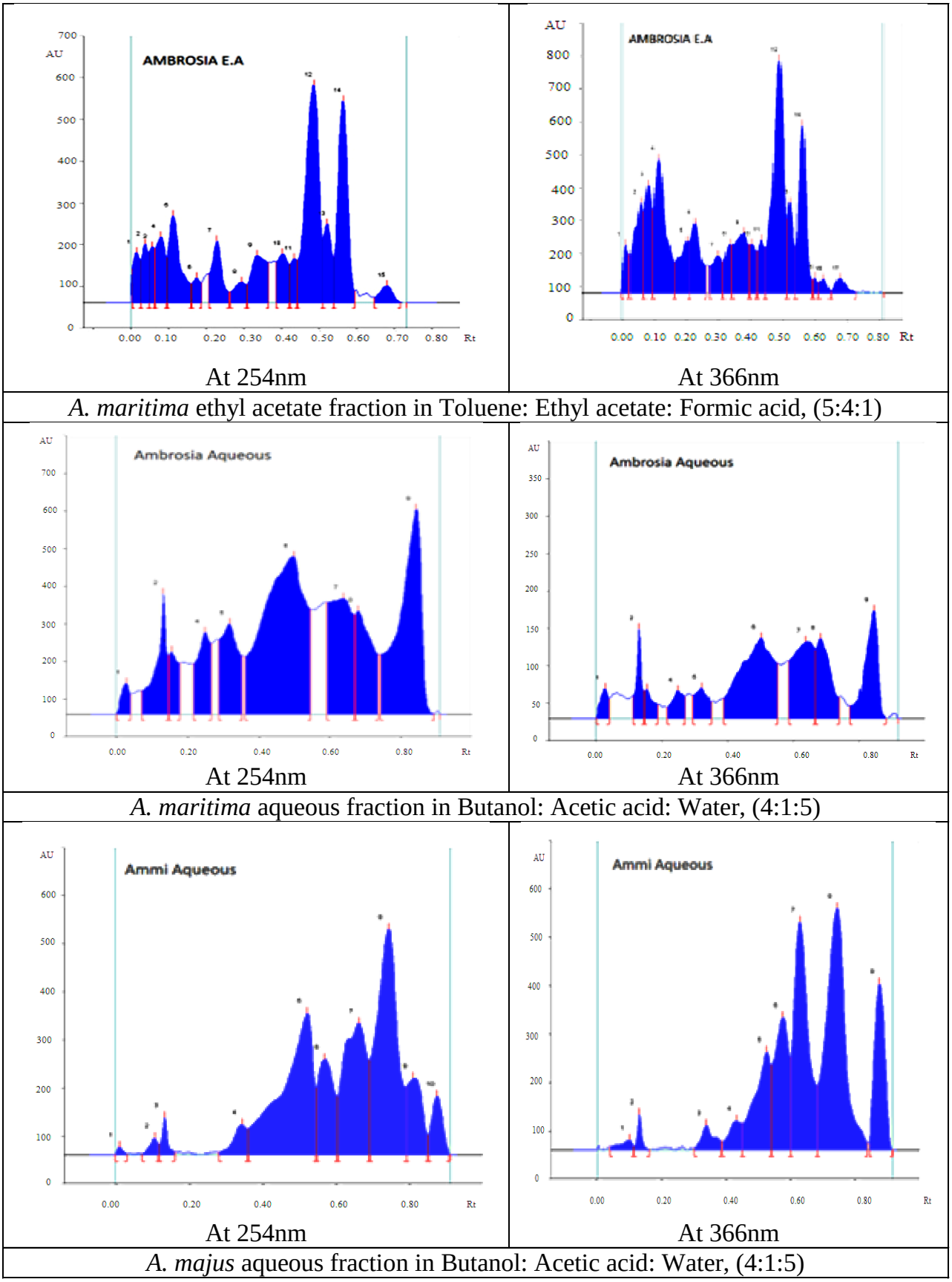

Figure 1. Fingerprint of *A. maritima* and *A. majus* active fractions at 254 and 366 nm.

Table 4. Gallic and ellagic acid content of the active fractions.

Fraction	Ellagic acid %	Gallic acid %
A. maritima ethyl acetate	0.61	0.54
A. maritima aqueous	0.79	0.00
A. majus aqueous	2.54	0.00

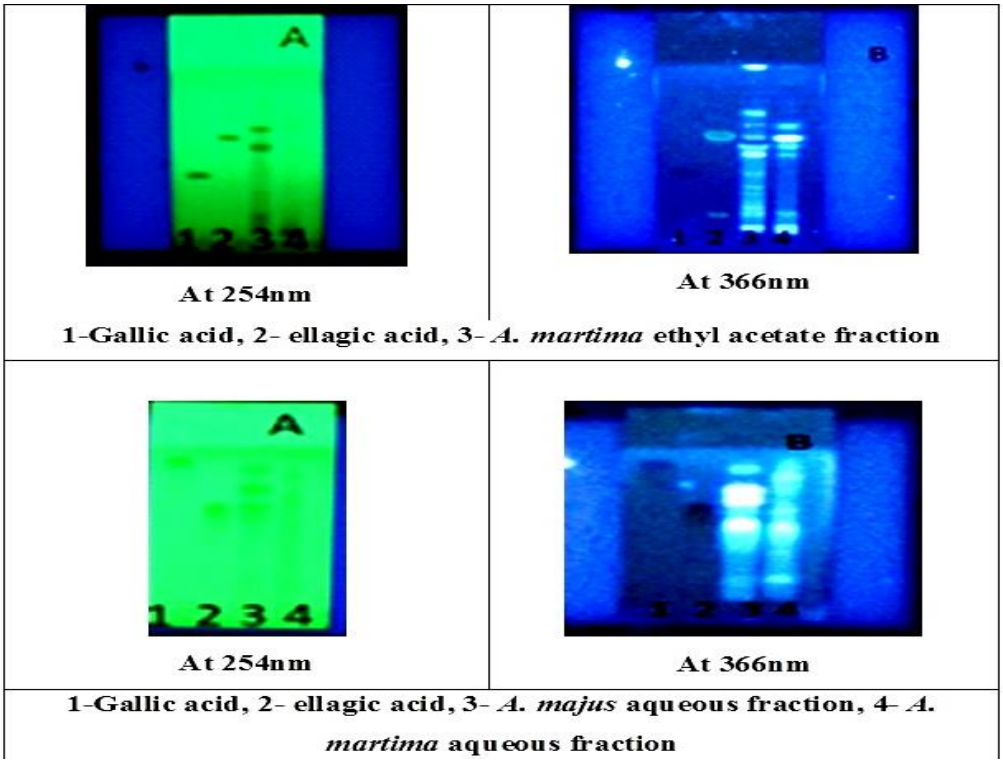

Figure 2. TLC chromatogram of *A. maritima* and *A. majus* active fractions at 254 and 366 nm.

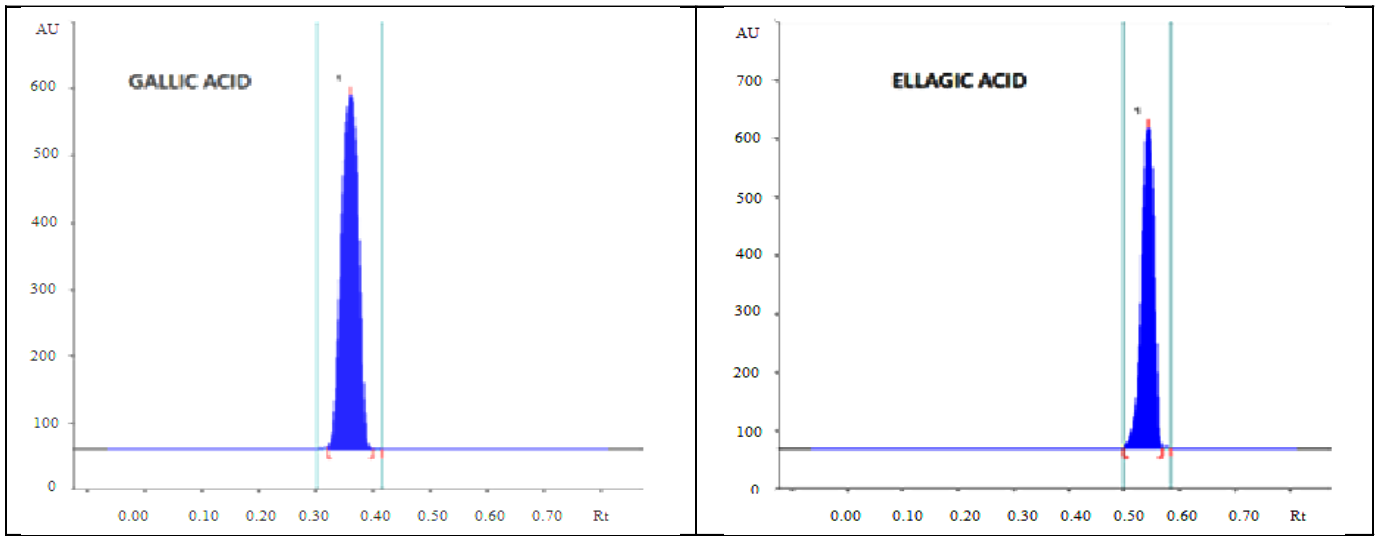

Figure 3. Chromatograms of gallic acid and ellagic acids.

Table 5. List of compounds identified from *A. maritima* ethyl acetate fraction with their R_t and molecular weight.

S/N	R_t	Mol.wt	Compound name
1	0.82	102.85	2-Aminobutyric acid
2	0.96	118.12	(R)-3-Amino-4-hydroxybutyric acid
3	2.72	227.22	Resveratrol
4	3.01	448.59	Kaempferol-3-O-glucoside
5	3.59	453.55	Scutellarein tetra-acetate
6	4.12	340.42	Esculin
7	4.59	280.19	Linoleic acid
8	5.65	111.11	Apomorphine
9	9.06	282.44	2',5-Dimethoxyflavone
10	11.24	280.32	Psilostachyin A

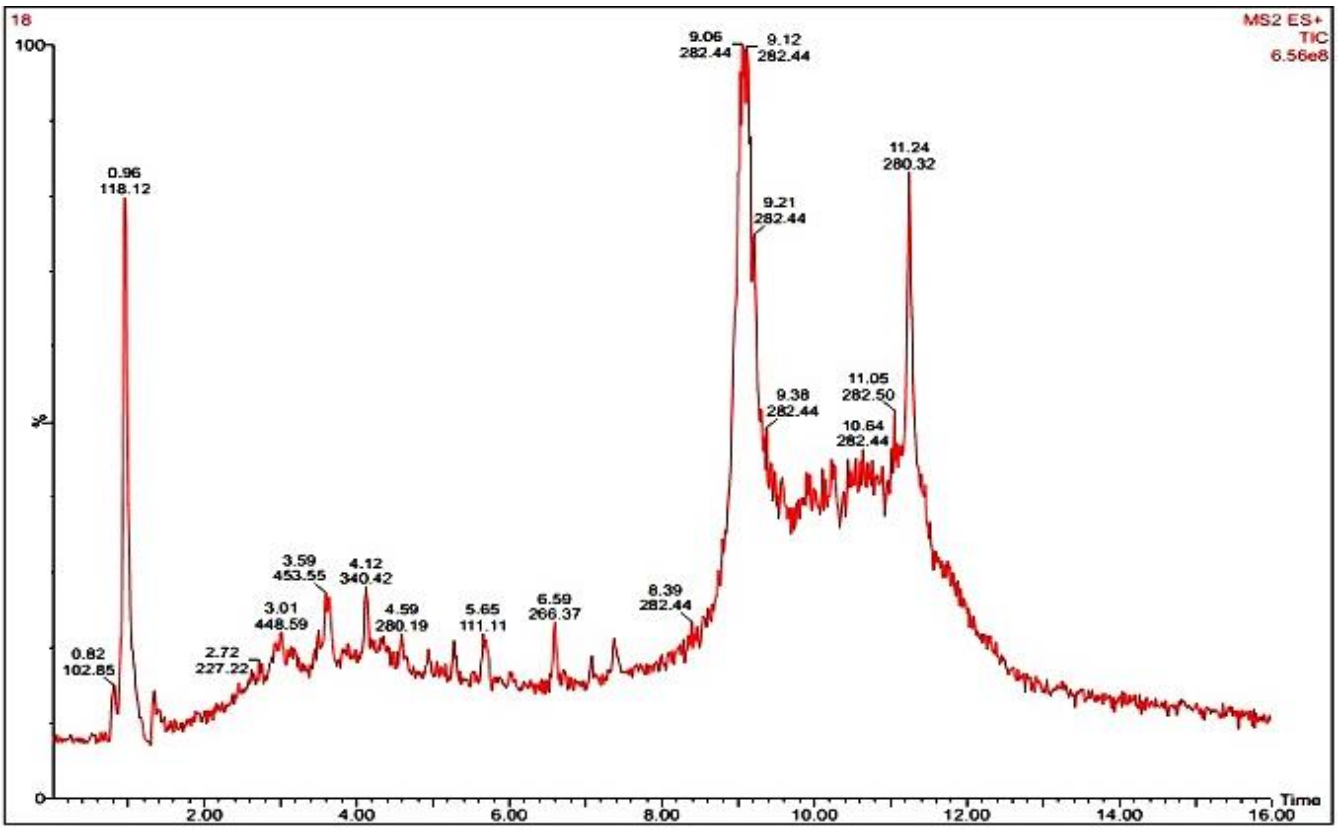

Figure 4. UPLC spectrum of *A. martima* ethyl acetate fraction.

Table 6. List of compounds identified from *A. maritima* aqueous fraction with their R_t and molecular weight.

S/N	R_t	Mol.wt	Compound Name
1	1.34	130.16	Isoleucine
2	2.99	448.40	Kaempferol-3-O-glucoside
3	3.63	453.55	Scutellareintetraacetate
4	4.13	340.49	Esculin
5	5.68	247.19	Matrine
6	11.24	280.52	Psilostachyin A

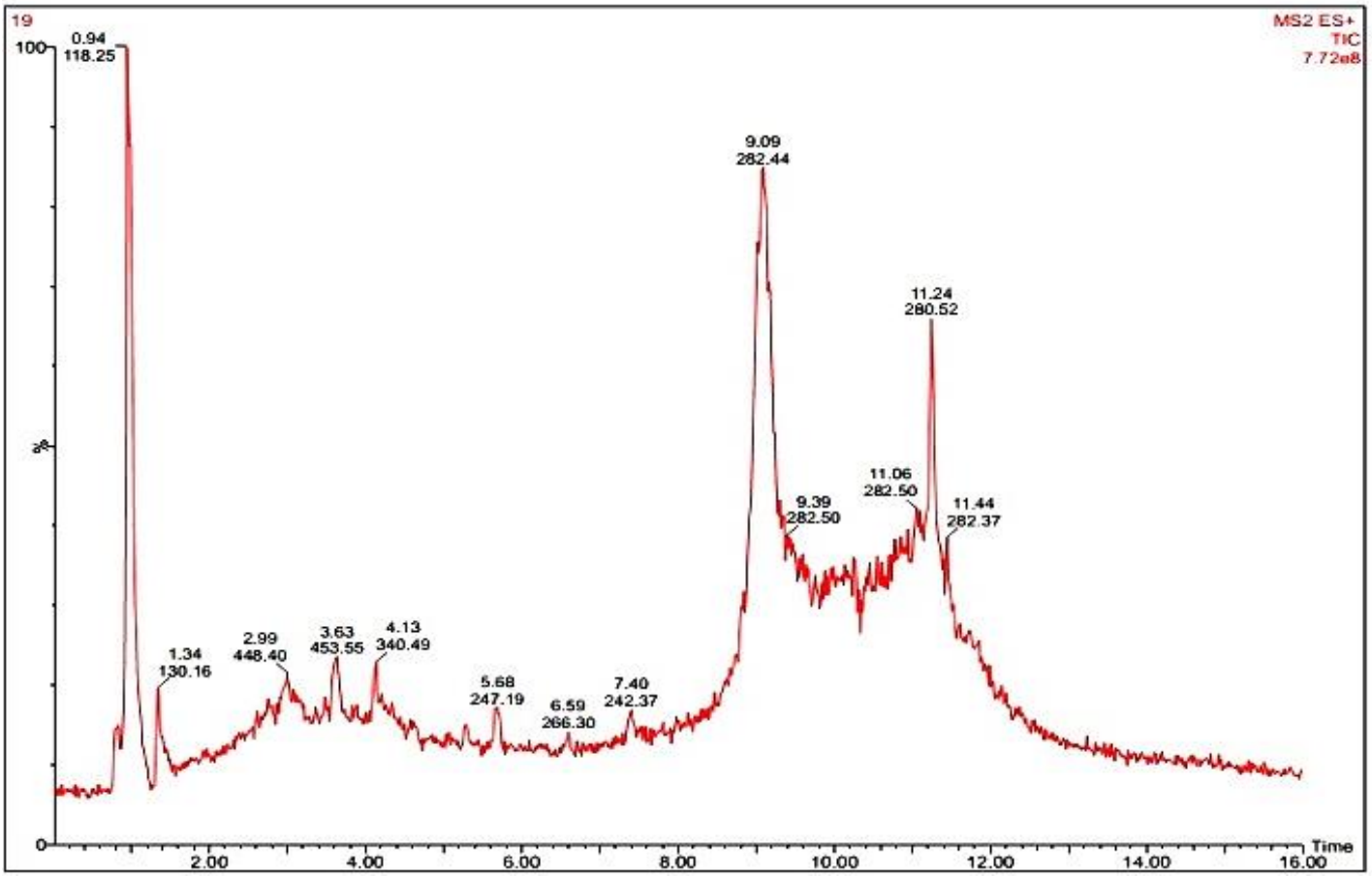

Figure 5. UPLC spectrum of *A. martima* aqueous fraction.

Table 7. List of compounds identified from *A. majus* aqueous fraction with their R_t and molecular weight.

S/N	Rt	Mol.wt	Compound name
1	0.81	128.04	3-Thiophenecarboxylic acid
2	0.99	116.14	Betaine
3	1.34	130.22	4-Hydroxy-L-proline
4	2.60	163.09	2-Coumaric acid
5	3.49	409.19	Mangostin
6	4.04	407.40	Nodakenin
7	5.61	261.21	Tryptophylglycine
8	6.55	275.36	Eserine

DISCUSSION

Herbal medicines have a long therapeutic history. However, the quality control and quality assurance of herbal drugs, singularly and in combinations, still remains a challenge because of the high variability of chemical compounds. This creates a challenge in establishing quality control standards for raw materials and standardization of finished herbal drugs (Chandrakar, 2018). Fingerprint analysis approach using HPTLC has become the most potent technique not only an alternative analytical tool for authentication, but also for quality control of complex herbal medicines (Lalhriatpuii, 2020), as well as qualitative and quantitative estimation of chemicals and bio-chemical markers (Chandrakar, 2018).

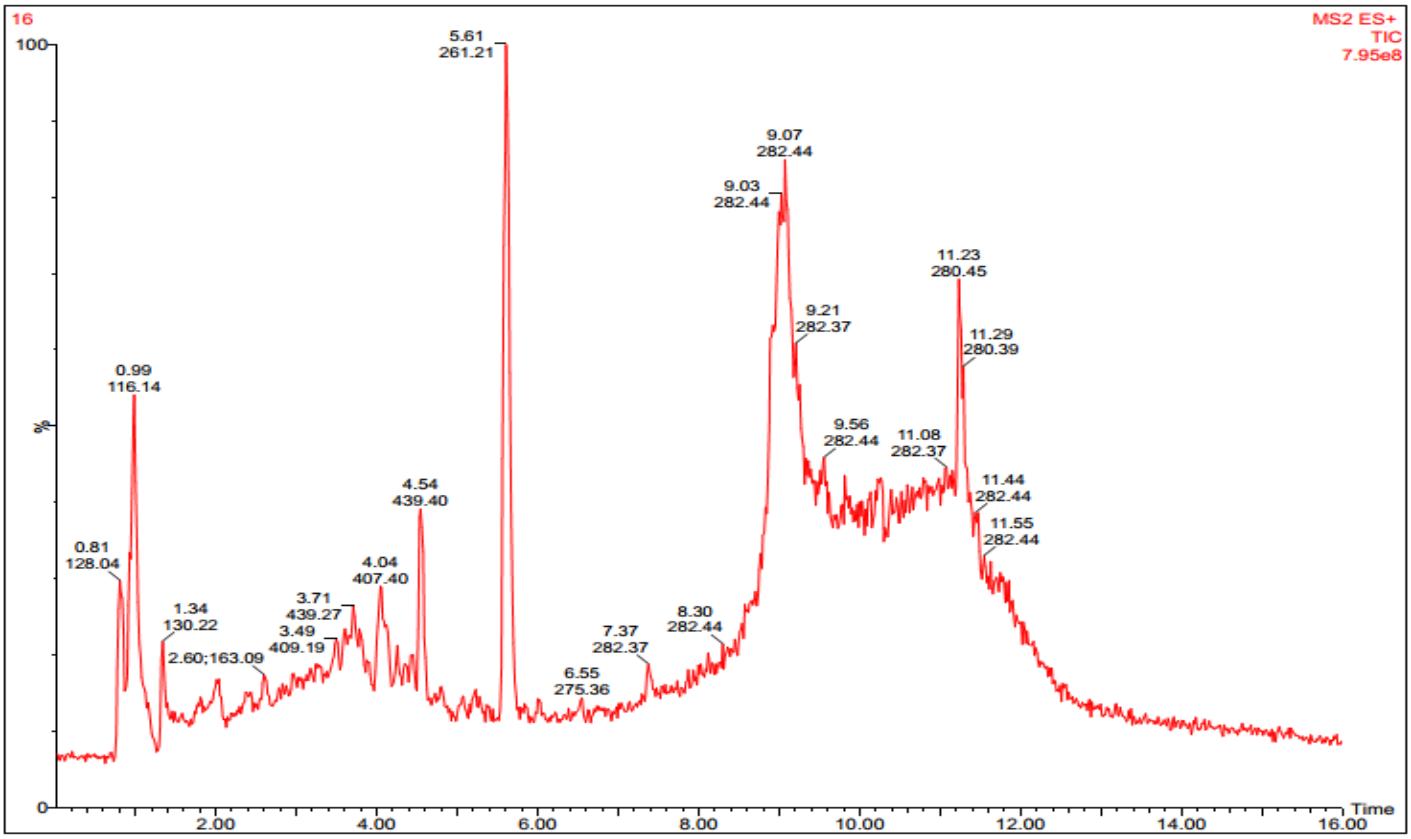

Figure 6. UPLC-MS spectrum of *A. majus* aqueous fraction.

Methanolic extract of *A. maritima* and *A. majus* showed acceptable antioxidant radical scavenging activity (87 and 58%) respectively compared with standard antioxidant agent Propyl Gallate (90%). The ethyl acetate, aqueous fractions of *A. maritima* and the aqueous fraction of *A. majus* showed the highest antioxidant activity (86, 82 and 81% respectively) compared to other fractions (Tables 1 and 2).

The HPTLC profile of the active fractions indicated different number of compounds (twelve for *A. majus* aqueous, nineteen for *A. maritima* ethyl acetate and nine for *A. maritima* aqueous) with different R_t values ranging from 0.01-0.87 (Table 3 and Figure 1). From HPTLC profile, the ethyl acetate fraction of *A. maritima* contain gallic and ellagic acid with different percentage (0.54 and 0.61% respectively) while the aqueous fractions of *A. maritima* and *A. majus* contain only ellagic acid with different percentage (0.79 and 2.54% respectively) (Table 4 and Figures 2 and 3).

UPLC-MS analysis of the active plant fractions indicated presence of ten compounds from *A. maritima* ethyl acetate with R_f values ranging from 0.82 - 11.24 (Table 5 and Figure 3), six compounds from aqueous extract of *A. maritima* with different R_f values ranging from 1.34 -11.24 (Table 6 and Figure 5) and eight compounds from aqueous extract of *A. majus* with R_f values ranging from 0.08 -6.55 (Table 7 and Figure 6).

Conclusion

HPTLC fingerprints and UPLC-MS analysis of the active fractions from *A. maritima* and *A. majus* have been done for the first time, and can be used as rapid and reliable methods in the quality control of the target plants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Diallel analysis for the inheritance study of phytic acid along with morpho-yield traits in bread wheat

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In the kernels of wheat, a macro molecule called phytic acid acts as an inhibitor of nutrients. Phytic acid in high concentration is undesirable as it hinders the absoption of other molecules. Regression analysis, the model of additive-dominance and Hotelling's t^2 test were adequate for biological yield, grains spike^{-1} and phytic acid, while partially adequate results were found for grain yield and 1000-grain weight. Greater values of H_1 and H_2 than D for biological yield, grain yield, 1000-grain weight, grains spike^{-1}, and phytic acid concentration indicated that these traits were under the control of non-additive gene action. Same results were also confirmed by average degree of dominance. Estimates of heritability for broad and narrow sense varied greatly for the traits of biological yield (0.89, 0.10), grain yield (0.98, 0.13), 1000-grain weight (0.68, 0.25) grains spike^{-1} (0.680, 05), and phytic acid concentration (0.86, 0.01). Phytic acid concentration ranged from 0.56 to 3.43% among F_1 crosses while for parental genotypes the range was 1.06 to 3.67%. Some of the F_1 hybrids like Ps-2005 × Ghaznavi (0.56%), AUP-4006 × Ps-2004 (0.74%), Janbaz × Ps-2004 (0.89%) and Janbaz × Ps-2005 (1.01%), indicated the lowest concentration of phytic acid. This research confirms that F_1 hybrids with low phytic acid concentration could yield desirable segregants.

