African Journal of Biotechnology

Volume 13 Number 49, 3 December, 2014
ISSN 1684-5315

Academic Journals

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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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African Journal of Biotechnology

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academicJournals

Vol. 13(49), pp. 4476-4491, 3 December, 2014
DOI: 10.5897/AJB2014.13692
Article Number: 7AF832548903
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of lycopene on hydroponic growth and productivity of *Amaranthus hybridus* L. under aluminium toxicity induced stress

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Received 3 February, 2014; Accepted 18 November, 2014

Aluminium (Al) toxicity is an important stress factor for plants in acidic environments. Currently, there exists a dearth of research information on sustainable approaches that can mitigate the problem of Al toxicity in sub Saharan Africa. It is often assumed that since plants have the ability to synthesize their native antioxidants, they may not need supplementation to cope with stresses. To challenge this assumption, the present study explored the effects of 50 and 200 µM concentrations of lycopene (ly) on the growth and productivity of *Amaranthus hybridus* subjected to 3 × 10^{-2} mM concentration of Al at pH 4.6 and 25°C. High performance liquid chromatography (HPLC) was used to determine the peak absorbance at 375 nm and the thiobarbithuric acid (TBA) method was used to determine antioxidant activity of ly. Pre- and post-antioxidant treatments were hydroponically applied to *A. hybridus* seedlings before and after Al treatment for 72 h. Results show that post-lycopene treatments significantly alleviated Al stress in *A. hybridus* more than pre-lycopene treatments. It is concluded that though *A. hybridus* could synthesize its own antioxidants, it is susceptible to Al toxicity-induced stress and post-lycopene supplementation could alleviate the stress situation and enhance growth and productivity. However, *A. hybridus* plants appear not to need lycopene while not under Al stress.

Key words: *Amaranthus hybridus,* growth and productivity, hydroponics, aluminium toxicity, stress amelioration, lycopene.

INTRODUCTION

Soil acidity limits agricultural production globally especially in the Sub-Saharan African region where food security is a global concern. Different kinds of stresses, mainly Al stress, generated from acid soils affect plant growth and result in food shortage and production (Carvalho et al., 1980; Roy et al., 1988; Foy, 1992). Trivalent Al (Al^{3+}) is one of the most toxic forms of soluble Al and is known to damage root cells at sites in the apoplast and cytosol that rapidly inhibit root growth (Kochian, 1995; Hede et al., 2001). Al is the third most abundant metallic element and is readily available to plants when the soil pH drops below 5.5. Some

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agricultural practices, such as leaching of nitrogen below the plant root zone, inappropriate use of nitrogenous fertilizers and increased excessive accumulation of organic matter, are major sources of soil acidification (Mohan et al., 2008). Fatur et al. (2002) explained that Al toxicity could be transferred to animals and man by consuming plants that accumulated Al. Amaranth species are erect or spreading fast growing annuals, with heights varying between 0. 3 and 2 m, ideal for plant studies in the tropic. Reports indicate that plants grown in pH 6.4 soil were significantly taller with more branches, leaves and greater leaf area than plants grown in pH 5.3 or 4.7 soil. Soil acidity impeded both top and root growth of the plant (Singh and Whitehead, 1992; Palada and Chang, 2003).

The study of Al toxicity has elicited divergent views, if not controversies, over the years and this may continue for a long time. It has been generally observed that plants grown in acid soils due to Al solubility, at low pH, have reduced root systems and exhibit a variety of nutrient-deficiency symptoms, with a consequent decrease in yield. In many countries with naturally acid soils, which constitute about 40% of world arable soil (Le Noble et al., 1996), Al toxicity is a major agricultural problem, and is intensively studied in plant systems. The effects of aluminium on plant growth, crop yield, up take and nutrients distribution in vegetative and reproductive parts are still not fully understood (Mossor-Pietraszewska, 2001). In their own contribution, Aniol and Gustafson (1984) observed that genetic variation in the response to Al toxicity has been found not only among plant species but also among cultivars within species. Other views on the complex nature of Al toxicity studies have been expressed (Kinraide, 1991; Lazof and Holland, 1999; Zatta et al., 2002; Kidd and Proctor, 2001; Exley, 2004). The role of Al in rhizotoxicity is a complex one and in spite of the enormous amount of information gathered from several studies, a lot of things are yet to be known about the complex process. A renowned authority in rhizotoxicity, Kinraide (1991), remarked that Al hydrolysis creates considerable uncertainty in the study of Al rhizotoxicity. A strict attribution of toxicity to Al^{3+} is not possible because Al^{3+} is always in equilibrium with its hydrolysis products.

Osaki et al. (1997) classified some tropical plants based on their Al tolerance. Plants in which growth was reduced by low and high Al applications were designated as Al-sensitive plant and Al-medium tolerant plants, respectively, while plants in which growth was not affected or was stimulated by Al application were designated as Al-tolerant plant and Al-stimulated plants, respectively. Plants tolerant to or stimulated by Al were further classified based on the criteria of Al accumulation: (1) Al-excluders (2) Al root-accumulators and (3) Al-accumulators. Al toxicity is always associated with H^+ which is often ignored by researchers. Some workers have observed that the solubility of Al is appreciable only at pH values below 5.5. Thus, toxicity to plants occurs only at these low pH values, with the possible exception of the toxicity of $Al(OH)_3^{4-}$ at higher pH values (Kinraide, 1990). Despite this, and in contrast to the large amount of literature on Al toxicity, very little attention has been given to H^+ toxicity, even though the latter is well known to be directly detrimental to root growth (Kidd and Proctor, 2001; Koyama et al., 2000). As in Al toxicity, H^+ toxicity is most severe in solutions of low ionic strength and low cation concentrations, and increasing the concentration of Ca^{2+} and other cations in the external solution reduces or even abolishes the detrimental effects of acidity (Marschner, 1991). An evaluation of these low-pH effects is necessary for greater understanding and correct interpretation in studies of Al toxicity, but this is rarely undertaken (Lazof and Holland, 1999; Samac and Tesfaye, 2003).

Ryan et al. (1993) found the root apex to be the most Al-sensitive zone and Sivaguru and Horst (1998) identified the distal transition zone (DTZ) as the specific site of Al sensitivity. The mechanisms of Al-induced inhibition of root growth, however, are still not clearly established (Kochian, 1995; Barcelo et al., 1996; Matsumoto, 2000; Barcelo and Poschenrieder, 2002; Kochian et al., 2004). Pioneer work by Clarkson (1969) revealed Al induced alterations of root development, indicating that cell division is a primary site for Al-induced root growth inhibition. Further studies reporting Al-induced inhibition of cell division in root tips and the observation that Al binds to nucleic acids supported the view of Al-induced inhibition of root cell proliferation as a primary target for Al-toxicity (Matsumoto et al., 1976; Yamamoto et al., 2001). Lycopene (Ly) and other carotenoids partake in the crucial process of photosynthesis by absorbing energy across a wider spectrum of light and then transferring the absorbed energy to chlorophyll in order to drive the events of photosynthesis (Polivka and Frank, 2010).

Ly is synthesized in plants that produce the yellow and red colours in leaves and fruits most frequently during ripening. Cazzonelli (2011) described that ly and other carotenoids play crucial roles in protecting chloroplasts of plants as well by regulating the amount of light absorbed by plants. In essence, excess light could destroy the photosynthetic apparatus without the presence of carotenoids. Additionally, plants regulate the amount of light being absorbed by absorbing reactive oxygen species (ROS) derived from triplet chlorophyll. Such behaviour is mediated by the action of ly and other carotenoids contained in plants (Cazzonelli, 2011). At the molecular level, Al stress is known to cause drastic changes in the expression patterns of genes, some of which are quite important in the mediation of responses to oxidative stress (Maron et al., 2008). It is thus inherent that exposure of plants to Al elicits the production of ROS, which may damage cellular components if antioxidant defences are jeopardized (Darko et al., 2004;

Sharma and Dubey, 2007). Other pertinent functions of ly include the attractive colours it imparts to leaves, fruits and flowers, thus facilitating plant pollination and seed dispersal (Walter et al., 2010). Ly is one of the most potent natural antioxidants (Miller et al., 1996) and has been suggested to prevent carcinogenesis and atherogenesis by protecting critical biomolecules including lipids, low-density lipoproteins (LDLs), proteins and DNA (Hodis et al., 1995; Agarwal and Rao, 1998; Rao and Agarwal, 1998). Several studies have indicated that ly is an effective antioxidant and free radical scavenger. Ly, because of its high number of conjugated double bonds, exhibits higher singlet oxygen quenching ability when compared to β-carotene or α-tocopherol. In *in vitro* systems, ly was found to inactivate hydrogen peroxide and nitrogen dioxide (Rao and Agarwal, 2000). Using pulse radiolysis techniques, Rao and Agarwal (2000) demonstrated its ability to scavenge nitrogen dioxide ($NO_2^{\cdot}$), thiyl ($RS^{\cdot}$) and sulphonyl ($RSO_2^{\cdot}$) radicals. Ly is highly lipophilic and is most commonly located within cell membranes and other lipid components. It is, therefore, expected that in the lipophilic environment ly will have maximum ROS scavenging effects. Oxidative modification of LDLs is hypothesized to be the key step in the atherogenic process, and LDL-associated antioxidants provide protection against this oxidation (Rao and Agarwal, 2000). *In vitro* ly and other carotenoids are able to inhibit oxidation of LDLs (Agarwal and Rao, 1998).

According to Gong et al. (2005), environmental stresses increase the formation of ROS that oxidize membrane lipids, protein and nucleic acids. Plants with high levels of antioxidants, either constitutive or induced, have greater resistance to oxidative damage (Sudhakar et al., 2001). Aftab et al. (2010) noted that all the treatments in which Al was applied to experimental plants had raised the activities of antioxidant enzymes. With increasing amounts of Al in the soil medium, a concomitant increase in the activities of catalase (CAT), peroxidase (POX) and superoxidase dismutase (SOD) antioxidant enzymes were observed in the exposed plants (Boscolo et al., 2003; Siminovicova et al., 2004; Sharma and Dubey, 2007; Zhen et al., 2007; Aftab et al., 2010). The fact that despite the increase in the activities of elevated levels of the antioxidant enzymes under Al toxicity, as many workers have reported; Al sensitive plants are still damaged, even under low Al concentrations, suggests that both the constitutive and induced high levels of antioxidants may be insufficient to combat the stress situation. Such a situation will undoubtedly lead to crop failure and aggravation of food security problems in the sub Saharan region. It is against this backdrop, and to contribute to the study of the complex problem of Al toxicity, that the present study was undertaken, with the focus of exploring the possible effects of supplemented antioxidant (ly) on both the vegetative and reproductive growth of *A. hybridus*, subjected to mild Al induced stress.

MATERIALS AND METHODS

Soil analysis

The top soil for raising the nursery was collected from the Botanic garden, University of Nigeria, Nsukka. A sample of the soil was air dried and taken to the Department of Soil Science Laboratory, University of Nigeria, Nsukka for analysis. The analysis was done using the standard method of the Association of Official Analytical Chemists (2005).

Lycopene extraction and purification

Fresh, matured and ripe, De Rica cultivar, tomatoes weighing 10 kg were purchased from Nsukka local market. The tomatoes were washed with tap water and then ground with a national electric blender without addition of water. This was allowed to run until a homogenous paste was obtained. Water was separated from the homogenous paste using a large separating funnel (20 cm) with filter paper wrapped within. The residue obtained was air dried at room temperature. The filtrate was discarded after filtration. The red paste obtained after filtration was extracted with 1.25 L of methanol and 2.5 L of petroleum ether and vortexed to aid the extraction of ly. Pressure was not allowed to build up in the container through the release of excess gas produced from the mixture. After extraction, petroleum ether was evaporated to dryness. The tomato paste extract was purified by dispersing into 2-propanol (1: 5, wt/wt) at 60°C for 1 h. Then, 37 wt. % KOH solution (1: 4, v/ v) was added and the mixture was stirred at 50°C for 2 h. The mixture was finally washed with distilled water and filtered to obtain ly crystals following the methods of Yaping et al. (2002). The procedure was repeated to re-extract more ly from the mixture. Extracted ly was stored at 2°C and protected from light to prevent transformation to inactive isomers.

Determination of antioxidant activity

The antioxidant activity of ly was determined by obtaining its thiobarbithuric acid (TBA) value. One hundred milligrams (100 mg) of ly were thoroughly mixed with 5 mL aliquot of ethanol and poured into a 5 mL vial. A control was set up by pouring 5 mL aliquot of ethanol into another vial. From each vial, 1 mL of sample solution was measured out and mixed with 2 mL of 20% trichloroacetic acid (TCA) solution and 2 mL TBA solution. The mixture was placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 3,000 rpm for 10 min and absorbance of the supernatant was measured at 532 nm. The readings were taken thrice. The antioxidant activities were calculated as below:

$$\% \text{ Antioxidant activity} = \frac{ABcontrol - ABsample}{ABcontrol} \times 100$$

Where, AB_{sample} = Absorbance of sample and $AB_{control}$ = Absorbance of control (Hanachi and Golkho, 2009).

High performance liquid chromatography analysis (HPLC)

The percentage (%) purity of the extracted ly was determined using HPLC in the Department of Pure and Industrial Chemistry, UNN. UV spectrometry of each antioxidant was determined using UV-visible spectrophotometer. Five millilitre aliquot of ly dissolved in ethanol was poured into a 1 cm vial and its absorbance was

Table 1. Details of the 8 treatments given to the experimental plants.

Type of Treatment	Abbreviation	Symbol	Details
Lycopene (50 µM)	ly_1	T1	*Amaranthus* plants (AP) were grown in Hoagland's nutrient solution (HNS) and 50 µM ly_1 for 21 days
Lycopene (200 µM)	ly_2	T2	AP were grown in HNS and 200 µM ly_2 for 21days
Pre-lycopene (50 µM)	(ly_1-Al)	T3	AP were grown in HNS and 50 µM ly_1 for 72 h before transfer into HNS and 3 x 10^{-2} mM Al for 18 days.
Pre-lycopene (200 µM)	(ly_2-Al)	T4	AP were grown in HNS and 200 µM ly_2 for 72 h before transfer into HNS and 3 x 10^{-2} mM Al for 18 days.
Post-lycopene (50 µM)	(Al-ly_1)	T5	AP were grown in HNS and 3 x 10^{-2} mM Al for 18 d before transfer into 50 µM ly_1 for 72 h.
Post-lycopene (200 µM)	(Al-ly_2)	T6	AP were grown in HNS and 3 x 10^{-2} mM Al for 18 d before transfer into 200 µM ly_2 for 72 h.
Aluminium (3 x 10^{-2} mM)	Al	T7	AP grown into HNS and 3 x 10^{-2} mM for 21 days
Control	Ctrl	C	AP grown into HNS for 21 days

Table 2. Physical and chemical composition of top soil in the Botanic Garden, University of Nigeria, Nsukka, used for seed germination and seedling production.

Parameter	Value
pH (H_2O)	6.200
pH(KCl)	5.200
Fine soil (%)	29.000
Silt (%)	5.000
Clay (%)	27.000
Coarse soil (%)	39.000
Organic matter (%)	5.779
Organic Carbon (%)	3.352
Total Nitrogen (%)	0.168
Available P (ppm)	37.370
Exchangeable cations (mg/100 g)	31.200
Calcium (mg/100 g)	8.800
Magnesium (mg/100 g)	15.200
Sodium (mg/100 g)	0.501
Potassium (mg/100 g)	0.131
Hydrogen ion (mg/100 g)	3.400

measured in the spectrophotometer using ethanol as a blank. Triple readings were taken for each sample. Spectra of ly standard in ethanol was plotted to confirm the peak absorbance of the extracted ly.

Stock preparations

Fresh 1 M stock solutions of lycopene and $AlCl_3$ as well as full Hoagland's nutrient solution were prepared daily, as shown below. They were stored at 4°C in a refrigerator before use.

Lycopene stock solution

One gram of ly was properly mixed with 10 mL of ethanol before the addition of 990 mL of distilled water. A 1% alcohol dilution of ly was used in this study following the protocol of Fiskesjo (1981); who showed that 1% alcohol dilutions of lipophilic solutes were not toxic to *Allium* roots.

$AlCl_3$ stock solution (1 M)

This was prepared by dissolving 133.5 g of $AlCl_3$ in little distilled water and the volume made up to 1000 mL with same. The pH was kept at 4.6.

Al treatment concentration

Al treatment concentration of 3 x 10^{-2}mM was achieved through serial dilution and pH 4.6 was through adjustments with H_2SO_4.

Hoagland's nutrient solution

This was prepared using the formulation of Hoagland and Arnon (1950 revised).

Determination of actual Al concentration in solution

This was determined using the suggested modified Aluminon method for aluminum determination by Shull (1960).

Treatments

Details of the eight treatments are presented in Table 1.

Growing *Amaranthus hybridus*

Viable *A. hybridus* seeds from the *Amaranthus* germplasm maintained in the Botanic garden, University of Nigeria, Nsukka (which were originally obtained from National Institute for Horticultural Research, Ibadan Nigeria) were planted in baskets containing top soil obtained from the Botanic garden, University of Nigeria, Nsukka. Both the physical and chemical constituents of the top soil were analysed in the Soil Science Analytical Lab., University of Nigeria, Nsukka; the results are presented in Table 2. The baskets were placed in the screen house in the Botanic garden. Seeds were watered with full strength Hoagland's nutrient solution. After 54 days of planting, 120 healthy seedlings each were

transplanted into separate one hundred and twenty, 50 Cl transparent plastic bottles, each containing full strength Hoagland's nutrient solution and laid in a randomized complete block design (RCBD) in the screen house of the Botanic garden. Five plants per treatment, replicated three times, were used to monitor the growth of plants. Each *A. hybridus* plant was held upright in each bottle using sterilized cotton wool in such a way that shoots propped out of the container while the roots were immersed into the nutrient solution. The 120 bottles were wrapped with opaque black water-proof cellophane bags to prevent the roots from receiving light. Ten days after stabilization in the nutrient solution, the 8 treatments were applied, with Hoagland's nutrient solution as the source of nutrient as detailed in Table 1. In total, all individual plants received treatments for 21 days. All treatments were renewed daily since the hydroponic method used was non continuous flow, to ensure uniformity of treatment and adequate supply of nutrients. At the end of this period, the experiment was terminated. Fresh and dry weights of shoots, roots and inflorescences were recorded. Other growth parameters such as number of leaves, plant height, root length, number of inflorescence, and length of inflorescence, were evaluated. The temperature of the screen house was monitored using a thermometer to ensure that the plants grew under normal temperatures.

Statistical analysis

Data collected from growth studies were analysed with one-way analysis of variance (ANOVA). Least significant difference (LSD) was used to separate means at $P \leq 0.05$ level of significance. SPSS v16, Microsoft excel 2010 and Gen-Stat packages were used for computation, data analysis and graphics.

RESULTS

Soil analysis

The result of the analysis of the soil from the Botanic garden used in the raising the *A. hybridus* nursery showed no presence of Al (Table 2).

Antioxidant activity

Antioxidant activity of the extracted ly was found to be 71.9%. This was significantly different from that of the ethanol blank, which exhibited a lower percentage activity of 18.5%. According to Rhee (1978), the TBA test is a colorimetric technique that measures the absorbance of a red chromogen formed between TBA and malondialdehyde (MDA). Peak absorbance of the extracted ly at 375 nm was 1.266.

Hydrogen ion concentration

Adopting the formula of Stephenson (2010); the H^+ concentration of 3×10^{-2} mM of Al used for the studies (pH 4.6) was calculated to be 2.5×10^{-5} mol L^{-1} while that of extracted ly (pH 5.8) was calculated to be 1.5×10^{-6} mol L^{-1}.

Determination of actual Al concentration in solution

The actual Al concentration in solution based on the 3×10^{-2} mM Al used for the study was found to be 1.85 mg/L, using the Aluminon protocol.

Vegetative and reproductive data

Average growth of *A. hybridus* plants was stunted after subjection to Al stress. Visible symptoms such as yellowing, wilting and loss of leaves, as well as inhibition of root growth were characteristic of the experimental plants (Figures 13 and 15).

Number of leaves (NOL)

All the treatment significantly reduced the NOL when compared with the control. Al treatment reduced the NOL by 44% in comparison with the control. There was a significant difference between the NOL produced by the Al treatment (T7) and that of ly (50 µM) (T1) and Al-ly (200 µM) (T6). There was no significant difference between T7 and all the other treatments (Figure 1).

Plant height (PLH)

Similar to the situation in Figure 1, all the treatments in Figure 2 significantly reduced PLH in comparison with the control(C) (Figure 12). For T7, reduction of PLH was 36.5% when compared with C. Reduction of PLH by T7 differed significantly with that of T1 and C. The response of T6 did not differ significantly with that of Al-ly (50 µM) (T5) and T7 but differed with all the other treatments.

Length of inflorescence (LOI)

Figure 3 show that T7 decreased LOI by 37% in comparison with C. The response of T7 differed significantly with that of T1, ly (200 µM) (T2) and ly-Al (50 µM) (T3), but failed to differ significantly with those of ly-Al (200 µM) (T4), T5 and T6. Also, the response of T4, T5 and T6 did not differ significantly.

Number of inflorescence (NOI)

All the treatments in Figure 4 differed significantly with C by reduction in the NOI produced. T7 reduced the NOI by 33.5% in comparison with C. Response of the plants to T7 treatment differed significantly with that of T1, T2 and T4. It did not differ significantly with that of T3, T5 and T6. Also, the response of T6 differed significantly with that of T1, T2, T3 and T4.

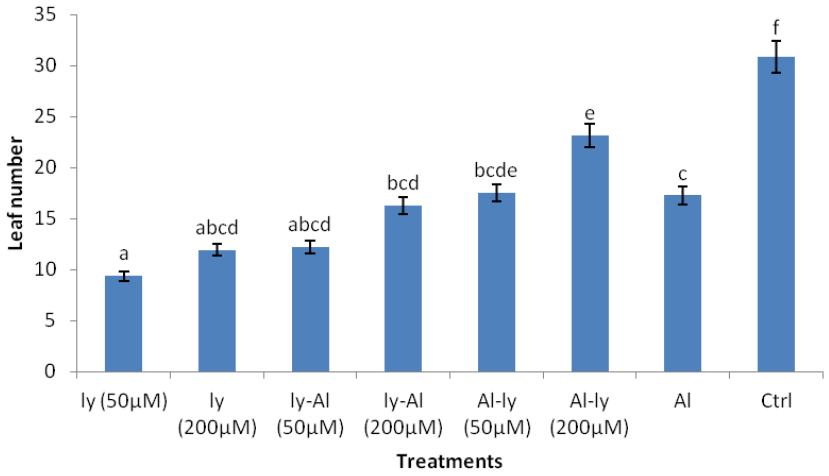

Figure 1. Effects of treatments on number of leaves (NOL). *Bars bearing different letters differ significantly (LSD.05).

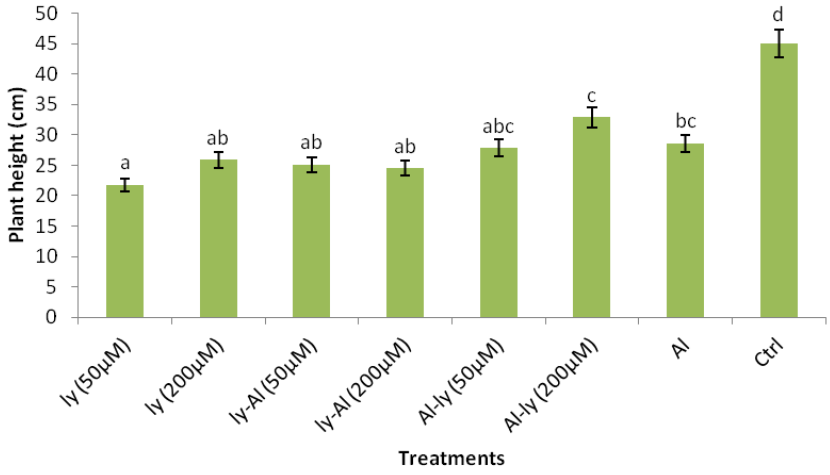

Figure 2. Effects of treatments on plant height (PLH). *Bars bearing different letters differ significantly (LSD.05).