Key words: Bread wheat, phytic acid, biological yield, grain yield, diallel analysis, inheritance, heritability.

INTRODUCTION

Bread wheat (*Triticum aestivum* L.) is a member of Poaceae (Graminae) belonging to monocoyledonae class of angiosperms which covers 2/3 area of cereals in the world and proves its most importance as a food. In Pakistan, bread wheat occupies first rank of cereal crop both in consumption and production and it is the richest source of carbohydrates. Wheat plays dual role on important food crop and a source of stabilizing indicator for the economy of Pakistan. Self sufficiency level has been reached by Pakistan due to increase in total production of wheat in the past few decades but yet we need to produce more wheat in order to earn earn foreign exchange by exporting wheat grain. In order to compete in the international market, we must focus and concentrate on the

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nutritional quality of wheat grain before export (Ijaz et al., 2013). In cereal grains, phytic acid is one of the constituents that is greatly present in the bran. Absorption of $Fe^{2+/3+}$ from the flour can vastly decline if humans consume food with high amount of phytate (Brune et al., 1992). Proteins, minerals and vitamins that make interaction are important factors which limit the nutritive value of wheat. Divalent (Mg^{2+}, Ca^{2+}) and trivalent (Fe^{3+}) metallic ions forms complexes with phytic acid in gastrointestinal tract which cannot be absorbed and these elements are no more available to cells, and are ultimately leading to nutritional disorders (Walter et al., 2002). Zn deficiency is direcly proportional with high phytic acid in diet (Linnerdal, 2000).

Five wheat genotypes were crossed in 5 × 5 full diallel on the basis of pre-screening results of 10 wheat genotypes, with the objective to develop low phytic acid segregants of wheat. The specific objectives of the present study were to determine phytic acid profile along with morpho-yield traits of different bread wheat genotypes and to estimate their heritabilities.

MATERIALS AND METHODS

In order to investigate "Inheritance of phytic acid and other morpho-yield traits in bread wheat", laboratory and field experiments were carried out jointly in the Department of Plant Breeding and Genetics, at The University of Agriculture, Peshawar and Nuclear Institute for Food and Agriculture (NIFA) Peshawar, Khyber Pakhtunkhwa-Pakistan during 2007-08 to 2008-09. Primarily, 10 bread wheat genotypes (AUP-4006, Janbaz, Saleem-2000, AUP-5006, Tatara, Uqab, Ghaznavi, Fakhre Sarhad, Pirsabak-2004 and Pirsabak-2005) were screened for phytic acid concentration in 2007 at the Nuclear Institute for Food and Agriculture (NIFA) Peshawar (Masud et al., 2007). On the basis of preliminary data of 10 bread wheat genotypes for phytic acid, two contrasting groups (Group 1 with low phytic acid concentration, that is, Pirsabak-2004 and Ghaznavi) (Group 2 with high phytic acid oncentrations, that is, Pirsabak-2005, Janbaz and AUP-4006) were identified. Both groups (5 genotypes) were crossed to generate a full diallel set by using 5 × 5 full diallel in 2007.

At least 15 spikes of each variety were manually emasculated and bagged in order to prevent contamination by foreign pollens during flowering season of 2007. Receptive ovaries of the female spikes were pollinated by applying fresh pollens from the desirable male spike within two or three days after emasculation. Each variety was used as male and female and generated 20 F_1 hybrids, with enough seed for planting experiment in 2008. All F_1 hybrids (20) along with parental genotypes (05) were planted with a plant to plant and row to row space of 25 cm to maintain 160,000 plants/ha for investigating phytic acid and other morpho-yield traits of bread wheat. An experiment was planted in randomized complete block design with triplicate. Each entry comprised of one row having a length of 3.75 m.

Urea and DAP fertilizers at the rate of 120 and 60 kg ha^{-1} were added to experimental field for maintaining standard nutrients status of soil. Full dose of DAP and half dose of urea fertilizer were added to soil during prepration of seed bed whereas the remaining half dose of urea was applied along with first irrigation. Standard agricultural practices like weeding, irrigation and hoeing were carried out for decreasing experimental inaccuracy.

Observations

Data were recorded on five randomly selected plants of each population for the following traits:

1) Grains $spike^{-1}$
2) 1000-grain weight (g)
3) Biological yield (kg ha^{-1})
4) Grain yield (kg ha^{-1})

For each of the above mentioned trait, already adopted standard procedure was used.

5) Phytic acid: An adequate amount of sample of wheat kernels was drawn for phytic acid determination from each entry after manual harvesting and threshing. Kernels were grinded by blender and 0.06 g of flour was collected for phytic acid determination by adopting the sensitive method of Haug and Lantzsch (1983).

Determination of phytic acid

A very minute fraction of sample (0.06 g) was taken by weighing with the help of electronic balance and digested with 0.2 N HCl in test tube. Sample in the test tube was heated with an acidic iron-III solution of known Iron content. The decrease in the iron content was the measure of free phytic acid in supernatant.

Reagents

Phytic acid reference solution: Sodium salt of phytic acid ($C_6H_6O_{24}P_6Na_{12}$) was used for the preparation of reference solution. Stock solution was prepared by dissolving 0.15 g sodium phytate in 100 ml distilled water. The reference solution was prepared by diluting the stock solution with HCl in a range from 3 to 30 micro-grams (ug ml^{-1}) phytic acid phosphorus.

Ferric solution: Ammonium Iron-III sulphate $12H_2O$. Ferric solution was prepared by dissolving 0.2 g of Ammonium Iron-III sulphate $12H_2O$ in 100 ml of 2 N HCl and the volume was made up to 1000 ml with distilled water.

2, 2-Bipyridine solution

This solution was prepared by dissolving 10 g of 2, 2-bipyridine and 10 ml of thioglycolic acid in distilled water and the final volume was raised up to 1000 ml.

Protocol

From each entry of the experiment, a representative grain sample (10 g) was weighed by an electronic balance and grinded by blinder for getting fine grade of flour. A minute quantity (0.06 g) was weighed and added in dry and clean screw cap test tube with a volume of 15 ml from each entry. Sample within test tube was digested by 0.1 N HCl (10 ml) by shaking for 1 h in shaker. From this extract, 0.5 ml in duplicate was taken into dry and clean screw cap test tubes. A quantity of 1 ml ferric solution (concentration = 23 ug ml^{-1} or 23 ppm solution) was added to these test tubes and closed by screw caps. These tubes were heated (105°C) in boiling water bath for 30 min and allowed to cool at room temperature. Reaction mixture was provided by 2 ml of 2, 2-biphyridine solution (concentration = 1% 2, 2 bipyridine solution) and mixed thoroughly by shaking. Reaction mixture was transferred to cuvet of spectrophotometer (UV-1800, Japan made) and optical density (OD 510 nm) was recorded. The absorbance was measured within 4 min. A standard curve was made in phytic acid and was determined by the following formula

Phytic acid = Phosphorus phytic acid × 4.97

Table 1. Means of parents and F_1s for biological yield (BY) (kg ha^{-1}), grain yield (GY) (kg ha^{-1}) grains $spike^{-1}$, 1000-grain weight (TGW in g), and phytic acid percentage (PA%) in 5 × 5 diallel cross of bread wheat.

S/N	Genotype	BY	GY	GSP^{-1}	TGW	PA%
1	AUP-4006	6958.90	1833.00	88.67	32.33	3.42
2	Janbaz	6668.03	2381.68	72.00	32.00	1.61
3	Ghaznavi	7278.08	2476.74	77.66	37.00	1.25
4	Ps-2004	6194.52	2138.61	94.67	31.00	1.66
5	Ps-2005	6593.21	1919.25	69.00	41.00	2.48
6	AUP-4006 × Ps-2004	8877.16	1838.31	69.00	36.00	0.74
7	Ps-2004 × AUP-4006	8485.38	2146.77	57.67	34.00	2.60
8	Ps-2004 × Ghaznavi	8296.81	1994.63	77.00	38.00	2.48
9	Ps-2004 × Ps-2005	7428.31	1892.37	64.00	35.00	1.46
10	AUP-4006 × Janbaz	6513.24	2443.89	84.00	35.00	2.83
11	AUP-4006 × Ghaznavi	7527.39	2220.89	80.00	39.00	2.81
12	AUP-4006 × Ps-2005	7575.34	2149.51	75.33	35.00	2.55
13	Janbaz × AUP-4006	8478.99	1926.66	75.33	40.00	2.65
14	Janbaz × Ghaznavi	8278.53	1956.16	73.00	36.70	1.63
15	Janbaz × Ps-2004	8098.32	2737.12	78.00	36.00	0.89
16	Janbaz × Ps-2005	7836.53	2330.82	74.00	31.00	1.01
17	Ghaznavi × AUP-4006	8173.97	2056.21	92.33	40.70	3.43
18	Ghaznavi × Janbaz	7962.92	2166.66	66.00	39.00	1.52
19	Ghaznavi × Ps-2005	8377.16	1818.32	95.00	36.33	2.81
20	Ghaznavi × Ps-2004	7834.74	2435.75	85.33	38.70	2.32
21	Ps-2004 × Janbaz	8391.32	1882.05	71.00	31.00	2.53
22	Ps-2005 × AUP-4006	8668.69	2530.95	70.33	38.33	2.83
23	Ps-2005 × Janbaz	7191.32	2058.31	78.66	35.00	2.77
24	Ps-2005 × Ghaznavi	7734.10	2068.21	79.66	40.70	0.56
25	Ps-2005 × Ps-2004	7616.43	2023.61	69.00	40.70	1.58

Statistical analysis

Analysis of variance

Analysis of variance (Steel et al., 1997) was performed for data of all traits.

Diallel analysis

Diallel-98 software was used for the analysis of 5 × 5 diallel cross, for calculating Griffing, ANOVA and estimates of genetic components of all traits.

RESULTS AND DISCUSSION

Data of phytic acid and other morpho yield traits for all genotypes (20 F_{1s} + 05) were subjected to diallel analysis for getting genetic information about various aspects.

Analysis of variance for F_{1s} and parental genotypes

All traits revealed significant differences after computing ANOVA. Means for the traits under study are presented (Table 1).

Data concerning biological yield revealed highly significant variations. Maximum biological yield was recorded for Ghaznavi and minimum Ps-2004 (Table 1). Among the F_1 progenies, maximum biological yield was recorded for AUP-4006 × Ps-2004 followed by Ps-2005 × AUP-4006, Ps-2004 × AUP-4006 and Janbaz × AUP-4006. Analysis of variance for grain yield was highly significant. Among the parents, highest grain yield was recorded for Ghaznavi followed by Janbaz while lowest was for AUP-4006. Amongst the F_1 hybrids, highest grain yield was recorded for Janbaz × Ps-2004 and Ps-2005 × AUP-4006.

Analysis of variance regarding grains $spike^{-1}$ was found significant. Among the parental genotypes, maximum grains $spike^{-1}$ was recorded for Ps-2004 and minimum grains $spike^{-1}$ for Ps-2005. Among the F_{1s} crosses, Ghaznavi × Ps-2005 produced more grains $spike^{-1}$ followed by Ghaznavi × AUP-4006 while cross combination, Ps-2004 × AUP-4006 yielded less grains $spike^{-1}$. Analysis of variance revealed significant differences for 1000-grain weight. Among the parents, Ps-2005 was found with maximum score for 1000-grain weight, followed by Ghaznavi. Janbaz and AUP-4006

Table 2. Additive–dominance model for phytic acid and other morho-yield traits in bread wheat for 5 × 5 diallel cross.

Parameter	t^2	Regression analysis (t value of b)		Conclusion
		b= 0	b= 1	
Biological yield	0.35^{ns}	4.89*	2.32^{ns}	Model was adequate
Grain yield	3.55^{ns}	1.17^{ns}	4.19^{ns}	Model was partially adequate
Grains spike^{-1}	0.07^{ns}	5.08*	0.46^{ns}	Model was adequate
1000-grain weight	0.28^{ns}	0.02^{ns}	0.46^{ns}	Model was partially adequate
Phytic acid	-0.065^{ns}	0.98*	1.64^{ns}	Model was adequate

were at par for the mentioned trait whereas Ps-2004 with lowest score was observed for the said trait among the parents. Maximum value of 1000-grain weight was observed for Ps-2005 × Ghaznavi, Ps-2005 × Ps-2004 and Ghaznavi × AUP-4006 and was at par among the crosses (Table 1).

Phytic acid indicated highly significant differences after ANOVA. High concentration of phytic acid was found in AUP-4006 while low concentration was observed in Ghaznavi among the parental genotypes. Among the hybrids (F_{1s}), highest phytic acid concentration was observed in cross combination Ghaznavi × AUP-4006, followed by AUP-4006 × Janbaz whereas lowest concentration was recorded for Ps-2005 × Ghaznavi and AUP-4006 × Ps-2004 and Janbaz × Ps-2004 among the F_1 hybrids (Table 1).

Diallel analysis

Data collected for phytic acid and other morpho yield traits was subjected to analysis of variance. Significant genotypic differences for all traits provided a full justification for diallel analysis. Diallel analyses (5 × 5) were performed by using Dial-98 software.

Biological yield

Biological yield indicated highly significant differences for an item after diallel analysis. Item **b** was also highly significant, displaying the contribution of overall dominance. Presence of directional genes ($\mathbf{b_1}$) for biological yield was justified by its significant value. Value of genetic item $\mathbf{b_2}$ with highly significant differences was the justification for the distribution of asymmetrical genes among parents whereas $\mathbf{b_3}$ was held responsible for the existence of specific gene effect. Maternal effect (**c)** was highly significant and it was a prerequisite for retesting of component **a**. Significant value of item (**a)** after retesting supported additive gene effect for maternal effect (Table 3).

Biological yield was adequate for diallel analysis due to non-significant values of adequacy tests (t^2 test and Regression analysis (Table 2). The estimates of genetic components **D, H_1, H_2, F, h^2** and **E** showed significant differences for biological yield. Less value of **D** than the values of both $\mathbf{H_1}$ and $\mathbf{H_2}$ suggested more contribution of dominance for biological yield. Average degree of dominance $(H_1/D)^{1/2}$ was 3.37 which is greater than unity, and is a clear indication of over dominance for the control of biological yield. Estimated value of **F** was positive with significant variation pleading for the existence of more dominant genes for the said trait. Both narrow sense and broad sense 0.10, 0.89 heritability estimates for biological yield are presented (Table 4).

Grain yield

Genetic component **a** which is responsible for the measurement of additive gene action and generally considered as a chief portion of total variation was found significant after conducting diallel analysis for 5 × 5 diallel cross for grain yield (Table 3). Item **b** which is a measure of overall dominance revealed highly significant differences which indicate the vital function of dominance for grain yield. Existence of directional genes for grain yield was mentioned by the significant value of $\mathbf{b_1}$. Distribution of asymmetrical genes among the parents was indicated by the significant value of genetic component $\mathbf{b_2}$ while there was non-significant value of item $\mathbf{b_3}$ which accounted for presence of specific gene effect. Maternal effect (**c**) was significant which is an important factor for retesting of genetic component **a**. Retesting of gentic component **a** decreased its value to non-significant level, thus showing that maternal effect suppressed the additive gene effect. Significant value of reciprocal effect (**d**) is mandatory for the retesting of **b**, $\mathbf{b_1}$, $\mathbf{b_2}$ and $\mathbf{b_3}$ which made them non-significant apart from **b** which kept its original value after retesting by **d**.

Regression analysis and t^2 test showed non-significant values (Table 2) for grain yield, justifying the adequacy of Additive-Dominance model. Significant differences were found for **D, H_1, H_2, F, h^2** and **E** genetic components (Table 3) for grain yield. Greater value $\mathbf{H_1}$ and $\mathbf{H_2}$ than additive gene effect specified crucial function of dominance for the abovementioned trait. Additive genes with dominance type nature are confirmed by the highly significant differences of **D** and $\mathbf{H_1}$ and $\mathbf{H_2}$ which showed

Table 3. Mean squares and degree of freedom for the analysis of variance of 5 × 5 diallel for gains spike^{-1}, 1000-grain weight, biological yield and phytic acid.

SOV	Grains spike^{-1}		1000-grain weight		Biological yield		Grain yield		Phytic acid	
	df	Ms	df	Ms	Df	Ms	df	MS	df	Ms
a	4	153.83^{NS}	4	19.72**	4	9235520**	4	74796**	4	0.39^{NS}
b	10	361**	10	13.65**	10	2452982**	10	195519.90**	10	2.92**
b_1	1	254.47^{NS}	1	20.80*	1	17649870**	1	112410**	1	3.85**
b_2	4	541.26**	4	13.91*	4	705571**	4	72530**	4	3.39**
b_3	5	237.52*	5	12.01*	5	811531**	5	108194**	5	2.36**
c	4	118.28^{NS}	4	10.25*	4	1124303**	4	163342*	4	4.29**
d	6	282.28**	6	16.86**	6	155731.50**	6	75210**	6	1.65**

*P=0.05, **P=0.01. a = additive gene effect, b = dominance gene effect, b_1 = directional dominance deviation, b_2 = genes distribution among parents, b_3 = effect of specific gene, c = maternal effect, d = reciprocal effect.

Table 4. Estimates of genetic components of variation for phytic acid, grains spike^{-1}, 1000-grain weight, biological yield and grain yield.

Component	Phytic acid		Grains spike^{-1}		1000-grain weight		Biological yield		Grain yield	
	MS	SE	MS	SE	MS	SE	MS	SE	MS	SE
D	0.89*	±0.26	95.32^{NS}	±60.82	7.91*	±3.69	144228^{NS}	±103889	2968.52*	±149.10
H_1	2.43*	±0.46	285.26*	±111.6	8.47^{NS}	±4.57	1641443*	±300437	144838.60*	±977.32
H_2	1.80*	±0.33	193.70*	±75.20	6.58*	±3.34	1535619*	±269391	130338.10*	±854.71
F	1.50*	±0.39	178.13*	±92.26	7.50^{NS}	±4.57	151804*	±146298	7501.76*	±380.3
h^2	0.777	±0.40	41.22^{NS}	±65.71	3.68^{NS}	±4.06	3735413*	±697825	239805*	±21103.
E	0.07*	±0.01	24.52*	±5.00	1.31*	±0.27	51910*	±10516	7.33*	±1.48
$(H_1/D)^{1/2}$	1.65		1.72		1.05		3.37		6.98	
Heritability (ns)	0.01		0.05		0.25		0.10		0.13	
Heritability (bs)	0.86		0.68		0.66		0.89		0.98	

* = Value is significant when it exceeds 1.96 after dividing by its standard error.

the aforesaid trait. Occurence of dominant genes for the trait of grain yield can be confirmed by F genetic item which was positive and significant. According to estimation of genetic components, the value of 6.98 for the average degree of dominance $(H_1/D)^{1/2}$ was responsible for over dominance type of gene action with additive effect. Values estimated for narrow and broad sense 0.13 and 0.98 heritability respectively were found for grain yield (Table 4).

Grains spike^{-1}

Diallel analysis (5 × 5) for item **a** was non-significant (Table 3) for the trait of grains spike^{-1}. The value **b**, with highly significant variation indicates the major role of dominance for mentioned trait. Dirctional genes are missing due to non-significant value of $\mathbf{b_1}$ for grains spike^{-1}. Existence of asymmetrical genes among parents for grains spike^{-1} is prominent from the significant value of $\mathbf{b_2}$. Specific gene effect was present due to significant value of $\mathbf{b_3}$. The value of **c** appeared with non-significant score. Reciprocal effect was significant and hence retesting of **b**, $\mathbf{b_2}$ and $\mathbf{b_3}$ was performed which rendered them non-significant except $\mathbf{b_2}$ which became significant.

Adequacy tests for additive dominance model showed partial adequacy of the data for grains spike^{-1} (Table 2). Estimated values of **D** and $\mathbf{h^2}$ were non-significant for grains spike^{-1} indicating lack of additive genes for this trait while $\mathbf{H_1}$, $\mathbf{H_2}$, **F** and **E** with significant variation demonstrated dominance gene effect for the said trait. Controlling of grains spike^{-1} is also evident from the significant and positive value of **F** genetic component. Over dominance type of gene action for grains spike^{-1} was also supported by average degree of dominance $(H_1/D)^{1/2}$ which was greater (1.72) than 1. Estimates of narrow sense heritability were 0.05 and broad sense heritability 0.68 for grains spike^{-1} (Table 4).

1000-grain weight

Diallel analysis for 1000-grain weight indicated major role of additive gene effect due to the significant value of **a**.

Genetic components b, b_1, b_2, b_3 were also highly significant for 1000-grain weight which pleaded for dominance type of gene action. Genetic component **d** was highly significant and hence retesting of **b**, $\mathbf{b_1}$, $\mathbf{b_2}$ and $\mathbf{b_3}$ was conducted which made them non-significant (Table 3).

Scalling tests indicated partial adequacy for 1000-grain weight (Table 2). Estimate of genetic component **D** was significant which suggested that 1000-grain weight is controlled by additive genes. More dominant genes for for 1000-grain weight are also supported by the significant value of **F**. Average degree of dominance $(H_1/D)^{1/2}$ was greater than unity, indicating over dominance type of gene action of additive nature for 1000-grain weight. Narrow and broad sense heritability estimates were 0.25 and 0.66, respectively, for 1000-grain weight (Table 4).

Phytic acid

Diallel analysis (5 × 5) for phytic acid revealed (Table 3) non-significant variation for additive gene effect (**a**). Overall dominance (**b)** was highly significant, indicating the importance of dominance for phytic acid. Directional gene distribution ($\mathbf{b_1}$**)** among parents was also found significant for the said trait. Distribution of asymmetrical genes ($\mathbf{b_2}$) among parents and existence of specific gene effect ($\mathbf{b_3}$**)** for phytic acid were also recorded with significant differences. Maternal effect (**c)** and reciprocal effect (**d**) score was also significant for phytic acid.

Additive dominance model was adequate for phytic acid due to non-significant values of t^2 test and regression analysis (Table 2). Estimation for genetic components of variations, D, H_1, H_2, F, h^2 and E revealed significant differences. Additive gene action was less important as it was less than both H_1 and H_2 indicating a more role of dominance for phytic acid. Postive and significant value of F also pleaded for dominant type of genes for phytic acid. Mean degree of dominance was more (1.65) than unity, a clear cut proof of dominant type of gene action for phytic acid. Narrow sense 0.01 and broad sense 0.86 heritability estimates were found for phytic acid (Table 4).

Conclusion

From the present research, it can be concluded that phytate can also be decreased by breeding bread wheat genotypes up to the greater extent and even up to to the desirable level. By developing genotype/cultivar or line by breeding with low phytate, it would be a great step towards the improvement of bread wheat quality because in the international market now, the competition is on quality not on quantity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Improving peanut protein quality: Expression of a synthetic storage protein

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Peanut is an affordable legume used in most households. It represents one of the most important protein supplies worldwide. However, peanut proteins are deficient in several essential amino acids (EAA), like most plant proteins; whereas plants are the main source of dietary proteins consumed by humans and livestock. This could lead to protein malnutrition in areas where people diet relies on one or two staple foods. Based on its high nutritional value, peanut is a good candidate for genetic biofortification. This study is aimed at expressing an EAA-rich artificial storage protein (ASPx) into peanut seeds for increased nutritive value. The ASPx derived gene was introduced into peanut via *Agrobacterium*-mediated transformation. Molecular analysis of regenerated kanamycin resistant plants using polymerase chain reaction (PCR) and Southern hybridization indicate the stable integration of one copy ASPx gene in transgenic plants. The expression of the ASPx in transgenic peanuts seeds was detected by mass spectrometry (multiple reaction monitoring). Amino acids analysis showed an increase of 12 to 19% of most EAA (Val, Tyr, Phe, Iso, Leu, Met) in a transgenic line. The results show that the nutritional quality of peanut could be improved.

Key words: Genetic biofortification, essential amino acid, storage protein, peanut.

INTRODUCTION

With the growth of the world's population, global food demand is tremendously increasing. The increase of the production of animal-based protein would require more water and land, resulting in negative environmental effect (Henchion et al., 2017). Legumes are not only the second most important food source (Kouris-Blazos and Belski, 2016), but also good sources of dietary proteins (Singh, 2017), and cheap alternatives for populations, especially in developing countries where many cannot afford meat or dairy products (Maphosa and Jideani, 2017). With the increased incidence of metabolic diseases, plant proteins are appropriate source of protein without the concern of cholesterol (Maphosa and Jideani, 2017). However, with respect to human and animal nutrition, most seeds do not provide a balanced diet in protein because of deficiencies in one or several essential amino acids (EAA). This last decade estimates indicate that at least half of the world's population suffer from diseases caused by insufficient

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supplies of essential nutrients (Zhu et al., 2010; McGuire, 2015; Tien Lea et al., 2016). The lack of EAA in human diet can lead to protein deficiency, which negatively affects the growth of children, therefore hinders the development of affected countries. Possible solutions include dietary diversification and food supplementation but, these strategies are difficult to implement in developing countries, because poverty is widespread, and populations' dietary habits rely, most of the time, on couple of food crops. Thus, biofortification becomes a good alternative for sufficient and sustainable production of nutritionally improved foods (Saltzman et al., 2013).