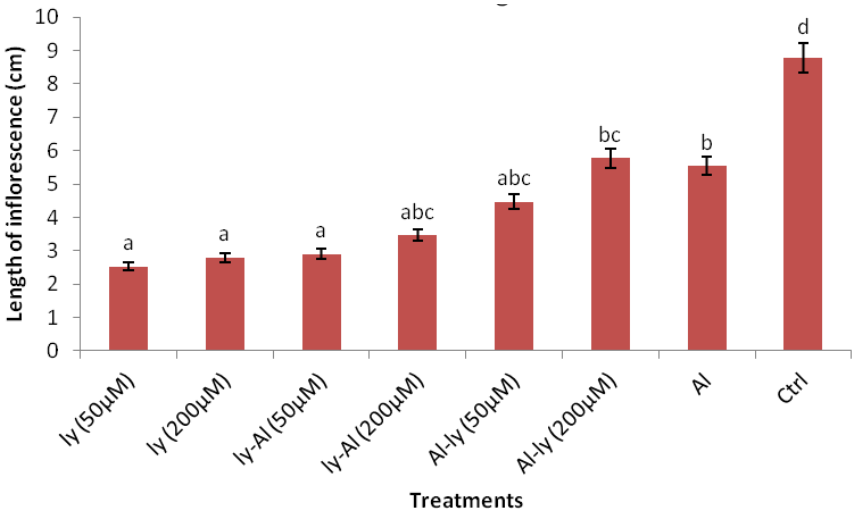

Figure 3. Effects of treatments on length of inflorescence (LOI). *Bars bearing different letters differ significantly (LSD.05).

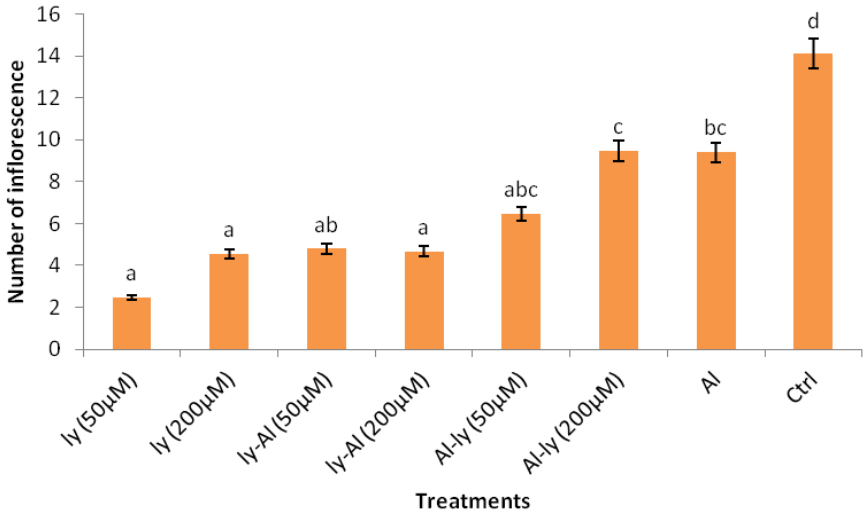

Figure 4. Effects of treatments on number of inflorescence (NOI). *Bars bearing different letters differ significantly (LSD.05).

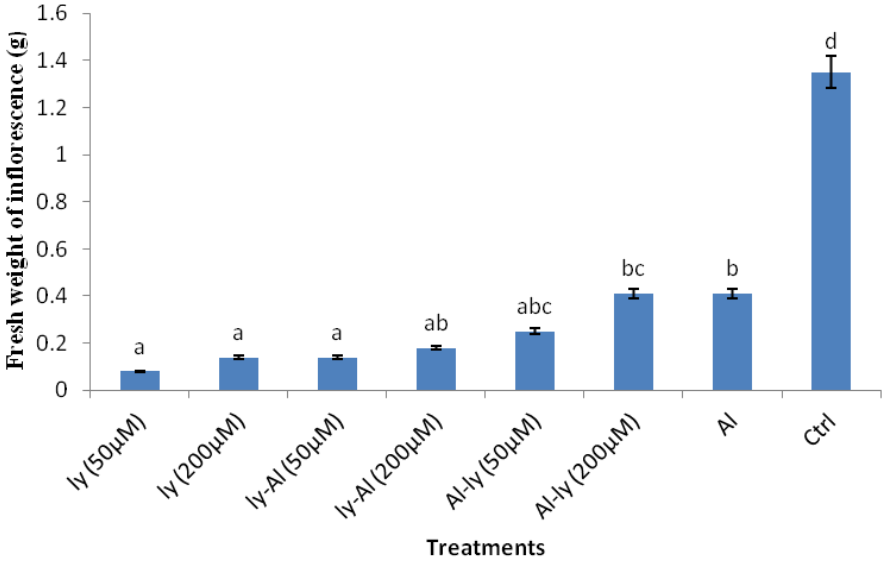

Figure 5. Effects of treatments on fresh weight of inflorescence (FWI). *Bars bearing different letters differ significantly (LSD.05).

Fresh weight of inflorescence (FWI)

As shown in Figure 5, all the treatments significantly reduced the FWI. For T7, the level of reduction in comparison with the control was a staggering 69.9%. The response of T7 differed significantly with that of T1, T2 and T3, but it did not differ significantly with that of T4, T5 and T6. Also while T6 differed significantly with T1, T2 and T3, it did not differ significantly with T4, T5 and T7.

Dry weight of inflorescence (DWI)

The response of all the treatments in Figure 6 differed significantly with that of C except for T7. For T7, the response of the plants, in comparison with the control, indicate a 31.3% decrease in DWI. The response of T7 did not differ significantly with that of T6 and C, but it differed significantly with that of the rest of the treatments. While response of plants to T6 differed significantly with responses to T3 and C, it did not differ significantly with that of T1, T2, T4, T5 and T7.

Fresh weight of shoot (FWS)

Figure 7 shows are similar to the other parameters, all the treatments significantly reduced the FWS in comparison

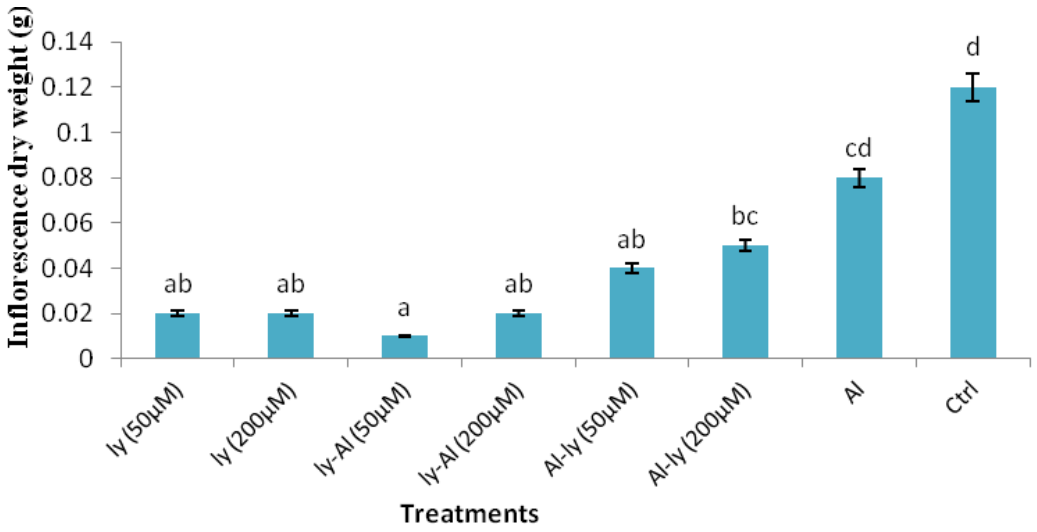

Figure 6. Effects of treatments on dry weight of inflorescence (DWI). *Bars bearing different letters differ significantly (LSD.05).

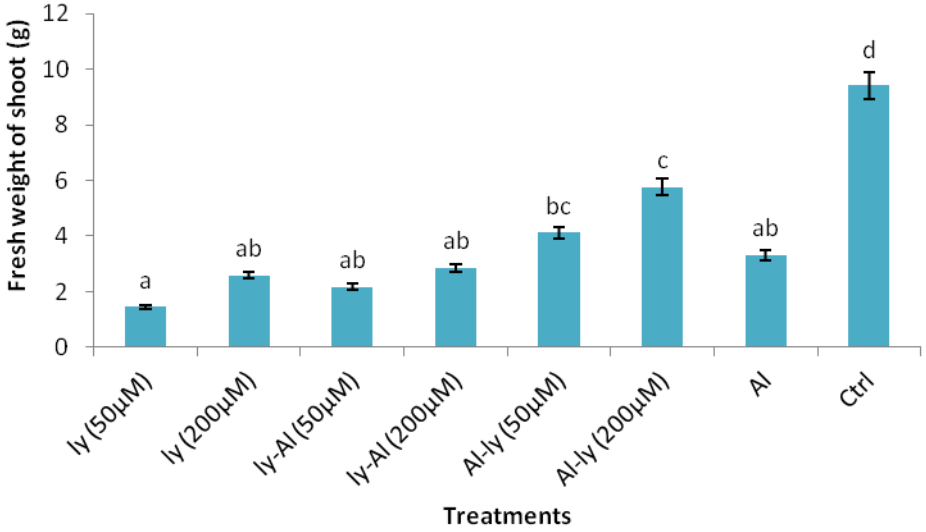

Figure 7. Effects of treatments on fresh weight of shoot (FWS). *Bars bearing different letters differ significantly (LSD.05).

with C. The level of reduction, for T7, was as high as 65%. This response differed significantly with responses from T6 and C but did not differ significantly with the rest of the treatments. T6 did not differ significantly with T5, but differed significantly with the rest of the treatments. While response to T5 differed significantly with that of T1 and C, it did not differ significantly with the rest of the treatments.

Dry weight of shoot (DWS)

Similarly, all the treatments significantly reduced the DWS when compared with C (Figure 8). A comparison between T7 and C showed a 54.9% level of reduction in DWS. T7 differed significantly from T6 but did not differ significantly with the rest of the treatments. While the response of T6 differed significantly from that of T1, T3, T4, and T7, it did not differ significantly with that of T2 and T5.

Root length (ROL)

The response of the plants to the treatments with respect to ROL (Figure 9), indicate that C (Figure 14) differed significantly with the rest of the treatments. T7 reduced root length by 57% in comparison with C. This response

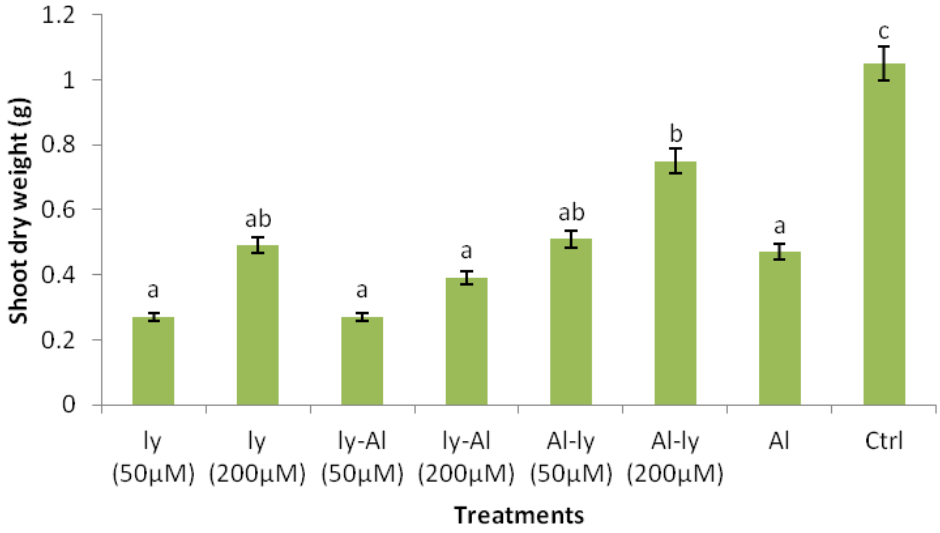

Figure 8. Effects of treatments on dry weight of shoot (DWS). *Bars bearing different letters differ significantly (LSD.05).

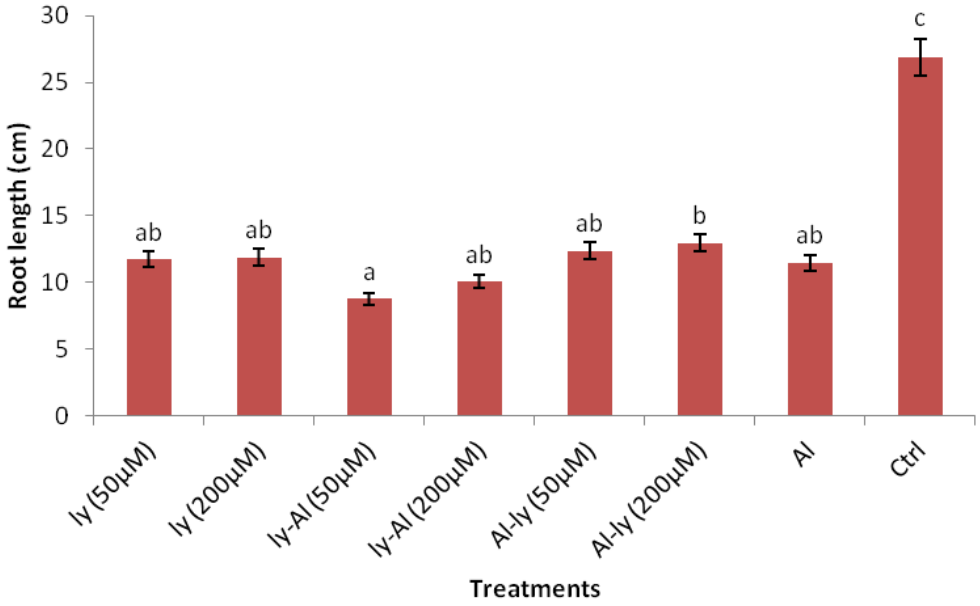

Figure 9. Effects of treatments on root length (ROL). *Bars bearing different letters differ significantly (LSD.05).

did not differ significantly with that of all the other treatments. T6 differed significantly with only T3.

Fresh weight of root (FWR)

The response of all the treatments measured in terms of FWR (Figure 10) turned out to be insignificant in comparison with each other (including C). Though T7 in comparison with C reduced FWR by 59.9%, but this turned out to be insignificant. Same is true for T1 and T5.

Dry weight of root (DWR)

Similar to the situation with FWR (Figure 10), the response of plants to all the treatments with regards to DWR (Figure 11) turned out to be completely insignificant. Incidentally, the response of the plants to T7 treatment was higher than that of all the treatments (including C) though this did not differ significantly with the other treatments. This shows that T7 treatment resulted to increase in DWR more than any other treatment. This was 13.5% higher than that of C. Also, T2

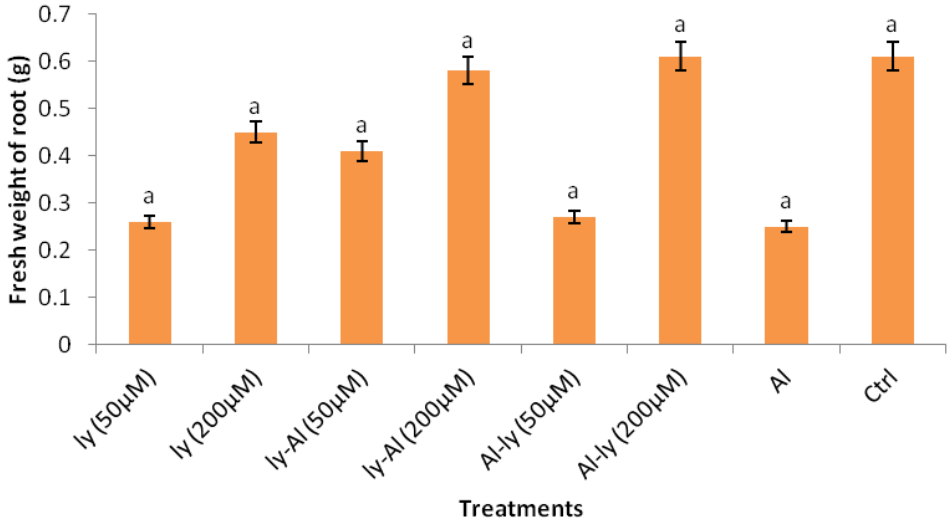

Figure 10. Effects of treatments on fresh weight of root (FWR). *Bars bearing different letters differ significantly ($LSD_{.05}$).

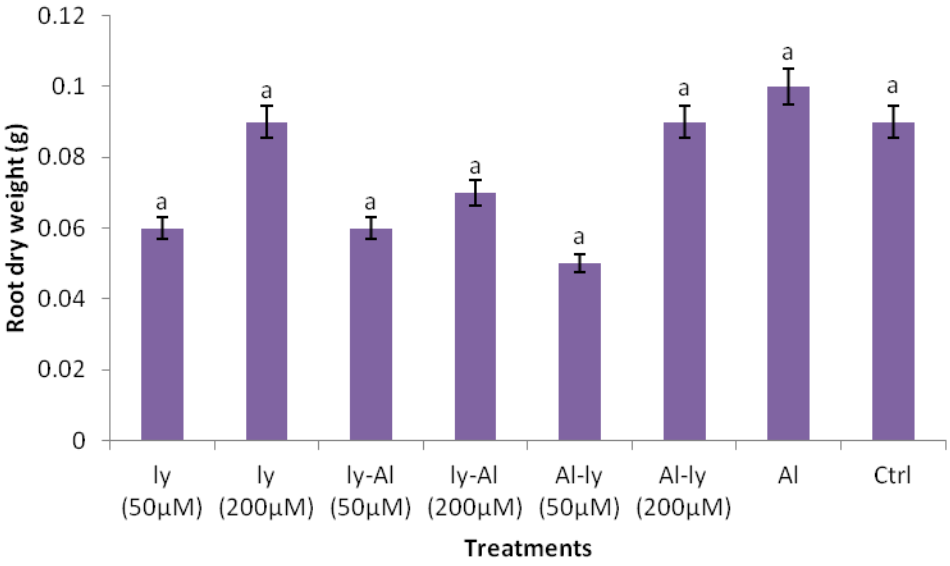

Figure 11. Effects of treatments on root dry weight (DWR). *Bars bearing different letters differ significantly ($LSD_{.05}$).

and T6 resulted to 1% increase in DWR in comparison with C.

DISCUSSION

The results of the analysis of the soil used for the raising of the seedlings showed complete absence of Al. This is an indication that the only source of Al in the study came from the Al treatments (T3-T7). Generally, as can be seen from the eleven parameters studied, Al treatment (which was the main focus of the study) significantly reduced both the vegetative and reproductive growths of *A. hybridus* at the low concentration of 3 x 10^{-2} mM used. This is an indication that the popular local *A. hybridus* cultivar, “Inine oma” used for the study is sensitive to low Al concentrations and is placed under the Al-sensitive group based on the classification of some tropical plants by Osaka et al. (1997) according to their Al tolerance capacity. Other workers have reported sensitivities of some plants to low Al concentrations. While Taylor et al. (1998) reported that in cowpea a toxicity threshold was observed at 0.1 µM Al and complete growth inhibition recorded at > 40 µM concentrations; Aftab et al. (2010)

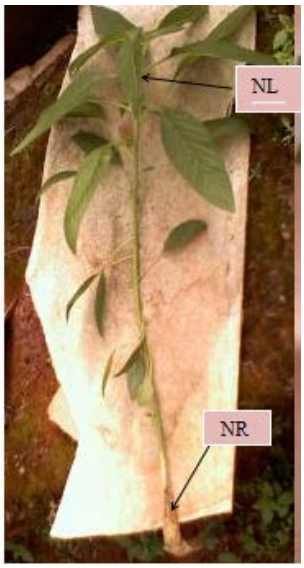

Figure 12. Control *Amaranthus* plant. NL = Normal leaves; NR = normal roots.

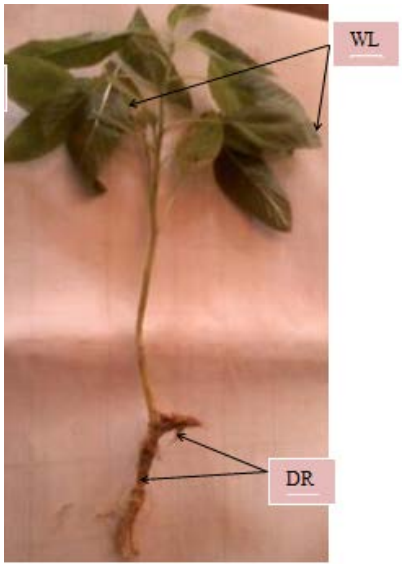

Figure 13. Aluminium toxicity stressed plant. WL=Wilting of leaves; DR= damaged roots.

observed that application of 0.01 and 0.10 mM Al concentrations on *Artemisia annua* resulted to a mild stress situation that gave rise to a higher production of artemisinin. This shows that low concentrations of Al could generate stress and reduce growth in some plants while it can enhance growth/ productivity in some others. A comparison between the effects of Al toxicity (T7) in

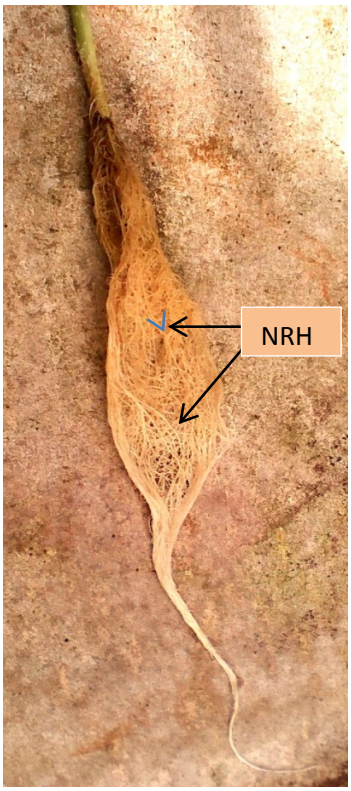

Figure 14. Roots of control plant enlarged. NRH= Normal Roots from Hydroponics.

Figure 15. Roots of Al toxicity stressed normal plant enlarged. DMLR = dense mesh of lateral roots.

relation to T5 and T6 treatments (which were the post-ly treatments) showed that there were more growth decreases among the vegetative parameters studied than the reproductive parameters. The difference in the decrease of number of leaves, fresh weight of shoot and dry weight of shoot was significant between T7 and T6 treatments, while it was non-significant for plant height, root length and fresh weight of root, respectively. This is thought to be caused by decrease in growth resulting from growing the plants, in T7 treatment for 21 d at 4.6 pH and higher H^+ concentration of 2.5 $x10^{-5}$ mol L^{-1}, while the ones given T5 and T6 treatments were first of all grown in the same 3×10^{-2} mM Al (T7) for 18 d, at the same 4.6 pH, before they were transferred into the ly (T1) and ly (T2) solutions for only 72 h, at a pH of 5.8 and lower H^+ concentration of 1.5 x 10^{-6} mol L^{-1}. Stressing the plants for 18 d (3 d less than T7) gave results closer to T7 and led to the reduction in growth of all the parameters. Growing them in the ly treatments, (T5 and T6), at a less acidic pH and lower H^+ concentration enabled them to recover non-significantly for plant height, root length and fresh weight of root but significantly for number of leaves, fresh weight of shoot and dry weight of shoot. The ameliorative role of ly was dose dependent with ly T2 showing better recovery than ly T1. Probably, if the plants were allowed to grow longer in the two ly concentrations, after Al treatment, better recovery results may have been achieved. This calls for further research to explore the possible effect of other concentrations of ly, as well as different durations of treatment, after Al stress. Aslanturk and Celik (2005) reported that ly had preventive effect on chromosome aberrations particularly at 1 and 3 µM concentrations but the effect decreased at 5 and 10 µM concentrations.

It is pertinent to emphasize that the observed decrease in growth should not be ascribed to the Al^{3+} in solution alone but also to the presence of relatively higher H^+ concentration of 2.5 $x10^{-5}$ mol L^{-1} based on the 4.6 pH of the solution. Soil Survey Division Staff- [SSDS] (1993) defined a solution with a pH of 4.6, as very strongly acidic. It is our view that the potential effects of H^+ under acidic conditions in rhizotoxicity studies have not been given adequate attention leading to gross exaggeration of the toxic effects of Al on plant roots. Based on the fact that Al^{3+} becomes available in solutions, under very strong acidic conditions, pH<5.5 (Kinraide, 1990; 1997; Harter, 2007; Silva, 2012), observed that growth defects are often attributed purely to the effect of Al^{3+}, completely ignoring the toxic effects of H^+, which at 2.5 $x10^{-5}$ mol L^{-1} could on its own totally disrupt growth, even in the absence of Al^{3+}. Koyama et al. (2000) and Kidd and Proctor (2001), noted that despite the large amount of literature on Al toxicity, very little attention has been given to H^+ toxicity, even though the latter is well known to be directly detrimental to root growth. Further reports indicated that as in Al toxicity, H^+ toxicity is most severe in solutions of low ionic strength and low cation concentrations, and increasing the concentration of Ca^{2+} and other cations in the external solution reduces or even abolishes the detrimental effects of acidity (Marschner, 1991). Furthermore, it was emphasized that an evaluation of these low-pH effects is necessary for greater understanding and correct interpretation in studies of Al toxicity, but regrettably this is rarely undertaken (Lazof and Holland, 1999; Samac and Tesfaye, 2003). Our finding are the actual Al concentration in solution at the 3×10^{-2} mM used for the study, using the Aluminon protocol, was 1.85 mg/L further buttressed the view that H^+ concentration of 2.5 $x10^{-5}$ mol L^{-1} of the Al solution used at 4.6 pH, played a key role in the observed decrease in yield of *A. hybridus*. The poor performance of *A. hybridus* plants treated with sole ly and ly (T1 and T2), as well as the two pre-ly (T3 and T4) treatments, in almost all the parameters studied, could be attributed to the effect of the H^+ concentration of 1.5 $x10^{-6}$ mol L^{-1} at the 5.8 pH of the solutions which is higher than the H^+ concentration of 4 x 10^{-7} mol L^{-1} at pH of 6.4 reported to be optimal for the growing of Amaranth plants (Singh and Whitehead, 1992; Palada and Chang, 2003). The 5.8 pH level was classified by SSDS (1993) as strongly acidic.

The observed dose dependent ameliorative effect of ly indicate that the Al stressed plants absorbed the supplemented ly antioxidants apparently because both the intrinsic and possibly stress-induced higher activities of antioxidant enzymes (not monitored) of the Al stressed plants were not enough for the plants to recover from the stress condition caused by Al toxicity. This view is supported by the findings of Gong et al. (2005), who stated that environmental stress increased the formation of reactive oxygen species (ROS) that oxidize membrane lipids, proteins and nucleic acids.

According to Sudhakar et al. (2001), under such conditions it is known that plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage. Additionally, it has been reported that with increasing amounts of Al in the soil medium, a concomitant increase in the activities of catalase (CAT), peroxidase (POX) and superoxidase dismutase (SOD) antioxidant enzymes were observed in the exposed plants (Boscolo et al., 2003; Siminovicova, 2004; Sharma and Dubey, 2007; Zhen et al., 2007; Aftab et al., 2010). The decrease in growth of the post- Al stressed (T3 and T4 treatments) plants (though not statistically significant in almost all the parameters) were more than those of the pre-Al (T5 and T6) treatments. The possible reasons for this is that growing the non-stressed plants in T1 and T2 treatments for only 72 h before transferring them to Al for 18 d (T3 and T4) had insignificant ameliorative effects for almost all the parameters in comparison with the T1, T2 and Al treatments. As already discussed under T1 and T2 treatment, *A. hybridus* plants that are not under stress may not need supplemented antioxidants, probably because they are adequately protected by their intrinsic

antioxidants. The essence of the antioxidants was to assist in mitigating stress conditions not to serve as growth stimulants. The 5.8 pH initial environment of the T3 and T4 treatments stopped the growth retardation process because of the H^+ in solution, despite the fact that the duration of treatment was only 72 h. The transfer of the plants to Al (T7) condition that lasted for 18 d subjected the plants to a greater stress conditions caused by both Al^{3+} and H^+, under a more acidic condition (pH 4.6 and H^+ concentration of 5.9), as against the milder acidic condition of pH 5.8 and H^+ concentration of 5.1 (for the T3 and T4 treatments). This can account for the non-significant difference between all the T3 and T4 treatments and Al (T7) treatment. In cases like plant height, fresh weight of shoot, dry weight of shoot and root length, growth suppression by Al was insignificantly lower than T3 treatment. The double growth decrease resulting from the initial growth in T1 and later in Al could be responsible for this. Additionally, the lower insignificant growth decrease of T4 over T3, treatment, as can be seen from leaf number, fresh weight of shoot, dry weight of shoot, root length, fresh weight of root; and dry weight of root can be as a result of the higher concentration of T1 in T2 being able to better prevent H^+, arising from the 5.8 pH of ly, from retarding the growth of the plants. Further studies will be needed to confirm this assumption.

The response of *A. hybridus* to the various treatments with respect to dry weight of root merits a separate discussion from the other vegetative parameters. Though the responses to all the other treatments were non-significant, this is the only case where there was growth enhancement based on Al treatment. The reasons why this should occur only in this parameter is not known. The number of roots could not be counted because of the mesh of deformed lateral roots formed due to Al toxicity. Despite the extensive wealth of information available about the effects of Al toxicity on the roots of plants, controversies still persist, meaning that much still need to be understood. Zobel et al. (2007) reported both root growth inhibition and increase in root diameter in roots exposed to Al. It has been suggested that the tolerance level of a genotype may not always be based on the number of primary roots and root length because both parameters may have similar results in stressed and stress-free environments. In this case, root vigour, root growth pattern, total root area, or total root mass of the corresponding genotypes under stressed and stress- free environments may be considered (Famoso et al., 2010; Roy and Bhadra, 2014).

On the other hand, it has been reported by other workers that Al in low concentrations could stimulate growth in some aspects of growth and productivity and not in others. Ou-yang et al. (2014) found that with the increasing of $AlCl_3$ concentration up to 1 mM, the fresh weight of cotyledons of *Jatropha curcas* had a little increase. On the other hand, the fresh weight of hypocotyls decreased gradually with increasing Al concentration up to 3 mM and the fresh weight of radicles showed a similar trend, but when Al concentration was higher than 1 mM, the development of radicles was completely suppressed. According to Aftab et al. (2010), the addition of Al to the soil medium significantly reduced the yield and growth of *Artemisia annua* plants, but the artemisinin content were higher when 0.10 mM Al was applied (51.8% more), as compared to untreated plants, however a decrease in artemisinin content was noted when further higher doses of Al were applied. The response of the reproductive parameters of length of inflorescence, number of inflorescence, fresh weight of inflorescence and dry weight of inflorescence, indicate that the decrease in the yield of these parameters by Al treatment were less than that of the other treatments, though the differences were not statistically significant. Though the concentration of Al in the different parts of the plant did not form part of this study, it is suspected that a possible reason for this could be reduced Al content of the reproductive parts of the plant which is probably a survival strategy to ensure conservation of the species germplasm, needed for the continuity of the species. The leaves, and not the reproductive parts, have been reported to be the major area of Al accumulation, especially in Al-accumulator species. Ma et al. (1997) remarked that Al ions translocate very slowly to the upper parts of plants and most plants contain no more than 0.2 mg Al g^{-1} dry mass. However, some plants known as Al accumulators, may contain over 10 times more Al in their leaves without any injury. Watanabe and Osaki (2002) reported that many Al accumulator species, especially woody plants, accumulate more than 10,000 mg Al kg^{-1} in the leaves, without injury to the plants. Specific classical examples of hyper-accumulators include tea plant (*Camellia sinensis*, in older leaves), *Hydrangea* and members of the Rubiaceae family; unfortunately, there is not much information in the literature as to mechanisms, cellular localization and chemical form of Al which accumulates in these plants (Vitorello et al*.,* 2005). The issue of the optimal concentration of ly needed to achieve highly significant ameliorative effect merits discussion. From the results obtained, it is suggestive that the higher dose of (T2) given to plants after Al stress yielded better ameliorative results even for the short duration of 72 h and extending the duration may possibly yield even better result. This merits a follow up investigation. This ameliorative effect of 50 and 200 µM ly on *A. hybridus* differs markedly from the situation in human and animal antioxidant supplementation results, as perhaps expected. Lowe et al. (1999) reported that low doses (1 to 3 µM) ly or β-carotene protected DNA from damage induced by xanthine/xanthine oxidase. By increasing the concentration to (4 to 10 µM) of the test substances, the opposite effects were observed at concentrations that were higher than physiological concentrations seen *in vivo* (Hwang and Bowen, 2005).

The bioavailability of ly to the experimental plants *in*

vitro is yet another curious issue. It is not known whether it is the cis or the trans-isomer of ly that could be more bioavailable to *A. hybridus* under Al stress. Again studies with human and animal models indicate that although the major form of ly in the diet is all trans-isomer, representing about 80 to 97% of total ly in tomatoes and related products (Nguen and Schwarz, 1998; Shi and Le Maguer, 2000), human blood and tissues contain mainly cis-isomers. Other studies have indicated that the cis-isomers are more bioavailable (Tyssandier et al., 2003; Khoo et al*.,* 2011) in humans and animals. This situation may again be different in plants and merits investigation. On the other hand, based on the reports of Lee and Chen (2002) that heating ly at 60 and 80°C favoured the isomerization of ly, it is suspected that in the course of this study, that processing of the tomato paste at 60°C, for the extraction of ly may have converted the naturally occurring trans-isomer to the cis-isomer. It is then hypothesized that it was the cis-isomer that the stressed *A. hybridus* plants absorbed that resulted to the observed ameliorative effect after Al stress. Perhaps once inside the plants they could be re-converted to the trans-isomer that make up almost the total amount of ly in tomatoes as reported by (Nguen and Schwarz, 1998; Shi and Magner, 2000). This however needs to be investigated through further studies. The report of Krinsky (1998) has provided good insight into the mechanism of the action of ly. Though ly is generally known to be a lipophilic substance; which may raise the possible question about its solubility and hence bioavailability in the 1% ethanol solvent used in this study, following the protocol of Fiskesjo (1981). Van Breemen et al. (2008) reported that besides its radical reactions, ly has also been shown to up-regulate the so-called antioxidant response element (ARE). Cellular enzymes, like glutathione S-transferase, super-oxide dismutase or quinone reductase, are activated by ly, resulting in another way of protecting cells against highly reactive oxygen species. Additionally, Linnewiel et al. (2009) found that hydrophilic oxidation products of carotenoids, rather than the intact lipophilic carotenoid molecules, were responsible for the stimulation of the ARE system, detected *in vitro* using LNCaP and MCF-7 cells.

Oxidized ly derivatives, built, due to the instability of these molecules, are present in tomatoes, as well as in human serum and tissues (Holzapfel et al*.,* 2013). From the foregoing, it is opined that the *A. hybridus* plants were able to possibly benefit from the lipophilic as well as the hydrophilic oxidation products of ly. This is also an area for further investi-gation in the study of the ameliorative role of supplemented ly in plants.

The present study has raised more questions than answers in this apparent controversial area of study; where many questions are still begging for answers. With further studies, this area of supplementing Al stressed plants, or plants subjected to other forms of environ-mental stress, either through soil application or spraying of the aerial parts of plants, may be found to contribute positively towards the alleviation of environmental stress in plants, leading to the reduction of the food security challenges in sub Saharan Africa and perhaps other parts of the world.

Conclusion

In conclusion, this present study has shown that the local cultivar of *A. hybridus* (Inine oma), used for this study is an Al sensitive plant. The vegetative parts were more sensitive than the reproductive parts and as such recorded more decrease in growth. *A. hybridus* plants appear not to need ly while not under Al stress. Pre-lycopene treatment had little or no ameliorating effect on Al stressed plants while post-lycopene treatment recorded more ameliorating effect in most of the parameters studied though only few were statistically significant. The ameliorating effect of ly was found to be dose dependent which calls for further research to find the optimal dose. This area of study has received little or no scientific attention, apparently because plants are known to possess their intrinsic antioxidants for fighting ROS. However, the fact that crop losses (even by elite cultivars) continue to be recorded due to crops succumbing to diverse and complex environmentally based stresses; argues strongly in favour of exploring alternative ways of improving on the capacity of plants to withstand stress situations. Antioxidant supplementation may turn out to be equally beneficial to plants under stress, similar to the enormous role it has been playing in ameliorating stress conditions in man and animals.

Conflict of Interests

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

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academicJournals
Vol. 13(49), pp. 4492-4500, 3 December, 2014
DOI: 10.5897/AJB2014.14157
Article Number: 330B62A48911
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Response of stay-green quantitative trait locus (QTL) introgression sorghum lines to post-anthesis drought stress

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Received 5 September, 2014; Accepted 17 November, 2014

An experiment was carried out to evaluate the response of sorghum stay-green quantitative trait locus (QTL) introgression lines under induced post-flowering drought stress. The QTL introgression was done in 2006 to 2008 from known stay-green lines to the locally adapted varieties through marker assisted backcrossing. The field experiment was carried out in 2009/2010 and involved two irrigation levels and 14 genotypes organized in a split plot design with three replications. Analysis of variance showed significant difference among the genotypes for all the measured nine morpho-physiological quantitative characters. Significant differences were also observed in leaf area, head exsertion, grain yield and hundred seed weight for irrigation indicating that the two irrigation levels were able to differentiate the genotypes for these characters. Genotype-by-irrigation interaction was significant only for head weight and 100 seed weight. In general, a yield reduction of up to 49% was observed in an induced post-flowering moisture deficit. Grain yield had strong positive correlation with head weight (r=0.66) and hundred seed weight (r=0.52) under conditions of moisture deficit. Most of the stay-green introgression lines included in this experiment maintained at least 25% of their green leaf area until maturity though some showed early leaf senescence, but there was no associated increase in grain yield. Possible reasons are discussed.

Key words: Drought, introgression, post-anthesis, QTL, stay green, sorghum.

INTRODUCTION

Drought is the single greatest problem limiting crop productivity in the semi-arid tropics (SAT) where most of the production systems are rain-fed (Serraj et al., 2005). The trade-offs between the trends of increased population size and the associated increased demand for food on one hand and the decrease in availability of water on the other hand call for the development of more water efficient (climate smart) crops (Balota et al., 2008). Sorghum is one of the most important food, feed and bio-energy crops in the world, which tolerates drought stress better than most other cereals. It is also an important food crop in sub-Sahara Africa including Ethiopia on which millions of poor people depend. In Ethiopia, it grows in a wide range of agro-ecological zones, but it is more important in the dry lowlands where crop failure due to recurrent drought stress is common (Adugna, 2007). Cates (1922) has applied the name "crop camel" to kafir (one of the grain sorghums) because the plants can stand for considerable dry periods without apparent suffering from a deficiency of moisture. Since the crop is

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both drought tolerant and highly responsive to added water, it is adapted to both dryland and irrigated conditions (Eck and Musick, 1979).

Drought may occur at any stage during the growth cycle of the sorghum crop. Sorghum exhibits two distinct responses to drought stress (Rosenow et al., 1983). One occurs when plants are stressed during the head development stage prior to flowering, called *preflowering,* and the second occurs when plants are stressed after anthesis and during grain development, called post-flowering (Walulu et al., 1994). When it occurs as preflowering during the first growth stage (GS1), the period between seedling emergence and panicle initiation (Quinby, 1974), it affects the vegetative growth and the time of floral initiation. Drought at second growth stage (GS2), the period between panicle initiation and anthesis, affects the number of seeds that will be produced, whereas at the third growth stage (GS3), the period between floral anthesis and physiological maturity, it affects seed weight as the grain does not fill well. Therefore, grain yield reduction becomes relatively worse when drought occurs at the last two growth stages (GS2-pre flowering and GS3-post-flowering) than at GS1 since seed number and weight are the most important grain yield components in sorghum. The reason why terminal drought stress might cause yield reduction is that, the affected plants mature early as a result of the induction of premature leaf senescence (forced maturity) (Gregersen et al., 2013). Genetic variation for resistance to drought at each stage has been observed in sorghum germplasm (Walulu et al., 1994). Hence, many genotypes with a high level of resistance at one stage were found to be susceptible at the other stage (Rosenow et al., 1983). Until recently, the global sorghum improvement approach towards alleviating the effect of drought has been focused on the development of short duration varieties, those that can escape drought occurs late during the season (terminal drought). Based on this approach a number of early maturing varieties have been released and are still being produced globally including Ethiopia. However, even these early maturing types can fail to produce if drought occurs at the critical stages. For example, in pearl millet, Mahalakshmi et al. (1987) found that when drought occurred during the midseason, early genotypes, which were proved to effectively escape a terminal drought stress suffered greater grain yield reduction than the later flowering genotypes.

Recently, the focus of research has been changed to the development of drought tolerant varieties helped by marker assisted selection. The best example of marker assisted selection against drought in sorghum is for the stay-green trait (Rosenow et al., 1983). The term stay-green refers to delayed senescence, which is associated with post-flowering drought tolerance irrespective of the maturity of the genotype (Ejeta and Knoll, 2007). It focuses mainly on alleviating the effect of drought at grain filling as drought stress during this stage in sorghum usually results in rapid premature plant senescence (Stout and Simpson, 1978). Sorghum genotypes with functional stay-green continue to fill their grain normally under drought (Rosenow and Clark, 1981; Borrell et al., 2014). Such stay green trait, producing healthier stems on plants, may indirectly contribute to higher crop performance by providing disease resistance, standability, resistance to lodging, and higher seed quality characteristics (Cukadar-Olmedo and Miller, 1997; Cattivelli et al., 2008).

Moreover, previous reports suggest that the Stay-Green trait in sorghum and rice might contribute to increased post-anthesis biomass production under drought stress (Borrell et al., 2000; 2001). Therefore, stay-green has been suggested as an indirect selection criterion for post-flowering drought tolerance (Rosenow et al., 1983). Molecular markers linked to the already mapped sorghum stay green QTLs are available (Hash et al., 2003; Harris et al., 2007) and with which marker assisted backcrossing has been undertaken (Kassahun et al., 2010). This study was, thus intended to evaluate the response of stay-green QTL introgression sorghum lines developed using marker assisted backcrossing and their parents for induced post-anthesis drought stress.

MATERIALS AND METHODS

Plant materials

Sorghum varieties adapted to the dry lowlands of Ethiopia; 76T1#23, Teshale, Gambella 1107, and Meko were selected for the backcrossing program. All of them, except Gambella 1107 were selections from ICRISAT introduced regional trials. Gambella 1107 is a selection from landraces in Gambella area, Ethiopia. These varieties have maturity days ranging from 90 to 130 days. So far, the sources of the stay-green QTLs that are widely used in sorghum have been B35 and E36-1 lines, which were originally obtained from Ethiopia (Reddy et al., 2009). B35 is a BC_1 derivative of landrace germplasm accession IS 12555, which is a durra sorghum (Rosenow et al., 1983). A Marker assisted backcrossing program was held in Ethiopia between 2006 and 2008 and carried out at Melkassa Agricultural Research Center in collaboration with ICRISAT, Nairobi to introgress stay-green QTLs from B35 and E36-1 to the locally adapted early maturing, but senescent sorghum varieties (76T1#23, Teshale, Gambella 1107, and Meko). Starting from the first backcrossing, all plants in a 5 m nursery plot were tagged and leaf samples were taken from each of them and sent to the Bio-Sciences for East and Central Africa (BECA) laboratory in Nairobi, Kenya for marker assisted selection of the stay green QTLs. Based on the marker information, those plants which had one or more stay-green QTLs from the donor parents were backcrossed to the respective locally adapted lowland sorghum varieties used as recurrent parents. In total, six stay-green QTLs conferring post-anthesis drought stress tolerance: *StgA*, *Stg1*, *Stg2*, and Stg4 from B35 and *SBI-01* and *SBI-10* from E36-1 were introgressed into the four elite local varieties in three molecular marker (SSRs) assisted backcrosses and the resulting populations were self-pollinated three times. Thus, all of the introgression lines used in this study had one or more of the stay-green quantitative trait loci (QTLs) (Table 1). Hence, eight backcross populations (BC_3F_3) here after referred to as stay-green QTL introgression lines resulted from the four lowland sorghum

Table 1. Genotype means for the measured quantitative characters (in parenthesis are stay-green QTLs).

Genotype	HL	HW	LA	PE	GY	HW	HSW
Teshale x B35 (*Stg2*)	19.8	11.0	368.6	13.86	316.0	65.8	3.1
Meko x B35 (*Stg1*, *Stg4*)	23.3	11.4	423.3	2.08	438.0	115.2	3.9
Gambella 1107 x B35 (*StgA*)	22.5	9.6	582.3	1.74	490.4	116.3	3.5
76T1#23 x B35 (*StgA*)	20.4	9.3	357.5	10.10	307.5	78.5	3.1
Teshale x E36-1 (*SBI-01*)	21.4	11.5	483.4	11.48	443.0	92.7	3.1
Meko x E36-1 (*SBI-01*)	23.7	12.5	517.1	1.24	626.3	128.7	3.8
Gambella 1107 x E36-1 (*SBI-01*)	21.1	10.3	604.6	4.49	588.5	117.0	3.7
76T1#23 x E36-1 (*SBI-10*)	19.2	9.7	393.8	7.74	392.8	91.9	3.0
Teshale	19.9	13.3	417.8	10.57	486.9	108.6	3.4
Meko	21.4	10.8	570.1	2.91	611.5	121.9	3.6
Gambella 1107	21.1	10.5	585.0	2.17	554.5	122.9	3.4
76T1#23	20.6	9.7	371.1	7.84	417.5	89.7	3.2
B35 (*Stg1, Stg2, Stg4, StgA*)	23.2	9.6	393.1	18.11	317.5	89.7	2.8
E36-1 (*SBI-01, SBI-10*)	21.9	12.7	449.8	6.76	469.0	118.4	3.9
Mean	21	11	466	7.22	461.4	104.1	3
LSD (0.05)	1.1	1.5	68.1	2.70	58.5	10.5	0.2

PH, plant height recorded at physiological maturity; LA, leaf area; HL, head length, HW, head width; PE, Peduncle exsertion; HWT, head weight; GY, grain yield; HSW, 100 seed weight.

varieties and the two stay green source lines (B35 and E36-1) were included in the experiment. Moreover, the 6 parents (donor and recurrent) were included in the experiment for comparison (Table 1).

Experimental setup

The field experiment was carried out during the off-season (from 16 December 2009 through 24 April 2010) at Melkassa Agricultural Research Center located in the Central Rift Valley of Ethiopia (39°21′E, 8°24′N, altitude=1550 m) by inducing drought at the post-anthesis stage. The soil at the experimental site is silty clay loam Andosol with a pH of ~7.8. It was a split plot design with three replications. The main plot factor was irrigation with two levels (I_0 = irrigation withheld after anthesis and I_1 = irrigation continued after anthesis until maturity) and the sub-plot factor was the genotypes with 14 levels (consists of 8 introgression lines, 2 donor, and 4 recurrent parents). The two groups of the irrigation (flood) treatments differed in three frequencies during the post-flowering stage. In the first case (I_1), all of the 14 genotypes were irrigated every seven days throughout their growth stages. In the second (I_0), however, the replicas of all of the genotypes did not receive irrigation after anthesis (the last irrigation was given just at anthesis). Hence, the total number of treatments was 2 × 14 = 28. The border of each block was sown to the variety Melkam to avoid border effect. Each plot had a single row of 4 m length and the inter-row space was 75 cm. the space between the two main plots was 1.5 m. The treatments were randomized within each main plot. The seeds were sown by hand drilling and later thinned to 15 cm between plants to give a total population density of 88 888 plants per hectare. The management practices including fertilizer and weeding except irrigation were done as per the recommendation for sorghum. Accordingly, DAP fertilizer was applied at the rate of 100 Kg/ha during planting in the seed furrow and Urea was applied at the rate of 50 Kg/ha as top dressing before booting. The field was kept free from weeds throughout the experiment. To control shoot flies and stem borers, Karate 5% EC was applied at the rate of 320 mm/ha two weeks after emergence.

Data recording and statistical analysis

As there is heavy weaver bird (*Quelea quelea*) pressure at Melkassa during the off-season, it was difficult to measure plot yield. Therefore, five plants were bagged with cloth bags from each row plot after anthesis and used for data recording. Data on days to flowering (DTF), plant height (PH) recorded at physiological maturity, leaf area (LA), head length (HL), head width (HW), Peduncle exsertion (PE), head weight (HWT), grain yield (GY) (Kg per plot, in this case mean of 5 plants), and 100 seed weight (HSW) at 12% moisture content, were measured from the five plants based on the new key access and utilization of sorghum descriptors (Bioversity International, 2010). In addition, the visual leaf senescence data scoring was modified from the descriptors for sorghum (IBPGR/ICRISAT, 1993) as 0 = ~100% green leaf area; 1=75% green leaf area; 2= 50% green leaf area; 3= 25% green leaf area; 4 = 100% leaves and stalk dead. This data were recorded at physiological maturity. Leaf area was calculated as Leaf length × Leaf width × 0.71 following Krishnamurthy et al. (1974). The data were subjected to analysis of variance to see individual treatment and interaction effects for all the measured quantitative characters using SPSS Release 17 Software. Moreover, paired t-test was performed in all means of the measured characters to evaluate the trend of the differences between irrigated and non-irrigated treatments using the protected LSD procedure at P = 0.05 (Carmer and Swanson, 1973).