Peanut (*Arachis hypogaea* L.) is a nutrient-dense legume providing over 30 essential nutrients and phytonutrients (Toomer, 2018). It is a major source of plant proteins in most tropical and subtropical regions of the world; and it is already used as base in some ready-to-use therapeutic food. However, the protein of peanut meal is of low quality as several EAAs (cysteine, methionine, threonine, lysine, isoleucine and valine) occur in limiting concentration (Andersen et al., 1998; Venkatachalam and Sathe, 2006). Because it is one of the cheapest staple foods and one of most consumed legumes in many countries of the world (Arya et al., 2016), peanut is a good candidate crop for protein biofortification. One of the strategies to enhance the level of aminoacids in food crops is to transfer genes encoding proteins with high content of essential amino acids (Zhang et al., 2003; Pérez-Massot et al., 2013). In this study, we report the successful genetic transformation of peanut hypocotyls using an amino acid rich artificial storage protein (ASPx) gene and the expression of the gene in the peanut seeds.

MATERIALS AND METHODS

Plant material

Seeds of peanut plant cultivar "Georgia Green" (Runner Market type) were obtained from Birdsong Peanut Co (Georgia, USA) and used in this study. The cultivar was selected, because it is the most commercially grown in the Southeast region of the USA.

Plasmids and bacterial strains

Vector construction

ASPx gene and protein: An ASPx and its corresponding gene were designed by Dr Jesse Jaynes (Tuskegee University, USA). The ASPx has been designed for stable expression *in vivo*. It is composed of 124 amino acids, arranged in 4 helical repeating monomers. It has about 75% of essential amino acid (EAA), including methionine (16%), lysine (13%), threonine (13%), isoleucine (10%), tryptophan (10%), valine (6.5%), phenylalanine (3%) and leucine (3%). In this study, the ASPx gene (420 bp) was synthesized by Operon Biotechnologies, Inc (Huntsville, AL USA) and carried into the pPCR-Script plasmid (Figure 1).

Plasmid pDK612 construction: The plasmid used for the genetic transformation of peanut in this study was constructed in two steps from the pPCR-Script. In the first step, the 420 bp ASPx gene was amplified using 2 primers, pf_ASPx and pr_ASPx, designed with *BamHI* and *BglII* restriction sites. The amplified polymerase chain reaction (PCR) fragment was cloned into TOPO TA vector (Invitrogen Inc, CA USA), resulting into the plasmid Topo-ASPx. In the second step, the 0.5kb *BglII*/*BamHI* fragment of ASPx gene from Topo-ASPx was subcloned into the plasmid pLAU2 (Idnurm et al., 2017) between the CVMV promoter and the E9 terminator. The resulting plasmid was pDK612. The gene arrangement from the right border to the left border of the T-DNA is p35S-nptII-NOS pCVMV-ASPx-E9. Plasmid amplification was done in TOP10 chemically competent *E. coli* cells from Invitrogen Inc.

Agrobacterium transformation: The plasmid pDK612 was mobilized into *Agrobacterium tumefaciens* strain EHA 105 by electroporation (Bio-Rad Gene Pulsar, Biorad, Hercules, CA). Bacteria were kindly donated by Dr. M. Egnin, (Tuskegee University, USA). Transformed *Agrobacterium* were used to infect peanut hypocotyls.

***Agrobacterium* preparation for peanut transformation:** For transformation, glycerol stocks of EHA105/pDK612 were prepared from a single colony of streaked plates grown at 28°C, divided into 300 µl aliquots and stored at -80°C. A day before transformation, the glycerol preps were used to inoculate liquid YEP medium supplemented with appropriate antibiotics (kanamycin or hygromycin 50 mg/l, along with rifampicin 15 mg/L). Cultures were incubated at 28°C for 16-20 h at 180-200 rpm (Gyromax 720 Orbital Shaker). For peanut transformation experiments, bacteria were pelleted for 10 min at 3500 *g* in a Beckman JA-20 rotor at 4°C. Pellets were resuspended in ½ MSi medium (half-strength MS -Murashige and Shoog- medium with 1 g/L myo-inositol and without sugar) (Egnin et al., 1998) and the OD_{600} was adjusted to 0.8-1.0 prior to the infection of peanut hypocotyl explants.

Genetic transformation, regeneration and selection of transgenic plants

Explants preparation

Mature peanut seeds were surface sterilized with 20% chlorox (2 times 30 min), followed by several rinses with sterile water. Embryo axes were excised from the seeds and germinated onto MS basal medium (Murashige and Shoog, 1962) supplemented with TDZ 10 µM (MSTDZ 10). Hypocotyl pieces (5-8 mm) from 5-6-day-old seedlings were used as explants for transformation. All cultures were incubated at 26±2°C under 16/8 h light/dark period.

Transformation and production of primary transformants

A modified method of Egnin et al. (1998), described by Dodo et al., (2008), was used in this experiment. Hypocotyl explants were rinsed three times for 20 min each in ½ MSi solution, and then immersed in an *Agrobacterium* suspension (OD = 0.9) for 10 min, blotted on sterile tissue papers, and co-cultivated inverted on MS0 medium (MS basal salts and vitamins, 100 mg/L myo-inositol, 30 g/L sucrose) for 5 days.

Selection: After the 5-day co-cultivation period, infected explants were transferred onto resting medium (MSTDZ and carbenicillin 400 mg/L) for 1-2 weeks, and then onto the selection medium (MSTDZ, carbenicillin 400 mg/L, kanamycin 200 mg/L) until appearance of shoot primordia. Elongated shoots were then transferred onto rooting medium (MS0, NAA 5 µM, Carbenicillin 100 mg/L, Kanamycin 50 mg/L). Explants were sub-cultured every 2-3 weeks. Well rooted putative transgenic shoots with well-developed roots were acclimatized for 1 week and transferred to the green house for

Figure 1. Aminoacids sequence of the ASPx.

Table 1. Sequences of primers used in the study.

Gene amplified	Primer name	Sequence	Amplicon length (Kbp)	References
ASPx gene	Pf_ASPx	5'-CTGGATCCGTTGATGATCGAGG-3'	0.5	This study
	Pr_ Aspx	5'-GGCTACTGACTCTAGAATTCGCG-3'	0.5	
CAMV35S	Primer 1 (35S-F)	5′-CAGAGGCAAGAGCAGCAGC-3′	1	Dodo et al. (2008)
	Primer 2 (35S-R)	5′-GCTGGGGTATCGATCACTGTCACAATGG-3′		
NPT2	NPT2 Fwd	5'-GCATACGCTTGATCCGGCTACC-3'	0.25	Matsumoto and Fukui (1996); Peyret et al. (2019)
	NPT2 Rev	5'-TGATATTCGGCAAGCAGGCAT-3'		
CAMV35S	Forward 35S	5'GAAGGTGAAGGTGACGACACTA3'	0.2	This study
	Reverse 35S	5'CTGTGGGTCAGCATTCTTTCTG3'		
	Probe 35S	5'FAM-TCACCACTGATAATGAGAAGGTTAGCC-TAMRA3'		
ASPx gene	ASPx-Fwd	5'GAAGGTGAAGGTGACGACACTA3',	0.2	This study
	ASPx- Rev	5'CTGTGGGTCAGCATTCTTTCTG3'		
ASPx mRNA	ASPx_FP	5'-TGGACGCATGATCGAGGAAAT-3'	0.1	
	ASP_RP	5'-TCGGCTTACACCCAGTAGGT-3'		

seeds production.

Production of subsequent generations of transgenic plants and seeds

Seeds from T0, T1 and T2 plants were germinated either through tissue culture or directly in soil to produce T1, T2 and T3 seeds.

Molecular and biochemical analysis

Genomic DNA was extracted from freeze-dried leaves using the DNeasy Plant kit (Qiagen Inc., Valencia, CA, USA). Young leaves of kanamycin resistant plants, as well as of wild type (WT) and non-transformed tissue culture control (TC) plants were used for the extraction. PCR and Southern blot analysis were performed following the standard protocols of Sambrook et al., (1989). The ASPx fragment was labelled using the AlkPhos direct labeling and detection system with CDP-Star and used as probe. For PCR amplification, different primers targeting the CAMV35S, NPT2 and ASPx gene were used (Table 1).

Taqman PCR amplification and analysis were performed using the Light Cycler 480 system (Roche Diagnostics, Corporation, Indianapolis, IN, USA), and following the manufacturer's procedure. The primers and probe were targeting the CaMV35S promoter. The sequences, presented in Table1, were designed using the software program Primer Express (Perkin-Elmer, Applied Biosystems, Foster City, CA).

Total RNA was extracted from T2 and T3 seeds using the Ambion RNAqueous-4PCR Kit (Life Technologies, Inc.). Reverse transcription was performed using the Superscript® II First-Strand Synthesis System for RT-PCR kit (Invitrogen, Inc.). About 400 ng of total RNA were used to synthesize the first strand of cDNA with the Oligo-dT primers. The first-strand cDNA obtained was amplified directly in a 25 µl reaction mixture containing primers and probe specific to the ASPx gene (FP 5'-TGGACGCATGATCGAGGAAAT-3'; RP 5'-TCGGCTTACACCCAGTAGGT-3'; probe FAM–TGAGACATGGATGAAAACCGTGATGGA-BHQ-1. Samples included a non-reverse transcriptase (nrt) control to ensure that amplification is not obtained from contaminated genomic DNA.

Crude protein was extracted from transgenic, as well as wild type seeds following a modified method described by Koppelman et al., (2001). Protein content of supernatants was determined using the Bradford assay kit (Bio-Rad) and equal amounts of proteins were loaded on each lane for gel electrophoresis (Bradford, 1976). Tris-glycine SDS-PAGE were run according to Laemmli, (1970) using a Bio-Rad Criterion cell system (Bio-Rad, Hercules, CA USA). Peptides bands around 15 kDa were excised from the 1-D gels to perform a multiple reaction monitoring experiment on the triple 5600 Qtrap mass spectrometer (Tandem MS/MS) (Applied Biosystems). Samples were digested *in vitro* using trypsin which cleaves on the carboxyl side of arginine (R) and Lysine (K). The digested peptides were then filtered by the quadrupole mass filter to select peptides. Based on the predicted cleavages of the ASPx protein with trypsin (Figure 2), the masses of resulting peptides (parent masses) and the fragmentation patterns (daughter ions) were predicted to

MIEEIMKKFETWMKTVMELWTKIMTYWVGPGRMIEEIMKKFETW

MKTVMELWTKIMTYWVGPGRMIEEIMKKFETWMKTVMELWTKI

MTYWVGPGRMIEEIMKKFETWMKTVMELWTKIMTYWV

Figure 2. ASPx protein tryptic cleavage pattern.

construct a diagnostic scan of the protein of interest. A positive control (expressed ASPx protein) was used to make method development scan. The first of the 3 quadrupoles selected for the parent mass (parent ion) of the desired analyte. The second quadrupole dissociated the parent ions by collision with an inert gas (N2) into daughter ions (b and y ions). The third quadrupole selected for one of the daughter ions. The parent–daughter ion combination allowed for a highly specific and sensitive diagnostic tool for detection and/or quantification for specific protein(s) in complex solutions. T3 seeds protein extracts were used for the experiment.

Nutritional analysis

Total protein and amino acids composition of transgenic peanut seeds from lines 2 and 6 were compared to those of the wild type (WT) peanut seeds. Total protein was analyzed by Kjeldahl according to AOAC 981.10. The amino acid composition of transgenic and wild type seeds was determined by using a C18 HPLC column equipped with an Alpha Plus Amino Acid Analyser (Biochrom, Cambridge, UK). The seeds were hydrolyzed with 6M HCl for 24 h at 110°C in an oil bath. Samples were then dried in an evaporator at 50°C, and dissolved in 5ml Na-Citrate buffer (0.2 M, pH 2.2) by vortexing for 30min. Samples were then ready to be loaded on the column for amino acid composition analysis (Lebet et al., 1994). Increment (%I) or decrement (%D) of aminoacids was calculated as followed:

(I%) = [(Transgenic seeds amino acid content − control seeds amino acid content) / control seeds
amino acid content] *100

Statistical analysis

Nutritional analysis was performed in triplicates. Means were calculated and compared by ANOVA using the SAS version 9.2 software. Tukey test was used to separate means.

RESULTS AND DISCUSSION

Production of transgenic plants and seeds expressing the ASPx gene

Primary transformants

Twenty-six independent kanamycin-resistant plants were obtained following the transformation of 479 hypocotyls explants. Eleven plants tested positive for the Southern blot of the PCR products. Ten T0 plants successfully rooted. Primary transgenic plants produced few pods (0 to 3). It has happened that primary transformants produced few seeds, but the main reason in our study was that plants were infected by the spotted-wilt disease.

Subsequent generations

From the T1 seeds, T2 seeds were produced with a germination success rate of 27.77%. Seeds from 2 transgenic lines (line 2 and line 6) successfully germinated along with a tissue culture and wild type control. T3 seeds from the same lines were produced from T2 seeds with about 50% germination success rate. The low germination rate of T1 seeds was primarily due to the small size of most seeds.

Molecular analysis of transgenic peanut plants

Putative transgenic T0 plants

During the selection for putative transgenic T0 plants, kanamycin-resistant plants were screened by PCR for the presence of the transgene. Primers specific to the CaMV35S promoter were used to amplify the putative transgenic plants along with the plasmids pDK612 and pDK30 as controls. A clear, defined and unique band was not obtained from the putative transgenic despite optimization of the PCR conditions. A smear along with unspecific bands showed on the PCR gels. So, to confirm the presence and the authenticity of the bands, PCR products were blotted onto nylon membrane for Southern hybridization (Figure 3). A 1 kb PCR amplified fragment of the CaMV35S promoter was used as probe. Out of 26 kanamycin resistant (KanR) plants, 11 plants were Southern blot positive, producing about 58% of escapes. The wild type and tissue culture control were Southern blot negative. Nine plants resulted from individual explants and 2 from the same explants, thereby 10 different transformation events occurred during the experiment, producing 10 transgenic lines.

The high number of escapes (58% of the kanamycin resistant) could be explained by the selection regime. Infected hypocotyl explants were cultured on a resting medium (MSTDZ) without kanamycin for 2 weeks before their transfer on the selection medium (MSTDZ + kanamycin). Dodo et al., (2008) also observed a high rate of escapes (over 50% due to the delay of the application

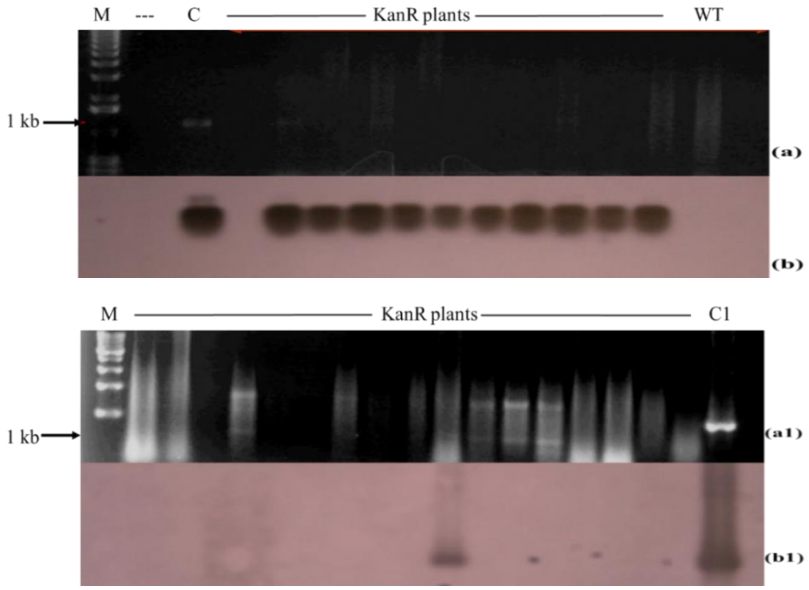

Figure 3. Molecular screening of transgenic T0 peanut plants. (a) and (a1) agarose gel of PCR targeting the CaMV35S promoter. (b) and (b1) PCR products probed with non-radioactive; Alkphos-labelled CaMV35S promoter. WT: wild type; M: 1kb step DNA ladder promega; C: plasmid pDK26; C1: plasmid pDK30.

of a selection pressure).

T1 plants

The inheritance of the ASPx transgene in the transgenic T1 plants was assessed by PCR and qPCR, targeting respectively a 250 bp fragment of the nptII gene and a 200 bp fragment of the transgene. Figure 4 shows the presence of the npt 250 bp and ASPx 200 bp fragments in the gels and Figure 5a shows the amplification curve obtained from the real-time PCR. Lines 2 and 6 plants tested positive.

Copy number analysis

Quantitative PCR: Transgene copy number was estimated by an absolute quantification of the ASPx transgene using a standard curve obtained by the amplification of the ASPx transgene in a 10-fold serial dilutions series of pDK612. Linearized plasmid has been used for estimation accuracy. The threshold value was plotted against the log DNA concentration. The standard curve was a linear regression line between Ct and log10 of transgene copies/reaction (calculated from standard plasmid DNA concentration). The amplification factor of the reaction was 1.95 and the resulting efficiency was 95%. The correlation R^2 between the Cp values and the log DNA concentration was 0.99. The amplification curves of the plasmid standard and transgenic plants are shown by Figure 5. Table 2 shows the results of the copy number obtained from the Taqman PCR. The precise number of integrated transgenic sequences was obtained from the ratio between the absolute quantity of the transgene and the genome copy number of each transgenic peanut line in T0 and T1 plants (Gadaleta et al., 2011). Transgene copy numbers ranged from 1 to 3 in the T0 transgenic lines analyzed. Lines 2 and 6 have 1 transgene copy. Results also showed that T0 plants were hemizygous and T1 plants homozygous.

Southern blot: Copy number of the ASPx transgene was confirmed by Southern blot. Ten (10) µg genomic DNA from T1 line 2 plants were digested with *BglII* which makes a single cut in the T-DNA region. A 400 bp ASPx fragment was labelled with Alkaphos direct DNA labeling kit (Amersham, Inc. USA) and used as probe to hybridize the blots. Results show that the analyzed plants carry a single copy of the transgene (Figure 6).

Expression of ASPx gene in the seeds

At the mRNA level

Expression of the ASPx at the RNA level was determined by RT-PCR using primers targeting a 100 bp fragment of the ASPx gene and total RNA extracted from T2 and T3 seeds in 2 lines. Figure 7 showed presence of the

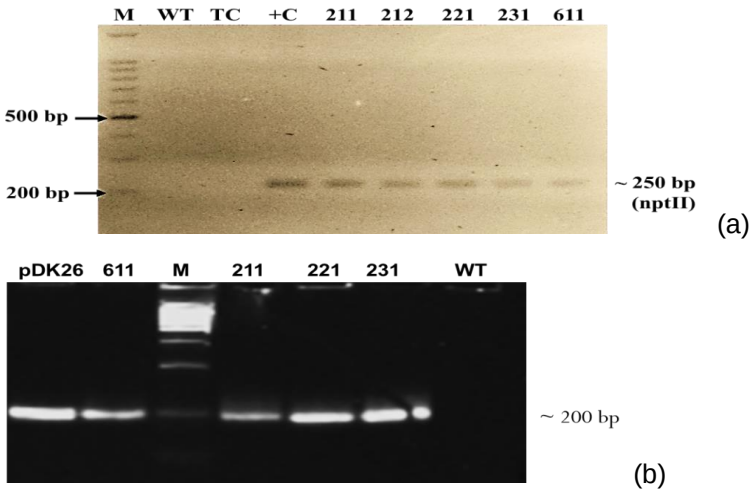

Figure 4. (a) PCR products T1 transgenic plants (targeting npt2 gene); (b) PCR products of T1 transgenic plants (targeting ASPx gene); M: 100 bp DNA ladder (Promega); WT: negative control; TC: Tissue culture control; +C & pDK26: Positive control; 211, 212, 221, 231 & 611: lines 2 and 6 - T1 plants.

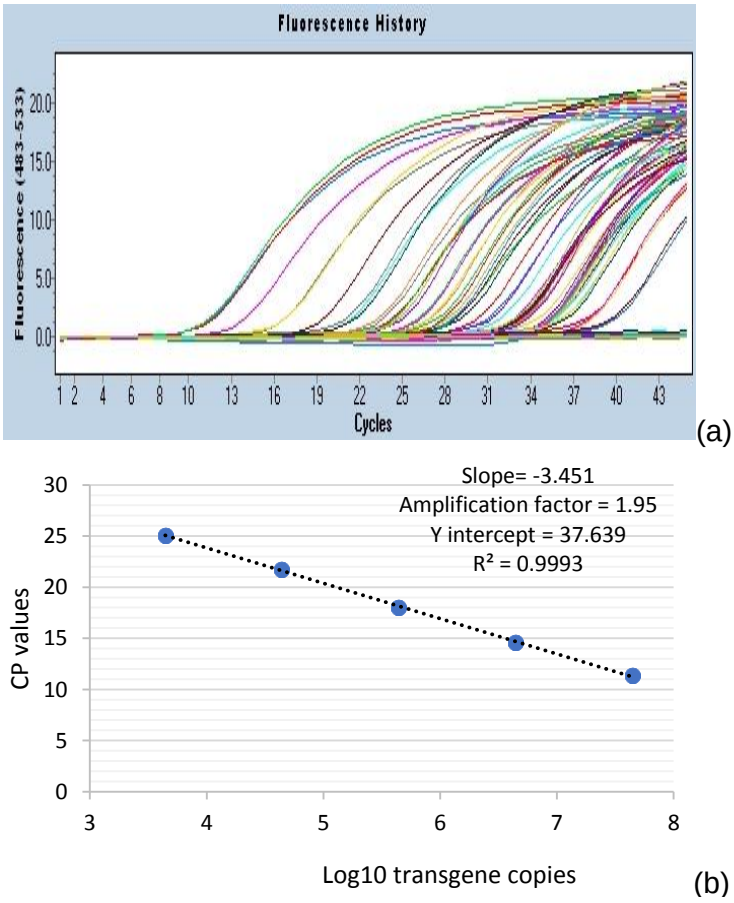

Figure 5. Copy number analysis of transgenic plants by qPCR. (a) Amplification curves of the serial dilutions of plasmid PDK26 (standard) and transgenic T0 and T1 plants. (b) Standard curve obtained from the serial dilutions.

Table 2. Determination of transgene copy number.

Lines or plants	Initial [(a)] absolute transgene copies / rxn	Initial [(b)] number of genomes /rxn	Estimated CN per number of genomes	Estimated CN per haploid genome	Zygosity
T0 line 1	12172	5273	1.2	0.6	
T0 line 2	**1478**	**710**	**1.0**	**0.5**	
T0 line 3	46228	10914	2.1	1	
T0 line 4	14869	4570	1.8	0.9	Hemizygous
T0 line 6	**10831**	**5516**	**1.0**	**0.5**	
T0 line 8	141813	23152	3.1	1.5	
T0 line 9	22413	9947	1.1	0.6	
T0 line 10	7090	1859	1.9	1	
T1 line2 (221)	751874	371520	2,0	1	
T1 line 2 (231)	15685	8551	1,8	0.9	Hemizygous
T1 line (661)	5941	2650	2,2	1.1	

a: Initial absolute transgene copies obtained from the standard curve; b: Initial genome copies calculated from the amount of transgenic plants DNA used in the PCR.

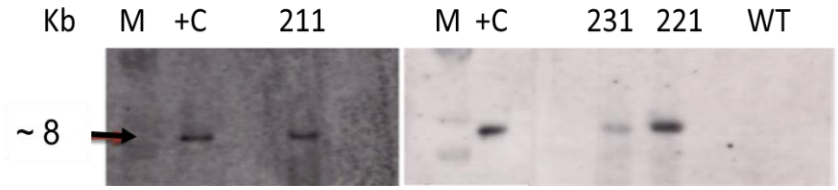

Figure 6. Molecular analysis of T1 progeny plants. Southern hybridization of *BglII*-digested DNA using ASPx probe. M-lambda *HindIII;* DNA marker (Promega); 5-negative control; C-positive control; 211, 231, 221: T1 transgenic plants.

fragment, indicating that the ASPx gene is actively transcribed to mRNA in transgenic seeds.