RESULTS

The result of this study showed a high significant difference in the genotypes for all the traits measured; indicating that the tested genotypes were diverse. Analysis of variance (ANOVA) showed highly significant difference ($p<0.01$) for genotypes factor for all the traits measured, which indicated that the tested genotypes were diverse. Highly significant differences were also

Table 2. Mean values of the measured characters for irrigation.

Irrigation	DTF	PH	HL	HW	LA	PE	GY	HW	HSW
I_0	79.04	159.08	20.99	10.67	423.39	8.28	433.40	94.33	3.28
I_1	79.81	161.37	21.79	11.04	507.68	6.17	489.37	113.84	3.48
LSD (0.05)	2.68	5.89	1.94	0.68	104.36	1.01	32.35	7.43	0.06

DTF, flowering; PH, plant height recorded at physiological maturity; LA, leaf area; HL, head length, HW, head width; PE, Peduncle exsertion; HWT, head weight; GY, grain yield; HSW, 100 seed weight.

Table 3. Genotype × irrigation interaction means of the stay-green QTL introgression lines and their parents for the different characters measured after harvest.

Genotype	HWT		GY		HSW	
	I_1	I_0	I_1	I_0	I_1	I_0
Teshale × B35	71.48	60.07	337.00	295.00	3.10	3.00
Meko × B35	119.20	111.23	449.00	427.00	3.90	3.80
Gambella 1107 × B35	134.44	98.11	524.33	456.50	3.57	3.47
$76T_1$#23× B35	82.13	74.81	368.00	247.00	3.15	3.13
Teshale × E36-1	118.67	66.64	463.00	423.00	3.55	2.65
Meko × E36-1	144.08	113.35	629.50	623.00	3.90	3.65
Gambella 1107 × E36-1	122.23	111.82	599.50	577.50	3.60	3.70
$76T_1$#23 × E36-1	95.69	88.03	419.00	366.67	3.13	2.90
Teshale	130.67	86.55	507.33	466.50	3.63	3.10
Meko	137.54	106.30	607.00	616.00	3.87	3.33
Gambella 1107	122.37	123.40	625.50	483.50	3.20	3.50
$76T_1$#23	99.40	79.94	451.50	383.50	3.30	3.03
B35	93.30	86.11	333.00	302.00	2.87	2.80
E36-1	122.60	114.28	537.50	400.50	3.97	3.83
Mean	113.84	94.33	489.37	433.40	3.48	3.28
SE± mean differences	4.372		12.573		0.079	
CV (%)	8.7		11		6.1	
Paired t test of the differences	4.46		4.45		2.57	
p(≤0.05) two-tail	0.001		0.001		0.023	

observed among the genotypes for leaf area, head length, head weight, grain yield, and hundred seed weight in response to the induced post-anthesis drought stress, which was further confirmed by paired t-test (Table 1). In these characters, the stressed plots showed reduced mean values. Although, significant differences were observed between mean grain yield, panicle exsertion and head weight of irrigated and non-irrigated sets of genotypes with higher values being in the former (Table 2), genotype-by-irrigation interaction was not significant for these traits except head weight (Tables 3 and 4).

Effect of induced post-anthesis drought stress on individual traits

Grain yield had significant positive correlation ($p<0.05$) with head weight ($r=0.66$) and hundred seed weight ($r=0.52$) under moisture stressed condition. The reduction in leaf area was significant and ranged from 8.3% (B35) to 45.6% (Gambella 1107 × B35) in response to the changed irrigation levels. Genotype-by-irrigation interact-tion was significant for head weight indicating differential response of the genotypes in response to irrigation levels for this trait. There was a wide range of head weight reduction between 0 and 78%. Head weight had positive correlation ($r=0.66$) with grain yield under non-irrigated condition and even the correlation was stronger ($r= 0.87$) under irrigated condition. Even though there is significant difference among the mean grain yield and panicle exsertion of irrigated and non-irrigated sets of genotypes, the absence of genotype-by-irrigation interaction in these traits may indicate that moisture stress at the post-anthesis stage affects these traits regardless of genotype. In a well-watered condition, Gambella 1107

Table 4. Genotype × irrigation interaction means of the QTL introgression lines and their parents for the different characters measured during pre-harvest

Genotype	DTF		PH		HL		HW		LA		PE	
	I_1	I_0	I_1	I_0	I_1	I_0	I_1	I_0	I_1	I_0	I_1	I_0
Teshale × B35	81.00	81.00	179.20	178.20	19.20	20.33	10.00	12.07	416.66	320.52	13.86	13.87
Meko × B35	77.67	77.00	153.73	151.07	23.87	22.80	11.33	11.53	481.35	365.15	0.97	3.20
Gambella 1107 × B35	86.00	84.33	164.18	167.73	23.00	22.07	10.60	8.67	690.42	474.10	0.78	2.70
$76T_1$#23 × B35	74.33	72.00	142.20	140.40	21.33	19.40	9.27	9.27	398.66	316.32	7.63	12.58
Teshale × E36-1	82.67	81.67	215.93	215.93	21.27	21.53	11.47	11.53	561.69	405.14	10.84	12.13
Meko × E36-1	80.67	80.00	157.20	158.28	24.07	23.40	12.93	12.13	553.61	480.66	0.78	1.70
Gambella 1107 × E36-1	84.50	83.67	176.07	171.43	22.07	20.20	10.67	9.87	632.01	577.23	2.87	6.11
$76T_1$#23 × E36-1	77.00	75.00	139.13	139.93	19.80	18.53	9.87	9.47	432.20	355.34	5.02	10.47
Teshale	80.33	79.67	209.87	203.40	19.93	19.80	14.80	11.80	439.26	396.37	9.23	11.92
Meko	81.33	80.33	166.20	146.93	21.80	20.93	10.67	10.93	603.57	536.58	3.33	2.50
Gambella 1107	82.67	82.50	162.00	160.13	21.40	20.73	10.60	10.47	626.24	543.78	2.72	1.63
$76T_1$#23	73.33	72.00	137.13	136.40	21.07	20.13	10.07	9.33	387.49	354.66	6.75	8.93
B35	78.33	79.00	90.07	87.10	24.27	22.20	9.53	9.60	408.79	377.36	16.13	20.10
E36-1	77.50	78.33	166.30	170.22	22.00	21.73	12.73	12.73	475.51	424.18	5.47	8.07
Mean	79.81	79.04	161.37	159.08	21.79	20.99	11.04	10.67	507.67	423.38	6.17	8.28
SE± mean differences	0.243		1.510		0.234		0.307		13.550		0.524	
CV (%)	3.17		5		4.6		12.1		12.6		32.3	
Paired t test of the differences	3.53		1.52		3.45		1.19		6.22		-4.03	
p(≤0.05) two-tail	0.007		0.153		0.004		0.254		0.000		0.001	

gave the highest and B35 the lowest yield of all the parental lines. Meko × E36-1 gave the highest yield of all the introgression lines under both water regimes. Meko and its introgression line, Meko × E36-1 gave comparative grain yield under both well watered and water deficit conditions. On the contrary, B35 and its introgression lines with 76T1#23 and Teshale gave relatively lower grain yield (Figure 1). Irrigation factor was significant ($p<0.05$) for hundred seed weight. When the data were pooled for all the genotypes, the range of reduction of hundred seed weight in response to the induced post-flowering drought was 0.5% (76T1#23 × B35) to 34.0% (Teshale × E36-1). All of the introgression lines, except Teshale × E36-1 and crosses of Gambella 1107, had lower sensitivity to the simulated drought stress response to seed weight than their recurrent parents (Table 3). Hundred seed weight had strong positive correlation (r=0.52) with leaf area. It had also very high positive correlation (r=0.79) with head weight under moisture stress and even higher positive correlation (r=0.84) under irrigated condition. Consequently, withholding irrigation during post-anthesis stage significantly reduced weight of the seeds by affecting grain filling (Table 2).

Genotype-by-irrigation interaction was also significant for hundred seed weight indicating differential response of the genotypes to the varying irrigation levels for this trait. However, hundred seed weight in the introgression lines alone had no correlation with leaf area (r=0.427, p=0.164) under stressed condition. It had also very high positive correlation with head weight under both moisture stress (r=0.887, p=0.003) and well watered (r=0.876, p=0.004) conditions. Irrigation factor was also significant ($p<0.05$) for peduncle exsertion. Twelve of the 14 introgression lines showed an increase in peduncle exsertion in response to moisture stress. The highest increase was observed in $76T_1$#23 × E36-1 followed by $76T_1$#23 × B35, both introgression lines of 76T1#23 with the two stay green source lines.

Leaf senescence is the most pertinent trait so far as this study was concerned. Five of the 14 genotypes, viz., Meko and Gambella 1107 and their introgression lines Meko × E36-1, Gambella 1107 × E36-1, Gambella 1107 × B35 showed early leaf senescence in one or more of the stressed plots. However, high level of average leaf senescence was visually observed in Gambella 1107 × B35, Meko, and Gambella 1107. The stay-green source line, B35 and its introgression lines 76T1#23 × B35 and Meko × B35 (Figure 2) maintained their green leaves until maturity.

DISCUSSION

Effect of induced post-anthesis drought stress on performance of the genotypes

Leaf characters

Even though no leaf size increase is generally expected

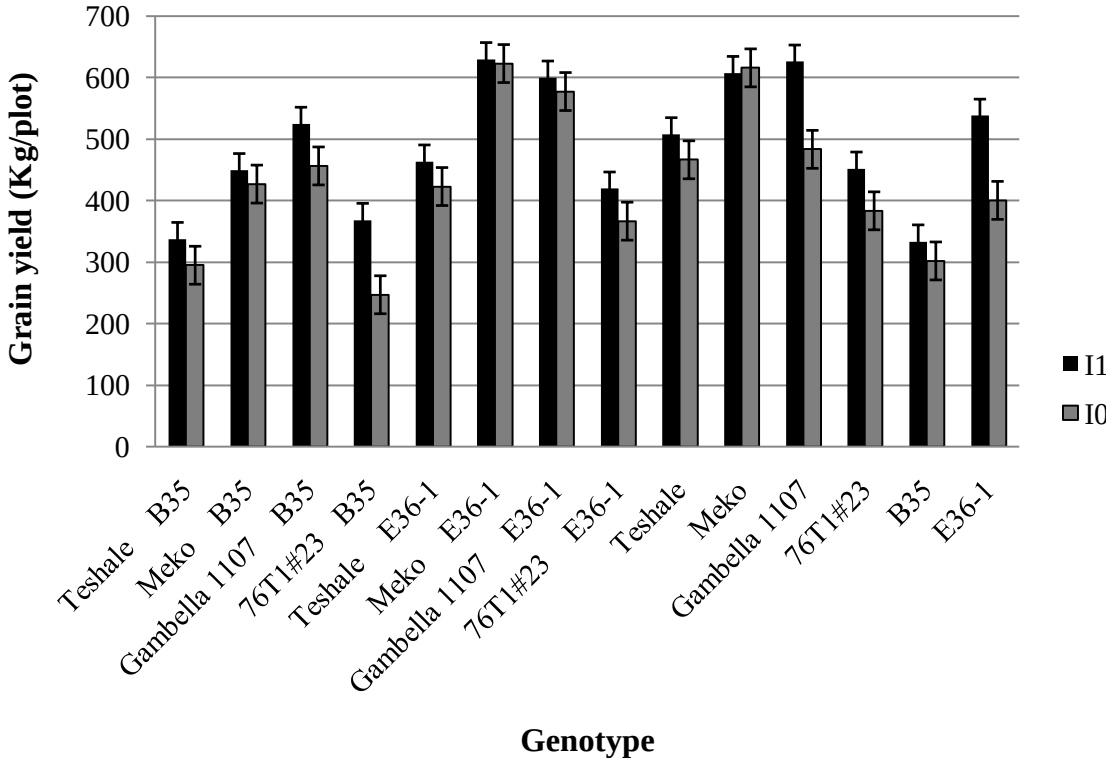

Figure 1. Comparison of the sorghum genotypes for grain yield under well watered (I_1) and water deficit (I_0) conditions (bars are standard error of means).

Figure 2. Differences in leaf senescence in the stay-green introgressed line, Meko × B35 (Left) and its recurrent parent Meko (Right) under water deficit condition (to the extreme left is a border row).

beyond flowering, the reduction in leaf area in this experiment was probably due to contraction of the full sized leaves in response to the induced moisture deficit. However, leaf area was highly and positively correlated with grain yield (r=0.885, p=0.004) under moisture deficit. It has been reported that decreasing leaf area reduces crop water use and results in reduced grain yield (Borrell et al., 2014; Vadez et al., 2014). Early studies by Swanson (1941) and Blum et al. (1989) showed that early maturing varieties having a small leaf area are the most efficient in the production of grain per unit of leaf area whereas later varieties have a higher biomass, higher daily transpiration and a longer transpiration period and are likely to suffering dry seasons. In the present study, most of the introgression lines showed higher sensitivity to moisture stress with respect to the change in leaf area. Perhaps that was one mechanism of drought tolerance beyond the maintenance of green leaf area that the stg QTLs are contributing. In their recent study aiming at investigating the positive effect of *Stg* QTLs on grain yield under drought, Borrell et al. (2014) concluded that leaf area and transpiration per unit leaf area could be some of the mechanisms that Stg loci have impacts to regulate transpiration on the demand for water. Although, the reduction in the mean values in the remaining characters is a disadvantage, the reduction in leaf area could be considered as an important adaptive physiological mechanism expressed in response to the changed osmotic potential contributing to drought tolerance. In agreement with this finding, Swanson (1941) reported that leaf area was the greatest when there was an abundant moisture supply and the influence of drought on leaf development was shown by the results when the rainfall was deficient throughout the growing season. The same study showed that leaf area per acre in droughty season was 73 to 81% less than when there was abundant moisture and very heavy leaf development.

Inheritance study of Walulu et al. (1994) suggested that the stay green traits in one of the known source lines, B35 is influenced by a major gene but, later, it has been mapped as a quantitative trait and four QTLs controlling the stay-green (*Stg1* through *Stg4*) were identified (Xu et al., 2000). Moreover, the effect of environment on expression of the stay-green trait in sunflower was suggested by Cukadar-Olmedo and Miller (1997). Therefore, these show that multi-location testing of the introgression lines would have helped the evaluation complete. The stay-green (non-senescence) trait in sorghum is reported to be often associated with good plant health and increased plant resistance to insects and diseases (Cukadar-Olmedo and Miller, 1997). Moreover, as it results in greater functional leaf area during grain filling and reduces the need for translocation of stored assimilates from the stem during grain filling, non-senescent sorghum accumulates more soluble sugars in the stem than does senescent sorghum, both during and after grain filling (McBee et al., 1983), which in turn improves the digestible energy content of the Stover (van Oosterom et al., 1996). In addition, stay-green QTLs may improve Stover digestibility by 3 to 5% units without negatively affecting grain and Stover yields (Reddy et al., 2012). This is also a very important trait in places like in Ethiopia where sorghum Stover is equally valued for cattle feed.

Yield and yield attributes

Although grain yield is a function of head weight and hundred grain weight, the significance of genotype × irrigation interaction in these characters in this experiment was not adequate to bring about a parallel significance in this major trait of importance. Eck and Musick (1979) found that a 27-day stress period (with average afternoon leaf water potential, - 22.7 bars) beginning at early grain filling reduced yields by 12% only. Similarly, in the present experiment whereby the non-irrigated treatments were exposed to a month of drought stress, the range of yield reduction in all the genotypes was from 1.0% (Meko × E36-1) to 49.0% ($76T_1$#23 × B35) with an average of 12.9%. The variety Gambella 1107 was the most sensitive local variety that showed a yield reduction of 29.4%. On the other hand, the variety Meko was the least sensitive, which performed similarly in both water regimes. Unexpectedly, the stay green source line, E36-1 showed 34.2% yield reduction under drought stress. However, B35 had very low yield reduction indicating its low sensitivity (stability) to the induced drought, which in turn confirms its tolerance to drought stress. The introgression line Gambella 1107 × E36-1 showed better grain yield than its recurrent parent, Gambella 1107 under moisture stress. Meko × E36-1 was the highest yielding of all the genotypes included in the experiment, but had no advantage over its recurrent parent, Meko (Figure 1). Earlier reports showed that yield increases in stay-green types have been directly associated with maintenance of photosynthetic capability during the grain filling period (McBee, 1984; Wolfe et al., 1988) and longevity of a leaf is intimately related to its nitrogen status or water availability (Thomas and Rogers, 1990; Borrell et al., 2001). In the present preliminary experiment, although $76T_1$#23 × B35 maintained >50% of its green leaves until maturity, its yield reduction by 49% was probably because it possessed a cosmetic type (types C and D) of stay-green (Borrell et al., 2014).

Reduction in yield components as a result of drought stress was previously reported. For instance, similar to the present study (Table 4), Hooker (1985) found a decrease in head weight and weight of the 100 seeds associated with reduction in soil moisture by affecting grain filling. In their comparative study of senescent and non-senescent sorghum genotypes, Duncan et al. (1981) also found that the non-senescent (stay-green) genotypes

had higher test weight. However, genotypic differences do exist for the period they take to fill their grain due to their efficiency in using the available moisture during post anthesis deficit. For instance, in the present study the variety Meko and its introgression line with E36-1 showed more or less similar grain yield in both moisture regimes. Kassahun et al. (2010) reported the low yield potential of B35 due to its small panicle size, non-tillering and small number of seeds per panicle. The lower grain yield in B35 and its introgression lines than their respective elite recurrent parents was in agreement with the findings of Kassahun et al. (2010) and may be an indication of yield drag caused by one or more of the stay-green QTLs of B35. Gambella 1107 × B35 had the same stay-green QTL (*StgA*) as that of $76T_1$#23 × B35, but it senesced earlier, which is an indication of the possibility of existence of QTL × Genotype or QTL × Genotype × Environment interaction for phenotypic expression of the trait, which calls for multilocation testing.

Previous reports showed that increased peduncle exsertion in rice was found to be associated with drought resistance and is controlled genetically (O'Toole and Cruz, 1983). This was in agreement with the present study and can be considered as an indirect selection criterion for drought tolerance in sorghum.

Conclusion

The scarcity of water associated with the impending climate change demands introduction and development of climate smart crops. Sorghum is one of these crops due to its adaptation to survive in severely stressed environments. Among the cope up strategies that sorghum as a climate smart crop possesses are drought and heat tolerance. Stay-green is a post-anthesis drought tolerance mechanism. In this experiment, stay-green QTLs were introgressed from known sources to the farmer preferred Ethiopian local sorghum varieties. Eight of the resulting introgression lines those possessed at least one *Stg* QTL were organized in an experiment to observe their performance under well watered and water deficit conditions. The result of this single season and single location experiment has indicated that most of the introgression lines maintained their green leaf area until maturity under conditions of post anthesis moisture deficit. However, most of them did not show better grain yield than their recurrent parents probably because the introgressed stay-green was cosmetic type. The reduction in leaf area was significant in the *Stg* QTL introgression lines under moisture stress perhaps because the QTLs play significant role in leaf area dynamics. Multienviroment testing of the performance of the introgression lines may give better understanding of the effect of the introgressed QTLs on the various characters.

Conflict of Interests

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

ACKNOWLEDGEMENT

The introgression part of the study was supported by the project from Syngenta Foundation for Sustainable Agriculture through ICRISAT. We thank the research and technical staff of the national sorghum research program at Melkassa Agricultural Research Center for handling the backcross populations.

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Vol. 13(49), pp. 4501-4507, 3 December, 2014
DOI: 10.5897/AJB2014.14059
Article Number: 0C75A1348918
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Synergistic action of *Azospirillum brasilense* combined with thiamethoxam on the physiological quality of maize seedlings

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Received 20 July, 2014; Accepted 25 November, 2014

Plant growth promoting substances, either from biological or chemical origin, are widely used in modern agriculture. In this context, this study evaluated the physiological quality of maize seed treated with thiamethoxam and inoculated with *Azospirillum brasilense*. Initially, we tested bacterial growth on medium with increasing concentrations of thiamethoxam. We also evaluated the physiological quality of seeds through morphometric measurements, emergence speed index and chlorophyll content by SPAD through a 2x3 factorial arrangement with seed inoculation with *A. brasilense* (100 mL per 25 kg of seed) and three doses of thiamethoxam (0, 80 and 120 ml per 60,000 seeds). Bacterial population showed a linear reduction according to increasing doses of thiamethoxam. The dose of 120 ml thiamethoxam (42 mg active ingredient) caused a decrease in several variables, while, the inoculation proved a positive activity in seedlings physiological quality. Thus, the inoculation combined with 80 ml thiamethoxam (28 mg active ingredient) show synergistic action in early development in maize seedlings.

Key words: Thiamethoxam, inoculant, plant growth promoting bacteria.

INTRODUCTION

Maize (*Zea mays* L.) is a cereal grown on all continents, with high economic relevance due to the variety of forms of use (Fancelli and Dourado Neto, 2003). This is possible because this cereal is used in numerous products, ranging from food and feed to the high-tech industry (Paes, 2008). Recent data indicate the high importance of this crop in Brazil, because only in the 2013/2014 season the country produced about 75.18 million tons of this cereal in a planted area of 15.12 million hectares (National Supply Company, 2014). These numbers are the result of years

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of research, producing improved cultivars, inputs and appropriate farming techniques, coupled with expansion of the planted area (Paes, 2008). Plant growth promoting substances have propelled the recent increases in productivity. These substances have been widely used in modern agriculture, and have biological or chemical origin (Busato et al., 2010). One of the most frequently growth promoting substances used today is the molecule thiamethoxam, a synthetic insecticide with effect of plant bioactivation by triggering several physiological reactions. It promotes the activation of membrane transport proteins, and enzymatic activation, which increases plant metabolism through the synthesis of amino acids, precur-sors of proteins, and endogenous synthesis of plant hormones (Carvalho et al., 2011), but without exhibit activity of plant regulation (Castro et al., 2007).

Biochemically, this insecticide stimulates the activity of peroxidases, whose activity focuses on combating reactive forms of oxygen, before they damage biomolecules, preventing plant cell death, being indirect effects of their mechanisms of action observed by increased biomass, increased photosynthetic rate and formation of deeper roots (Almeida et al., 2012). Likewise, plant growth promoting substances may also be of biological origin, such as the plant growth promoting bacteria. Microorganisms of the genus *Azospirillum* are capable to survive, both in the rhizosphere and inside plants belonging to the family Poaceae (Huergo et al., 2008). These microorganisms stimulate plant development especially due to biological nitrogen fixation (Hungria, 2011), phosphate solubilization (Rodriguez et al., 2004), hormone production (Perrig et al., 2007) and reduction of ethylene activity through the action of the enzyme ACC deaminase (Blaha et al., 2006).

However, many researchers combine the effects of *Azospirillum* mostly to production or stimulation of hormones, promoting an increase in the plant size and root development verified by Rampim et al. (2012) and Dartora et al. (2013), being results mainly associated with indole-3-acetic acid (IAA) and gibberellins (Bashan et al., 2004; Perrig et al., 2007; Radwan et al., 2004). According to Hungria et al. (2010), the greater root development can also provide better absorption of macro and micronutrients by the plant.

In this context, considering the use of chemical and biological products to promote an increase in plant development, it is necessary to check if the effects are synergistic when applied together. Thus, this study aimed to evaluate the physiological quality of maize seedlings in the association of thiamethoxam seed treatment and inoculation with *Azospirillum brasilense* strain AbV5, as well as the effect of the insecticide on the bacterial population.

MATERIALS AND METHODS

The work was developed in the Laboratory of Plant Physiology and Laboratory of Phytopathology, State University of West Paraná – Unioeste, Campus Marechal Cândido Rondon, Paraná State, in October 2013. Strain of *A. brasilense* AbV5 used in the study was obtained from the collection of growth promoting bacteria of Federal University of Paraná - Campus Curitiba, Paraná, Brazil. Bacterial cells were grown in enlermeyers flask with 250 mL containing 100 mL of NFb medium (Dobereiner et al., 1976) at 30°C under constant agitation at 80 rpm, until exponential growth phase at optical density ($OD_{450\ nm}$) of 0.5, corresponding to population of 6.67 x 10^7 colony-forming units (CFU) ml^{-1} in NFb-agar. Initially, we tested the toxicity of the insecticide thiamethoxam on the bacterial population. To this end, solid medium DYGS (Baldani, 1996) were added with doses of Cruiser® (350 mg thiamethoxam ml-1) at 0; 40; 80; 120; 160 and 200 ml of commercial product (c.p.), corresponding to 0; 14; 28; 42; 56 and 70 mg of thiamthoxam per 60,000 seeds, while the medium was at 45 to 55°C, so that the active ingredient was not inactivated. Thiamethoxam was sterilized by filtration through Millipore filter (0.25 µm) and homogenized with the medium in a continuous air flow chamber, and distributed in 20 ml Petri dishes.

The inoculant containing *A. brasilense* strain AbV5 was serially diluted by adding 100 ml of inoculant to 900 µl of saline solution (0.85% NaCl), and so forth, comprising dilutions from 10^{-1} to 10^{-8}. Then, dilutions were distributed in Petri dishes using the microdrop technique with a volume of 10 µl per drop, with three replicates per dilution on each plate. Through mathematical relationships, each microdrop of the dilutions occupied an area upon the culture media corresponding to the area of a seed where bacteria were grown in the same *Azospirillum*/thiamethoxam ratio. We used four replications in a completely randomized design, in a total of 24 plates. Subsequently, they were incubated at 30°C for 48 h, when we counted the colonies, and were expressed as CFU ml^{-1} inoculum. To evaluate the influence of the association of thiamethoxam with the inoculation with *A. brasiliense* on seed germination, we adopted a completely randomized design in a 2x3 factorial arrangement. In the first factor, we assigned the seed inoculation with *A. brasiliense* (with and without inoculation) at 100 ml per 25 kg seed. In the second factor, we assigned the doses of thiamethoxam (Cruiser®) with 0; 80 and 120 ml c.p. per 60,000 seeds, as recommended by the manufacturer. Treatments were added to seeds by hand shaking in polyethylene bags until visual homogeneity. The inoculants had an initial bacterial population of 6.67×10^7 CFU ml^{-1} *A. brasilense* AbV5. We used commercial seeds of the cultivar Dekalb DKB 240 VT PRO as the standard for the test.

On germitest paper moistened with distilled water at a volume equivalent to 2.5 times its mass, four experimental replicates were performed with 50 seeds each, placed in a BOD germination chamber at 25 ± 1°C, with photoperiod of 12:12. Four days later, we determined the number of seeds that had produced radicle longer than two millimeters and straight and well developed coleoptile, composing the variable vigor. At day seven, we held the same procedure, regarding the variable viability, according to the Rules for Seed Analysis (Brasil, 2009), both expressed in percentage. Alongside, 25 seeds were sown in polyethylene trays, with four replicates per treatment; the substrate was sand sterilized by autoclaving for 20 min at 121±1°C. The trays were placed in a BOD germination chamber at 25 ±1°C, 12:12 (light: dark) photoperiod. Trays were weighed daily and watered until reaching field capacity. We counted the seedlings emerged for seven days, according to the Rules for Seed Analysis (Brazil, 2009), determining the Emergence Speed Index (ESI) as described by Maguire (1962). At the end of seven days, we determined the chlorophyll content through digital chlorophyll meter SPAD-502-Plus based on sampling ten seedlings per plot, obtained using the arithmetic mean of three measurements per plant. The same seedlings were selected for determination of morphometric characteristics. We counted the number of roots. The root length and shoot length were measured with a graduated scale. The steam diameter was measured with a digital caliper. We calculated the shoot

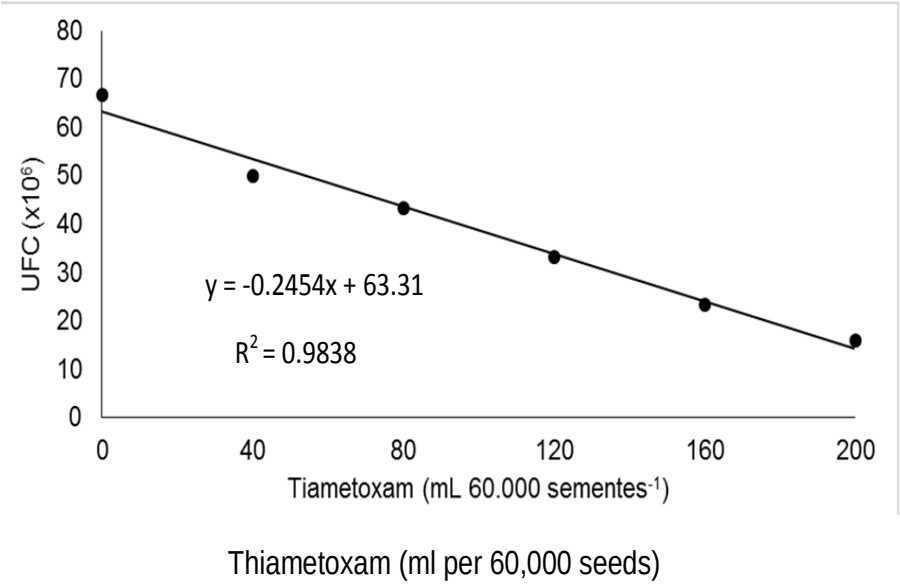

Figure 1. Colony forming units (CFU) based in the dose of thiametoxam.

length/stem diameter ratio, by dividing the length by stem diameter, expressing the robustness of the seedling. Finally, with the aid of a graduated cylinder, we identified the volume of the root system using the water column displacement technique, based in difference of water volume before and after the immersion of the roots.

After the morphometric measurements, plants were separated into shoot and roots and oven dried at 65 ± 2°C for 72 h until constant weight, when they were weighed on an accurate analytical balance. Each value of shoot dry weight and roots was obtained by dividing the total weight of the organ by the number of seedlings used in the test; the results were expressed in g.seedling^{-1}. The sum of the shoot dry weight and roots represented total dry matter. The root/shoot ratio was obtained by dividing the root dry weight by the shoot dry weight. Data were tested for normality (Shapiro-Wilk), and then subjected to analysis of variance with the software Sisvar 5.1 Build 72 (Ferreira, 2011). In the case of significant effect at 95% significance, mean values were compared by Tukey's test at 5% probability of error.

RESULTS AND DISCUSSION

The mortality caused by thiamethoxam to bacterial population of *A. brasilense* (Figure 1) showed a linear decrease according to the increase in the dose of insecticide, with mortality of 35 and 50% for doses of 80 and 120 ml c.p. per 60,000 seeds (doses recorded in maize, by the manufacturer), respectively. In agreement with Andaló et al. (2004), thiamethoxam has low toxicity to microorganisms, with no effect on germination, growth and production of conidia of *Beauveria bassiana*. Also, for phylogenetically close organisms such as the bacterium *Herbaspirillum seropedicae*, the same insecticide showed no effect on generation time and lag phase of growth, exhibiting a behavior similar to the control (Fernandes et al., 2012). Nevertheless, Gomez et al. (1998) found susceptibility of *A. brasilense* to chemicals as bromopropilate and methidathion, showing the same linear decreasing behavior observed in this study. However, Dartora et al. (2013) observed compatibility between inoculation and use of chemicals in seed treatments, evaluating the early development of maize seedlings. Thus, it is shown that the sensitivity of *A. brasilense* depends on the interaction with the chemical compound to which it is subjected.

ANOVA detected interaction between thiamethoxam doses and inoculation (Table 1) for ESI, stem diameter, root volume, shoot length, shoot/stem ratio, SPAD content, and root/shoot ratio. Vigor, viability and number of roots were not affected by the treatments with thiamethoxam and by the inoculation with *A. brasilense*. As to ESI (Table 2), the lowest value was found when the inoculation was conducted with the highest dose of thiamethoxam. When maize seeds were inoculated with *A. brasiliense*, higher emergence speed index were obtained without the use of the insecticide, suggesting its effect on the population and action of bacteria. When there was no inoculation, the highest emergence speed for the higher doses of thiamethoxam was registered. These results corroborate those obtained by Lauxen et al. (2010), who found increased ESI in cotton seeds treated with this insecticide.

In the same way, Almeida et al. (2012) evaluated the influence of thiamethoxam on physiological performance in oat and observed that the product is capable of stimulating the performance of the crop, especially at a dose of 300 ml of commercial product per 100 pounds of seeds. Comparing the effect of inoculation within each dose, even with no statistical difference, there was a

Table 1. Analysis of variance and F-test for analyzed variables.

Parameter	Mean square (QM)				
	Tiamethoxam	Inoculation	Interaction	Error	CV (%)
Vigor	1.166667	16.666667	7.166667	6.111111	2.57
Viability	2.666667	20.166667	2.666667	6.277778	2.60
ESI	0.162554	0.005400	1.127187**	0.820720	5.79
Indíce SPAD	11.616867**	9.151350**	21.497550**	1.042208	3.10
Shootlength	1.497088*	0.018150	7.960363**	0.316364	5.02
Stemdiameter	0.041679**	0.025350*	0.028213**	0.003492	2.28
Numberof root	0.016250	0.060000	0.008750	0.048611	5.62
Root length	5.012904*	0.297038	2.416512	0.911635	5.03
Root volume	0.011629	0.006667	0.016529*	0.004442	13.51
Shoot/stemratio	0.469029**	0.108004	0.687079**	0.054938	5.41
Shootdrymass	0.000015	0.000012	0.000076**	0.000006	6.22
Root drymass	0.000994**	0.000536**	0.000118	0.000057	12.56
Total drymass	0.001049**	0.000710**	0.000115	0.000073	8.66
Root/shootratio	0.642141**	0.251843*	0.316697**	0.034789	11.73

**significant at 1% by F-test; *significant at 5% by F-test; ^{ns} non-significant by F-test; CV, coefficient of variation.

Table 2. Emergence speed index and SPAD index of maize seedlings derived from seeds treated with thiamethoxam (0; 80 and 120 mL per 60,000 seeds) and inoculated with *A. brasilense* (0 and 100 mL per 25 kg seeds).

Thiametoxam dosage (ml)	Emergence speed index			SPAD Index		
	NI	I	Mean	NI	I	Mean
0	4.77Ab	5.41Aa	5.09	31.41Ab	35.34Aa	33.38
80	4.82Aa	5.07Aa	4.94	32.68Ab	34.87Aa	33.78
120	5.21Aa	4.40Bb	4.81	32.73Aa	30.31Bb	31.52
Mean	4.93	4.96	4.95	32.27	33.51	32.89

Means followed by different letters, uppercase in the column and lowercase in the row, are significantly different by Tukey's test at 5%; NI, Non-Inoculated; I, Inoculated.

positive increase in ESI when seeds were inoculated with bacteria at 0 and 80 ml of the insecticide. Araújo et al. (2010) stated that the inoculation of five out of six strains of diazotrophic bacteria promotes higher speed of germination in seeds of two rice cultivars. Similarly, Cassán et al. (2009) argued that the inoculant based on *A. brasilense* strain Az39 is capable of promoting faster seed germination and the early development of maize plants. In addition, this plant growth promoting bacterium is able to excrete indole-3-acetic acid, gibberellic acid and zeatin, hormones that can contribute to increased emergency speed index. Thus, nitrogen-fixing bacteria may contribute to plant growth through auxin production (Radwan et al., 2004), leading to higher values of emergence speed index. For the chlorophyll content in seedlings (SPAD index) (Table 2), there was a positive increase following the increase in the thiamethoxam dose, without inoculation, indicating a beneficial effect of the insecticide for this variable. Thiamethoxam works physiologically by activating membrane transport proteins and elevating the enzyme activation, increasing metabo-lism, synthesis of amino acids precursors of new proteins and the endogenous synthesis of plant hormones (Carvalho et al., 2011). Therefore, with the increase of its dose, the production of secondary metabolites increased, diverting photosynthates that would be used in growth, particularly nitrogen and consequently reducing the size and color of the plant. When inoculation was performed, the chlorophyll content was higher combined with doses of 0 and 80 ml of thiamethoxam.

Studies have presented conflicting results regarding the relationship between chlorophyll and nitrogen (Wolff and Floss, 2008). The majority found positive correlations between the SPAD index and the nitrogen content (Guimarães et al., 1999; Lima et al., 2001), but is not very accurate for the determination of this element in the initial stages of the maize crop, being reliable only for chlorophyll concentration (Argenta et al., 2001). However, nitrogen is an element directly related to chlorophyll, and is an essential component of the chemical structure of

Table 3. Shoot length, stem diameter, shoot/stem ratio, root length and root volume of maize seedlings derived from seeds treated with thiamethoxam (0; 80 and 120 mL per 60,000 seeds) and inoculated with *A. brasilense* (0 and 100 mL per 25 kg seeds).

Thiametoxam dosage (ml)	Shoot length (cm)			Stem diameter (mm)			Shoot/stem ratio		
	NI	I	Mean	NI	I	Mean	NI	I	Mean
0	10.77 Bb	12.06 Aa	11.41	2.42 Bb	2.60 Aa	2.51	4.46 Aa	4.64 Aa	4.55
80	11.07 ABb	11.96 Aa	11.51	2.60 Aa	2.66 Aa	2.63	4.26 Aa	4.49 Aa	4.37
120	11.89 Aa	9.55 Bb	10.72	2.66 Aa	2.61 Aa	2.63	4.47 Aa	3.66 Bb	4.07
Mean	11.24	11.19	11.22	2.56	2.62	2.59	4.40	4.26	4.33

	Root length (cm)			Root volume (cm^3)		
	NI	I	Mean	NI	I	Mean
0	18.29	19.53	18.91AB	0.50Aa	0.49ABa	0.49
80	19.64	20.01	19.82A	0.46Ab	0.60Aa	0.53
120	18.72	17.78	18.25B	0.47Aa	0.44Ba	0.46
Mean	18.88	19.10	18.99	0.48	0.51	0.49

Means followed by different letters; uppercase in the column and lowercase in the row are significantly different by Tukey's test at 5%. NI, Non-Inoculated; I, Inoculated.

chlorophyll *a* and *b* (Streit et al., 2005). Dietrich et al. (2005) suggest that when the plant is subjected to any induction involving high metabolic costs, it is necessary to complement the fertilization with higher nitrogen levels in order to compensate for the extra consumption of the element, thus explaining the lower values of the SPAD index for the application of thiamethoxam at 120 ml per 60,000 seeds associated with inoculation. In relation to shoot length (Table 3), we detected an interaction between doses of thiamethoxam and inoculation with *A. brasiliense*. In the case of non-inoculation, plants showed higher values for the highest dose of thiamethoxam, and an opposite effect was verified when plants were inoculated, where plants were greater in length at a dose of 80 ml and in the absence of the insecticide (0 ml). According to Almeida et al. (2011), increased shoot length with the use of thiamethoxam, depending on the dose applied, can increase the uptake of water and stomata resistance to water loss, favoring the metabolism and enhancing the resistance to environmental stress; in addition, it can also increase efficiency in the uptake, transport and assimilation of nutrients.

Stem diameter increased when inoculated with *A. brasiliense*, only in the absence of thiamethoxam (0 ml). For the other doses, no difference was found for inoculation. Several studies have highlighted the positive effect of thiamethoxam on physiological quality in seed germination of several crops (Almeida et al., 2009; Almeida et al., 2012; Lauxen et al., 2010), demonstrating increased rooting and development of shoots. Thus, higher values of stem diameter are also possible due to the stimulation of seedling growth. Shoot/stem ratio was reduced at the highest dose of thiamethoxam (120 ml) associated with inoculation. This is as a result of the relationship between smaller height in the same treatment, without changing stem diameter. The root length was affected only by thiamethoxam doses. In absolute numbers, the roots were longer at the dose of 80 ml of thiamethoxam, but this dose was not different from the control. The dose of 120 ml reduced the development of roots. Similar results were obtained by Corrêa Junior et al. (2013) when worked with different chemicals on germination of maize seeds. According to these authors, these results show that the application of the insecticide thiamethoxam at high doses produced a phytotoxic effect for root growth in maize seedlings.

There was interaction between the doses of thiamethoxam and inoculation with *Azospirillum* for the root volume. When seeds were not inoculated, there was no effect of thiamethoxam doses. As well as for root length, the phytotoxic effect of the insecticide was observed when applied at high doses. Tavares et al. (2007) examined the effect of thiamethoxam on soybean seed germination, and observed higher root and leaf volume when the product was applied. In accordance with Almeida et al. (2011), this difference between the dose zero and the dose providing the highest response, containing thiamethoxam, can be because this insecticide enables a greater expression of the germination potential, leaf area, root length and seedling length. With regard to the inoculation with *Azospirillum* combined with the dose of 80 ml of thiamethoxam, the inoculation promoted an increase of 30% in root volume. In the other treatments (0 and 120 ml), there was no effect of inoculation. Quadros (2009) registered a root volume of 60 to 80% higher in seedlings whose seeds were inoculated with *Azospirillum* compared with non-inoculated seedlings. For the shoot dry weight (Table 4), the treatments with the lowest doses of thiamethoxam inoculated with *A. brasiliense* were better than the non-inoculated. Likewise, Dartora et

Table 4. Shoot dry weight, root dry weight, total dry weight and root/shoot ratio of maize seedlings derived from seeds treated with thiamethoxam (0; 80 and 120 mL per 60,000 seeds) and inoculated with *A. brasilense* (0 and 100 mL per 25 kg seeds).

Thiametoxam Dosage (ml)	Shoot dry weight (g seedling^{-1})			Root dry weight (g seedling^{-1})		
	NI	I	Mean	NI	I	Mean
0	0.0360Ab	0.0410Aa	0.0384	0.0476	0.0483	0.048B
80	0.0370Ab	0.0420Aa	0.0394	0.0622	0.0772	0.07A
120	0.0400Aa	0.0340Bb	0.0367	0.0568	0.0695	0.063A
Mean	0.0374	0.0389	0.0382	0.056b	0.065a	0.0603
	Total dry weight (g seedling^{-1})			Root/shoot ratio		
	NI	I	Mean	NI	I	Mean
0	0.0832	0.0893	0.0863B	1.35Aa	1.18Ba	1.26
80	0.0992	0.1188	0.1090A	1.68Aa	1.85Aa	1.76
120	0.0963	0.1033	0.0998A	1.44Ab	2.05Aa	1.74
Mean	0.0929b	0.1038a	0.0984	1.49	1.69	1.59

Means followed by different letters; uppercase in the column and lowercase in the row are significantly different by Tukey's test at 5%. NI, Non-Inoculated; I, Inoculated.

al. (2013) observed increased yield of root dry weight for treatments inoculated with *Azospirillum* spp. The high dose of thiamethoxam associated with the inoculation was the treatment with the lowest increase in weight.

Considering the root dry weight, between the three doses of thiamethoxam, the lowest values were found at the dose of 0 ml. The inoculated treatments exhibited the highest mean value of root dry weight than non-inoculated treatments, corroborating the results of Santos et al. (2008) inoculating the growth promoting bacteria in melon plants, and Dawwam et al. (2013) working with seven isolates in potato. For the root/shoot ratio, all treatments showed mean values above 1, indicating that seedlings invested more in root than shoot. At the highest dose of thiamethoxam (120 ml), the non-inoculated treat-ment showed the lowest ratio compared with the inocu-lated treatment. This is because, when inoculated, *Azospirillum* affects the plant cell membrane activity, leading to changes in the morphology of roots, resulting in an increase in the root system (Bashan et al., 2004).

Conclusions

The chemical compound thiamethoxam is toxic to *A. brasilense*, reducing its population. Seed inoculation with *A. brasilense* results in significant gains on the physiological quality of maize seedlings, represented by increases in various morphometric variables along with increased emergence speed. The dose of 80 ml thiamethoxam per 60,000 seeds performs best when combined with inoculation acting synergistically, due to the reduction in bacterial mortality and to the moderate effect of bioactivation, showing better results of growth in roots and shoots.

Conflict of Interest

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

ACKNOWLEDGMENTS

Authors are grateful to Araucaria Foundation of Support, the Scientific Development and Technological of Paraná, affiliated to the State Secretariat of Science, Technology and Higher Education - SETI, CAPES/PNPD, INCT-FBN for the financial support and to CNPq for researcher on productivity to Vandeir Francisco Guimarães.

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Vol. 13(49), pp. 4508-4514, 3 December, 2014
DOI: 10.5897/AJB2014.14141
Article Number: 2BB75DB48923
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization of *in vitro* regeneration and microcorm induction in elephant foot yam (*Amorphophallus paeoniifolius*)

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Received 31 August, 2014; Accepted 28 November, 2014

Elephant foot yam (*Amorphophallus paeoniifolius)* is a vegetatively propagated stem tuber crop. In this investigation we describe a highly competent and reproducible *in vitro* propagation of the plant from corm bud, petiole and young leaf explants. Friable callus was initiated from all the explants on modified MS medium (half the concentration of NH_4NO_3 and KNO_3) supplemented with 0.5 mg l^{-1} each of benzyl amino purine (BAP), α-naphthalene acetic acid (NAA) and 2,4-dichloro phenoxy acetic acid (2,4-D). Shoot regeneration from calli was optimal on modified MS medium supplemented with 5.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA. Microcorms, capable of producing micro shoots all over the surface, were induced from the callus at a frequency of 90% on shoot regeneration medium supplemented with 5% sucrose. Rooting was 100% on modified liquid MS medium augmented with 5.0 mg l^{-1} Indole 3- butyric acid (IBA). A 100% survival rate of plantlets on transplantation to soil: sand: coir pith mixture was recorded.

Key words: Callus, elephant foot yam, *in vitro*, microcorms, regeneration, somatic embryo.

INTRODUCTION

Elephant foot yam or *Amorphophallus paeoniifolius* (Dennst.) Nicolson is a tropical tuber crop of South-East Asian origin belonging to the family *Araceae*. The plant is distributed throughout India, Australia, Philippines, Madagascar and Sri Lanka (Chandra, 1984). The plant is cultivated as an intercrop with banana, turmeric and coconut (Ravi et al., 2009). It is a cash crop due to its high production potential (50 to 60 t ha^{-1}), farm income and export potential (Misra et al., 2001; Srinivas and Ramanathan, 2005). The corms and pseudostems of elephant foot yam are used popularly as vegetables. The tubers are used for preparing ayurvedic medicines as they are anti-inflammatory, anti-haemorrhoidal, astringent, haemostatic, digestive, appetizer, anodyne, rejuvenating and tonic (Misra et al., 2002). They are used in the treatment of tumors, elephantiasis, inflammations, cough, asthma, vomiting, flatulence, colic, dyspepsia, constipation, fatigue and anaemia (Nair, 1993). The plant starch is easily extractable and is with good viscosity, stability and suitability for many applications in food industry (Moorthy et al., 1994). The high incidence of mosaic disease, corm dormancy and non-availability of quality planting materials are the major production constraints in elephant foot yam (Khan et al., 2006; Venkatram et al., 2007). The use of

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Table 1. Effect of PGRs on callusing response of different explants of elephant foot yam.

PGRs in modified MS media (mg l^{-1})			Days to callus induction			% Callusing		
BAP	NAA	2,4-D	Corm bud	Petiole	Leaf	Corm bud	Petiole	Leaf
0.0	0.0	0.0	0	0	0	0	0	0
0.5	0.0	0.0	0	0	0	0	0	0
0.0	0.5	0.0	0	0	0	0	0	0
0.0	0.0	0.5	25.43[a]	0	0	56[d]	0	0
0.5	0.5	0.0	21.52[b]	29.00[a]	59.00[a]	72[b]	40[c]	20[c]
0.5	0.0	0.5	25.46[a]	0	0	52[e]	0	0
0.0	0.5	0.5	25.84[a]	0	0	52[e]	0	0
0.25	0.25	0.25	21.35[b]	29.35[a]	50.70[b]	60[c]	56[b]	48[b]
0.5	0.5	0.5	13.91[c]	15.56[b]	30.40[c]	88[a]	72[a]	60[a]

[a]Means with a same letter within a column do not differ significantly according to the LSD test (p≤0.05) following ANOVA using the SAS system. Plant growth regulators (PGRs).

disease free planting material can be a cultural measure of immediate success as proved in many previous studies (Hollings et al., 1965). High quality planting material of elephant foot yam can be obtained through tissue culture but the method, specifically the process of regeneration is far from being routine due to its recalcitrant nature (Mukherjee et al., 2001).

Also, the plant being a stem tuber and the corm bud being one of the most studied explant, obtaining contamination free starter cultures is the most challenged part in the tissue culture. However, tissue culture of *A. paeoniifolius* has been achieved earlier with limited success (Mukherjee et al., 2009). The *in vitro* regeneration through callus and/ somatic embryogenesis can be employed for effective regeneration thereby mass propagation through tissue culture (Hu et al., 2005; Zhao et al., 2012). In the present study, an efficient *in vitro* callus multiplication and regeneration protocol in elephant foot yam was developed using corm bud, petiole and leaf explants. The callus cultures were used as a source for microcorm induction for enhanced mass propagation of this plant.