Protein profile of the transgenic seeds by SDS-PAGE

The expression of the APSx protein (presence of the 15 kDa ASPx protein) was first investigated by SDS-PAGE of proteins extracted from transgenic seeds along with WT and TC controls. The presence of the ASPx protein was not clearly distinguishable between negative controls (WT, TC) and transgenic seeds at the expected size, due to the presence of existing proteins at the same size in non-transgenic peanut seeds, especially the Arah2 peanut protein (Figure 8). However, differences are observed in the complete profile. Most transgenic seeds, if not all, showed an altered profile compared to the WT and TC. Some seeds showed reduction of proteins bands at the size of major allergens in peanut, especially Arah1 and Arah3 (between 75-35kDa). Most seeds showing reduction around the Arah1 protein area, showed accumulation of protein between 10 and 20 kDa. Even though the mechanism of storage protein accumulation in the proteins bodies of legumes is not fully understood, it seems to exist a compensatory effect between the different storage proteins in a crop. As storage proteins are source of nitrogen for the new developing seed, reduction of the expression of some key proteins might increase the expression of others and vice versa, in order to maintain a minimum pool of storage proteins necessary for the germination process..

Multiple reaction monitoring (MRM)

Since SDS-PAGE results did not clearly show the presence of the ASPx protein, mass spectrometry was chosen as a tool to detect the expected ASPx protein in the transgenic seeds. Crude proteins extracted from transgenic and wild type seeds were resolved on SDS-PAGE and proteins bands around 15 kDa were cut for

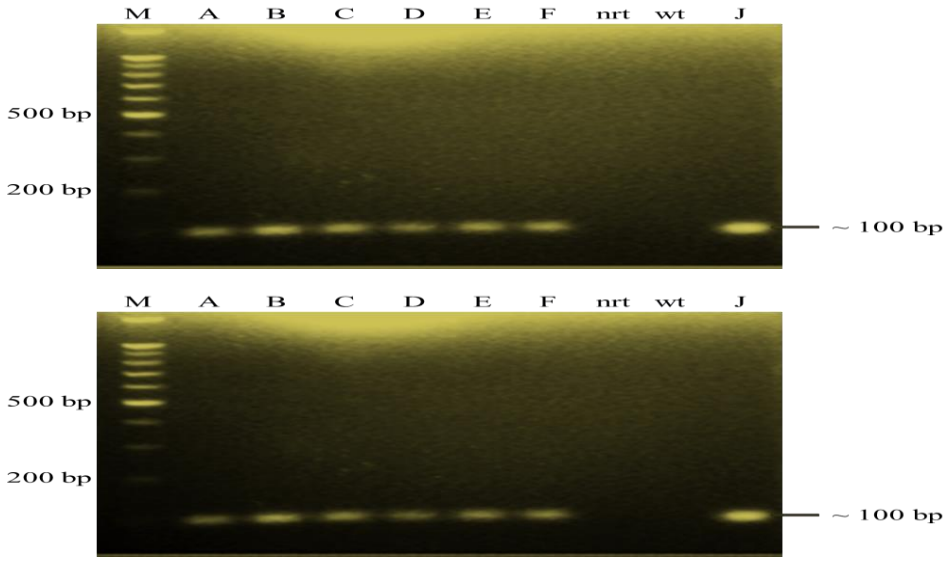

Figure 7. RT-PCR (Reverse-transcriptase polymerase chain reaction) of T2 and T3 seeds. 2% Agarose gel of products. M: 100 bp DNA ladder (Promega); A-F: transgenic T2 seeds; 1-6: transgenic T3 seeds; wt: wild type; J: ASPx positive control. nrt: non-reverse transcriptase negative control.

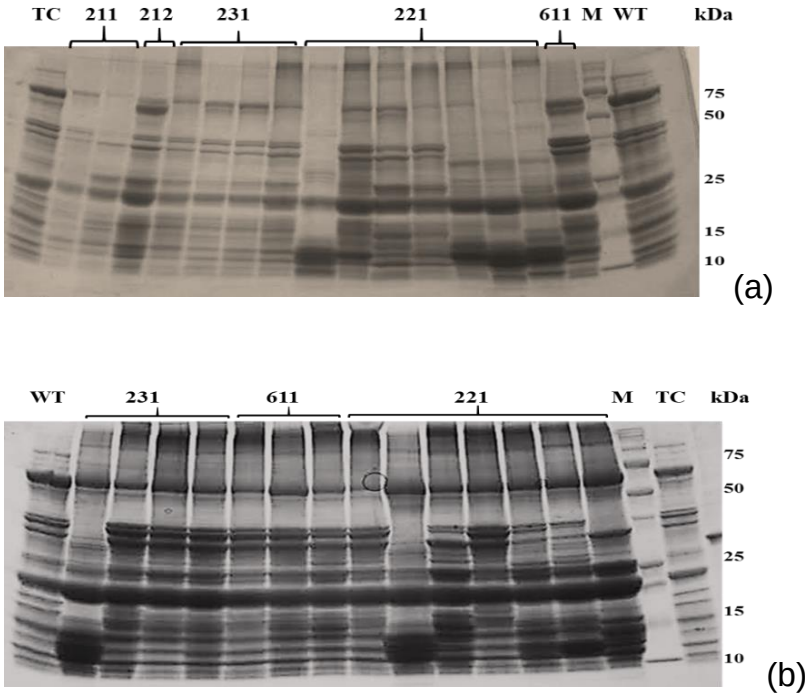

Figure 8. Protein profile of T2 (a) and T3 (b) seeds. WT: wild type; M: Protein standards; TC: Tissue culture 211, 212, 221, 611: Transgenic seeds.

targeted analysis of the ASPx protein using the MRM method on the triple 5600 Qtrap mass spectrometer (Tandem MS/MS) (Applied Biosystems). Spectrometer profile obtained showed the presence of the ASPx protein in the protein extracts analyzed. Figure 9 represents an example of chromatograms obtained during the analysis.

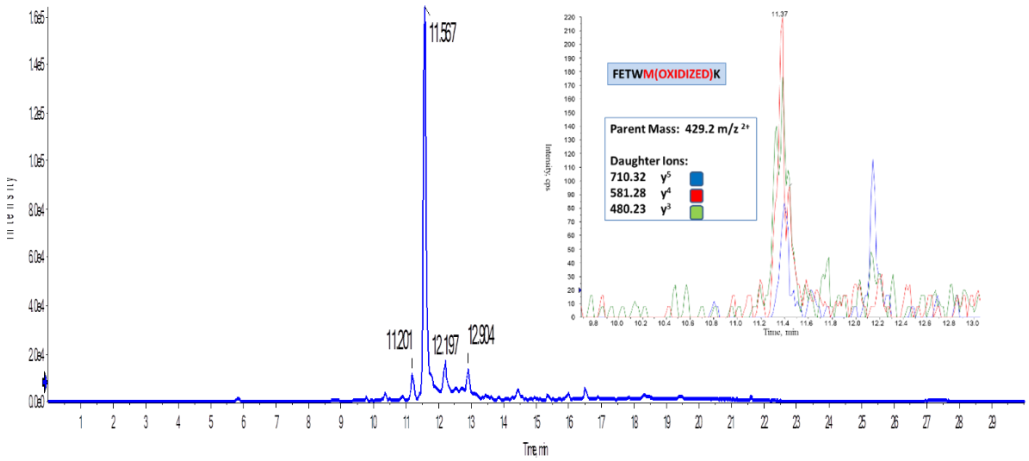

Figure 9. MRM scan profile ofthe ASPx-derived peptide sequences (positive control and T3 sample).

Table 3. Amino acid composition of peanut from transgenic and non-transgenic seeds (%).

Amino acids	WT	Line2	Line6
Aspartic Acid	2.42^{b}	2.88^{a}	2.55^{b}
Threonine	0.63^{a}	0.71^{a}	0.66^{a}
Serine	0.92^{b}	1.06^{b}	0.93^{b}
Glutamic Acid	3.42^{b}	4.24^{a}	3.95a^{b}
Proline	0.91^{a}	1.07^{a}	0.94^{a}
Glycine	1.11^{a}	1.22^{a}	1.15^{a}
Alanine	0.73^{a}	0.84^{a}	0.77^{a}
Valine	0.88^{b}	1.05^{a}	0.91^{ab}
Isoleucine	0.75^{b}	0.88^{a}	0.78^{b}
Leucine	1.47^{b}	1.68^{a}	1.51^{b}
Tyrosine	0.98^{a}	1.11^{a}	0.96^{a}
Phenylalanine	1.23^{b}	1.42^{a}	1.21^{b}
Lysine	0.77^{b}	0.88^{a}	0.86^{a}
Histidine	0.52^{a}	0.61^{a}	0.54^{b}
Arginine	2.58^{a}	3.02^{a}	2.66^{a}
Methionine	0.39^{b}	0.44^{a}	0.42^{a}
Cysteine	0.06^{a}	0.05^{a}	0.05^{a}
Total Amino Acids	19.77^{b}	23.16^{a}	20.85^{b}
Crude protein			

WT: wild type; Line2: Transgenis seeds from line 2; Line 6: Transgenic seeds from line 6; Values are means and numbers with different superscript are statistically different ($p<0.05$) according to Tukey's studentized test.

Nutritional analysis of transgenic seeds

Total protein content and the amino acids composition of transgenic seeds from lines 2 and 6 and from WT negative control seeds were analyzed (Table 3). Protein content of WT seeds (23.94%) is not statistically different from line 2 (24.52%), and line 6 (25.92%) ($P\geq0.05$) seeds. Unlike the total protein content, line 2 has a significantly higher total amino acids content (23.16%) than transgenic line 6 (20.85%) and WT (19.77%) at level $P<0.05$. Line 6 and WT are not statistically different. ($P\geq0.05$). Overall, line 2 showed a significant increase in individual amino acids, especially in EAA compared to the line 6 and WT. EAA presented between 12 and 19% increase for line 2,

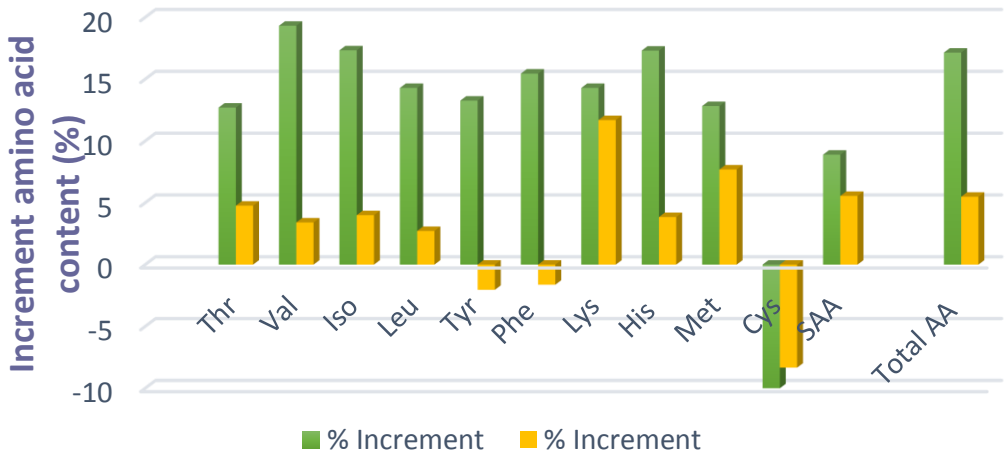

Figure 10. Percentage of the essential amino acids above the wild type control. Line 2: In Green; Line 6: in yellow; Increment is calculated by (transgenic seeds amino acid content − control seeds amino acid content) ÷ control seeds amino acid content.

especially in five of the limiting amino acids in peanut, Val, Iso, Leu, Met and Threonine. Line 6 had lower increase compared to line 2.

The difference in the expression levels of the ASPx protein in the transgenic plants is a phenomenon that has been reported in several transgenic crops and might be due various factors including position effects, transgene rearrangements and developmental stage of the tissue (Zhang et al, 2003; Yang et al., 2016). However, several authors obtained increase level of aminoacids in tubers and roots (Zhang et al., 2003; Wong et al., 2015). Concerning total protein content, some authors have also noticed either no significant difference in transgenic seeds, leaves or tubers, or very slight increase, even there was significant difference in the amino acids (Zhang et al., 2003; Yang et al, 2016). Results suggest that the overall protein content must be regulated in peanut seeds, or the expression of ASPx might have caused the suppression or the down-regulation of the expression of some other proteins.

We have noted a decrease (1-2.5%) in the level of Phe and Tyr, the aromatic amino acids in line 6. (Zhang et al, 2003) made the same observation in some biofortified transgenic cassava roots. A decrease in cysteine level was also noted in both transgenic (-10% in line 2 and -8% in line 6) compared to the WT. Molvig et al., (1997), Wong et al. (2015) and Yang et al. (2016) reported a decrease of some amino acids following the expression of a sunflower seed albumin gene in lupins seeds or the increase of lysine in transgenic rice grains. The increase of one sulphur-containing amino acids can make up the decrease of the other, because both cysteine and methionine can be synthesized from one another (Bin et al., 2017; Cohen et al., 2017), as they are synthesized via the aspartate (Asp) family pathway. Increments and decrements are better shown by Figure 10.

Conclusion

In this study, the ASPx gene encoding a protein with 75% EAA was successfully integrated into the peanut genome and expressed into seeds via *Agrobacterium*-mediated transformation and direct organogenesis. For stable expression levels in transgenic plants, the ASPx gene was linked to the KOZAK translational enhancer. An increase of at least 4 limiting amino acid in peanut was obtained for transgenic lines, along with few decreases in the level of some amino acids. This result shows that the protein quality of peanut seeds could be improved. For further study, better increase level of amino acids should be investigated by coding the ASPx gene under the control of dicots seed-specific promoters such as the bean β-phaseolin, the β-conglycinin, the napin, and the soybean lectin promoters. Also, the allergenic status of the transgenic seeds should be evaluated, as well as the bioavailability of the new protein.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Bio-appraisal of three strains of *lactobacillus* based probiotics on the growth traits and semen characteristics of local toms

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The effect of three strains of *Lactobacillus* based probiotics on the growth performance and semen quality of toms was conducted in a thirty-four week study. Eighty day-old (d 1) poults were randomly divided into 4 groups, replicated twice with 10 birds per replicate and assigned to four treatments; T1 (*Lactobacillus delbrueckii* subsp *Bulgaricus*), T2 (*Lactobacillus acidophilus*), T3 (*Lactobacillus sporogens*) and T4 (control), in a completely randomized design (CRD). Feed and water were supplied *ad libitum*. Results showed significant differences (P<0.05) in the birds' mean values for average daily weight gain, average daily feed intake, feed conversion ratio and final body weight. Birds on T2 had significantly (P<0.05) higher final body weight than other treatment groups. Toms on T4 had significantly (P<0.05) higher values for feed conversion ratio than birds on probiotic treatment with T2 recording the least value. However, the effect of treatments on the semen physical characteristics of the birds were also found to be significant (P<0.05). The birds on probiotic treatment recorded higher values for the semen quality indices under study than the control group, with T2 having the highest values. It was thus concluded that of the three strains of *Lactobacillus* based probiotics used in the current study, T2 (*L. acidophilus*) improved the growth performance and semen quality of local toms.

Key words: Artificial insemination, direct-fed microbial, growth, nutrition, turkey production.

INTRODUCTION

Artificial insemination (AI) is one of the animal production technologies commonly referred as Assisted Reproductive Technology (ART), where off-springs are generated from parents by facilitating the union of gametes (Morrell, 2011). It simply involves the collection of semen from choice male(s) and transferring the semen into the vagina of the females for fertilization to take place. AI has proved to be an indispensible tool in turkey production owing to the selective breeding for a heavier and broader-breasted commercial turkey, and the consequent incapacitation of toms to natural mating due to their larger body size compared to the hens (Bakst and Dymond, 2013). Moreover, it has also been found to enhance cross breeding programs, improve productive

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performance and ensure rapid sustainable economic gains from turkey birds (Ngu et al., 2014).

However, to achieve a successful AI program, the need for an outstanding quality of semen cannot be overemphasized (Harstine et al., 2018). Semen of good quality, typified by the quality parameters of semen such as; volume, concentration, color, and motility, guarantees its usefulness in any AI program (Kotlowska et al., 2005). Although the quality of poultry semen can be influenced by a myriad of factors ranging from;age of the bird (Murugesan et al., 2012), breed/strain (Abas et al., 2018), temperature (Karaca et al., 2002), nutrition (Mahmood and Hazim, 2011; Hudson and Wilson, 2003), environmental conditions (Elagib et al., 2012; Santiago-Moreno et al., 2015), etc., it is pertinent to note that poor quality semen represents a great economic loss to production (Khatun et al., 2013). Hence, the need to improve and maintain a superior semen profile in any poultry AI program becomes imperative.

Throughout the last decade, animal nutritionists continued to search and document several dietary managements that promoted poultry semen (Heydari et al., 2015; Sharideh et al., 2015; Deivendran and Yeong, 2015; Murugesan et al., 2016; Amin et al., 2019) and body weight (Dim et al., 2018), as both are positively correlated. These documented researches also included the dietetic use of probioticsto improve poultry semen (Inatomi and Otomaru, 2018; Aalaei et al., 2019). Probiotics have been defined as direct-fed live microbials that confer beneficial effects on the host when administered amply (FAO, 2009). They are principally composed of bacteria and/or fungi organisms, but can also be of protozoan origin (Chabe et al., 2017).

The lactic acid bacteria are the most dominant class of colonizing bacteria species used as probiotics to improve production traits in farm animals (Ezema, 2013; Panda et al., 2007; Ehrmann et al., 2002). Reports of Duncan et al. (2004) suggests that they exhibit the cross-feeding mechanism, where the lactic acids they produce are utilized by the strictly anaerobic butyrate producing bacteria for the assemblage of large concentrations of butyric acid. These assembled butyrate function to change the intracellular pH, thus leading to the exclusion of the pathogenic bacteria cells in the gut (Panda et al., 2009). Also, the development of epithelial cells with improved gastrointestinal health has been linked to butyric acid metabolism in the gut (Bron et al., 2002). These protocols justify why lactic acid bacteria administration can significantly enhance performance indices in food animals. Conversely, Triplett et al. (2016), Kiess et al. (2016) and Haines et al. (2015) made some interesting observations on the decreased quality of poultry semen in-relation with lactic cid bacteria. They suggested that *Lactobacillus* organisms negatively affected the quality of poultry semen under the conditions of their respective studies.

However, there is a dearth of credible literatures on the improvement of the indigenous local turkey (*Meleagris gallopavo*) despite its hardy and resilient adaptation features to the tropical humid environment. These birds adapt under unfavorable environmental conditions and meager nutritional status better than most poultry species (Perez-Lara et al., 2013; Yakubu et al., 2013). The meat percentage of the toms is better expressed through their massive, stocky and long-legged indices (Damaziak et al., 2014). Therefore, a study designed to bio-assess the growth rate and semen quality of local toms raised in the tropics, fed three selected strains of *Lactobacillus* based probiotics is prompt and of paramount significance.

MATERIALS AND METHODS

Location and duration of study

The study was conducted at the turkey unit of Veterinary Teaching and Research Farm, University of Nigeria, Nsukka. Geographically, Nsukka is located within longitude 07°54 ^{1}E and latitude 05°22 ^{1}N, with annual rainfall range of 966- 2098mm (Momoh et al., 2010). Agbagha et al. (2000) reported the study area to be typically tropical, having a mean daily temperature of 26.8°C, and relative humidity percentage values that ranges from 65-80%.Duration of study lasted a period of 28 weeks.

Test strains

The three selected strains of the *Lactobacillus* – based probiotics used in the study included *Lactobacillus sporogenes* NRRL – 4496 (1×10^{8} CFU/ml), *Lactobacillus delbrueckii* subspecies *bulgaricus* NRRL B – 4527 (1×10^{8} CFU/mL)and *Lactobacillus acidophilus* NRRL – 4495 (1×10^{8} CFU/ml). They were obtained and constituted in an MRS broth from the Microbial Genomics and Bio-processing Research Unit, National Centre for Agricultural Utilization Research, Agricultural Research Service, United States Department of Agriculture, University St., Peoria, Illinois. Upon receipt, the probiotics were maintained in the broth medium and kept in a cold room at a temperature of 18°C.

Experimental diets

The experimental diets comprised starter, grower and finisher diets, with their respective percentage compositions are presented in Table 1. The poults were fed the starter diets from day-old (d-1) to eight weeks of age before carefully replacing their feed gradually to the grower diets. The birds were placed on the finisher diets from sixteen weeks of age till the completion of the study.

Management of experimental birds

The conduct of the current study adhered strictly to the provisions of the ethical committee on the use of animals and humans for biomedical research of the University of Nigeria. 80 day old (d-1) male poults were randomly divided into 4 groups of 20 birds each. Each group was randomly assigned to four treatment groups, tagged; T1, T2, T3 and T4, in a completely randomized design (CRD). Each treatment group was replicated twice with 10 birds per replicate, placed in a deep litter system. Birds on T1, T2 and T3 received probiotics of *L. delbrueckii* subspecies *bulgaricus, L. acidophilus* and *L. sporogenes,* respectively at inclusion levels of

Table 1. Percentage composition of experimental diets.

Ingredients	Starter (%)	Grower (%)	Finisher (%)
Maize	45.00	48.00	50.00
Soybean meal	40.00	37.00	24.10
Fishmeal	7.30	4.50	1.50
Wheat offal	0.00	4.00	17.55
Bone meal	4.30	3.20	3.65
Oyster shell	2.50	2.50	2.50
Vitamin Premix*	0.25	0.25	0.25
Salt	0.25	0.25	0.25
DL-methionine	0.30	0.20	0.10
Lysine	0.10	0.10	0.10
Total	100	100	100
Calculated composition			
Crude protein (%)	26.00	22.40	19.50
Crude fibre (%)	3.90	4.26	4.65
Ether extract (%)	5.00	4.52	4.31
ME (K cal/Kg)	2845.00	3100.00	3150.00

*Each 2.25 kg of vitamin premix contains; 10,000,000 I.U Vitamin A, 2,200,000 I.U Vitamin D3, 10,000 mg Vitamin E, 2000 mg. Vitamin K3, 1500 mg Vitamin B1, 5000 mg Vitamin B2, 1500 mg, Vitamin B6, 10 mg Vitamin B12, 15,000 mg Niacin, 20 mg biotin, 125,000 mg Anti-Oxidant, 500 mg, Folic acid, 5000 mg Calpan.

0.5ml/L in their drinking water for 3 consecutive days from 1 - 3, 10 - 12 and 21 - 23 days of age to achieve the recommended microbial concentration (10^6 CFU/ml) as stipulated by the supplier. 0.5 kg of milk powder/1000 L of the birds' drinking water served as substrates to protect the lactic acid bacteria from oxidative damage during the duration of administration. T4 served as control with no strain of *Lactobacillus* inclusion. Feed and water were provided *ad libitum*. Birds were weighed at the start of the experiment and also at weekly intervals to obtain their weekly bodyweight gain. Daily feed intake was also recorded for the birds all through the period of study.

Semen analysis

The Burrows and Quinn method of abdominal massage as outlined by Yahaya et al. (2013) was used for the semen collection. Semen volume was graduated using a conical test tube. The progressive motility and spermatozoa count was determined using a haemocytometer (450 × magnifications) and a light microscope with warm stage. The Ernst and Ogasawara technique as cited by Ngu et al. (2014) was used to determine the percentage values of live/dead and normal/abnormal sperm cells.

Statistical analysis

One way analysis of variance (ANOVA) was used to analyze the data generated in the current study, using a statistical package (SPSS) windows version 20.0. The differences in the mean values were separated using the Duncan New Multiple Range Test as put forward by Obi (2002). The experimental model of the CRD used in the current study is:

$$X_{ij} = \mu + T_i + \sum_{ij}$$

Where X_{ij} = any observation or measurement taken, μ = population mean, T_i = Treatment effect, $\sum_{ij}$ = Experimental error, i= number of treatments, j= number of replicates.

RESULTS AND DISCUSSION

Effect of three strains of *Lactobacillus* based probiotics on the growth traits of local toms

Table 2 shows the growth performance of local toms fed three strains of *Lactobacillus* based probiotics (LBP). Results showed significant differences (P<0.05) in the birds' values for final body weight, average daily weight gain, average feed intake, and feed conversion ratio (FCR) across the treatment groups. Birds on LBP treatments were observed to be heavier (P<0.05) than those on the control group, with toms on T2 (*L. acidophilus*) recording the most superior values. Despite the differing numerical values recorded for birds on T1 (*L. delbrueckii* subspecies *bulgaricus*) and T3(*L. sporogenes*), their mean values were found to be statistically similar (P<0.05). However, toms on LBP treatments consumed less feed (P<0.05) than birds on the control group (T4), with birds on T2 recording the least feed intake values across the treatment groups under study. Toms on T1 and T3 also had comparable (P<0.05) average daily feed intake values while varyingnumerically. Hence, it follows that birds on LBP treatments ate less feed to weigh more than the birds on

Table 2. Growth performance of toms fed three strains of *Lactobacillus* based probiotics.