MATERIALS AND METHODS

Plant material and establishment of aseptic starter cultures

The source plants of elephant foot yam were grown in open field of ICAR-Central Tuber Crops Research Institute (CTCRI), Kerala, India. 50 explants each of corm buds, petioles and young leaves collected from the field grown plants were used for getting aseptic starter cultures. Apical buds as well as lateral dormant buds of field grown corms of elephant foot yam were scooped out and rinsed under running tap water for 30 min and surface sterilized with 0.1% (w/v) bavistin for 30 min followed by 0.05% (w/v) streptomycin for 1 h, 0.1% (w/v) mercuric chloride for 1 min and 70% (v/v) ethanol for 1 min. Each treatment was followed by washing three times with sterile distilled water. The petiole and leaf explants were surface sterilized with bavistin (0.1% w/v) for 1 h followed by streptomycin (0.05% w/v) for 1 h, mercuric chloride (0.1% w/v) for 5 min and ethanol (70% v/v) for 1 min. As an alternative, one step surface sterilization procedure was done by dipping in absolute ethanol and flaming the unopened leaves covered by cataphylls to get petiole and young leaf explants. The explants were inoculated on modified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with half the concentration of NH_4NO_3 and KNO_3 supplemented with different plant growth regulators (PGRs).

Effect of different PGRs on callus induction

Modified MS medium supplemented with various combinations of 6-benzylaminopurine (BAP), α-naphthalene acetic acid (NAA) and 2,4-dichloro phenoxy acetic acid (2,4-D) were used for callus induction (Table 1). For each treatment, 25 explants each of corm buds, petioles and young leaves were used. The experiments were repeated thrice for reproducibility and accuracy. All the components of the medium including 3% sucrose and PGRs were mixed and adjusted to pH 5.6 prior to adding 0.7% plant agar for solidifying the media. The cultures were incubated under light intensity of 2500 lux with 16 h light and 8 h dark cycle. The temperature was maintained at 25 ± 2°C throughout the incubation period. Observation on the days to callusing, callusing % and the nature of callus were recorded. The effect of PGRs on Growth Index (GI) was calculated by taking 10 sets of callus cultures obtained from different explants. Calli were cut into pieces to make the initial fresh weight (FW) 100 mg and the GI was recorded after 60 days of growth. The GI was calculated as:

GI = (Final callus FW - Initial callus FW)/ Initial callus FW.

Shoot regeneration, microcorm induction and *in vitro* rooting

Shoot regeneration was optimised by culturing the calli on modified MS medium with various combinations of BAP and NAA in the ratio 5:1 along with control (without PGRs). For each treatment, 24 sets of calli of FW 250 mg were used and the days taken for shoot initiation, number of shoots per mass of calli and percentage response was recorded. A total of 20 callus mass of size 1 cm^2 were inoculated on optimal shoot regeneration medium supplemented with various concentrations of sucrose ranging from 1 to 10% to find its effect on microcorm induction. The experiment was repeated twice. Subculture of microcorms was done in every 60 days. Rooting of the regenerated shoots was attempted on regeneration medium as well as on modified MS medium supplemented with various auxins. Trials with liquid medium instead of agar solidified medium were carried out for rooting.

Acclimatization and hardening

Well rooted plantlets as well as rooted/unrooted microcorms were removed from the culture bottle and were treated with 0.2% bavistin for 5 min. The unrooted microcorms were treated *ex vitro* with 5.0 mg l^{-1} Indole 3- butyric acid (IBA) for 5 min. Transplantation was done on potting mixture [(soil: sand: coir pith, 1:1:1 (v/v/v)]. The plantlets were kept covered in trays/pots using transparent poly covers with holes and were allowed to grow in the net house. After a week, or when the plants started showing signs of healthy growth, the plants were transferred to normal external environmental conditions, but in shade. The plants were thereafter transferred to soil kept in pots or in the field. Survival rate of the *in vitro* hardened plants on monthly interval up to 6 months were recorded.

Statistical analyses

Analysis of Variance (ANOVA) and comparison of the mean value of different treatments using Least Significant Difference (LSD) was performed with the SAS software (SAS, 2010).

RESULTS AND DISCUSSION

Initiation and multiplication of callus from different explants

The method of surface sterilisation followed for corm bud, petiole and leaf explants of elephant foot yam proved to be effective in getting 70% of contamination free cultures. The use of fungicide, mercuric chloride and ethanol in surface sterilisation has proved effective in initiating *in vitro* cultures using explants taken from underground parts (Nongalleima et al., 2014). The method of dipping in ethanol and flaming proved to be effective in getting 75% aseptic starter cultures from leaf and petiole explants, thus avoiding long steps of surface sterilisation normally followed for these explants. The treatment of ethanol dip and flame has been utilised successfully in establishing aseptic starter cultures from difficult to sterilise explants (Sugii, 2011). In the present study, modified MS medium with half the concentration of NH_4NO_3 and KNO_3 was used instead of basal MS medium (with normal nutrient concentration) due to the prominent occurrence of necrosis and yellowing in the initial stages of development observed in preliminary studies, which inhibited the callus induction and further growth. The positive effect of altered concentrations of NH_4NO_3 and KNO_3 on *in vitro* culture growth has been reported in tobacco (Ramage and Williams, 2002), *Gossypium hirusutum* (Ikram-ul-Haq and Zafar, 2004) and *Azadirachta indica* (Srinidhi et al., 2008). In this experiment the tissue culture response of elephant foot yam differed with the explant type as well as with the combinations of PGRs used in the medium. Small sprouts initially appeared from the corm bud explants which then bulged leading to callus initiation (Figure 1a). Enormous bulging followed by callus initiation was observed in petiole and leaf explants (Figure 1b and 1c). Time taken for callus initiation and the percentage of explants responded differed significantly depending on the explant type and the combinations of PGRs used (Table 1). The use of 2,4-D alone in the medium resulted in callus induction in corm bud explant within 56 days while it failed to induce callus from other explants. Callus was induced from corm bud, petiole and leaf explants in 21, 29 and 59 days, respectively, with a callusing percentage ranging from 20 to 72 depending on the explant type on medium supplemented with 0.5 mg l^{-1} each of BAP and NAA. The BA/NAA combination has been used with great success in the callus induction of *Amorphophallus* spp. (Hu and Liu, 2008; Anil et al., 2012). A combination of all the 3 PGRs (BAP, NAA and 2,4-D) in 0.25 mg l^{-1} concentration resulted in increased callusing % in petiole and leaf explants.

However, the combination was not effective in the case of corm buds. The increase in the concentration of all the 3 PGRs from 0.25 to 0.5 mg l^{-1} decreased the callus initiation time and increased the callusing %. Thus, modified MS medium supplemented with 0.5 mg l^{-1} each of BAP, NAA and 2,4-D (Callus Induction or CI medium) was found to be optimal for callus induction as well as callus multiplication and was therefore used in further studies. Yellowish white or pink friable callus was initiated within 15 to 30 days of inoculation from corm bud, petiole and leaf explants on CI medium (Figure 1d). In an earlier report, it took more than 20 to 24 weeks for callus induction from apical bud explants of the crop with limited survival/multiplication rate while the petiole gave rise to callus in a month time (Anil et al., 2012). The effect of PGRs on the nature of callus and GI recorded is shown in Table 2. Irrespective of the explant type, GI as high as 8 was observed on callus induction medium. While on MS medium with half of the concentration of PGRs (0.25 mg l^{-1} each of BAP, NAA and 2, 4- D), the GI was markedly reduced to ~ 3 and did not differ significantly with the explant type. When 0.5 mg l^{-1} of BAP and NAA was used without 2,4-D, the GI decreased to 2.5 in all the 3 different explants. Medium without any of the 3 PGRs either failed to induce callus or if induced showed reduced GI. Hence, for elephant foot yam, irrespective of the explants, the combination of the 3 PGRs (BAP, NAA, 2,4-D) at 0.5 mg l^{-1} concentration is essential for callus induction with enhanced GI. In *Amorphophallus konjac* also friable calli was induced when appropriate combinations of BAP, NAA and 2,4-D was used, although the callus induction rate was as low as 35.5% (Zhao et al., 2012). Subculture of callus was done in 60 days interval on CI medium.

Shoot regeneration and microcorm induction from friable callus

Vigorous growth followed by emergence of greenish micro shoot like structures was observed within 25 to 40 days when friable calli were sub cultured on MS medium augmented with various combinations of BAP and NAA in

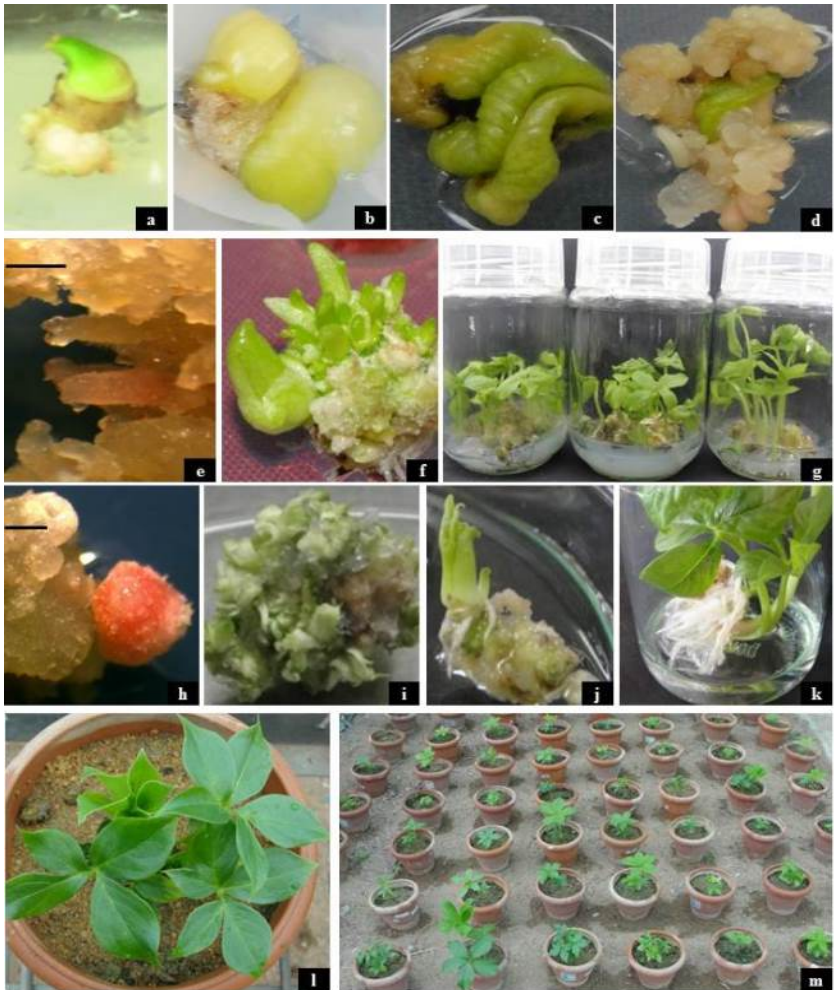

Figure 1. Various stages in the *in vitro* propagation of elephant foot yam. **a.** sprouted corm bud. **b.** bulged petiole. **c.** expanded leaf, **d.** callus. **e.** Somatic embryos**. f.** Shoot initials arising from callus. **g.** Shoot multiplication. **h.** Microcorm arising from callus. **i.** Microshoots arising all over the surface of microcorm. **j.** Rooted microcorm. **k.** *In vitro* rooted plants. **l.** Plant clusters arising from microcorm lacking shoots and roots on transplantation. **m.** Hardened plants kept in net house. Bar = 2 mm.

Table 2. Effect of PGRs on callus nature and growth index (GI) of different explants of elephant foot yam.

PGRs in modified MS media (mg l^{-1})			Nature of callus			GI after 60 days (100 mg Initial FW)		
BAP	**NAA**	**2,4-D**	**Corm bud**	**Petiole**	**Leaf**	**Corm bud**	**Petiole**	**Leaf**
0.0	0.0	0.0	NC	NC	NC	0	0	0
0.5	0.0	0.0	NC	NC	NC	0	0	0
0.0	0.5	0.0	NC	NC	NC	0	0	0
0.0	0.0	0.5	W,C	W, C	W, C	2.25[d]	0	0
0.5	0.5	0.0	W/P, C	W/P, C	W/P, C	2.58[c]	2.38[c]	2.51[c]
0.5	0.0	0.5	W, C/F	W, C	W, C	2.17[d]	0	0
0.0	0.5	0.5	W, Pu/F	W, Pu	W, Pu	1.32[e]	0	0
0.25	0.25	0.25	YW, F	YW, F	YW, F	3.00[b]	2.90[b]	3.08[b]
0.5	0.5	0.5	YW/P, F	YW/P, F	YW/P, F	8.83[a]	8.83[a]	8.47[a]

[b]Means with a same letter within a column do not differ significantly according to the LSD test (p≤0.05) following ANOVA using the SAS system. NC- Callus not induced, W-White, P-Pink, YW-Yellowish white, C-Compact, Pu-Puffy, F-Friable. Benzyl amino purine (BAP), α-naphthalene acetic acid (NAA), plant growth regulators (PGRs).

Table 3. Effect of combination of PGRs on *in vitro* shoot regeneration in elephant foot yam from callus.

Medium number	PGRs (mg l^{-1})		Days to shoot initiation	% Shooting	Mean number of shoots per cluster	Mean shoot length (cm)
	BA	NAA				
Control	0	0	0	0	0	0
1	1.0	0.2	38.83[a]	16.37[g]	1.33[f]	3.83[c]
2	2.0	0.4	27.67[b]	70.75[d]	7.33[e]	4.25[b]
3	3.0	0.6	27.25[bcde]	69.87[d]	9.00[d]	3.25[d]
4	4.0	0.8	25.33[f]	91.12[b]	18.67[b]	3.67[c]
5	5.0	1.0	24.58[g]	95.87[a]	22.00[a]	6.17[a]
6	6.0	1.2	26.92[e]	75.50[c]	12.67[c]	0.70[e]
7	7.0	1.4	27.08[de]	57.62[f]	12.67[c]	0.29[f]
8	8.0	1.6	27.17[cde]	57.25[f]	12.17[c]	0.27[f]
9	9.0	1.8	27.50[bcd]	60.75[e]	9.00[d]	0.22[f]
10	10.0	2.0	27.58[bc]	61.75[e]	9.00[d]	0.19[f]

[c]Means with a same letter within a column do not differ significantly according to the LSD test (p≤0.05) following ANOVA using the SAS system. Benzyl amino (BA), α-naphthalene acetic acid (NAA) plant growth regulators (PGRs).

the ratio 5:1 (Table 3). Somatic embryos developed within 15 to 20 days on the same medium (Figure 1e) which followed the micro shoot appearance. The length of somatic embryos ranged from 2 to 8 mm. The friable nature of callus was found to be essential for somatic embryo induction. In *Amorphophallus rivieri* and *A. konjac* somatic embryos were induced from compact and incompact callus, respectively (Hu et al., 2005; Zhao et al., 2012). For somatic embryogenesis, no special incubation was required; as against the necessity of dark incubation in many crops (Ozias-Akins and Vasil, 1983; Compton, 1999). There was no significant difference (at $p \leq 0.05$) between the response of calli developed from different explants on further growth and plant regeneration. The medium devoid of any of PGRs (basal medium) failed in shoot regeneration, while there was a steady increase in the shooting % with increase in the concentrations of BAP and NAA in the 5:1 combination from an initial concentration of 1 mg l^{-1} BAP and 0.2 mg l^{-1} NAA. The days for shoot initiation decreased from 38 to 24 with the increase in the BAP: NAA concentration from 1: 0.2 to 5:1. However, a further increase in the concentration did not show any improvement in the shooting %. Thus, modified MS medium containing 5.0 mg l^{-1} BAP and 1.0 mg l^{-1} NAA (Shoot Regeneration or SR medium) was found to be optimum for shoot regeneration (Figure 1f) of up to 95%. On SR medium, a mean shoot number of 22 with an average shoot length of 6 were recorded in 3 months. The maximum shoot regeneration percentage reported so far is 78% in *Amorphophallus albus* (Hu et al., 2004) on medium containing BAP and NAA in 4:1 ratio. In the present study, the number of shoots produced depended on the callus subculture rate rather than the number of explants. Shoots of length 3 to 9 cm developed within a period of 3 months (Figure 1g). The callus from lateral bud explants of *Amorphophallus campanulatus* yielded less number of plantlets as well as shooting percentage even after a prolonged period of culture (Irawati et al., 1986). In elephant foot yam, a maximum of 15 shoots per segmented explant or 2 to 3 shoots per whole explant were obtained through direct organogenesis by Mukherjee et al. (2009). The method explained in this study is efficient in producing about 25 to 30 plants per subculture per 250 mg callus. Thus, the number of planting materials available from a single explant depends on the subculture frequency. The corm bud was found to be the most suitable explant for mass production in elephant foot yam due to its high callusing % and less time for plantlet establishment as against the previous reports on the need of long retention time in medium for positive response (Anil et al., 2012).

The effect of sucrose concentration in SR medium on callus response and microcorm induction was studied. The callus proliferated enormously on SR medium supplemented with 1 and 2% sucrose while there was a tendency towards the plant regeneration with 3% sucrose. Pink, off white or dark brown microcorms were induced as hard globular structures in between friable callus in SR medium with sucrose concentrations between 3 and 7% (Figure 1h). They were induced with a frequency of 90% on SR medium supplemented with 5% sucrose while it was only 10% with 3 or 4% sucrose. With 6% sucrose, the induction % was 70 whereas with 7%, there was a tendency to microcorm enlargement rather than its multiplication. The sucrose concentrations beyond 7% lead to darkening of the callus while 10% sucrose was detrimental to growth. Various studies show the importance of sucrose concentration on microcorm/microtuber induction (Dantu and Bhojwani, 1995; Gopal et al., 1998; Sinha and Roy, 2002). In *Amorphophallus* spp., the concentrations of PGRs were reported to play a role in the formation of corm-like structures (CLS) (Liu et al., 2001; Hu et al., 2006; Anil et

Table 4. Effect of auxins on rooting in elephant foot yam under *in vitro* conditions.

PGRs (mg l^{-1})			Days to root initiation	% Rooting	Number of roots	Mean root length (cm)
BA	NAA	IBA				
5.0	1.0	0.0	60.30[a]	50[c]	8.55[c]	3.81[b]
0.0	5.0	0.0	21.20[b]	80[b]	13.3[b]	3.52[b]
0.0	0.0	5.0	8.65[c]	100[a]	20.9[a]	5.07[a]

[d]Means with a same letter within a column do not differ significantly according to the LSD test (p≤0.05) following ANOVA using the SAS system, plant growth regulators (PGRs).

al., 2012) rather than the sucrose concentration as shown in this study. CLS had been observed on organogenic calli derived from the petioles of *Amorphophallus* spp. *in vitro* (Hu and Liu, 2008; Anil et al., 2012). However, a prolonged retention time in the medium was required for the CLS formation in the callus derived from corm bud explants (Irawati et al., 1986; Anil et al., 2012). The microcorms were separated from the callus and subcultured on the SR medium. They enlarged when kept on the same medium for more than 60 days. The enlarge-ment was enormous when the sucrose concen-tration in the SR medium was increased from 3 to 5%. However for microshoot regeneration, the normal sucrose concentration (3%) remained the best. The large sized microcorms were cut into pieces for further multiplication. They were capable of producing microshoots all over the surface (Figure 1i).

In vitro rooting and transplantation of plantlets to soil

In about 50% of the cultures, roots developed on the SR medium itself after 60 days. But the root length as well as the root number was enhanced when a separate rooting media was used. The inclusion of 5 mg l^{-1} NAA or IBA in the modified basal MS medium increased the rooting % to 80 and 100, respectively. The days to root was decreased from 60 to 8 with the supplementation of IBA in the medium. Therefore, modified MS medium augmented with 5.0 mg l^{-1} IBA (Rooting or R medium) was found to be the most potent rooting medium for elephant foot yam (Table 4). A mean number of 21 roots with an average root length of 5 were recorded in 25 days. IBA had been reported to be the most favourable root inducer compared to Indole 3- acetic acid (IAA) and NAA (Caboni and Tonelli, 1999). IBA acts as a slow release reservoir of a more easily metabolized auxin, whereas NAA, blocks the root emergence as it remains in the tissue in free form due to its stable nature (Fogaca and Fett-Neto, 2005). However in *A. konjac*, NAA proved to be efficient in root induction (Zhao et al., 2012). The number of roots obtained in the present study is much better than those reported in previous studies on this plant (Mukherjee et al., 2009). The use of liquid medium in the rooting phase considerably decreased the time required in agar removal and post hardening contamination (due to the presence of poorly removed agar from finely honed root hairs). The microcorms as such or with plantlets rooted within 15 days when kept on liquid R medium (Figure 1j). In *A. albus*, rooting percentage of *in vitro* corm like structures was not significantly affected by the presence of auxins (Hu and Liu, 2008). The plants rooted in liquid medium are shown in Figure 1k. The unrooted microcorms from the SR medium on direct transplantation after dipping in 5 mg l^{-1} IBA gave rise to plant clusters (Figure 1l). It took approximately 7 months from explant inoculation to reach the hardening stage. Poor survival rates on hardening have so far been reported in this plant (Anil et al., 2012). However in the present study, a survival rate of 100% was observed when the plants/microcorms were trans-ferred to sand: soil: coir pith mixture. The hardened plants were maintained in the net house, ICAR-CTCRI (Figure 1 m).

A high frequency *in vitro* mass propagation of elephant foot yam, a stem tuber crop, using different explants was developed. The present study can be extended for virus elimination and transformation for inducing desirable traits in this plant. As the protocol was found efficient in producing large number of plantlets from less number of explants, it can be commercially exploited for medicinal purpose as well as for its value addition.

Conflict of Interests

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

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Director, CTCRI for extending all the facilities to carry out this work, Dr. S. Chandra Babu for his guidance in corm bud excision, Dr. J. Sreekumar for his help with statistical analysis and Council for Scientific and Industrial Research, India for providing Junior Research Fellowship to the first author.

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Vol. 13(49), pp. 4515-4522, 3 December, 2014
DOI: 10.5897/AJB2014.13831
Article Number: D685FBC48927
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antioxidant activity, phenols and flavonoids contents and antibacterial activity of some Moroccan medicinal plants against tomato bacterial canker agent

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Received 3 April, 2014; Accepted 20 November, 2014

Medicinal and aromatic plants (MAP) belonging to 16 species, currently used in southern Moroccan traditional medicine, were evaluated here, firstly, for the action of their aqueous extracts against the bacterial canker agent of tomato *in vitro*. Then the phenolic contents, flavonoids contents and antioxidant capacities of these MAP extracts were estimated. Results obtained show that tested species exhibited biological activity toward the pathogen studied here; the inhibition zone diameter was between 0.5 and 4.88 cm. Furthermore, tested species exhibited a board range of phenolic contents varying from 55.58 ± 5.07 to 3.98 ± 0.16 mg of Cafeic acid equivalents (CAE) per gram of dry weight (DW). The flavonoids contents varied from 19.82 ± 0.65 to 1.74 ± 0.34 mg of Rutine equivalents (RE) per g of DW. Significant and positive linear correlations were found between total phenolic contents (R = 0.87 and R^2 = 0.76), flavonoids contents (R = 0.96 and R^2 = 0.93) and the biologic activity (IZ diameters) of the aqueous extracts. The antioxidant capacity expressed as Trolox equivalents antioxidant capacity (TEAC) varied from 550.67 to 1.18 mM per 100 g dry weight. Significant and positive linear correlation was found also between antioxidant capacities and both phenolic and flavonoids contents. These results proved that the richness of MAP with phenols and flavonoids was involved in there antibacterial activity and there antioxidant capacity. This finding is useful and can contribute to the development of potent and natural bio pesticides in the future for the control of bacterial canker of tomato as well as other phytopathogens by exploiting MAP compounds accepted by consummators and environmentalists.

Key words: Bacterial canker, tomato, medicinal plants, biological control, flavonoids, Trolox equivalents antioxidant capacity (TEAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH).

INTRODUCTION

The tomato crop is subject to attack by a multitude of pathogenic microorganisms and its intensive culture has generated and amplified the phytosanitary problems. In addition to fungi, viruses and deleterious organisms,

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pathogenic bacteria are important factors that reduce the quality and performance of this culture (Gartemann et al., 2003). The bacterial canker caused by *Clavibacter michiganensis* subsp*. michiganensis* (Smith) (Davis et al., 1984), account among the most important phytosanitary tomato problems in California, Ohio and Morocco (Fatmi, 1989; Fatmi and Schaad, 2002). It is a very contagious and destructive disease in tomato crop both under controlled and field cultures (Utkhede and Koch, 2004). The bacterial canker can cause significant damage which may go up to the destruction of 100% crop (Gitaitis, 1990; Chang et al., 1992). In Morocco, all tomato production areas are contaminated by this pathogen, whose seriousness varies by the following regions (Fatmi, 1989). In the Souss-Massa Draa region, it has become the main cause of the premature death of tomato (Fatmi, 1989). The seeds and infected transplants are the primary sources of primary inoculums of *C. m.* subsp*. michiganensis* (Bryan, 1930; Gleason et al., 1991). Bacteria can survive in the soil (Bryan, 1930; Chang et al., 1992; Strider, 1967; Farley, 1971), in crop residues and multiple alternative hosts (Fatmi and Schaad, 2002). The spread of the disease is ensured by various manipulations like transplantation (Gitaitis, 1990; Gitaitis et al., 1991; Gitaitis and Walcott, 2007), phytosanitary treatments and irrigation systems (Strider, 1969; Chang et al., 1992). The chemical treatments recommended for this disease only reduced the population of the pathogen in the surface of the infected plants (Hausbeck et al., 2000).