Parameter	Treatments				SEM
	T1	T2	T3	T4	
Initial Body Weight (g)	60.01	60.03	60.02	60.00	1.63
Final Body weight (g)	9450.00[b]	9900.00[a]	9500.00[b]	8000.00[c]	200.51
Average Daily Weight Gain(g)	39.70[b]	41.59[a]	39.91[b]	33.61[c]	1.02
Average Daily Feed Intake (g)	125.45[b]	120.61[c]	124.91[b]	134.45[a]	2.80
Feed Conversion Ratio	3.16[b]	2.90[c]	3.13[b]	4.00[a]	0.96

[abc]Means on the same row with different superscripts are significantly (P<0.05) different. SEM= Standard error of Mean. T1= *L. delbrueckii*subspecies *bulgaricus*; T2= *L. acidophilus;* T3= *L. sporogenes*; T4= control.

the control group. This translated to the improved FCR values (P<0.05) observed in birds on LBP treatments, with toms on T2 recording the best-quality values. The increased growth performance indices observed in birds on LBP treatments can be linked to the role of these bacteria in enhancing bio-availability of nutrients, nutrient assimilation and metabolism. Literature evidence suggests that lactic acid bacteria utilize carbohydrates as substrates for growth (Watson et al., 2013), while most pathogenic bacteria primarily employ secreted proteins as their substrate-media (Figaj et al., 2019; Cezairliyan and Ausubel, 2017). By producing bacteriocins and a gut pH that limits the favorable proliferation of the pathogenic bacteria in the gastrointestinal tract, the lactic acid bacteria competitively excludes the harmful bacteria from the tract, thus allowing for total absorption of their secreted substrate proteins by the body of the birds. This consequently transformed to the amplified growth response observed in birds on the *Lactobacillus* based probiotics (LBP) treatments. These resultsconfirm the observations of Li et al. (2014) and Vantsawa et al. (2017), who documented a major increase in bodyweight and decrease in FCR for birds fed/treated with *L. acidophilus.* The outcome of the present study substantiates the reports of Ahmad (2006) and Eckert et al. (2010) who demonstrated the positive effect of probiotics on poultry birds.

Effect of three strains of *Lactobacillus* based probiotics on semen quality of local toms

Table 3 shows the effect of the three strains of *Lactobacillus* based probiotics (LBP) on semen characteristics of local toms. Results from the study illustrated significant differences (P<0.05) in the toms values for all the semen quality indices studied, ranging from semen volume, semen concentration, progressive motility, percentages of live, dead, normal and abnormal spermatozoa. Toms on LBP treatments (T1, T2 and T3) produced more concentrated semen (P<0.05) with greater percentages of live and normal sperm cells that are highly motile than the control group. Birds on T2 (*L. acidophilus*) recorded the top values (P<0.05) for volume of semen, concentration of semen, and percentage live spermatozoa among the treatment groups, with birds on T1 (*L. delbrueckii* subspecies *bulgaricus*) and T3 (*L. sporogenes*) recording similar (P<0.05) comparable statistical values irrespective of their contrasting numerical values. However, toms on T1 and T2 were observed to produce the most progressive motile spermatozoa (P<0.05) across the groups under study. Also, percentage values for dead and abnormal spermatozoa were observed to be higher (P<0.05) in birds on the control group, with birds on T2 having the least values. Moreover, toms on *L. acidophilus* administration had the best-quality values (P<0.05) for normal sperm cells across the groups understudy. These quality values were also found to be statistically the same (P<0.05) with the values recorded for birds on *L. delbrueckii* subspecies *bulgaricus* and *L. sporogenes*. Nevertheless, birds on the control group also had the lowest values (P<0.05) for normal sperm cells, which were however observed to be statistically similar (P<0.05) with the values recorded for birds on T1 and T3. The improved semen quality indices observed in birds on the LBP treatments can however be linked to the role of these bacteria in the synthesis of trace minerals and vitamins in the birds' gut that supported quality of semen. Reports have it that *Lactobacillus* bacteria synthesize trace minerals (Nagy et al., 2016), antioxidant vitamins (E and C) and B-complex vitamins (LeBlanc et al., 2011). These vitamins (B_{12}, E and C) have been found to guarantee improved spermatozoa motility and countin food animals (Surai et al., 2001; Eid et al., 2006; Deivendran and Yeong, 2015; Banihani, 2017). Trace minerals like selenium, zinc and manganese produced by these bacteria also work to boost spermatogenesis in poultry (Barber et al., 2005). Furthermore, the secreted substrate proteins absorbed by the birds' gastrointestinal tract as a result of the pathogenic exclusion exhibited by the lactic acid bacteria, ultimately supported the improved quality of semen observed in birds on LBP treatments. It is evident from the results observed in the current study, that probiotic organisms support superior quality of semen as opined by Khan et al. (2012) and Emmanuel

Table 3. Effect of three strains of *Lactobacillus* based probiotics on semen quality of local toms.

Parameter	Treatments				SEM
	T1	T2	T3	T4	
SV (ml)	0.39[b]	0.48[a]	0.38[b]	0.27[c]	0.03
SC (x10^9/ml)	5.11[b]	5.82[a]	5.13[b]	4.78[c]	0.09
PM (%)	82.20[a]	82.93[a]	81.17[b]	70.13[c]	1.57
LS (%)	93.20[b]	94.13[a]	93.07[b]	87.73[c]	1.08
DS (%)	6.80[b]	5.87[c]	6.93[b]	12.27[a]	0.63
NS (%)	90.00[ab]	91.38[a]	90.32[ab]	82.33[b]	0.18
ABS (%)	10.00[b]	8.62d	9.68[c]	17.67[a]	0.12

[abc] Means on the same row having different superscripts are significantly different (P<0.05); SEM = Standard Error of Mean.*SV = Semen volume; SC = Sperm concentration; PM = Progressive motility; LS = Live sperm; DS = Dead sperm; NS = Normal sperm; ABS = Abnormal sperm. T1= *L. delbrueckii*subspecies *bulgaricus*; T2= *L. acidophilus;* T3= *L. sporogenes*; T4= control.

et al. (2018). These results debunk the findings of Triplett et al. (2016), Haines et al. (2015) and Kiess et al. (2016) who documented decreased quality of poultry semen when associated with lactic acid bacteria. The differences might be attributed to a collage factors like breed/strain of the experimental birds, strain of the lactic acid bacteria used, climatic condition of the study area, among others. However, the superiority of semen observed in the present study validates the report of El-Deep et al. (2011), who observed improved semen quality indices in birds fed probiotic organisms.

CONCLUSION AND RECOMMENDATION

It is glaring from the results obtained in the present study that birds on T2 (*L. acidophilus*) recorded the most superior values for the birds' growth traits and semen quality indices studied among the treatment groups. *L. acidophilus* based probiotics should therefore be used in local toms' nutrition to improve their growth performance and of course, the quality of semen. Nevertheless, further research should be carried out to understand how these bacteria affect their host beneficially even at the genomic level. A better understanding of how they affect the genes responsible for production traits in birds will bring poultry research closer to the field of nutrigenomics.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison of crop production efficiency of compost leachate with chemical fertilizer and evaluating its effect on germination and growth of wheat crop

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Increasing public concern in relation to health and environmental issues of organic waste leads to many solutions of waste management. Many different methods are discovered for proper waste management and disposal. Organic fractions of solid waste are treated with aerobic composting. It is one of the better treatments for organic waste. The important issue of concern here is the production of compost leachate with organic waste. In this study, to evaluate the competency of produced leachate as fertilizer and making the composting process more cost effective and environmentally friendly, liquid fertilizer prepared by Lahore Compost Company was used. Different percentages of leachate (0.2, 0.5, 1.5, 3.5, 5.5, 10.5, 15.5, and 25.5%) were prepared and germination rate of wheat was checked by evaluating its fertilizing quality compared to fertilizer. Results showed high germination and growth rate at lower concentration of leachate as compared with control and commercial fertilizer due to the presence of many suitable nutrients. This study was designed to compare the crop production efficiency of compost leachate with commercial fertilizer. It was revealed in this study that low leachate percentage give better crop production in case of wheat.

Key words: Compost, leachate, recycling, organic waste.

INTRODUCTION

Solid waste management has become a core issue in under-developing countries. International recommendations implement the strategy to recycle waste in these countries. Main emphasis was provided on aerobic composting. Composting is a natural process but can be altered by changing various physical and chemical parameters (Turjillo e al., 2006). In this method, the organic content of municipal solid waste is converted into humus-like material called compost. To increase food production in modern agriculture, the use of agricultural chemicals such as fertilizers, herbicides, and insecticides has become an integral part and will continue in future (Azizullah et al., 2011). Most farmers wrongly believe that applying more fertilizer will lead to higher production but this result comes with contamination of natural resources (Zia et al., 2008). Many fertilizers have very slow degradation rate, complicates the calculation of nutrient availability and may have properties that magnify their

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pollution potential. In addition, some chemical fertilizers contain substances which are toxic to living organisms such as inorganic acids, heavy metals and organic pollutants. Worldwide, there are sufficient evidences of harmful effects of long term application of these chemicals on aquatic and forest ecosystems (Howarth et al., 2002). The benefits of using compost leachate as fertilizer are obvious. Its utilization as fertilizer would lower the consumption of commercial fertilizers for which a huge amount of energy and production cost is required. Use of leachate in agriculture would also reduce the waste treatment plants cost which is needed for the removal of excessive substances such as nitrogen and phosphorous (Romero, 2013). Leachate includes a number of considerable potential pollutants for contaminating the environment such as high and variable concentrations of oxidizable organic substances, inorganic salts, ammonia, metals and toxic compounds (Brown et al., 2013). Leachate can be defined as a potentially polluting liquid that may lead to harmful effects on the public health, surrounding aquatic ecosystems and ground water resources. Solid waste compost leachate is a liquid that percolates through the organic solid waste and brings out a complex variety of dissolved and suspended organic compounds and materials such as heavy metals, fatty acids, humic substances and many hazardous chemicals (Zazouil and Yousefi, 2008). Many parameters effect on leachate e.g. seasonal weather variations, pilling and compacting method used during its production. If compost leachate is not properly managed it can place local and public ecosystems at risk by contaminating the surface and ground water resources (Cumar and Nagaraja, 2011). Since leachate is potentially harmful, leachate management system is required by the composting facility municipalities. A well-managed system is required for the collection, monitoring, control and treatment of leachate before its disposal to the environment (Brown et al., 2013). Municipal Waste Compost Leachate (MWCL) is considered in compost production, as it may be utilized as a source of water and nutrients (Romero et al., 2013). MWCL composition is characterized by several parameters in high concentrations such as boron, carbon, phosphorous, potassium, zinc, calcium, magnesium and nitrogen. These elements are important micro- and macronutrients required by the plant, thus leachate may be useful as fertilizer for the growth of plants (Dimitriou, 2006). Leachate obtained from vermicomposting comprises of larger amounts of plant nutrients so it can be utilized as liquid fertilizer. As leachate usually contains higher amounts of plant nutrients, it should be diluted to prevent plant damage (Gutierrez-Miceli et al., 2008). In addition to these nutrients, leachate also contains sufficient amount of organic matter which helps in the maintenance of soil structure and fertility. Soil organic matter (SOM) is taken as a key indicator for assessing soil quality (Riley et al., 2008).

The potential of using municipal solid waste compost as a fertilizer was evaluated by observing the effect of different concentrations of leachate on the germination and growth rate of wheat.

MATERIALS AND METHODS

This study was designed to check the efficiency of municipal solid waste compost leachate as fertilizer. Lahore Compost Company was selected as the basic site for the collection of municipal solid waste compost leachate. Lahore Compost Company at Mahmood Booti has their composting plant in Pakistan.

Leachate preparation

Samples were collected from the leachate collection tank of Lahore Compost Company during fall season for one week and stored in 1.5 L labelled glass bottles. After collecting 5 samples from different windrows, the samples were transported from composting plant to the laboratory and preserved at 3°C for further analysis. Samples of commercial fertilizer were obtained from a certified shop. They were stored in air tight transparent polyethylene bags and transported to the laboratory for further analysis. Municipal solid waste compost leachate and commercial fertilizer were analysed for chemical parameters. Some important parameters were selected to increase the fertility and quality of leachate. Leachate sample (100-ml) digestion was poured into the beaker and heated using hotplate without boiling it until the sample volume was reduced to 50 mL. 15 mL of concentrated nitric acid and HCl was added and then heated for 30 min. Thereafter solution was filtered to remove any insoluble material. After filtration, the sample was transferred to 200 mL flask and volume was raised up to 150 mL by adding distilled water (Abu Dabees et al., 2013). Compost sample was digested as follows: 10 g compost was dissolved in 20 ml HCl and heated for 30 min. Samples of different percentage of leachate (0.2, 0.5, 1.5, 3.5, 5.5, 10.5, 15.5, and 25.5%) were prepared in duplicates by using 10 g compost per sample for different leachate percentage preparation. The prepared leachate was diluted further to make different percentages (0.2, 0.5, 1.5, 3.5, 5.5, 10.5, 15.5, and 25.5%). Commercial fertilizer was used in its original form as purchased from the company.

Seed collection

The seeds of wheat (*Triticum aestivum L*.) were collected from Seed Corporation in Lahore. Proper morphology of seeds was examined and healthy seeds were screened out for further analysis.

Seed germination experiment

Seed germination experiments were conducted by using 10 cm glass Petri dishes. The seeds of wheat species were surface sterilized with 0.4% mercuric chloride solution for 8 min and washed with distilled water (Zhou et al., 2012). Samples of municipal solid waste compost leachate and commercial fertilizer (Urea) were diluted to concentration series with distilled water. Petri dishes (10 cm diameter) were lined with filter paper which was moistened with 4 mL of sample. In case of control, the seeds were placed on two filter discs moistened with water instead of samples. Throughout the experiment filter papers were moistened whenever needed with the respective solutions. In the experiment, 10 seeds of each species were placed in a Petri dish, using three replicates for each treatment. The Petri dishes were incubated at 26°C in an oven.

Measurement of primary root and shoot length

The seeds with germination effect were examined after 2 days interval in a week. Initial root and shoot length of the germinated seeds were measured after one week. Proper radicle protruded more than 1.92 mm from the seed coat (Zhou et al., 2011). Data was analyzed and interpreted by using SPSS. The descriptive analysis was done to express the percentage of germination rate. Standard error of mean was fined by using SPSS (Nath et al., 2005; Hema and Subramani, 2013).

RESULTS AND DISCUSSION

This study deals with the effect of different leachate and fertilizer concentrations on seeds of wheat crop. The purpose of the study was to check the potential of leachate as a liquid fertilizer. For this purpose, germination rate, primary root and shoot lengths were calculated.

Effect of leachate and fertilizer on seed germination of crops

There are many factors which contribute to the yield of the crop, and one of them is seed germination (Buriro et al., 2011). Seed germination is a sensitive stage of plant's life. It is affected by the variations in internal conditions and environmental parameters. Seed germination and vigorous seedlings are important characteristics for any crop which could provide advantages for its establishment. For the achievement of better growth and high yield of crop, rapid and steady field emergence is necessary. In this study, potential of MSW compost leachate as liquid fertilizer is checked by noticing its effect on wheat seed germination and comparing it with a commercial chemical fertilizer. Wheat seeds were used for bioassay test because they show a more sensitivity to low concentration of phototoxic substances and salts. Salinity is one of the major abiotic stresses. It has many negative effects on morphology, physiology and biochemical activities of crop. High salt concentrations cause slow germination rate. Osmotic pressure causes imbalance in enzyme activities and the results also showed that wheat seeds were more sensitive towards different concentrations of leachate (Hasanuzzaman et al., 2017). By comparing with control results, significant difference between leachate and fertilizer was observed. Maximum seedling length was observed in 0.5% leachate sample and lowest was observed in 25.5% leachate sample.

Effect of leachate on germination rate of crops

Both leachate and fertilizer had affected the growth of wheat. Results indicated that on lower concentrations, leachate promoted the seed germination and seedling growth but on higher concentrations it resulted in reduced seed germination rate and seedling growth. Analysis of data regarding germinated wheat seeds in different leachate concentrations collected is shown in the Tables 1 and 2.

The results from the study provide evidence of micro and macro nutrients present in compost leachate (Quaik et al., 2012; Dimitriou, 2006). This indicated that retarded growth of wheat exist with decreased and increased dilution. Excessive amount of nutrients are present in sample for crop benefits (Savage and Tyrrel, 2005). Dosage of leachate should be balanced according to the need of crop.

Effect of commercial fertilizer on germination rate of crop

In Table 2, low concentration leachate has promoted the growth. This might be due to dilution of leachate which resulted in the dilution of toxic substances too, thus reducing their adverse effects and the negative impacts which were produced due to leachate irrigation.

On higher concentration, leachate caused wilted root to grow due to higher amounts of salt and toxic substances. High levels of leachate concentration were toxic for plant growth because leachate also contains some toxic substances and high amount of heavy metals. Leachate treatment from 0.5 to 5.5% concentration has an impact on seed germination and length when compared with control (Table 1). Maximum increase in concentration of leachate up to 25.5% is responsible for the reduction in the seed germination, root, and shoot length as compared to control. In wheat, 0.2% leachate solution showed average root and shoot lengths were noticed about 6.4 and 5.7 cm which were less compared to control. Maximum root, shoot seedling length 11.8, 6.2 and 16.5 cm were observed in 1.5 and 5.5% leachate concentration and almost these same results were observed in 3.5% leachate concentration (Table 3). Minimum root and shoot length about 0.7 and 0.2 cm were noted in 25.5% leachate solution. The results from these studies revealed that at low concentration of leachate, maximum growth was observed. Dilution of leachate up to maximum value also decreases the amount of toxic substances (Zhou and Wang, 2010; Romero et al., 2013). The results confirmed the hypothesis that due to the presence of essential plant macro and micro nutrients such as nitrogen, carbon, potassium, phosphorous and other trace elements like zinc, calcium, boron, magnesium (Zhou and Wang, 2010), leachate could be used as liquid fertilizer (Miceli et al., 2008; Dimitriou, 2006). Higher concentration of leachate up to 5.5% and above showed minimum growth of the crop and the growth of both primary shoot and root were stopped (Table 3). On higher concentration, leachate caused wilted root to grow due to higher amounts of salt and toxic substances.

Table 1. Weekly analysis of germination rate of wheat seed in leachate.

Leachate (%)	Wheat seed germination						
	Day 1	Day 2	Day 3	Day4	Day 5	Day 6	Day 7
0.2	62±1.2	76±0.5	82±0.5	91±0.5	92±0.5	91±0.5	92±0.5
0.5	31±1.0	58±0.5	83±0.6	90±0.5	90±0.5	90±0.5	90±0.5
1.5	30±1.0	45±0.5	53±0.53	83±0.5	83±0.5	83±0.5	83±0.5
3.5	61±1.2	78±2.5	90±0.5	97±0.7	97±0.7	97±0.5	97±0.5
5.5	21±1.0	56±0.5	69±0.6	80±0.5	80±0.5	80±0.5	81±0.5
10.5	32±1.0	66±0.5	79±0.6	82±0.5	83±0.5	83±0.5	83±0.5
15.5	09±1.1	30±1.5	52±0.5	63±0.5	63±0.5	63±0.5	63±0.5
25.5	00±0.0	20±0.5	37±0.5	40±0.5	40±0.5	40±0.5	40±0.5
Control	**40±0.5**	**63±0.5**	**77±0.5**	**87±0.5**	**87±0.5**	**87±0.5**	**87±0.5**

The standard error of mean value is shown in the above table. Mean difference is significant at the level of ($p \leq 0.05$) by Duncan's new multiple range test.

Table 2. Weekly analysis of germination rate of wheat seed in commercial fertilizer.

Fertilizer (%)	Wheat seed germination						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
0.2	23±1.2	56±0.5	82±0.5	90±0.5	90±0.5	91±0.5	91±0.5
0.5	43±1.0	63±0.5	80±0.6	84±0.5	84±0.5	84±0.5	84±0.5
1.5	03±1.0	10±0.5	13±0.53	13±0.5	13±0.5	13±0.5	13±0.5
3.5	NG	NG	NG	NG	NG	NG	NG
5.5	NG	NG	NG	NG	NG	NG	NG
10.5	NG	NG	NG	NG	NG	NG	NG
15.5	NG	NG	NG	NG	NG	NG	NG
25.5	NG	NG	NG	NG	NG	NG	NG
Control	**40±0.5**	**63±0.5**	**77±0.5**	**87±0.5**	**87±0.5**	**87±0.5**	**87±0.5**

The standard error of mean value is shown in the above table. Mean difference is significant at the level of ($p \leq 0.05$) by Duncan's new multiple range test. *NG = Not germinated.

Table 3. Effect of low and high percentage of leachate on root, shoot and seedling length of wheat on 1st week.

Leachate treatment	Root length (cm)	Shoot length (cm)	Seedling length (cm)
Low % of leachate			
0.2	6.4±1.9	5.7±1.1	12.1±1.2
0.5	11.8±2.8	6.2±1.1	16.0±1.0
High % of leachate			
1.5	9.7±1.1	4.1±0.7	15.2±0.6
3.5	10.2±1.7	6.4±1.1	17.1±1.2
5.5	9.2±1.5	5.1±1.3	14.5±0.9
10.5	4.8±1.3	1.1±1.0	6.0±1.0
15.5	2.4±0.6	0.6±0.7	2.7±0.6
25.5	0.7±0.1	0.2±0.0	0.9±0.0
Control	**8.7±2.4**	**5.9±2.3**	**16.1±2.1**

The standard error of mean value is shown in the above table. Mean difference is significant at the level of ($p \leq 0.05$) by Duncan's new multiple range test.

In leachate concentration of 0.2 and 0.5%, wheat crop germination rate was less than control. Concentrations of leachate up to 1.5 and 3.5% of the germination rate were better. 3.5% leachate treatment showed highest growth. The concentration of 5.5% leachate sample showed germination rate that was approximately equal to the control trial. Larger quantity of leachate 10.5 and15.5% retarded the growth. In this study, highest germination rate was noted in less (3.5%) leachate treatments and lowest was noted in high (25.5%) leachate treatment.

Conclusion

The results from this study revealed that the leachate produced from solid waste processing have fertilizer efficiency. It provides better results at low concentrations when compared to commercial fertilizer. Leachate promoted the growth due to the high basic nutrient content e.g. nitrogen, phosphorous, humid acid and organic matter etc.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Effects of biochar and sewage sludge on spinach (*Spinacia oleracea* L.) yield and soil NO_3^- content in texturally different soils in Glen Valley, Botswana

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The effects of biochar and sewage sludge application on spinach (*Spinacia oleracea* L.) yield and soil NO_3^- content were investigated in typical soils of Botswana (Luvisol, Cambisol) under field conditions. Ten treatments with 3 levels of biochar (0, 2.5, 5 tons ha^{-1}) and sewage sludge (0, 6, 12 ton ha^{-1}) were applied in 2 subsequent seasons. Significant ($p < 0.05$) yield increase on the Luvisol occurred if sewage sludge was added at 12 Mg ha^{-1} with or without biochar. A combination of 6 Mg ha^{-1} sludge and 5 Mg ha^{-1} biochar application resulted in the highest crop yield over 2 seasons. On the Cambisol, only marginal yield increase occurred upon high rates of sole organic amendments and chemical fertilizer, while co-applications decreased yields. Decrease in soil NO_3^- content caused yield declines in the second season, while P uptake increased significantly ($p < 0.05$). Correlations between yields, soil NO_3^- and leaf N contents were insignificant ($p > 0.05$). On the Cambisol, a significant regression model for sludge and soil NO_3^- was determined. Therefore, one – time combined application of 6 Mg ha^{-1} sewage sludge and 5 Mg ha^{-1} on the Luvisol, and 12 Mg ha^{-1} sewage sludge are recommended for spinach production on the Luvisol and Cambisol, respectively. In subsequent seasons, crop productivity could be maintained by application of mineral N in order to mitigate over-application of P.

Key words: Biochar, sewage sludge, soil NO_3^-, luvisol, cambisol.

INTRODUCTION

Soil fertilization with sewage sludge is an effective way to recycle nutrients and combat nutrient deficiency in agricultural systems (Sharma et al., 2017). Spinach is one of the most important vegetable crops in Botswana, but good crop yields are constrained by poor soil fertility, especially N and P deficiency. Many studies have reported high spinach yield response to mineral fertilizers and sewage sludge applications (Ngole, 2010; Biemond

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et al., 1996; Wang and Li, 2004; Lefsrud et al., 2007; Stagnari et al., 2007; Rodríguez-Hidalgo et al., 2010; Türkmen* et al., 2004). These studies showed that adequate N availability from sewage sludge is critical for high quality and yields of spinach. When contents of heavy metals, pathogens, and toxic organic compounds in sludge are within the WHO limits, such as the case for the Glen Valley sludge (Ngole, 2010; Mosekiemang and Dikinya, 2012), application rates of sludge to agricultural soils is based on the crop nitrogen (N) demand (Gilmour and Skinner, 1999; Correa et al., 2006).