Although, the control of this bacterial disease continues to be difficult, prevention was the first defense line. Given the inefficiency of chemical treatments and their impact on health and the environment, research and development of alternative methods are recommended. The biocontrol is a promising way and much research works have been made worldwide against bacterial canker agent and various plant pathogens and encouraging results were found (Amkraz et al., 2010; Basim et al., 2006; Umesha, 2006; Boudyach et al., 2004; Boudyach et al. 2001; Daferera et al., 2003). With a view to developing effective natural treatments, naturally extracted substances have been obtained from plants as privileged axes of the biological control of plant pathogens both in crop treatments and in post-harvest manages (Talibi et al., 2012; Askarne et al., 2012; Talibi et al., 2011; Blaestra et al., 2009; Taqarort et al., 2008; Ameziane et al., 2007). Daferera et al. (2003) tested the antimicrobial activity of essential oils from aromatic plants against several plant pathogens. The results obtained by these authors showed that *C. m.* subsp. *michiganensis* is very sensitive to essential oils of thyme and oregano. Blaestra et al. (2009) evaluated the antibacterial activity *in vitro* and *in vivo* of the aqueous extracts of two medicinal plants, *Allium sativum* and *Ficus carica*, against several pathogens including the tomato canker agent. The effects obtained were very satisfactory since the extracts of *A. sativum* and *F. carica* helped to control the bacterial rates ranging, respectively, up to 65 and 38% , in comparison with a standard treatment with copper (Balestra et al., 2009). Also, Kasselaki et al. (2011) have reported a lack of effective seed treatments against pathogenic bacteria. Indeed, compounds based on copper (the only chemical treatments permitted under organic farming standards) provide only partial control.

Goufo et al. (2008) reported the effectiveness of extracts of plants from Camarón to the agent of tomato late blight. Recently, Ravikumar and Garampalli (2013) have shown that aqueous extracts of 13 plants among the 39 they used have an interesting antifungal activity against *Alternaria solani*. These extracts can be used as potential fungicides in organic cultivation of tomato (Ravikumar and Garampalli, 2013). Furthermore, Boulogne et al. (2012) reported in their review that, the potential use of secondary metabolites from plant extracts as antifungal and insecticidal. Indeed, those secondary metabolites were the support of the biological activity of plant extracts (Boulogne et al., 2012).

The objective of this study was to select among 16 medicinal and aromatic plants (MAP) harvested in different regions of Morocco those which aqueous extracts inhibit the growth of *C. m.* subsp*. michiganensis in vitro.* The last step of this study aimed the assessment of the total phenolic contents, the flavonoids contents and the antioxidant capacity of the MAP tested using the classical Folin-Ciocaleteu reagent, the $FeCl_3$ method and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, respectively. These data will be helpful for understanding the bioactivity of these MAP and also useful for synthesize potent bio pesticides based on the bioactives molecules implicated (Cai et al., 2004; Surveswaran et al., 2007).

MATERIALS AND METHODS

Plant sampling and preparation of extracts

Sixteen Medicinal and aromatic plants (MAP) were collected from several regions of Souss-Massa-Draa, south of Morocco, between March and April 2011 and 2012. The plants harvested were placed in clean plastic bags and numbered. After identification in the laboratory, the samples were air dried in the darkness for a week, followed by a drying in an oven at 35°C for one night. Different parts of the plants were then grinded to powder. Aqueous extracts were prepared as described by Ali-Emmanuel et al. (2002) and Talibi et al. (2011).

Twenty grams (20 g) of each powder were put into suspension in one beaker 250 ml containing 60 ml of distilled water. All were brought to a boil during 5 min. Then, the obtained suspensions were double filtered utilizing the gauze followed by filter paper (Wathmann n°1). The obtained filtrate was dried in an oven at 60°C. The stock solution of each extract corresponded to a concentration of 0.5 g dry extract in 1 ml of distilled water. Extracts were conserved at -4°C in dark medical bottles (30 ml) for further utilization.

Antibacterial activity of the MAP extracts

The paper disc diffusion method was used to detect the antibacte-

rial activity of plants extracts as described by Talibi et al., 2011. One suspension of *C. m.* subsp. *michiganensis* JS 07 isolate (Amkraz et al., 2010) was prepared in the sterile physiological water from a young culture of 72 h on the NBY medium. The optical density of bacterial suspension was adjusted to 0.46 at 610 nm to have 10^8 cfu ml^{-1} (Umesha, 2006). Aliquots (100 µl) of the suspension were speared in the medium surface with sterile glass beads. Cultures prepared were subsequently placed in an incubator at 25°C for 30 min. Sterilized filter paper discs (5 mm in diameter) were soaked with 10 µl of each plant aqueous extract at a concentration of 0.5 g ml^{-1}. Discs were placed in the middle of Petri plates containing pathogen dried cultures prepared previously. Distilled water was used as negative control. The plates were incubated at 25°C and observed after 48 to 72 h. At the end of the incubation period, inhibition zones around the paper discs were measured and compared to the controls. All tests were conducted in triplicate.

Phytochemical screening of medicinal and aromatic plants studied

In order to determine the chemical compounds capable of beings responsible for the antibacterial activity of PAM tested. We were interested in the determination of total phenols, flavonoids and the determination of the antioxidant activity of PAM that showed a significant growth inhibition of *C. m*. subsp. *michiganensis*.

Extracts preparation

For methanolic extraction, 2 ml of methanol 80% was added to 100 mg of plants' powders in a conical flask, which was kept to room temperature overnight with occasional shaking. The extract was than centrifuged using centrifugation apparatus. The supernatant was recuperated and stored at 4°C until use.

Determination of total phenol content

The total phenol contents were estimated using the classical Folin-Ciocalteau colorimetric method according to Alemanno et al. (2003). 25 µl of each sample were reacted with 110 µL of Folin-Ciocalteau reagent for 3 min at room temperature. The reaction was then neutralized with 200 µl of saturated sodium carbonate (20%) and 1.9 ml of distilled water. After homogenisation, reaction was allowed to stand for 30 min in the dark at 60°C. Later the absorbance of the resulting blue colour was measured at 750 nm with a spectrophotometer (Janeway 6400). Appropriate dilutions were made before measure. The blank was prepared using 2 ml of methanol. Quantification was done on the basis of a standard curve with Cafeic acid solutions. This calibration curve was done by preparing scale of Cafeic acid concentration from 0.1 to 0.5 mg/ml in methanol 80%. Results were expressed as microgram of Cafeic acid equivalents (CAE) per milligram dry weight (DW). All measures were done in triplicate and values were means ± SD.

Determination of flavonoids contents

To estimate the flavonoids contents, the method of Singh et al. (2010); Harnafi et al. (2007) was utilized with minor modifications. The $FeCl_3$ reagent was prepared by mixing 400 mg of sodium acetate with 133 mg of $FeCl_3$ powder in 100 ml of 80% methanol. 300 µl of this reagent was added to 600 µl of the sample (plant extract 5% in 80% methanol). After homogenisation, the mixture was left to stand for 30 min in the dark at room temperature. Thereafter, the absorbance was measured at 430 nm with a spectrophotometer. Dilutions were made before measure if necessary. Total flavonoids contents were calculated as µg of Rutine equivalents from a calibration curve. The calibration curve was prepared with solutions of Rutine at concentrations scale from 0.1 to 0.5 mg/ml in methanol 80%. All measures were done in triplicate and values were means ± SD.

Determination of antioxidant capacity

The determination of antioxidant activity was performed according to the protocol, slightly modified as described by Rawat et al. (2011). This method is based on the degradation of DPPH radical (2, 2 diphenyl-1-picrylhydrazyl). In the presence of free radical scavengers, DPPH (purple color) is reduced to 2, 2-diphenyl-1-picrylhydrazine (yellow color). The DPPH radical ($DPPH^{\bullet}$) solution (0.1 mM) was prepared in 80%v/v methanol. The $DPPH^{\bullet}$ solution (1.9 ml, absorbance of 0.47 ± 0.13 at 517) was added to 100 µl of tested extract. The reaction for scavenging DPPH radicals was carried out at room temperature in the dark for 30 min, and then the reduction in absorbance was recorded at 517 nm. A calibrate Trolox standard curve was also made in the same condition of the experiment with concentration scale of Trolox from 0.25 to 1 mM in 80%v/v methanol. The results were expressed as TEAC units (mM Trolox equivalents per 1 milligram dry weight of sample). All measures were done in triplicate and values were means ± SD.

Statistical analyses

Data from phenolic contents, favonoids contents and antioxidant capacity were means of triplicates ± SD. The correlation coefficient R and the determination coefficient R^2 were calculated using Microsoft Excel 2007.

RESULTS

Evaluation of the antibacterial activity of plant extracts

The antibacterial efficacy of tested plants was determined *in vitro* using the agar plate's methods. Table 1 illustrates the summary of results obtained. The most efficient MAP were *Lavandula coronopifolia* (78), *Rubus ulmifolius* (7), *Rosa canina* (43), *Cistus monspliensis* (114), *Pistacia atlantica* (26), *Anvillea radiata* (83), *Cistus crispus* (107), *Lavandula stoechas* (25) and *Ighermia pinifolia* (65) for the aqueous extracts tested here (IZ ≥ 2.59 cm). We can also conclude that the bacterium was more sensitive to extracts containing a large board of molecules suggesting that the antibacterial activity of MAP results in a synergetic activity more than one kind of compounds. However, our results suggest that the plants tested here will be a potent source for natural compounds to combat the bacterial canker agent in tomato crop managements.

Phytochemical screening of medicinal and aromatic plants studied

Results obtained are summarized in Table 1. If these results are analyzed in a comprehensive manner, we

Table 1. Content of total phenols, flavonoids and antioxidant activity of medicinal and aromatic plants selected for their antibacterial activity against the agent of bacterial canker of tomato.

Tested plants	[Phenols] (mg CAE [b]/g DW)	[Flavonoides] (mg ER [c]/g DW)	IZ [d] (cm)	TEAC [e](mM/100g DW)	Parts used
Lavandula coronopifolia (78)	55.58 ± 5.07	19.51 ± 0.49	4.88	550.67	Stem, leaves and flowers
Rubus ulmifolius (7)	37.52 ± 6.64	19.82 ± 0.65	4.05	430.50	Stem and leaves
Rosa canina (43)	44.68 ± 0.573	17.58 ± 0.98	4.04	333.73	Stem and leaves
Cistus monspliensis (114)	37.21 ± 0.63	19.79 ± 2.07	3.86	233.27	Stem, leaves and flowers
Pistacia atlantica (26)	62.78 ± 1.51	18.38 ± 1.53	3.70	460.49	Leaves
Anvillea radiata (83)	24.13 ± 5.02	15.39 ± 1.43	3.55	166.89	Stem, leaves and flowers
Cistus crispus (107)	27.62 ± 0.29	16.53 ± 0.31	3.20	118.63	Stem, leaves and flowers
Lavandula stoechas (25)	32.58 ± 4.10	6.30 ± 0.26	3.07	90.80	Stem, leaves and flowers
Ighermia pinifolia (65)	45.41 ± 5.70	14.72 ± 0.60	2.59	73.49	Stem, leaves and flowers
Artemisea inculta (89)	21.99 ± 1.18	10.31 ± 1.15	2.11	35.22	Stem and leaves
Ceratonia siliqua (62)	21.20 ± 0.99	7.44 ± 2.26	1.58	55.38	Leaves
Fagonia zilloïdes (93)	6.55 ± 0.78	5.56 ± 1.6	1.27	11.08	Leaves
Zygophyllum gaetulum (104)	3.98 ± 0.16	2.26 ± 1.8	1.25	1.18	Stem and leaves
Colocynthis vulgaris L. (67)	6.55 ± 0.35	2.13 ± 0.72	0.82	9.82	Leaves
Sanguisorba minor (19)	4.47 ± 0.28	1.74 ± 0.34	0.68	3.64	Stem and leaves
Reseda alba (99)	5.01 ± 0.13	1.99 ± 0.28	0.50	3.59	Stem and leaves

[a]Values are means of three replicates ± SD. [b]Results are expressed in equivalent mg caffeic acid (CAE) per g dry weight (DW) of plants. [c]The results are expressed in mg Rutin equivalents (RE) per gram dry weight (DW) of plant. [d]Diameters of inhibition zones of aqueous extracts in cm. [e]TEAC, Trolox equivalent antioxidant capacity, results are expressed as mM Trolox equivalents per 100 g dry weight.

Table 2. Correlation (R) and determination (R^2) indices between the content of phenols (Ph), the content of flavonoids (Flav), antioxidant capacity (TEAC) and the antibacterial activity of aqueous extracts (diameters of the inhibition zones, IZ).

Parameter	Ph/Flav	Ph/IZ	Flav/IZ	Ph/TEAC	Flav/TEAC	TEAC/IZ
R	0.88852516	0.87339293	0.96832161	0.84607994	0.78914914	0.86716811
R^2	0.78947696	0.7628152	0.93764673	0.71585126	0.62275636	0.75198053

noted that the following species: *L. coronopifolia* (78), *R. ulmifolius* (7), *R. canina* (43), *P. atlantica* (26), *A. radiata* (83), *C. crispus* (107), *L. stoechas* (25) and *I. pinifolia* (65) had levels of phenols, flavonoids and anti-oxidant activities much higher than the other tested plants. Furthermore, it is important to note that these parameters change, usually, in proportion to the diameters of the inhibition zones. More precisely, the content of total phenols species tested ranged from 3.98 ± 0.16 to 62.78 ± 1.51 mg of caffeic acid equivalent (CAE) per g dry weight (DW), that of flavonoids ranged from 17.4 ± 3.4 to 19.82 ± 6.5 mg of rutin equivalent (RE) per g of dry weight (DW) and the antioxidant activity ranged from 1.18 to 550.67 mM Trolox equivalent per 100 g of dry weight (DW) (Table 1). Further, a significant positive correlation was observed on one hand, between the phenol content ($R = 0.87$ and $R^2 = 0.76$), the flavonoid content ($R = 0.96$ and $R^2 = 0.93$), and the antimicrobial activity of aqueous extracts and secondly between the phenols content, the content of flavonoids and antioxidant activity of aqueous extracts (Table 2). These results confirm the richness of selected MAP on phenols and flavonoids, which are likely involved in their antibacterial activities and their anti-oxidant capacity. The positive correlation between the antibacterial activity and the antioxidant one is that, more extract had antioxidant capacity, is more interested in terms of biological control. Furthermore, the antioxidant capacity of the samples may explain the activation of the germination already observed in our previous study (Talibi et al., 2011; Amkraz, 2013). This activation of the germination would be due to the action of the antioxidant molecules on the pH of the surrounding medium of seeds. Indeed variations in the pH affect germination of seeds of several plant species (Chachalis and Reddy, 2000).

DISCUSSION

The researches undertaken in our laboratory aimed to

evaluate the antibacterial potential of medicinal and aromatic plants (MAP) from the Souss-Massa Draa valley, against *C. m.* subsp. *michiganensis* and other pthytopathogens both in crop and post-harvest (Talibi et al., 2013; Askarne et al., 2013; Askarne et al., 2012; Talibi et al., 2012a; Talibi et al., 2012b; Talibi et al., 2011; Ameziane et al., 2007). In fact, the aqueous extracts of several MAP (40 species) were studied firstly by measuring their reduction capacity, or even elimination of the inoculums of this pathogen on tomato seeds by Talibi et al. (2011). Effect of these extracts on the seed germination was also evaluated and results obtained confirmed that MAP extracts tested had a positive action on the germination rate. This improved seed germination could be explained by an acceleration of the process of solubilization of seed husks on the one hand and the effect of these extracts on the pH of the medium (Chachalis and Reddy, 2000; Talibi et al., 2011; Amkraz, 2013).

In this paper, we aimed to find eventual relation between the biological activity of the MAP studied previously and their phytochemical composition. To do this, we chose some plants belonging to the three categories defined by Talibi et al. (2011) and Amkraz (2013) based on the inhibition zone diameters obtained against the bacterial canker agent. So, we have chosen 16 MAP for this study. We have firstly repeated the *in vitro* antibacterial activity of these MAP and secondly assessed the relative richness of the MAP with phenolic compounds and we have also assessed their antioxidant activity. This approach could be a promising antibacterial alternative to chemicals whose multiple disadvantages constitute a major constraint for both the consumer and the environment. This is more important because we still do not have, to this day, any commercial cultivars resistant to the bacteria causing bacterial canker in tomato. The results of the preliminary screening showed that aqueous extracts of all tested MAP have antibacterial activity toward the pathogen studied here. The most effective species belong to the following families: Lamiaceae, Rosaceae, Asteraceae, Anacardiaceae and Cistaceae. The results obtained with species belonging to the genus *Lavandula* are remarkable and entirely consistent with those of Daferera et al. (2003) reporting that the *Lavandula* genus includes several species with antimicrobial activity against some plant pathogens. In addition, these authors showed that the essential oil of *Lavandula angustifolia* (600 mg ml^{-1}) completely inhibited the growth of *C. m*. subsp. *michiganensis*. According to these authors, the antibacterial activity of this essential oil is probably due to bioactive compounds, especially linaloole and linalyl acetate (Daferera et al., 2003). In addition, Teixeira et al. (2012) confirmed also the high antibacterial potential exercised by a representative of this genus. They have indeed demonstrated that methanolic extracts of *L. stoechas* exhibit significant antibacterial properties toward *Staphylococcus aureus* and *Staphylococcus epidermidis*. This power is linked to the phenolic and terpene compounds of this plant. According to our results, the second species which significantly inhibited the growth of *C. m.* subsp. *michiganensis in vitro* was *R. ulmifolius*. In fact, Sisti et al. (2008) showed that the dry extract *R. ulmifolius* is of great interest in so far as it has biological activity against a number of human pathogens. In another study, Thiem and Goślińska (2004) had reported that extracts from the leaves of *Rubus chamaemorus* were endowed with antibacterial activity against some Gram negative bacteria.

Furthermore, Arima et al. (2002) showed that rutin (quercetin -3- rutinoside) is responsible for the antimicrobial activity against some Gram negative and Gram positive bacteria. Through our results, the growth inhibitory power of *C. m.* subsp. *michiganensis* exercised by *R. canina* was significant. This result agrees well with that of Montazeri et al. (2011) which showed that the aqueous and methanolic extracts of *R. canina* exhibit antibacterial activity against various pathogens (*S. aureus*, *Escherichia coli*, *Bacillus cereus*, *Candida albicans* and *Bacillus subtilis*). Moreover, this result is also consistent with those of Basim and Basim (2004), which showed that the essential oil of *Rosa damascena* is effective against *Erwinia amylovora*, the fire blight pathogen of Rosaceae.

In previous studies, the specific phenolic components of the genus Rosa such as catechins and procyanidins has been shown to be effective antioxidants. As regards the inhibitory effect toward *C. m.* subsp. *michiganensi*s exercised by *A. radiata*, the results correlate well with those of other authors. Indeed, El Hassany et al. (2004) showed the existence of a new terpene compound, obtained by extraction with chloroform, which is the main component involved in the antibacterial activity. This compound has a strong inhibition against *B. cereus*, *Streptococcus*, *Proteus vulgaris*, *Enterococcus faecalis*, *E. coli* and *Pseudomonas aeruginosa*. We also obtained a significant anti *C. m.* subsp. *michiganensis* activity with extracts of *P. atlantica*. This is in perfect agreement with the results of Hosseini et al. (2013) that showed the high inhibitory potential of *Streptococcus mutans* by the aqueous extracts and diethyl ether of this plant. Regarding the genus *Cistus*, we showed that *C. crispus* and *C. monspeliensis* induced a significant inhibition of the proliferation of *C. m.* subsp. *michiganensis*.

These results are in agreement with most of the work involving representatives of this kind. Indeed, Bouamama et al. (2006) reported that the sheets of *C. villosus* and *C. monspeliensis* have antibacterial activity against some Gram-negative bacteria responsible for certain human diseases. This activity is linked to the richness of this species in polyphenols which are known for their antibacterial property.

Furthermore, it has been shown repeatedly that the aqueous and methanolic extracts of *C. villosus* induce very high antimicrobial activity against the major pathogenic

of citrus in postharvest (Ameziane et al., 2007). Talibi et al. (2012a, b) also reported that aqueous extracts of *C. monspeliensis*, *C. villosus* and *C. crispus* from southern Morocco, reduce mycelial growth of *Geotrichum candidum*. This significant reduction is of the order of 100, 98 and 52%, respectively.

The content of total phenols varied between 3.98 and 62.78 mg of EAC (caffeic acid equivalents) per g dry weight (DW). This increase in the concentration of total phenols in some samples was also highlighted by Surveswaran et al. (2007). These authors reported levels ranging from 0.6 to 356.3 mg Gallic acid equivalents (GAE) per g dry weight in extracts of 113 Indian medicinal plants. In the same context, Bahri-sahloul et al. (2009) have shown, in the analysis of 8 MAP samples, which the amount of these secondary metabolites varies between 0.45 and 16.38 mg GAE per g of DW. This relative wealth of some MAP with polyphenols was also confirmed by Ebrahimzadeh et al. (2010) whose analysis of the samples revealed a total phenol content between 35.4 and 90 mg GAE per g dry weight. Finally, it is essential to clarify that a significant fluctuation, 52.8 to 16.65 mg GAE per g of DW was also discovered during the analysis of leaves of the same species, *Cinnamomum osmophleum*, harvested from eleven different locations (Wu et al., 2013).

As regards to the dosage of flavonoids, the results obtained revealed that the content of the compounds tested in these plants varies between 1.74 and 19.82 mg RE per g of DW. The great variability in rates of flavonoids at the MAP analyzed was reported by El Allagui et al. (2007). According to this team, the level of these compounds in the five studied samples varies between 2.90 and 36.20 mg Quercetin equivalent per gram of dry weight. Ebrahimzadeh et al. (2010) also showed that the plants they studied have relatively high contents in flavonoids, ranging from 22.80 to 48.20 mg Quercetin equivalent per g of DW. According to Bahri - Sahloul et al. (2009), the rate of flavonoids on the samples tested varies between 3.178 and 7.536 mg RE per g of DW. These results are of great interest because they reflect significant changes in concentrations of these metabolites according to locality, variety and even according to the maturity of the plant, and that for the same organ (flowers). In addition, a remarkable variability in levels of these compounds were identified in the comparative study of the same MAP samples; subjected to various types of extraction or fractionation protocols (Li et al., 2010; Montazeri et al., 2011).

The antioxidant activity of the 16 MAP studied here, expressed in mM TEAC per 100 g DW varies between 1.18 and 550.67 mM TEAC per 100 g of DW. This result is quite comparable to those described by other authors (Fu et al., 2010 etc). Indeed, Surveswaran et al. (2007) showed after extensive sampling and analysis of 133 species of medicinal and aromatic Indian plants that anti-oxidant activity varies between 0.16 and 500.70 mM TEAC per 100 g of DW. The substantial variability in the antioxidant activity between the different samples is probably linked to the presence of a different combination of chemical compounds having the antioxidant potential; this is not only determined by the nature and the concentration of each compound but linked also to the synergism that can establish certain components relative to others. A comparative analysis, case by case, for our best samples to representatives of the same kind described in the literature test was conducted. However, we encountered some heterogeneity in obtained results that perfectly explains the diversity of extrinsic factors (geography, climate) and intrinsic (variety, maturation stage, organ analyzed, method of extraction and assay technique) which the plant samples are submitted and inducing heterogeneous phytochemical profiles. Despite this, we must remember that at least six of our samples are remarkable because of their high levels of phenols, flavonoids but also by their significant antioxidant potential. This is even more interesting when we have only tested the aqueous extracts. Indeed, the methanol, ethyl or flavored extracts using other organic solvents reveal even greater potential as has been repeatedly demonstrated by several authors (Panizzi et al., 2002; Fu et al., 2012; Oliveira et al., 2012). The major advantage is that our samples is also reflected in the strong correlation both between the phenolic content (R = 0.84) and flavonoids (R = 0.789) and antioxidant activity and secondly between the phenols content (R = 0.87) but mainly flavonoids (R = 0.96) and antibacterial activity. These correlations agree well with the results of other teams for other models plant / pathogen (Cai et al., 2004; Bahri-Sahloul et al., 2009; Fu et al., 2010; Surveswaran et al., 2010). Furthermore, phenols appear to be more involved in the antioxidant activity, while anti-microbial activity appears more related to flavonoids (Bahri-Sahloul et al., 2009; Fu et al., 2010; Surveswaran et al., 2010).