On dry basis, sewage sludge contains 2 – 6% total N which is predominantly organic (Rigby et al., 2016) hence the rate of N mineralization influences potential plant-available N. In sludge-amended soils, this plant-available N varies between 20 to 63% of organic N in a crop year under field conditions (Magdoff and Amadon, 1980), depending on factors such as sludge application rates, timing, climate, soil properties and moisture dynamics (Weggler-Beaton et al., 2003). Thus, the application rate of sludge should consider the sum of the inorganic N and mineralised organic N in the soil and the added sludge. However, important soil processes such as microbial-mediated immobilization, leaching, ammonia volatilization and denitrification can decrease the amount of the plant available N pool (Clough et al., 2013).

At modest sewage sludge application rates (c.a. 10 tons ha^{-1}), sludge may not provide adequate N for optimum spinach yields because it has a high N demand. In addition, spinach typically prefers NO_3^- because high concentrations of ammonium (NH_4^+) ions can be toxic and suppress both root development and plant growth (Wang et al., 2009). Thus, the relative concentrations of NO_3^- and NH_4^+ in sewage sludge, and the nitrification rates determine N uptake and productivity of spinach. But, excessive levels of NO_3^- in spinach leaves may be noxious to humans (Citak and Sonmez, 2010), while leaching of NO_3^- into groundwater is linked to methemoglobinemia or “blue-baby” syndrome in infants, cancer and spontaneous abortions (Spalding and Exner, 1993). Nitrogen availability from sewage sludge in Botswana was sparsely explored by Ngole (2010) under controlled conditions but the results were confounded by lack of mineral fertilizer comparisons.

Biochar-induced changes of soil properties such as soil pH, cation exchange capacity (CEC), moisture dynamics, and microbial activity may in turn significantly influence N transformation reactions (Nelson et al., 2011; Clough et al., 2013; Anderson et al., 2011). Besides the potential direct N supply, biochar effects on soil organic matter decomposition rates could either decrease or increase organic N mineralization from organic amendments, retention of NH_4^+ on its surfaces, and therefore, soil NO_3^- and NH_4^+ ratio. Biochar-induced N deficiency due to net N immobilization have been linked to high biochar C/N and increased microbial activities (Deenik et al., 2010). Other studies showed that such microbial N immobilization and partly due to high biochar cation exchange capacity (CEC) improved N fertilizer use efficiency and plant productivity (Chan et al., 2008; Steiner et al., 2007). Nitrification rates in biochar-amended soils can either increase or decrease due to the biochar stimulatory or inhibitory effects, respectively (Nelissen et al., 2012; Clough et al., 2013; Zackrisson et al., 1996), with significant implications for spinach growth and yields as it prefers NO_3^- compared to NH_4^+ (Wang et al., 2009).

A growing number of studies showed significant synergistic effects of biochar and N fertilizers on N use efficiency and crop yields (Chan et al., 2008; Adekiya et al., 2019; Partey et al., 2014). Contrastingly, Lentz and Ippolito (2012) found no synergistic effects of hard-wood biochar and cattle manure on corn silage yields and nutrient concentrations, except for Mn. Others reported biochar-induced N deficiency due to N immobilization or decreased nitrification rates (Zheng et al., 2013; Cayuela et al., 2013).

The contrasting results suggest that the effects of biochar on N availability is heterogeneous, depending on biochar and soil properties, application rates for both biochar and organic N fertilizers and other experimental conditions. However, information regarding the combined effects of biochar and sewage sludge fertilization on N uptake and yields of spinach is scarce. We hypothesized that synergistic effects of biochar and sewage sludge application would significantly increase NO_3^- availability and spinach yields. The objective of this study was to determine the effects of co-application of biochar and sewage sludge on soil NO_3^- content, N uptake and spinach yields in typical soils of Botswana.

MATERIALS AND METHODS

Study sites

The experiments were established at Glen Valley, Botswana. A Calcic Luvisol (henceforth called Luvisol) and Vertic Cambisol (henceforth called Cambisol) were selected for the study. Some properties of the surface soils (0–15 cm) are shown in Table 1. The Luvisol was classified as a sandy loam textural class (sand – 73.3%, clay – 16.4% and silt – 10.3%) with a bulk density of 1.6 g cm^{-3}. The Cambisol had sandy clay texture comprising of the following size fractions; sand – 48.5%, clay – 39% and silt – 12.6%), and 1.4 g cm^{-3} bulk density.

Experimental design

The experiment comprised 10 different treatments with 3 replicates and so each site comprised 30 plots (1.8 m x 1.5 m) arranged in a randomized complete block design (RCBD). Table 2 shows the treatments structure and application rates in each season. The spinach (variety; *Fordhook Giant*) crop in the first season was planted in March and harvested in April, 2018. The second harvest was done in June, 2018. Similar amendments were applied before the plots were replanted in the second season with spinach seedlings in July, 2018. Harvests 1 and 2 were done in September and October, 2018, respectively.

Table 1. Pre-crop planting soil properties and basic characteristics of sludge and biochar used in the study (n=3).

Properties	Luvisol	Cambisol	Biochar	Sewage sludge
pH ($CaCl_2$)	7.5 ± 1.5	6.8 ± 1.3	7.7 ± 1.1	6.3 ± 0.3
EC (µS/cm)	54 ± 6	80 ± 11	1124 ± 204	2270 ± 318
CEC (cmolc kg^{-1})	8.4 ± 1.1	26.2 ± 3.2	12± 2.5	38 ± 6
Organic matter (%)	1.83 ± 0.37	2.30 ± 0.55	nd	24.6 ± 2.8
Total carbon (%)	nd*	nd	65.4 ± 5.1	80.1 ± 7.9
Organic Carbon (%)	1.0±0.2	1.8±0.3	nd	nd
Ash content (%)	nd	nd	34.7 ± 3.3	19.9 ± 2.2
Total P (ppm)	103 ± 27	91.3 ± 14.3	824 ± 123	5753 ± 525
Available P (ppm)	42.3 ± 5.1	24.0 ± 4.1	51.3 ± 8.1	272 ± 38
Total N (%)	0.08 ± 0.01	0.04 ± 0.01	1.1 ± 0.6	4.5 ± 1.2
C/N ratio	12.5	45	59	17.8
Exchangeable bases (cmolc kg^{-1})				
Ca	6 ± 2.3	17.4 ± 5.6	128 ± 22	159 ± 47
Mg	2.3 ± 1	8.5 ± 3	34 ± 8	66 ± 13
Na	0.06 ± 0.01	0.17 ± 0.02	3 ± 1	13 ± 4
K	0.06 ± 0.01	0.18 ± 0.07	153 ± 13	51 ± 7
Sand (%)	73.3 ± 8	48.5 ± 5	nd	nd
Clay (%)	16.4 ± 2.9	39 ± 3	nd	nd
Silt (%)	10.3 ± 1	12.6 ± 1.4	nd	nd
Bulk density (g/cm3)	1.60 ± 0.29	1.42 ± 0.66	nd	nd

*nd – Not determined.

Both sites were disc ploughed to about 30 cm depth before the study. Planting rows were constructed using hand-hoes before organic amendments were incorporated and mixed into soil (15 cm). Transplanting was done one day after irrigation. Mineral fertilizer (2:3:2, 22%) was applied by banding during transplanting at 300 kg ha^{-1} (Bok et al., 2006). Urea ammonium sulphate (46% N) was top-dressed on CHEM plots at 200 kg ha^{-1} after 2 weeks of transplanting and after each harvest. The trials were drip-irrigated based on soil moisture conditions for about 2 h per irrigation. In the second season, the plots were cleared of crop residues, but the soil was not ploughed. The planting lines and treatment plots were maintained as during the first season.

Air-dried sewage sludge was collected from the stock piles at the Glen Valley Waste Water Treatment Plant. The sewage sludge was crushed and sieved (2 mm) before analysis and soil application. Biochar was produced from mixed-wood chips via a home-made slow pyrolysis unit (535ºC, 6 h). After cooling, the biochar was air-dried, then mixed thoroughly before crushing and sieving (2 mm). The properties of the biochar (BC) and sewage sludge (SS) used for this study are presented in Table 1. The sludge was enriched in N, P, Ca, Mg and Na relative to the biochar, while the K concentration of biochar was over 3–fold that of sludge (Table 2). The biochar C/N ratio was 64:1, over 3-fold that of the sewage sludge, which indicates a potential for N immobilization during labile biochar C degradation by soil microorganisms. The biochar was characterised by slightly alkaline pH (7.7), high EC (1124 µS/cm), and high contents of available Ca (128 cmol kg^{-1}) and K (153 cmol kg^{-1}), medium concentrations of Mg (34 cmol kg^{-1}) and low contents of Na (3 cmol kg^{-1}) (Table 1).

Soil sampling and analysis

Soil samples (0 – 15 cm) from each plot were collected using the composite sampling procedure at each harvesting stage. Air-dried samples were sieved < 2 mm and analysed in triplicate. Total carbon (TC) of the biochar and sewage sludge was characterized by ashing in muffle furnace at 500°C for 48 h. Exchangeable cations and soil CEC were determined using the ammonium acetate method at pH 7, using a mechanical extractor on a 2.5 g sample (van Reeuwijk, 1993). Exchangeable cations were quantified using a 4210 MP-AES (Agilent Technologies).

The pH of soil and sludge samples was potentiometrically determined in a 1:5 distilled water and 0.01 M $CaCl_2$ solution. Biochar pH was measured on 1 g of sample as described by Wang et al. (2015). The pH values were determined in biochar-to-water ratio of 1:20 (w/v) via an Orion pH meter installed with a glass electrode. Total nitrogen (TN) of soil, sewage sludge, plant and biochar samples was analysed according to the micro-Kjeldahl procedure (van Reeuwijk, 1993). Plant-available phosphorus was determined as described by Ziadi and Tran (2008). Soil bulk density (BD) was determined using 100 cm^3 soil core samplers. Soil particle-size distribution of air-dried samples was measured according to the hydrometer method (van Reeuwijk, 2002). Soil pH and EC were determined at the end of each season while soil bulk density (BD) was measured in the second season only. Soil NO_3^- was quantified according to the Cadmium reduction procedure. Briefly, 3 g thawed soil samples were extracted with 2 M KCl at the soil-to-solution ratio of 1:10 (w/v), while simultaneously determining the moisture factors. The extracts were frozen until they were required for NO_3^- analysis using a Technicon Autoanalyzer II (Technicon Cooperation).

Plant sampling and analysis

The spinach plants were grown for about 60 days from the date of

Table 2. Treatments, their application amounts per season and abbreviations.

Treatment	Sewage sludge (Mg ha^{-1})	Biochar (Mg ha^{-1})	Chemical fertilizer (kg ha^{-1})
CT (Control)*	0	0	0
2.5BC	0	2.5	0
5BC	0	5	0
CHEM (NPK)	0	0	300
6SS	6	0	0
6SS+2.5BC	6	2.5	0
6SS+5BC	6	5	0
12SS	12	0	0
12SS+2.5BC	12	2.5	0
12SS+5BC	12	5	0

*Symbols represent additions of; CT – no amendment (control); 2.5BC – 2.5 ton ha^{-1} biochar; 5BC – 5 ton/ha biochar; CHEM – NPK mineral fertilizer at 300 kg ha^{-1}; 6SS – 6 ton ha^{-1} sewage sludge; 6SS+2.5BC – 6 ton/ha sewage sludge and 2.5 ton ha^{-1} biochar; 6SS+5BC – 6 ton ha^{-1} sewage sludge and 5 ton ha^{-1} biochar; 12SS – 12 ton ha^{-1} sewage sludge; 12SS+2.5BC – 12 ton ha^{-1} sewage sludge and 2.5 ton ha^{-1} biochar; 12SS+5BC – 12 ton ha^{-1} sewage sludge and 5 ton ha^{-1} biochar.

transplanting. Harvesting was done on plot basis. Randomly selected plants were cut at about 5 cm above the soil surface on each plot. Fresh weights of leaves from each plot were recorded at each harvest stage, before oven drying and sieving (2 mm). For total content of P and bases in sludge and plant, 1.25 g of sample was wet digested in 2.5 ml of sulphuric acid-selenium mixture according to van Reeuwijk (2002). Basic cations were determined in the diluted digests via 4210 MP-AES (Agilent Technologies). In the diluted digests, P was measured spectrophotometrically by the indophenol-blue method (van Reeuwijk, 2002). Total P was measured by the method of Murphy and Riley (1962). Determination of the total content of P, K, S, Mg and Ca in biochar was done according to the modified dry-ashing method (Enders and Lehmann, 2012).

Statistical analysis

A 2-way ANOVA was conducted using SAS version 9.4 (SAS Institute Inc, Cary, NC) with significant differences identified at 5%, unless specified otherwise. Mean differences due to treatments were evaluated using least significant difference (LSD) and were ranked according to Duncan's multiple range tests. The Pearson's correlation procedure was used to analyse relationships between variables at 5% level of significance. The values given at each entry for all the parameters, except soil pH, electrical conductivity and bulk density are the average analyses for each of the two separate harvests in each season.

RESULTS

Effects of amendments on soil NO_3^- content

The ANOVA showed that treatments and the interactions between soil type and treatments significantly ($p = 0.0001$) affected soil NO_3^- content. The unamended control on the Luvisol had significantly ($p < 0.05$) lower NO_3^- concentrations compared to the Cambisol in both seasons (Figure 1a and b).

Season 1

During the first season, the control on the Cambisol had higher NO_3^- content (13.1 mg kg^{-1}) compared to the same treatment on the Luvisol (9.2 mg kg^{-1}). These levels are consistent with commonly reported NO_3^- values (10 – 25 mg kg^{-1}) in agricultural soils (Tisdale et al., 1993). Except for application of low rate of sole sewage sludge (6SS), organic amendments significantly ($p < 0.05$) increased NO_3^- on the Luvisol while the effects were insignificant on the Cambisol during the first season. The highest NO_3^- levels during this season were caused by co-application of 6 Mg ha^{-1} sewage sludge and 5 Mg ha^{-1} biochar on the Luvisol (19.6 mg kg^{-1}) while on the Cambisol, sole sewage sludge application at 12 Mg ha^{-1} gave the highest NO_3^- level (15.1 mg kg^{-1}), which also coincided with the highest spinach yields for the respective soils.

Soil NO_3^- content insignificantly ($p > 0.05$) increased with the amount of applied soil amendments on the Luvisol during the first season (Figure 1a). The same trend was observed for sole sewage sludge on the Cambisol, while increasing biochar amount marginally decreased NO_3^- content from 14.1 to 13.2 mg kg^{-1} over the same period. On the Luvisol, sole sewage sludge application significantly increased NO_3^- content relative to the control only when applied at 12 Mg ha^{-1}, but when combined with both rates of biochar, the lower rate of sewage sludge (6 Mg ha^{-1}) resulted in a significant increase in soil NO_3^- (Figure 1a).

Co-application of amendments marginally increased soil NO_3^- compared to both rates of sole amendments on the Luvisol. With regard to the Cambisol, there were no significant ($p > 0.05$) treatment effects on soil NO_3^- content during the first season, but co-applications decreased soil NO_3^- content in comparison to the sole amendments and mineral fertilizer (Figure 1a). Mineral fertilizer (CHEM) did not significantly increase NO_3^- above

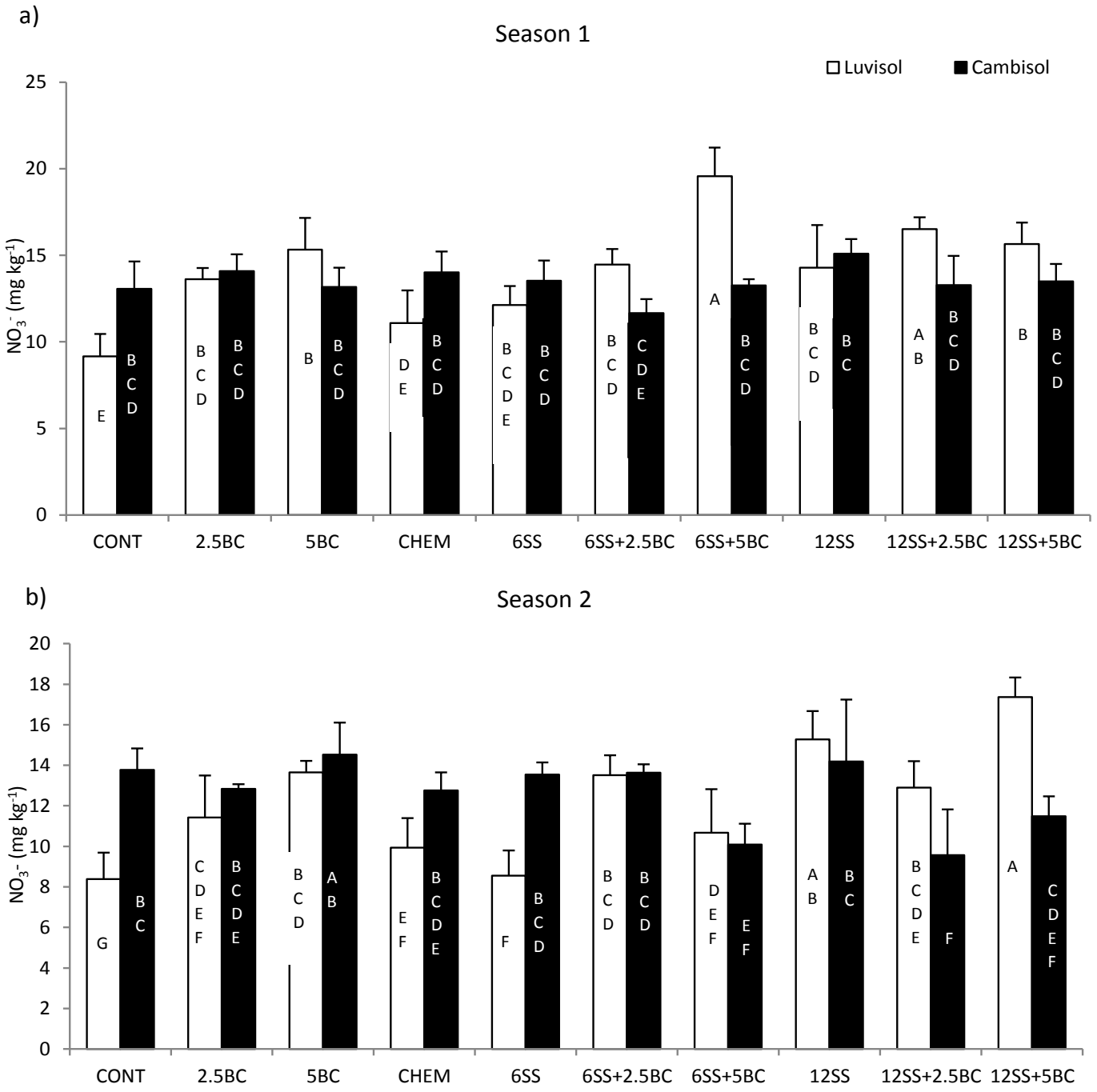

Figure 1. Soil NO_3^- content during two seasons; (a) Season 1 (March – June) and (b) Season 2 (July – Oct) 2018. Error bars denote standard error of the mean (SEM). Columns with different letters are significantly different ($p<0.05$).

the control on both soil types. Interestingly, all combinations of sewage sludge and biochar on the Luvisol significantly increased NO_3^- content relative to mineral fertilizer (Figure 1a), indicating a great potential of these organic amendments to substitute mineral fertilizers (Glaser et al., 2015; Dikinya and Mufwanzala, 2010).

Season 2

During the second season, all the treatments significantly ($p < 0.05$) increased NO_3^- content relative to the control on the Luvisol (Figure 1b). Soil NO_3^- content increased with increasing amount of each organic amendment on both soils, but the differences were only significant

Table 3. Regression functions for relationships between treatments and agronomic parameters.

Variable	Luvisol		Cambisol	
	Sewage sludge	***Biochar***	***Sewage sludge***	***Biochar***
Yield	$y = 1.9x + 17.2$	$y = 3.9x + 15.2*$	$y = 0.9x + 18.2$	$y = 0.58x + 18$
Soil NO_3^-	$y = 1.9x + 10*$	$y = 2x + 9.9$	$y = 0.9x + 13.4*$	$y = -0.8x + 13.4$
Leaf N	$y = 0.06x + 3.9$	$y = 0.08x + 3.8$	$y = 0.03x + 3.5$	$y = -0.03x + 3.5$

*Significant at p = 0.05 level.

($p < 0.05$) for sole sewage sludge on the Luvisol. As can be seen in Table 3, the regressions between NO_3^- content and sewage sludge amount on both soil types are significant ($p < 0.05$), and the regression coefficient is higher on the Luvisol. Conversely, biochar had an insignificant, but positive influence on NO_3^- content on the Luvisol, while on the Cambisol, increasing biochar amount resulted in a decrease for NO_3^-.

Soil NO_3^- content generally decreased during the second season for most of the treatments, on both soil types. Notably, substantial decrease was observed for 6 Mg ha^{-1} sewage sludge plus 5 Mg ha^{-1} biochar (19.6 to 10.7 mg kg^{-1}) on the Luvisol. With the few exceptions where NO_3^- content increased (e.g. 12SS, 12SS+5BC on the Luvisol, and CONT, 5BC, 6SS+5BC on the Cambisol; Figure 1a and b), the differences between seasons were marginal.

Effects of amendments on leaf N content

ANOVA indicated that soil type had significant ($p = 0.0004$) effects on spinach leaf N content, while the effects of treatments, and soil by treatment interactions were insignificant ($p > 0.05$). Generally, the spinach leaf N content in this study is similar to other studies (2 – 5%; Tisdale et al., 1993). In both seasons, treatments maintained statistically similar ($p > 0.05$) leaf N content compared to the control for both soil types (Table 4).

Increasing the amount of sewage sludge on the Luvisol marginally increased leaf N content during the first season, but the same trend did not occur on the Cambisol. Leaf N content generally increased in the second season for the corresponding treatments on both soils, except for co-application of 6 Mg ha^{-1} sewage sludge and 5 Mg ha^{-1} biochar on the Luvisol. Sole biochar at either rate also had no significant effects on leaf N on both soils (Table 4). In general, the effects of factors on leaf N content were marginal as shown by the small regression coefficients for both soil types (Table 3). The effects of biochar were positively related to both NO_3^- and plant leaf N contents on the Luvisol, while on the Cambisol, both parameters decreased with increasing biochar rates, as indicated by negative coefficients of the regression equations.

Effects of amendments on spinach yields

The spinach yield data for the 2 cropping seasons are presented in Figure 2. Treatment effects were significant ($p < 0.0001$; CV = 33.5%) on yield as indicated by the general ANOVA model. Spinach yields in the control plots were similar between seasons. In the first season, all treatments improved yields relative to CONT on the Luvisol, with the greatest yields resulting from 6 Mg ha^{-1} sewage sludge plus 5 Mg ha^{-1} biochar. During the second season, all organic amendments maintained higher yields than control on the Luvisol while CHEM resulted in slightly lower yields relative to the control. On the Cambisol, yields were statistically independent of treatments ($p > 0.05$).

The changes in crop yield and soil NO_3^- between seasons followed contrasting trends on the different soil types. An insignificantly negative correlation ($p = 0.43$: $r^2 = -0.22$) between yield and NO_3^- was determined on the Luvisol, while the correlations on the Cambisol was positive and significant ($p < 0.05$: $r^2 = 0.57$). Decreasing yield during the second season on the Luvisol coincided with decreasing soil NO_3^- content (except 12SS, 12SS+5BC), but marginal yields increases on the Cambisol followed an increasing trend of soil NO_3^- content.

Considering other plant nutrients, the decline in crop yield in the second season as already highlighted above corresponded with increasing content of both leaf P (Table 4) and available P (Table 5). Other studies (Bhattacharjee et al., 1998; Tisdale et al., 1993; Türkmen* et al., 2004) have reported higher spinach leaf mineral composition than observed in our study; hence, this could be a contributing factor to the decreased yields in the second season.

DISCUSSION

Effects of amendments on NO_3^- availability and leaf N content

Soil NO_3^- content in the control was significantly lower than the Luvisol compared to the Cambisol (Figure 1a and b), which confirms the lower N availability in this soil,

Table 4. Effects of amendments on leaf nutrient contents [mg kg^{-1}] of spinach in (A) season 1 and (B) season 2.

Treatment	N		P		Ca		K		Mg	
	A	B	A	B	A	B	A	B	A	B
Luvisol										
CONT	3.07±0.46A	4.99±0.31A	2068±392ABCD	2154±399IJ	55±9AB	94±8B	534±111EF	437±56C	80±6BC	95±8BC
2.5BC	2.78±0.00A	5.23±0.40A	2275±402AB	3297±393DEFG	75±10AB	100±9B	677±127BCDEF	532±73ABC	85±8ABC	96±8BC
5BC	3.12±0.34A	5.44±0.10A	1780±403ABCD	3525±394CDEF	57±8AB	110±10AB	532±54F	559±90ABC	71±9BC	105±89ABC
CHEM	2.78±0.10A	6.21±0.73A	1823±399ABCD	2514±401HIJ	68±7AB	124±13AB	672±72BCDEF	538±110ABC	83±7ABC	131±12AB
6SS	2.91±0.34A	6.28±0.67A	1900±393ABCD	3128±399EFGH	66±8AB	113±13AB	687±98BCDEF	591±59ABC	77±6BC	107±10ABC
6SS+2.5BC	2.63±0.12A	5.05±0.39A	1727±394ABCD	3751±397CDE	72±9AB	111±10AB	693±83BCD	560±92ABC	102±10AB	105±9ABC
6SS+5BC	3.98±0.39A	6.01±0.64A	2002±401ABCD	5136±389A	74±8AB	141±14A	663±129CDEF	652±110AB	91±8ABC	139±13A
12SS	3.32±0.08A	5.47±0.33A	1779±392ABCD	2818±395FGHI	60±7AB	116±14AB	546±96DEF	556±89ABC	82±7ABC	103±13ABC
12SS+2.5BC	2.75±0.00A	5.54±0.07A	1762±399ABCD	2657±392GHI	64±9AB	118±10AB	727±145BC	587±85ABC	97±9ABC	110±9ABC
12SS+5BC	2.84±0.14A	5.74±0.21A	2300±395A	1772±390J	67±8AB	110±10AB	693±123BCD	554±106ABC	89±9ABC	109±9ABC
Cambisol										
CONT	3.70±0.34A	3.84±0.31A	1423±407CD	2747±405GHI	69±8AB	100±9B	826±153AB	605±124AB	86±6ABC	93±9C
2.5BC	3.81±0.44A	3.34±0.25A	1721±386ABCD	3897±402BCD	76±9AB	96±12B	758±120BC	608±119AB	96±9ABC	102±10ABC
5BC	4.12±0.32A	3.61±0.59A	1858±368ABCD	4653±402AB	68±8AB	96±9B	786±97ABC	566±104ABC	80±8ABC	96±10BC
CHEM	3.99±0.00A	3.17±0.40A	2285±397AB	3585±398CDE	61±8AB	109±9AB	737±88BC	675±98A	64±9C	110±10.6ABC
6SS	3.76±0.10A	3.30±0.40A	1299±375D	3575±374CDEF	62±6AB	95±8B	829±134AB	592±104ABC	83±5ABC	96±10BC
6SS+2.5BC	4.04±0.00A	3.54±0.43A	1660±367ABCD	4028±409BCD	71±8AB	112±12AB	717±119BC	633±110AB	83±9ABC	101±8ABC
6SS+5BC	3.90±0.15A	3.54±0.07A	1608±399ABCD	4008±402BCD	65±7AB	102±9B	745±99BC	506±94BC	77±8BC	99±9.7BC
12SS	3.92±0.10A	3.78±0.13A	1883±405ABCD	4210±408BC	54±6B	110±9AB	691±68BCDE	644±98AB	86±7ABC	94±7.9BC
12SS+2.5BC	3.59±0.23A	3.50±0.33A	1488±407BCD	3333±376DEFG	92±6A	121±11AB	924±113A	643±116AB	118±10A	117±12.5ABC
12SS+5BC	3.87±0.10A	3.35±0.53A	2194±386ABC	5200±409A	71±8AB	100±9B	754.6±104BC	612±145AB	62±6C	92±8.9C

*Values followed by different letters in the same column for each season are significantly different ($p<0.05$), given error is standard error ($n=3$; $p<0.05$).

and is possibly due to the effects of past management practices and variability in the soil textural properties (Table 1). The Luvisol site was continuously cropped for the previous five years before the inception of the experiments, while the Cambisol was fallow during that time. Thus, exhaustion of mineral N by the crops preceding the trial on the Luvisol may have accounted for the comparatively lower NO_3^- content. Comparatively high soil NO_3^- content in the control of the Cambisol could also be explained by greater mineralization of N from soil organic matter, which was higher on this soil type (Table 4).

In addition, the Luvisol and Cambisol had sandy loam and sandy clay textures, respectively. Therefore, the potential movement of soil NO_3^- that is mineralized from native organic N or contained in the irrigation water down the profile (below the root zone) is higher on the Luvisol. As a result of these differences in NO_3^- content in the control plots, the increase in soil NO_3^- due to application of amendments was greater on the Luvisol.

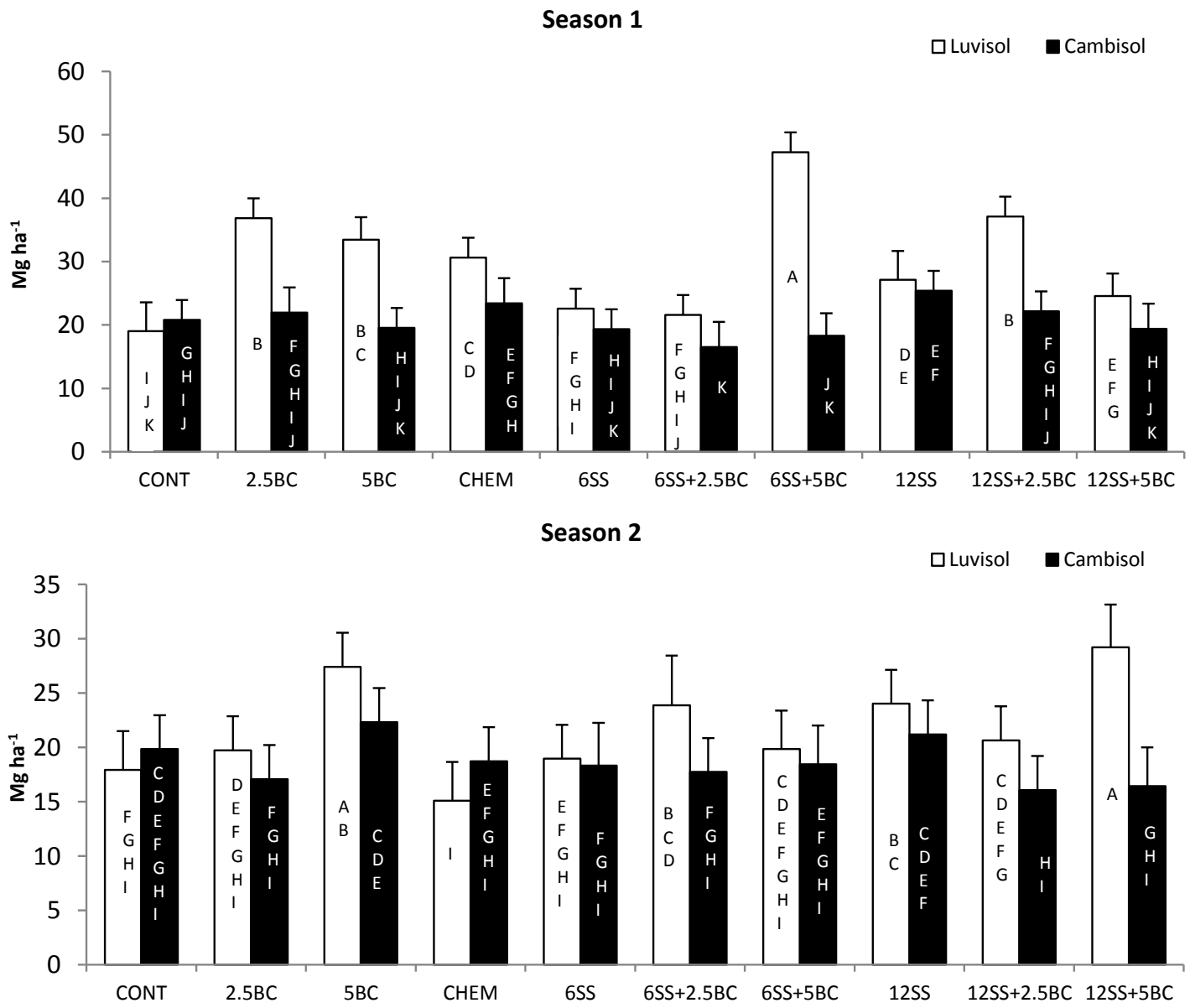

Figure 2. Spinach yields during two seasons; Season 1 (March – June) and Season 2 (July – Oct) 2018. Error bars denote standard error. Columns with different letters are significantly different ($p<0.05$).

Furthermore, organic amendments were more effective in increasing soil NO_3^- relative to application of mineral fertilizer on the Luvisol. In particular, significant treatment effects were detected when biochar was co-amended with sewage sludge compared to sole amendments on the Luvisol, whereas the opposite effects were determined on the Cambisol. These findings can be attributed to a number of reasons. Soil-specific effects of biochar on organic matter decomposition (priming), with potentially greater organic N mineralization on the sandy loam textured Luvisol is expected to play a significant role. The higher clay (39%) and native organic matter (2.3%) contents in the Cambisol could result in occlusion of biochar particles (Zackrisson et al., 1996; Wardle et al., 2008; Brodowski et al., 2006), thus restricting the interactions between biochar and sewage sludge particles. The complexation of humus by clay fraction has been linked to reduced soil C and N mineralization (Amlinger et al., 2003). Therefore, greater biochar and sewage sludge interactions on the Luvisol presumably enhanced mineralization of sludge-borne organic N and consequently higher nitrification of NH_4^+ into NO_3^-. This effect is well known and can be explained by the supply of nutrients, reduced soil bulk density and improved aeration, optimum soil pH and potential biochar sorption of nitrification inhibitory compounds such as terpenes

Table 5. Physicochemical properties of the sites after season 1 (A) and season 2 (B).

Treatment	pH ($CaCl_2$)		Available P (mg kg^{-1})		Organic C (%)		CEC (cmolc kg^{-1})	
	A	B	A	B	A	B	A	B
Luvisol								
CONT	7.7± 1.1A	7.5±2.3AB	28.6±3.3HIJ	53.6±7.1IJ	1.0±0.1DE	0.4±0.0G	8.7±3.1D	8.5±1.6E
2.5BC	7.5±2.3BCD	7.4±1.7ABC	64±8.1CDEF	106±9.8E	1.3±0.3ABCDE	1.0±0.2DEFG	7±2.7D	11±2.1DE
5BC	7.3±1.4D	7.1±0.9EF	87±7.3AB	150±12.6AB	1.0±0.1CDE	2.0±0.6BCD	9±3.1D	15±1.7D
CHEM	7.6±2.1ABC	7.6±1.1A	45± 4.5FGH	81±10.2FG	1.2±0.2ABCDE	0.4±0.0G	8±1.9D	8.5±1.1E
6SS	7.7±1.8A	7.5±1.0AB	33±3.2GHIJ	55±6.5HIJ	1.1±0.1CDE	0.5±0.0FG	7.9±2.3D	7.5±2.1E
6SS+2.5BC	7.3±1.5D	7.3±1.6CDE	81.7±5.4ABCD	133±12.6BCD	1.1±0.1BCDE	1.1±0.1DEFG	10±1.8D	11.5±3.6DE
6SS+5BC	7.4±1.2CD	7.3±2.0BCD	92±6.6A	128.9±13CD	1.5±0.2ABCDE	1.5±0.2CDEFG	8.6±1.4D	10±2.2DE
12SS	7.6±2.3AB	7.5±2.6AB	29.5±5.1HIJ	70±8.9GHI	0.9±0.1E	0.8±0.1EFG	9.2±2.9D	10.5±2.6DE
12SS+2.5BC	7.3±1.5D	7.4±1.9BCD	85±7.9ABC	132±11.2JBCD	1.2±0.4ABCDE	1.2±0.3DEFG	8±2.1D	11±3.4DE
12SS+5BC	7.4±1.8D	7.2±2.2DE	63±5.8DEF	177±22.4A	1.3±0.2ABCDE	2.5±0.5BC	8.4±1.7D	12.7±2.7DE
Cambisol								
CONT	6.7±1.6HI	6.9±1.6GH	15±1.3J	26.5±3.2K	1.7±0.4ABCDE	1.6±0.6BCDEF	26±4.9BC	27.5±4.6ABC
2.5BC	6.9±1.1FGH	6.8±2.1H	20.6±5.2IJ	58±6.6HIJ	1.9±0.3ABCDE	2.6±0.8B	30.6±3.2AB	30±3.5AB
5BC	6.5±1.8I	6.6±1.1I	53±7.9EFG	121.9±19.3DE	2±0.2ABCD	2.6±0.7B	26.5±2.9ABC	29±6.3ABC
CHEM	6.9±2.5FG	6.9±1.0GH	18±4.2IJ	58.6±5.5HIJ	1.8±0.3ABCD	2±0.4BCD	31.8±2.6A	31.8±6.5A
6SS	6.9±1.9FGH	6.9±1.7GH	19±5.4IJ	44.9±3.9JK	2.2±0.5AB	2.1±0.8BCD	31±4.1AB	31±3.9AB
6SS+2.5BC	6.5±0.8I	7±2.1FGH	38.8±6.6GHI	93.5±7.7F	2.1±0.4ABC	2.4±0.4BC	27.9±5.2ABC	32±2.7A
6SS+5BC	6.8±1.3GH	6.8±1.8HI	52.8±8.1EFG	130.7±10.6BCD	2.1±0.5ABC	2.1±0.6BCD	29.7±4.9AB	31±6.2AB
12SS	7±1.8E	7±1.4FG	19.7±3.2IJ	46.8±4.7JK	1.9±0.3ABCDE	1.8±0.5BCDE	24±3.3C	23.6±4.2C
12SS+2.5	7±1.1EF	6.9±1.3GH	37±4.8GHI	75.9±7.2FGH	1.9±0.4ABCDE	1.7±0.4BCDE	27.7±2.8ABC	26±4.4ABC
12SS+5BC	7±1.7EF	6.9±1.6GH	66±8.6BCDE	145.7±10.6BC	2.3±0.6A	3.7±1.1A	29.8±3.3AB	31±2.9AB

	Exchangeable cations (cmolc kg^{-1})							
	Ca		Mg		Na		K	
	A	B	A	B	A	B	A	B
Luvisol								
CONT	106±24C	117±14CD	40±5.5FGH	33.9±2.9G	1.0±0.1CDE	1.4±0.4CDE	1.0±0.0A	1.0±0.1AB
2.5BC	119±28ABC	124±12CD	50.5±6.7DEFG	35.9±4.3G	1.0±0.0CDE	1.4±0.1CDE	1.1±0.0A	1.1±0.1AB
5BC	130±23ABC	120±17CD	55±8.3DEF	33.9±2.7G	1.1±0.0BCDE	0.9±0.3E	1.1±0.2A	0.8±0.0B
CHEM	99±17C	108±10D	38.7±4.4GH	31.6±5.6G	1.0±0.0CDE	1.25±0.1DE	1.0±0.1A	1.0±0.2AB
6SS	102.5±24C	115±16CD	38±5.6H	31.8±3.9G	1.0±0.1CDE	0.9±0.1E	1.0±0.0A	0.9±0.1AB
6SS+2.5BC	104.9±16C	127±11CD	39±6.7GH	42±4.1G	0.9±0.1DE	1.3±0.2DE	1.0±0.0A	1.0±0.1AB
6SS+5BC	119±19.4ABC	121±20CD	48.7±8.2EFGH	34±5.2G	0.9±0.0DE	1.1±0.1DE	1.0±0.3A	1.0±0.0AB

Table 5. contd.

12SS	122±17ABC	132±11CD	52±5.9DEF	35.9±3.9G	1.0±0.0CDE	1.5±0.1BCDE	1.2±0.2A	1.1±0.1AB
12SS+2.5BC	109±19BC	125±10CD	44.5±5.1FGH	32.9±5.7G	1.3±0.2ABCDE	1.2±0.3DE	1.2±0.2A	0.9±0.1AB
12SS+5BC	116±18ABC	156±15BCD	48±6.3EFGH	41±6.2G	0.8±0.1E	1.7±0.1ABCDE	1.1±0.1A	1.1±0.2AB
Cambisol								
CONT	127±22ABC	153±24BCD	62±6.9CD	76±7.4CDEF	1.3±0.2ABCDE	1.8±0.2ABCD	1.2±0.2A	1.4±0.3AB
2.5BC	144±1.9ABC	190±29AB	72±5.4BC	92.5±8.3AB	1.7±0.0ABCD	1.9±0.1ABCD	1.2±0.1A	1.6±0.1AB
5BC	118±17ABC	162±32ABC	56.9±6.6DE	72.7±6.5EF	1.3±0.1ABCDE	1.7±0.1ABCDE	1.1±0.1A	1.4±0.1AB
CHEM	166±21A	200±18AB	85.7±7.8A	95±8.3A	2±0.2A	2.2±0.3ABC	1.4±0.1A	1.7±0.4A
6SS	158±21AB	188±20AB	80.9±10.2AB	85±7.9ABCD	1.8±0.1ABC	1.9±0.2ABCD	1.5±0.2A	1.7±0.3A
6SS+2.5BC	165±17AB	189±31AB	81.7±8.7AB	82±10.6CDE	1.9±0.2AB	2.2±0.2ABC	1.3±0.1A	1.5±0.1AB
6SS+5BC	150±23ABC	202±35AB	74±8.3ABC	92±7.8AB	1.7±0.1ABC	2.2±0.1AB	1.2±0.0A	1.5±0.2AB
12SS	114±19ABC	162±22ABC	55.5±6.9DEF	68±6.9F	1.1±0.0BCDE	1.7±0.3ABCDE	1.3±0.2A	1.5±0.3AB
12SS+2.5	134±18ABC	167±22ABC	62±9.3CD	74.8±5.2DEF	1.2±0.2ABCDE	1.7±0.2ABCDE	1.2±0.2A	1.4±0.4AB
12SS+5BC	151±20ABC	213±24A	71±8.4BC	88±10.1ABC	1.7±0.1ABC	2.3±0.4A	1.3±0.1A	1.7±0.5A

*Values followed by different letters in the same column for each season are significantly different ($p<0.05$), given error is standard error ($n=3$; $p<0.05$).

(Zackrisson et al., 1996).

Furthermore, biochar has greater effects on improvement of water retention on sandy soils than clay soils (Biederman and Harpole, 2013). This effect can result in higher responses in microbial decomposition and mineralization of sludge N on the Luvisol. The expected improvement in water retention could reduce leaching losses of soil NO_3^-, and this effect is likely to be more pronounced on the Luvisol because of its coarse texture, hence co-application of amendments had more NO_3^- content compared to sole sewage sludge treatments (Figure 1a and b).

Soil NO_3^- content generally decreased for most treatments in the second season. Significant ($p < 0.05$) differences were determined for application of sole sewage sludge at 6 Mg ha^{-1} on the Luvisol, or in combination with 5 Mg ha^{-1} on both soils. During the first season, both the highest leaf N (Table 5) and yields (Figure 2a) on the Luvisol were determined for combination of 6 Mg ha^{-1} sewage sludge plus 5 Mg ha^{-1} biochar. Thus, the significant decrease in NO_3^- content in the second season for 6SS+5BC could be a direct result of greater plant N assimilation which accounted for high crop yields during the first season. On the Cambisol, the decrease in soil NO_3^- content was also statistically significant for co-application of 2.5 Mg ha^{-1} biochar and 12 Mg ha^{-1} sludge (Figure 1b).

Such decreasing trends in NO_3^- content could be attributed to several factors including the variability between agro-climatic conditions between seasons (Figure 3a and b) as shown by the statistically significant ($p < 0.05$) seasonal effects from the ANOVA. Total rainfall in the first and second seasons as determined from a nearby weather station at Sebele was 160 and 24 mm, respectively. The mean minimum and maximum temperature was 16.2 and 31.3ºC, respectively during the first month (March) after application of organic amendments. On the other hand, these attributes were 4.5 and 21.6ºC in the second season.

Several studies have been conducted which indicate that under warm moist conditions, sewage sludge organic N mineralization is more than in low temperatures (Sierra et al., 2001; Magdoff and Amadon, 1980; Barbarika et al., 1985). N mineralization rates in the first month after sewage sludge application is critical as it precedes the period of high spinach N demand and is usually supplied via top dressing with mineral N fertilizers. Thus, the warmer and humid climatic conditions in the first season potentially contributed to the higher N mineralization of sludge N resulting in high crop yields than in the following season. Furthermore, these results indicate that a significant proportion of the sewage

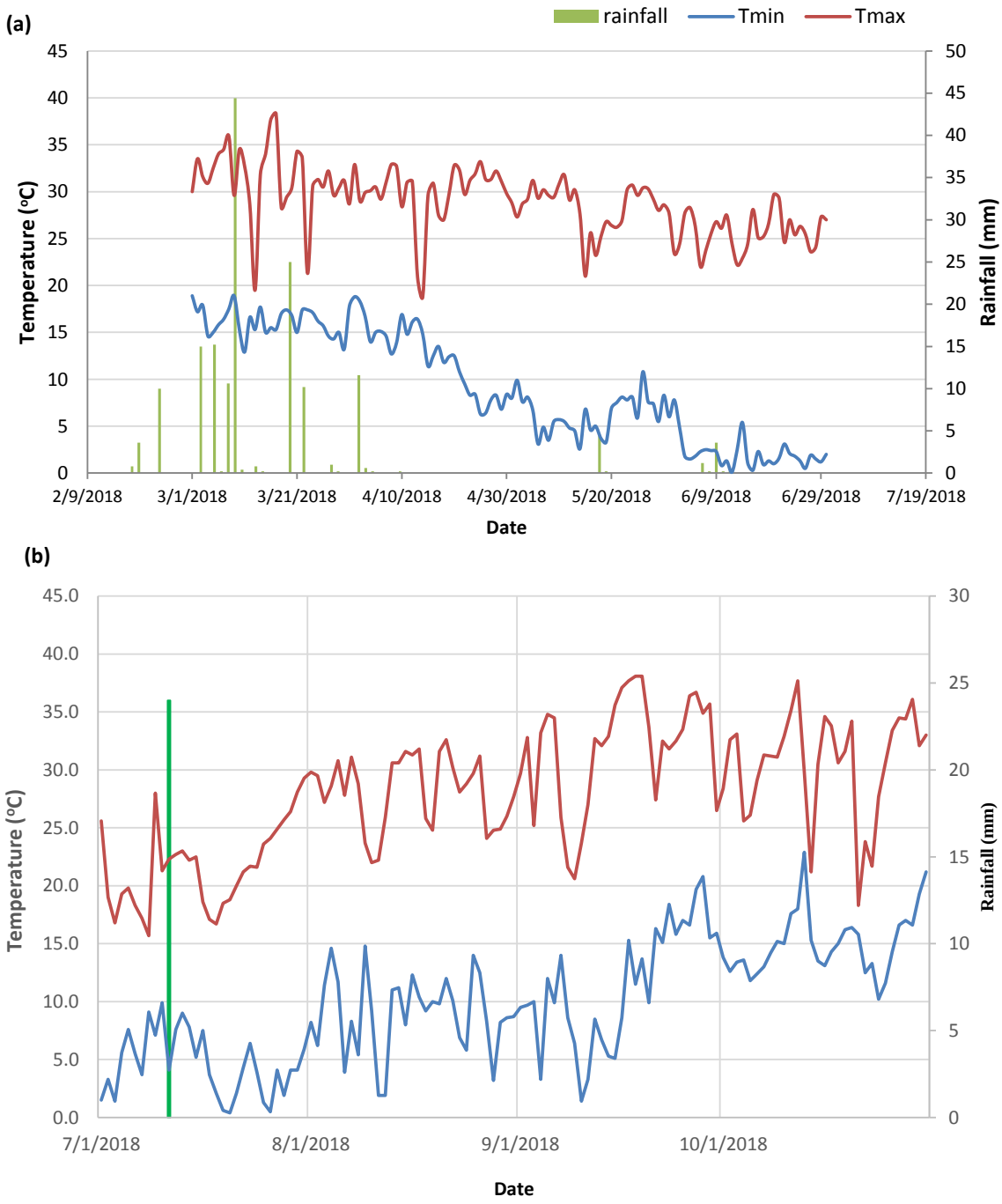

Figure 3. Precipitation and temperature variability during (a) season 1 and (b) season 2. TMPmax; Maximum temperature, TMPMin; Minimum temperature.

sludge used in this study was organic (Magdoff and Amadon, 1980) which required mineralization before plants N assimilation.

Moreover, it is worth considering that before the trial was established in March 2018, the land was disc ploughed whereas prior to the second season, reduced tillage was applied to retain the planting lines and plots. The differences in mechanical aeration of the soil due to tillage possibly contributed to higher decomposition of sewage sludge and biodegradable biochar C in the first season, leading to accumulation of N into the labile microbial N pool. Although N immobilization can result in reduced yields (Deenik et al., 2010), in a wet season such as the March – June period in this study, it probably mitigated NO_3^- leaching potential, which possibly resulted in higher N efficiency. However, from a long-term field

trial in Gottingen, Hoffmann et al. (1997) concluded that minimal tillage systems increased N – net mineralisation compared with conventional tillage systems, mainly due to the greater microbial biomass under reduced tillage. More research is therefore required to determine the factor(s) with overriding effects on sewage sludge N mineralization under Botswana conditions for development of sewage sludge management guidelines on agricultural soils.

There was insignificant correlation ($p > 0.05$) between soil NO_3^- and leaf N contents. While soil NO_3^- generally decreased among the majority of treatments in the second season, leaf N increased for all the treatments (Table 5). This disparity can be attributed to the dilution effects of greater yields in the first season. Furthermore, lower crop yields in the second season probably increased leaf N content to sufficiency levels (3%) and above compared to the first season.

Effects of amendments on spinach yield

Table 3 shows that applications of sole organic amendments had greater effects on spinach yields on the Luvisol than on the Cambisol as shown by the higher slope of the regression equations. Moreover, application of biochar on the Luvisol significantly ($p < 0.05$) increased spinach yield, while the relationship on the Cambisol was insignificant. Crop yields across the two seasons also indicate greater yield response on the sandy loam textured Luvisol, and these responses were better than under mineral fertilizer (Figure 2a and b). Contrastingly, yield responses to organic amendments on the Cambisol were similar to mineral fertilizer. Studies on biochar effects on spinach productivity are lacking, but the results of this study support findings by Boersma et al. (2017). In their study, *Eucalyptus polybractea* biochar application to a fertile red Ferrosol did not increase yields of several vegetable crops. These results demonstrate the greater prospects of organic amendments in improving the crop productivity of degraded sandy soils which are prevalent in the tropics.

Mean yield data across the two seasons (data not shown) indicates significant synergistic effects of biochar and sewage sludge on the Luvisol for most of the treatments but no significant complimentary effects on the Cambisol. That there was significant ($p < 0.05$) yield increase from combined application of high rates of amendments on the Luvisol in the second season, while yield declined for the majority of the treatments (Figure 2a), is evidence of the synergistic effects of the amendments on the relatively infertile soil. Contrasting results were reported in a comparative short-term pot study in Zimbabwe by Gwenzi et al. (2016). Working on a clayey soil, the authors reported synergistic effects of sewage sludge and its biochar amended at 15 Mg ha^{-1} in increasing maize biomass yields, while sole biochar application without mineral fertilizer was less effective in increasing biomass yields. In our study, sole biochar application on the clay soil had more positive influence on yields than combination of biochar and sewage sludge. The difference in performance of amendments with their results for a soil type with similar texture to the Cambisol can be attributed to the variability in experimental conditions (Glaser et al., 2015).

Crop yields were statistically independent from soil NO_3^- content ($p > 0.05$), which presumably was caused by high coefficient of variation of the yield data (CV = 33.5%). Nonetheless, the decrease in yields on the Luvisol closely followed the same trend as soil NO_3^- and leaf P contents between seasons (Figure 1a and Table 5), while leaf N increased. These data demonstrate that soil NO_3^- content was the limiting factor for yields in this study because available P increased above the critical range of 45 – 50 ppm (Ziadi and Tran, 2008) between seasons. Comparison of yield data between the two soil types indicates that there are more beneficial effects of application of organic amendments on the Luvisol than on the Cambisol.

Distinctly, 12SS+5BC consistently increased soil NO_3^- and plant N contents, and yields ($p < 0.05$) between seasons on the Luvisol (Figures 1 and 2). The average yields for 12SS+5BC (26.9) was less than that for 6SS+5BC (33.6 ton ha^{-1}), hence there is no added benefit of increasing the amount of sewage sludge combined with 5 Mg ha^{-1} biochar from 6 to 12 ton ha^{-1}. Also, available P increased well above the critical level to 177 mg kg^{-1} under 12SS+5BC (Table 3) on the Luvisol. Although P is not toxic and less mobile compared to NO_3^- in the environment, its potential loss into the nearby Notwane River could result in eutrophication and degradation of the aquatic life.

Biotic and abiotic oxidation of biochar surfaces increases the CEC (Liang et al., 2006; Glaser et al., 2000; Wiedner et al., 2015), and this presumably retained significant levels of ammonium (NH_4^+), thus suppressing nitrification (Clough et al., 2013; Nelson et al., 2011). This effect is hypothetically greater on the Cambisol due to its high clay content (Amlinger et al., 2003). Spinach typically prefers NO_3^- to NH_4^+ (Wang et al., 2009). Thus, in maintaining a relatively small pool of NO_3^-, formation and assimilation by spinach is suppressed, which could account for the yield declines in the second season for most treatments on both soils, specifically the biochar-amended treatments, because elevated levels of NH_4^+ can be toxic to aerobic plants and suppress both root development and plant growth (Wang et al., 2009; Deenik et al., 2010). Therefore, the decline in the yields under co-applications (Figure 3b) might be attributed to NH_4^+ toxicity in biochar treated plots, since the other nutrients were in adequate supply (Table 3). Biochar addition of 5 Mg ha^{-1} on the Luvisol significantly reduced spinach yields (mean = 23 Mg ha^{-1}) compared to application of biochar at 2.5 ton ha^{-1} (mean = 28 Mg ha^{-1}), emphasizing

the possibility of greater NH_4^+ accumulation under higher sole biochar application.

High yields for a combination of intermediate sewage sludge (6 Mg ha^{-1}) and biochar applications (5 Mg ha^{-1}) during the first season depleted soil NO_3^-, leading to decreased yields the following season. This hypothesis and the likely low mineralization rates of sludge organic N during second season due to cooler temperatures played a significant role in decreasing yields relative to the first season. Since available P was in adequate supply, crop yields on the Luvisol could be sustained by additional mineral N fertiliser instead of annual addition of sewage sludge to prevent excessive levels of P in the environment. This is true across all the treatments because P reached the critical level on this soil type for most of the treatments.

Conclusion

Overall, the results showed that co-application of biochar-sewage sludge had statistically similar effects on soil NO_3^- content on both soil types. Further, the improvement in N bioavailability and yields was greater on the Luvisol, while on the Cambisol, the effects of organic amendments were similar to mineral fertilizer. The decline in spinach yields in the second season was linked to the decrease in soil NO_3^- because biochar – sewage sludge addition supplied adequate P for spinach growth and leaf P reached sufficiency levels in the second season. Available P, SOC, CEC, soil bulk density, exchangeable bases all improved due to organic amendments. However, leaf micronutrients levels were comparatively lower than those reported for spinach in other studies, which should be the subject of future research. Therefore, Glen Valley farmers can reduce their fertilizer costs by using 5 Mg ha^{-1} of biochar on both soils, or combined application of 6 Mg ha^{-1} sewage sludge plus 5 Mg ha^{-1} biochar on the Luvisol. However, to prevent excess application of P, one-time application of organic amendments followed by mineral N fertilizer is necessary to maintain crop yields.

CONFLICTS OF INTERESTS

The authors have not declared any conflicts of interests.

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Antioxidant, cytotoxic and antimicrobial activities of *Dendropanax morbifera* and sweet potato extracts for production of health-oriented food materials

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The antioxidant, cytotoxic and antimicrobial effects of fermented and non-fermented extracts of 'the Korean shrub' *Dendropanax morbifera* and sweet potato were compared to assess the potential utility of these species in the development of health-oriented food. Non-fermented extract (NFDSE) was obtained from the leaves and branches of *D. morbifera* and the bodies of sweet potato using distilled water. The fermented extract (FDSE) was prepared by inoculating the above-obtained extracts with *Lactobacillus plantarum* and *Lactobacillus brevis.* The extracts of the two species combined *D. morbifera* and sweet potato exhibited substantial antioxidant activity. Moreover, NFDSE at 24 h exerted more antioxidant effects than FDSE (72.57% vs. 71.08%, respectively) at a concentration of 100 mg/ml. Comparison of the effects of the non-fermented and fermented extracts on HaCaT keratinocyte cell viability revealed that FDSE had a slightly higher cytotoxicity than NFDSE (94.8% vs. 102.7% viability, respectively) at a concentration of 500 μg/ml. It was further found that NFDSE and FDSE had the strongest antimicrobial effects against *Staphylococcus aureus* ATCC 25923 and *Staphylococcus epidermidis* (a Gram-positive coccidium). Therefore, it is obvious that extracts of *D. morbifera* and sweet potato represent 'novel candidates' for the production of functional anti-aging agents with minimal side effects.

Key words: *Dendropanax morbifera* and sweet potato, antioxidant activity, cytotoxicity, antimicrobial effects, health-oriented food.

INTRODUCTION

Due to rapid industrial developments and economic growth, research interest in health-related products and, consequently, life expectancy has greatly increased (Bergh and Nilsson, 2010). In addition, food habits have changed from the traditional form of consuming fermented foods to western diets. Excessive intake of calories and fat via meat-based diets and high quantities of processed food and food additives is associated with a higher incidence of chronic degenerative disorders, such as hypertension, stroke, diabetes, cancer, macular degeneration and chronic liver disease. Accordingly, substitution of meat-based diets with vegetarian options

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is the current diet trend. In addition, there is an increased tendency to ingest food extracts prepared from physiological components of vegetable material. A representative example is plant-derived fermented liquid consumed in large quantities in the private sector over recent years. Following age-induced fermentation of plant material, many components are activated and converted into forms that are easy to absorb. Fermented liquid plant extracts have been shown to exert multiple beneficial effects through regulatory functions along with antioxidant, anti-obesity and anti-cancer activities (Kim et al., 2011; Lee et al., 2012; Yang et al., 2011), although research on this topic is still in its infancy. At present, various plant materials are utilized in view of their effects on wellbeing. However, related functionalities and physiological aspects are yet to be investigated in detail, with the only knowledge so far being down to long-term experience and partial facts from word of mouth.

Recently, *Dendropanax morbifera* and sweet potatoes were suggested to be beneficial for human health. This species belongs to the genus *Dendropanax* in the family *Araliaceae.* Around 75 species of *Dendropanax* are distributed in East Asia, Malay Peninsula, Central and South America, with one species identified in Korea. This subtropical broad-leaved evergreen tree is economically important due to its utility in the production of golden varnish (Moon et al., 1999; Kim et al., 2006), and has been increasingly cultivated on Jeju Island and regions of the Korean coastline along the southwestern sea. In addition, its leaves, stems, roots and seeds are traditionally used in folk medicine for skin and infectious diseases, headaches and other maladies (Park et al., 2004). Various beneficial physiological activities of *D. morbifera* have been documented, such as improvement of lipid abnormalities, diabetic disease, immune activity, thrombosis and kidney loss protection effect (Tan and Ryu, 2015; An et al., 2014; Lee et al., 2002; Choi et al., 2015; Kim et al., 2015). The plant is additionally reported to exert a skin whitening effect (Park et al., 2014; Lee et al., 2015), indicative of a variety of physiologically active components, supporting its potential utility in the development of novel therapeutic drugs and functional materials.

Sweet potatoes are one of the most common seasonal foods and widely used as a major food resource along with cereals, such as rice and barley. Sweet potatoes mostly constitute starch, along with water and β-carotene, and are rich in minerals and dietary fiber. Research to date has focused on processing of foods from sweet potato, such as chips and noodles, and the techniques involved. However, to our knowledge, no studies have explored processing of beverages from sweet potato tubers.

The properties of fermented sugar extracts of *D. morbifera* and sweet potato are not known although the functions of specific raw materials and extracts have been investigated. Both *D. morbifera* and sweet potato are known to contain various physiologically active substrates but have rarely been used in processed foods so far. The main objective of the current study was to examine the potential utility of *D. morbifera* and sweet potato distilled water extracts fermented with the aid of lactic acid bacteria in health-oriented food products. Hydrothermal extraction and various *in vitro* experiments were applied to obtain a functional beverage from *D. morbifera* and sweet potato extracts with antioxidant, cytotoxic and antimicrobial activities.

MATERIALS AND METHODS

Preparation of *Dendropanax morbifera* and sweet potato extracts

Boughs of *D. morbifera* and sweet potato were collected from a natural habitat in South Korea, Jeju Island in February 2018. Samples were dried at room temperature and subjected to the extraction process. Collected boughs were cut into 1.0 cm sections. Sweet potatoes were finely pulverized and stored at a temperature of -20°C. The distilled water extract of *D. morbifera* and sweet potato (NFDSE) was obtained using 20 volumes of water at 95°C for 4 h. Fermented *D. morbifera* and sweet potato extract (FDSE) was prepared as follows: *Lactobacillus plantarum* and *Lactobacillus brevis* strains were inoculated in De Man, Rogosa and Sharpe (MRS) broth at 37°C for 24 h and diluted to obtain an initial population of 1-5 × 10^7 CFU/ml *D. morbifera*. For fermentation, the *D. morbifera* and sweet potato solution (5%) was inoculated with fresh bacterial subculture (4% v/v) at 37°C for 24 h, followed by sterilization and filtration. The filtered solution of fermented sample was concentrated and spray-dried.

Measurement of antioxidant activity of the extracts

The antioxidant capacity of extracts was analyzed by measuring free radical scavenging activity using the DPPH assay (Brand-Williams et al., 1995). Samples were prepared at concentrations of 1, 10 and 100 mg/ml, with vitamin C (Vit. C) treatment used as the positive control. After incubation at room temperature for over 30 min, free radical scavenging activity was determined by mixing with 500 μM DPPH solution (1:1) and incubating in the dark, followed by measurement of absorbance at 517 nm using a spectrophotometer.

Analysis of cytotoxicity of the extracts

HaCaT keratinocytes obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea) were employed for cytotoxicity experiments. Preadipocyte cells were sub-cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/ streptomycin (P/S; Gibco) every 24-36 h and seeded in 96-well plates at a density of 1.0 x 10^5 cells per well. Next, cells were treated with 200 μl NFDSE or FDSE at a range of concentrations (10, 50, 100, 200, 300, and 500 μg/ml) at 37°C for 24 h in 5% CO_2. Cell viability was determined according to the manufacturer's protocol. MTT reagent (20 μl) was added to individual wells and incubated under similar conditions for 1 h and absorbance read at 490 nm in a microplate reader. The number of viable cells was directly proportional to absorbance of formazan formed due to reduction of MTT. Cell viability was expressed as a percentage of control cells. All experiments were performed in triplicate.

Table 1. DPPH radical scavenging activity (%) of distilled water and fermented extracts of *D. morbifera* and sweet potato.

Sample	Concentration (mg/ml)		
	1	10	100
NFDSE	4.13±0.54	25.54±2.8	72.57±4.4
FDSE - 24 h	3.27±0.44	24.17±2.8	71.08±4.6
Vitamin C	21.63	100	100

Values represent means ± SD (n=3). Means not sharing a common letter were significantly different at $p<0.05$.

Antimicrobial activity measurement

Gram-positive *Staphylococcus epidermidis*, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecali*s and Gram-negative *Bacillus cereus* and *Escherichia coli* ATCC 26922 strains used for the antibacterial assay were purchased from the Korean Society of Microbiology. Nutrient broth and agar were used for culture of microorganisms. For measurement of antibacterial activity, growth medium containing 1.5% agar was spread on the bottom of a petri dish, followed by growth medium containing 0.6% agar, to prepare a 2-fold plate culture medium. After adding a dosage of 70 μl dosages of 100% extracted solution of NFDSE or FDSE to a 0.8 cm diameter disc, the strain was placed on a flat plate covered with growth medium and incubated at 37°C for 24 h to measure the growth inhibitory effect. Tetracycline and streptomycin as the standard drug were used in the experiment.

Statistical analysis

All data are presented as mean ± standard deviation of three replicates. Differences among treatments were assessed by analysis of variance (ANOVA), followed by Dunnett's test. *p*-value of < 0.05 was regarded as significant.

RESULTS

DPPH radical scavenging activity of *D. morbifera* and sweet potato extracts

The scavenging activities of NFDSE and FDSE for DPPH radicals increased with the treatment concentrations (Table 1). NFDSE exerted increasing inhibitory effects (4.13, 25.54, and 72.57%) at concentrations of 1, 10 and 100 mg/ml, respectively. Within this concentration range, the inhibitory effects of FDSE at 24 h were 3.27, 24.17, and 71.08%, respectively, indicating that both NFDSE and FDSE possess good scavenging activity for DPPH radicals at the concentrations tested. Vitamin C, the positive control, displayed excellent scavenging ability (21.63, 100, and 100%) within the same concentration range.

Effects of *D. morbifera* and sweet potato extracts on HaCaT keratinocyte cell viability

The potential use of NFDSE and FDSE as health-oriented food components was further ascertained based on specific bioactivities, such as cytotoxicity. To determine the effects of the extracts on cell viability, the MTT assay was performed on HaCaT keratinocyte cells treated with 10 to 500 μg/ml NFDSE or FDSE. The results are expressed as a percentage of surviving test cells relative to the control group (Figure 1). No significant toxicity of either the fermented and non-fermented extracts were observed within the range of concentrations examined against HaCaT keratinocytes.

Influence of *D. morbifera* and sweet potato extracts on antimicrobial activity

Antimicrobial activities of NFDSE and FDSE against five bacterial strains (*S. epidermidis*, *S. aureus* ATCC 25923, *E. faecali*s, *B. cereus*, and *E. coli* ATCC 26922) were examined using an agar well diffusion method. As summarized in Table 2, gram-positive *S. epidermidis*, *S. aureus* ATCC 25923 and *E. faecali*s strains showed susceptibility to both NFDSE and FDSE at a concentration of 200 mg/ml with inhibition zone diameters of 14.3, 12.1, and 8.2 mm for NFDSE and 15.2, 13.5, and 9.3 mm for FDSE, respectively. At the same test concentrations, the two gram-negative strains, *B. cereus* and *E. coli* ATCC 26922, showed susceptibility to NFDSE and FDSE with inhibition zone diameters of 6.7 and 6.3 mm for NFDSE and 7.0 and 6.8 mm for FDSE, respectively. Our results indicate that highest inhibitory activity of the extract in both fermented and non-fermented forms is against *S. epidermidis* and the weakest activity against *E. coli* ATCC 26922. The inhibition zone diameters with streptomycin and tetracycline, and the positive controls, ranged from 11.5-19.7 mm and 12.3-20.6 mm, respectively.

DISCUSSION

Oxidative stress is defined as intracellular damage caused by an imbalance between oxidants and antioxidants. Oxidants are produced during normal metabolism *in vivo* and highly generated in pathological conditions (Sies, 1997). Oxidative stress is known to be a

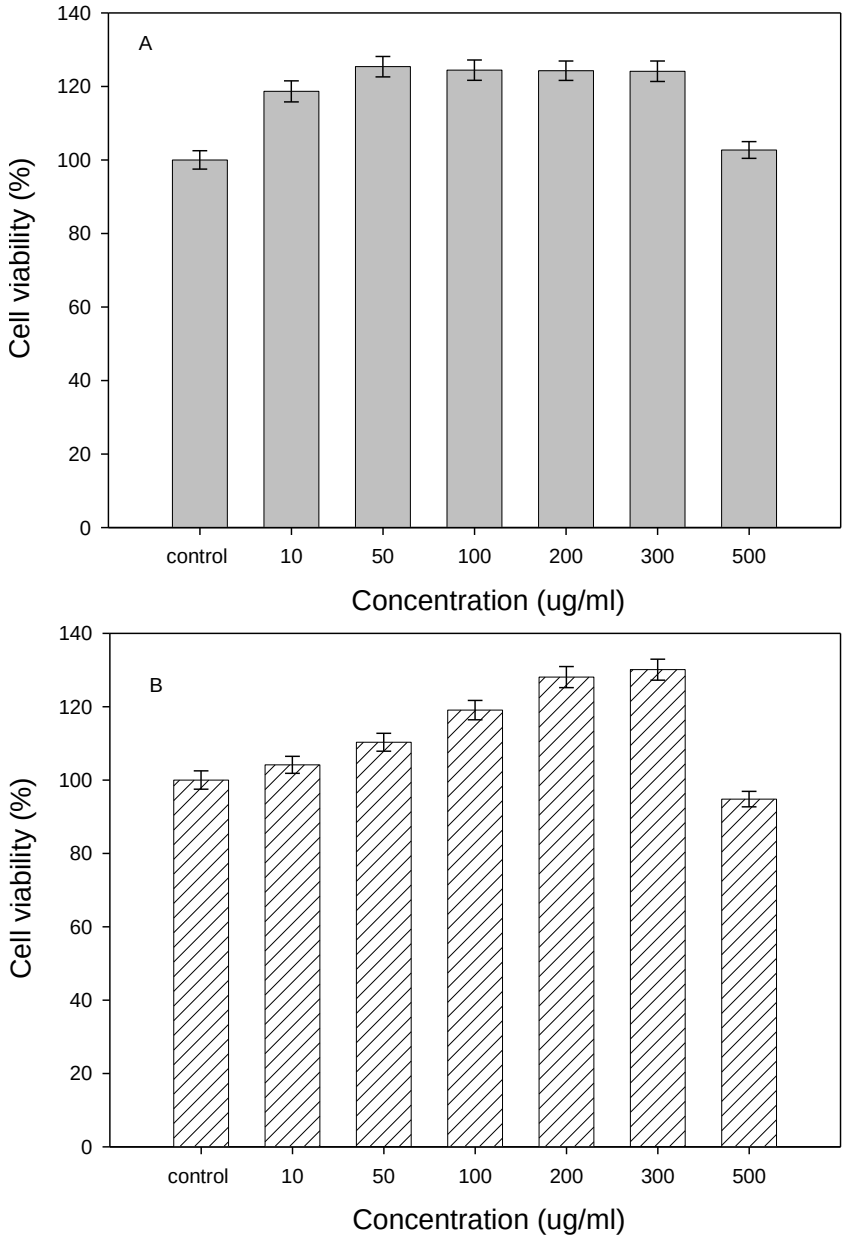

Figure 1. Effects of NFDSE and FDSE on viability of HaCaT keratinocyte cells. Cells were seeded at a concentration of 1 x 10^5 cells/well in a 96-well plate and differentiation allowed for 24 h, following treatment with a range of concentrations of NFDSE and FDSE. Following harvesting, cytotoxicity was determined with the MTT assay. Results are presented as means ± SD of experiments performed in triplicate. (A) Non-fermented extract (NFDSE), (B) Fermented extract (FDSE).

direct cause of aging and several chronic diseases, such as cancer and arteriosclerosis, and the discovery of food-derived antioxidant agents to control these conditions is a major research concern (Finkel and Holbrook, 2000). The purpose of the current study was to evaluate the antioxidative activity of extracts of *D. morbifera* and sweet potato, which are known to contain high levels of antioxidants.

Antioxidants are capable of reducing the stable DPPH radical (purple) to its non-radical form, DPPH-H (yellow). The DPPH scavenging activities of antioxidants are attributed to their hydrogen donating ability to ROS. In the DPPH radical scavenging assay, the antioxidant activities of NFDSE and FDSE were relatively good but still lower than that of Vitamin C, which showed excellent DPPH scavenging effects at all the concentrations examined.

Table 2. Diameters of inhibition zones of NFDSE and FDSE against selected microorganisms.

Pathogenic bacterial strain	Treatment			
	NFDSE (200 mg/ml)	FDSE (200 mg/ml)	Streptomycin (30 μg/ml)	Tetracycline (30 μg/ml)
	Clear zone (mm)			
Staphylococcus epidermidis	14.3±0.23	15.2±0.32	19.7±1.12	20.6±0.32
Staphylococcus aureus ATCC25923	12.1±1.05	13.5±1.05	18.1±0.23	19.3±1.03
Enterococcus faecalis	8.2±0.25	9.3±0.67	16.9±1.16	17.7±1.32
Bacillus cereus	6.7±0.25	7.0±0.25	11.8±1.05	12.5±1.07
Escherichia coli ATCC 25922	6.3±0.23	6.8±0.25	11.5±1.05	12.3±1.05

Treatment groups: NFDSE, non-fermented extract; FDSE, fermented extract. Values represent means ± SD (n=3). Means not sharing a common letter were significantly different at $p<0.05$.

The electron donating ability was evaluated as an index of antioxidant activity against phenolic acid, flavonoids and other phenolic substances, whereby higher reducing power was correlated with greater electron donating ability (Kang et al., 1995). This result is consistent with previous studies showing that the *D. morbifera* and sweet potato extracts showed the presence of antioxidants and the ability to scavenge free radicals (Hyun et al., 2013; Zou et al., 2012; Ghasemzadeh et al., 2012).

To investigate cytotoxicity, HaCaT keratinocyte cells were treated with different concentrations of *D. morbifera* and sweet potato extracts and the MTT assay was performed. Cell proliferation was observed at a range of extract concentrations (10 to 300 μg/ml), indicating weak toxicity or a cell protective effect. Optimal proliferation was detected at a concentration of 300 μg/ml NFDSE and FDSE, following which proliferative ability was decreased and toxicity evident.

Our experiments disclosed antimicrobial activity of NFDSE and FDSE against all five selected microorganisms, indicating relatively broad-spectrum antibacterial effects. Among these, highest antibacterial activity was observed against gram-positive *S. epidermidis* and *S. aureus* ATCC 25923 and lowest activity against gram-negative *E. coli* ATCC 26922, indicating greater resistance of gram-negative bacteria to antibacterial agents. One potential explanation for this difference in antibiotic resistance properties is variations in the cell wall structure between gram-positive and gram-negative bacteria (Epand et al., 2016).

Conclusion

This study has demonstrated antioxidant, cytotoxic and antimicrobial activities of extracts of *D. morbifera* and sweet potato. The antioxidant *D. morbifera* and sweet potato, indicating a high activity to remove harmful free radicals, have preventive and inhibitive effect on various diseases and aging. Our collective results support the utility of *D. morbifera* and sweet potato extracts as a future source of natural anti-aging and therapeutic agents with minimal side-effects.

CONFLICT OF INTERESTS

The author has not declared any conflict of interests.

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