Finally, we must note that all MAP samples tested here are available in the Souss-Massa Draa valley southern Morocco and grow very easily. In addition, the extraction method adopted is very simple using the most accessible and least expensive solvent, namely water (Talibi et al., 2011; Amkraz, 2013).

Conclusions and perspectives

The application of micro-organisms and/or natural substances for control or suppression of pest pathogens populations has recently been imposed by government guidelines to minimize or even eliminate the use of chemical pesticides seen concerns of the general public on the one hand, to the problem of resistance to chemical pesticides (Martineza et al., 2013) and secondly, to the adverse effects of chemical residues on both the environment and the human health (Whipps and Lumsden, 2001). Thus, several biopesticides have recently been

made (Hynes and Boyetchko, 2006). The research started in this framework allows us to conclude that southern Morocco and especially the region of Souss Massa Draa, is rich in MAP with significant antibacterial activity against *C. michiganensis* subsp. *michiganensis*. The best results are obtained with plants belonging to the genera: *Lavandula*, *Rubus*, *Rosa*, *Cistus*, *Pistacia* and *Anvillea*. A positive correlation was found between the antibacterial activity and the content of flavonoids and phenols. These results are promising and may contribute to the future development of natural biopesticides for the control of bacterial canker of tomato. The development of a reliable method of seed treatment with MAP, or their bioactive compounds to eliminate or reduce the initial inoculums of the pathogen on the seed is a very interesting application. The success of this process will help to get free seeds of this pathogen. This certification is essential to trade tomato seeds internationally. Further studies are needed to complete and confirm these results in green house level in order to estimate the power of the selected plants to prevent and control the bacterial canker in tomato. Indeed, the possibility of combining biological methods remains an important factor in promoting the use of biological control. This approach can provide powerful and reliable tools for biological control against the major pest problems in crops. Indeed, Bardin et al. (2008) reported that separated plant extract made of *Reynoutria sachalinensis* (Milsana) and mush-room *Microdochium dimerum* formulations exhibit high efficiency in the respective control of powdery mildew and *Botrytis cinerea* affecting tomato crops in greenhouse. These authors showed that the two treatments are compatible because the combination does not alter their respective efficiency.

Conflict of Interests

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

ACKNOWLEDGEMENTS

Authors are grateful to Pr. Saida Tahrouch for his assistance in the phytochemical screening of the MAP studied here.

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Vol. 13(49), pp. 4523-4528, 3 December, 2014
DOI: 10.5897/AJB09.749
Article Number: EAFC72D48940
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Biological active compounds from actinomycetes isolated from soil of Langkawi Island, Malaysia

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Received 25 May, 2009; Accepted 13 November, 2014

Actinomycetes which were categorised as beneficial microorganisms have long been studied for their potential in producing secondary metabolites either for pharmaceutical or agricultural industries. In this study, 160 isolates of actinomycetes had been isolated using soil suspension method. All the 160 isolates were later tested for their potential to secrete secondary metabolites such as cellulases, mannanases, xylanases, lipases, proteases and antifungal compounds. Prescreening of the 160 actinomycetes isolates showed that 73.1% of the actinomycetes produces xylanases, 69.4% produces cellulases, 65.0% proteases, 44.4% lipases and 9.4% mannanases. It was also observed that 43.1% (69/160) of actinomycetes showed antagonistic reaction towards *Colletotrichum capsici* while 18.8% (30/160) showed antagonistic reaction towards *Colletotrichum gloeosporioides*. Five (5) of the best producers of the bioactive compounds were identified using 16S rRNA primers. All of the isolates were identified to be originated from streptomyces genus. These potential actinomycetes need to be further tested for their application and have their potential fully characterized before being distributed or made known to interested industries.

Key words: Actinomycetes, anthracnose, bioactivity, biodiversity, Malaysia.

INTRODUCTION

Soil microbes which had been known to possess the ability to act as degradation and biocontrol agents have been widely studied by researchers around the world. One of these well-known soil microbes are the actinomycetes. Actinomycetes had been long known to be the main producer of antibiotics in the pharmaceutical industries. The used of actinomycetes for agricultural and medicinal purposes have also been studied by researchers in Malaysia as well as international (Johnson 1954; Vikineswary et al., 1997; Ismet et al., 2002; Lo et al., 2002; Jeffrey et al., 2011). According to Lo et al. (2002), there are about 100 genera of actinomycetes inhibiting in the soil. The abillity of actinomycetes to act as the biodegradation of cellulosic and hemicellulosic compounds and biocontrol agents would have been favored by consumers globally due to the rising conscious of consumers towards synthetic chemical compounds that may polluted that environment as well as

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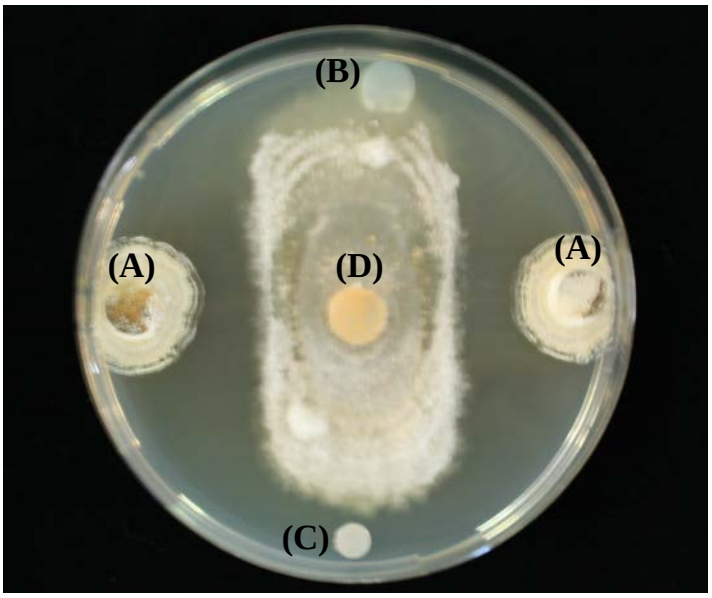

Figure 1. *Colletotrichum gloeosporioides*. screening against actinomycetes. (A) Actinomycetes, (B) negative control, (C) cycloheximide 50 mg/ml (positive control), D; *C. gloeosporioides*.

dangerous to human health. Studies have shown that *Streptomycetes* spp. gave significant control towards pythium root rot of corn and sugarcane (Johnson, 1954) and cotton wilting (Arjunarao, 1971). Work done by Lee and Hwang (2002) on actinomycete isolated from Korean soil showed strong antifungal activity of actinomycetes against *Alternaria mali, Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *cucumerinum* and *Rhizoctonia solani*. Managing pest and diseases using biological control would give long term advantages, such as cost saving and a sustainable agriculture although the effect is not immediately (Aghighi et al., 2004). All of these showed that, bioactive compounds isolated from actinomycetes were studied extensively by researchers through out the world. In this study, actinomycetes isolated from Langkawi Island soil were screened for their bioactivity. Potential isolates were later characterized and identified using molecular technique.

METHODOLOGY

Soil samples collection

Soil samples were collected randomly under the trees shrubs of few places at Langkawi Island. The soil was dug 15 cm from the surface and the soil samples were taken at that depth. The soil samples were put into a ziplock bag for transportation back to Serdang, Selangor.

Soil actinomycetes isolation and enumeration

The soil were left to air dried for about 7 to 14 days. Ten grams of dried soil were grinded before being put into a conical flask added with 100 ml of sterile distilled water. The conical flasks were then put onto an orbital shaker and agitated at 220 rpm for 1 h at room temperature (28±2°C). Serial dilution was performed for each soil samples until the factor of 10^6. A total of 150 µl of the soil suspension were pipetted and spread onto starch casien agar (SCA) plates supplemented with cycloheximide (50 µg/ml). Duplicate plates were prepared for each of the serial dilutions. The plates were then incubated at 28±2°C for about 10 days. The emerging colonies of actinomycetes were subcultured onto a fresh SCA plate.

Bioactivity testing for enzymatic activity

Plug of actinomycetes were innoculated onto Minimal Medium Agar (MMA) containing AZO-CM-Cellulose, AZO-Carob-Galactomannan or AZO-xylan (Oat) as substrate (Sahilah, 1991). Gelatin hydrolysis assay as described by Frazier (1926) was employed for the screening of protease activity. Lipase activity was conducted using method for determination of esterastic activity (Sierra, 1957) with modification. Tween 80 used in the esterastic assay test was replaced with Tween 20 for this purpose. Formation of halo zone indicates positive reaction for the entire test conducted. Measurement of the halo zones were taken at day 5 of the test. All the test were conducted under room temperature (28±2°C) condition.

Bioactivity testing of soil actinomycetes with for antifungal activity

Plug of actinomycetes was inoculated onto the test plate of potato dextrose agar (PDA). Cycloheximide 50 mg/ml was used as the positive control and plug of PDA was used as the negative control. Plug of tested plant pathogens (*C. gloeosporioides* and *Colletotrichum capsici*) was then innoculated at the middle of the test plate as shown as in Figure 1. Clear zone form was measured

Table 1. Inhibition profile produce by actinomycetes.

Pathogen	Isolates inhibition profile (x)			Total number of isolates which produce inhibition zone
	No inhibition (y= 0 mm)	Low inhibition (1 mm ≤ y ≤ 15 mm)	High inhibition (y > 15 mm)	
Colletotrichum capsici	91	45	24	69
Colletotrichum gloeosporioides	130	8	10	18

x was the number of isolates; y was the inhibition profile.

after seven days of the test and average size of each clear zone was calculated. All the tests were conducted under room temperature (28±2°C) condition. The inhibition profile was scored as no activity (0 mm), low antagonistic activity (1 to 15 mm) and high antagonistic activity (≥15 mm) (Table 1).

Identification of the best isolates

The isolates that gave the best results were chosen and molecular analysis was done. Deoxyribonucleic acid (DNA) was isolated using GF-1 bacterial extraction kit purchased from Vivantis. Protocol used was as suggested by the manufacturer (http://www.vivantis.com/doc/1112006164042.pdf). Polymerase chain reaction (PCR) was conducted using method stipulated by Jeffrey et al. (2008). PCR products were purified using kit obtained from Vivantis and protocol used was as suggested by manufacturer (http://www.vivantis.com/doc/225200716542.pdf). Purified PCR products were later sent to 1st Base sequencing for sequencing purposed. Sequencing results were later BLAST with database from NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Isolation and enumaration of actinomycetes

From the soil samples collected, 160 isolates of actinomycetes were isolated. From the total number of the isolates, 36 isolates were observed to possess grey colour aerial mycelium, while 16 in yellow, 71 in white, 1 in green, 21 in yellowish white, 3 in brown, 8 in red, 2 in orange and 2 in dark brown. The average colony forming unit / gram (cfu/g) of dry soil for Langkawi Island soils were estimated to be 1.85×10^7. Pigmentation was also observed from 12.5% (20/160) of the isolates. The highest colour of diffusible pigmentation observed was brown in colour (15 isolates), followed by yellow (four isolates) and green (one isolate).

Bioactivity testing for enzymatic and antifungal activity

From the 160 isolates of actinomycetes tested for enzymatic activities, 69.4% showed the ability to produce cellulases, 9.4% mannanases, 73.1% xylanases, 65.0% proteases and 44.4% lipases. While for the antifungal activities tested, 43.1% (69/160) showed antagonistic reaction towards *C. capsici* and 18.8% (30/160) showed antagonistic reaction towards *C. gloeosporioides*.

Identification of actinomycetes

All the 5 isolates of actinomycetes with high bioactivity producing activity were identified to be from the genus of Streptomyces, with three from the species *Streptomyces humidus*, 1 from the species of *Streptomyces hygroscopicus* and 1 just identified as *Streptomyces* spp. only (Table 2).

DISCUSSION

Isolation and enumaration of actinomycetes

From the morphological characterization of the isolated actinomycetes conducted, it was observed that the highest aerial mycelium observed were from the white series. This finding however were different from the reults obtained from studies conducted by researches in Malaysia and overseas (Ndonde and Semu, 2001; Barakate et al., 2002; Jeffrey et al., 2008). The result of diffusible pigmentation was low comparing to the results obtained by Ndonde and Semu (2001). In their study, Ndonde and Semu (2001) observed that 65.0% of their isolates produced soluble pigmentation but in this study only 12.5% of the actinomycetes were seem to produce soluble pigmentation. From the colour of aerial mycelium and the production of diffusible pigment it was observed that the distribution of actinomycetes could be considered to be more diversed in this study compared to study done by Jeffrey et al. (2008) and Lo et al. (2002). The average cfu/g of dry soil obtained in this study is 1.85×10^7 which is much more higher compared to cfu/g of dry soil for actinomycetes obtained from some garden and agricultural soils isolated from selected areas in Selangors, Johor and Kuala Lumpur (Jeffrey et al., 2007). However, the cfu/g was lower than the cfu/g of actinomycetes isolated from Semongok soil which was 8.0×10^7 (Jeffrey et al., 2008). Study by Lee and Hwang (2002), on the diversity of actinomycetes isolated from various vegetative soils in Korea, showed that cfu/g for the actinomycetes isolated were between 1.17 to 4.2 x 10^6 which is much lower than the cfu/g obtained in this study.

Table 2. Bioactivity produced by 5 best actinomycetes isolates.

Isolate number	Isolate Id	Aerial mycelium colour	Pigmentation (if any)	Bioactivity produced (Clear zone size, mm)						
				Cellulases	Mannanases	Xylanases	Proteases	Lipases	*Colletotrichum capsici*	*Colletotrichum gloeosporioides*
DF11	*Streptomyces hygroscopicus*	Grey	Nil	22.5	16	15	14	20	18	Nil
LB06	*Streptomyces humidus*	White	Nil	30	20	27	11	14	0	16
K17	*Streptomyces humidus*	Yellow	Green	12	0	15	12	34	18	14
DF22	*Streptomyces humidus*	Yellow	Brown	16	16	20	0	12	10	19
N07	*Streptomyces* spp.	White	Nil	30	0	20	8	16	16	18

Bioactivity testing

Approximately 85.0% (136 isolates) of the isolate produced one or more enzymatic activity. From the total isolates, 15.0% (24 isolates) did not produced any enzyme, while only 1.9% (3 isolates) produces all the 5 types of enzymes. Most of the actinomycetes produces 3 to 4 enzymes with the percentage of 26.9% (43 isolates) and 31.3% (50 isolates), respectively. This indicates that actinomycetes possess the potential to secrete broad range enzymes, which maybe the results from natural selection of this microorganism in order for it to survive in a competing environment. In a study conducted by Arifuzzaman et al. (2010), soil content, soil types, pH and soil moisture played a role in determining the type of microbes and their population thus influencing the type of enzymes secreted. From the results obtained, it was observed that actinomycetes isolated from Langkawi Island did not show a high number for mannanases producing isolates. However, this is not the only study done in Malaysia that showed a low mannanases activity from actinomycetes. A few studies conducted also showed that mannanases activities are low among soil actinomycetes compared to other polysaccarides produced (Jeffrey, 2006; Jeffrey et al., 2008). The low mannanases production by actinomycetes may indicate that actinomycetes isolated from Malaysian soils are not good producers for mannanase or the environment around these actinomycetes does not required for the production of mannanse from these microbes.

In a study conducted by Boontim and Lumyong (1999) on cellulases producing actinomycetes from Thailand, they observed that from a total of 125 isolates of actinomycetes isolated from Chiang Mai soil, only 1 isolate produces cellulase. This number however is lower than the percentage of actinomycetes producing cellulase obtained in this study (69.4%) and other study conducted by researchers in Malaysia (Jeffrey et al., 2007; Jeffrey, 2008). From the results obtained, it can be concluded that different demographic sample sites played its role in influencing the secretion of secondary metabolite by actinomycetes. For antifungal activities it was observed that a total of 3.1% of actinomycetes produces both antifungal. Many antimicrobial activities had been detected from actinomycetes by researchers all around the world in the past few decades for medical purposes (Moncheva et al., 2002, Aghighi et al., 2004, Oskay et al., 2004, Jeffrey, 2006). Little emphasis was given to the utilization of actinomycetes as a biological control in agriculture sector in Malaysia as the used of biological control does not give immediate results to the farmers.

In recent years, it was observed that soil borne actinomycetes have the potential of controling *C. gloeosporioides* in chilli (Suwan et al., 2012). Sacramento et al. (2004), showed that actinomycetes isolated from the Brazilian soils demonstrated antifungal activities against *Fusarium solani* with the inhibition zone of about 30 mm. Studies on the infection of *C. gloeosporioides* on orchid had been done by Prapagdee et al. (2008). Known antifungal from *Streptomyces* spp. had been isolated and identified by Intra and Panbangred (2008), showing high inhibitory activity towards *C. capsici* and *C. gloeosporioides*. These examples showed that actinomycetes had the potential to produce

antifungal compounds although the novelty of the compounds maybe unknown. In this study, we observed that actinomycetes isolated were able to secrete antifungal for both *C. capsici* and *C. gloeosporioides*. As we observed, some of the actinomycetes produces antifungal for both the *C. capsici* and *C. gloeosporioides* while other may be towards a specific species only. This maybe due to the reason that antifungal compounds secreted were non specific antifungal (broadrange antifungal) while other maybe specific antifungal (narrow range antifungal). The production of enzymes such as glucanases, proteases and β-1,3-glucanses might be the another factor influencing the inhibitory activity of the actinomycetes towards *C. gloeosporioides* (Anitha and Rebeeth, 2010).

The production of antifungal compounds by actinomycetes in this study need to be studied more extensively as the production of antibiotics are often influenced by few factors such as chemicals added to the agar media (salt, carbon and nitrogen sources), temperature and pH (Vasavada et al., 2006). By changing the composition of media used we may be able to increase or decrease the production of antibiotics form on these microorganisms. It is also possible that other useful antimicrobial compounds were produced and not screened in this study (Barakate et al., 2002). Porter (1971) hypothesized that all actinomycetes possessed some antimicrobial properties and can be assessed when cultured and study under suitable condition. Kokare et al. (2004) had demonstrated that antimicrobial activity by actinomycetes was dependent upon the media used for its cultivation.

Conclusion

Preliminary screening from 160 actinomycetes isolated from Langkawi Island showed that actinomycetes isolated from Langkawi Island were potent producers of bioactive compounds. Five (5) potential producers of bioactivities were identified as *S. humidus* (3 isolates), *S. hygroscopicus* (1 isolate) and *Streptomyces* spp (1 isolate). These potential actinomycetes should be further utilized in various industries such as agriculture and food for the benefit of human race.

Conflict of Interests

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

ACKNOWLEDGEMENT

The authours would like to thank the Malaysian government for the fund allocated under 05-03-08-SF0155 and RMK10(148).

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academicJournals

Vol. 13(49), pp. 4529-4535, 3 December, 2014
DOI: 10.5897/AJB2014.13717
Article Number: 7C8313548942
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Production and evaluation of instant emulsion base ("ncha") from oil palm biogenic waste

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30 January, 2014; Accepted 6 May, 2014

Instant emulsion base ("ncha") was produced from oil palm biogenic waste by extraction, concentration and encapsulation. Traditional products which were prepared and commercial product served as controls. The formulated instant product and the controls were analyzed for compositions, selected physico-chemical, microbial and organoleptic properties. Instant formulated product (IP) showed significantly ($p<0.05$) higher level of iron (Fe): 8.29 ± 0.014 mg/g than the controls (traditional palm waste product- TP: 0.52 ± 0.014 mg/g, traditional palm waste blend product- TPWB: 5.70 ± 0.283 mg/g, commercial palm waste product- CP: 0.52 ± 0.42 mg/g). The controls also showed comparable levels of K, Ca, Mg and Zn with the formulated instant product. Untrained panelists made up of 30 persons scored the emulsions when used for the preparation of African tapioca salad ("Abacha ncha") on a 9-point Hedonic scale for appearance, colour, smoothness and taste. Results indicated that the instant product was significantly accepted ($p<0.05$) with the controls which are already in use. Besides, all the emulsion based products had pH range of 9.0 to 11.6 and exhibited zero mould and reduced total microbial load counts indicating microbial stability even under storage period of 3 weeks at ambient conditions. The overall results indicated that instant formulated product even though an innovation to the controls was highly preferred and could be a convenient means of preparing African tapioca salads by both rural and urban dwellers.

Key words: Convenient foods, oil palm wastes, salad dressing, tapioca salads.

INTRODUCTION

Food waste management, recovery and utilization is the series of activities where discarded food materials are collected, sorted, processed and converted into non-edible/edible new products (Anonymous, 2013a). Besides, large amounts of waste products evolve during production, preparation and consumption of foods. Hence, to maintain a healthy environment, reduction in waste is universally acceptable but in practice the dream has not been accomplished. Oil palm as an economic crop is so useful that none of its by-products is considered a waste. The bunch of the oil palm fruits which may be considered a waste after the fruit removal, is used by some communities in Eastern parts of Nigeria to produce an edible emulsion base (a mild soap) called "Ncha". It is an African salad dressing–water in oil emulsion. This emulsion is used in the preparation and consumption of African salad ("Abacha

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ncha"), bitter yam and processed oil bean seed ("Ugba"). Many food products such as milk, salad dressings, gravies and other sauces, whipped dessert to pings, peanut butter and ice cream are emulsions of edible fats and oils. They affect the physical form of the food product in addition to impacting taste because emulsified oils coat the tongue, impacting "mouth feel" (Anonymous, 2010). In this study, "Ncha", which is a native food item, has an added value by making it instantly through extraction, concentration and encapsulation. The traditional products, palm waste (TP) and palm waste blend (TPWB) were prepared through modifications of local method. Commercial palm waste emulsion base product (CP) that was also prepared, acted as the control.

METHODOLOGY

The oil palm bunch wastes used for this experimental study were procured from a farm in Nsukka town of Enugu State, Nigeria. Fresh tender kola nut leaves were also collected from the above farm while commercial palm product was purchased from a market in the same town. Weighing balance (50 kg capacity, "Five goats" with model no. Z051099) was used.

Production of emulsion products from the ashes of wastes

The oil palm wastes were open sun dried and divided into two portions (Figure 1). The first portion which was 5.1 kg was burnt together with 1 kg of kola nut leaves (KL), after which it was allowed, to cool. The residue obtained was subjected to ashing in a furnace at a temperature of about 650°C for 4 h (Pearson, 1976). This led to the production of creamy white ash product without traces of carbon. The 200 g of ash obtained was mixed with 100 ml of palm fruit sludge (PFS), 20 ml of palm oil (PO) and 60 ml of kola-nut leaves extract. The sample was molded into round shaped balls and allowed to dry at ambient-temperature (27°C) for three days. The second portion of the dried palm waste was burnt and allowed to cool. The ash (250 g) was re-hydrated with 2.5 L of clean tap water and allowed to extract for 3 weeks. During the extraction period, the total soluble solid (TSS) of the supernatant was monitored. On the last day of the monitoring, supernatant was decanted, filtered and divided into two portions. The first portion was bottled (TP) while the second portion was concentrated in a water bath at 100°C. The concentrated extract was encapsulated with edible starch, cold extruded, dried and packaged as instant "Ncha".

Kola nut leaf extract

Healthy and tender fresh leaves (0.5 kg) were collected from a kola nut tree as mentioned above and washed thoroughly with clean salty cold water. The leaves were drained; coarsely cut using kitchen knife with one liter (1.0 L) of clean tap water added and the mixture blended with domestic blender (Ken wood model). The blended mixture was separated using cheese cloth.

Analyses of raw materials and finished products

Proximate analysis

Ash, moisture and fiber contents of the raw palm bunch waste were determined using AOAC (2010) method. Crude fat, nitrogen and protein contents of the raw material were also determined using soxhlet extraction and micro-Kjeldahl methods as described by AOAC (2010), respectively. Total carbohydrate for the raw material was calculated by difference (AOAC, 2010).

Vitamin C analysis

This was done on the fresh kola-nut leaves and palm waste. 5 g of each extract from the raw materials were placed in a 100 ml volumetric flask and 2.5 ml of 20% Meta phosphoric acid was added as a stabilizer. The entire mixture was diluted to 100 ml with distilled water. 10 ml of the solution was then pipetted into a flask and 2.5 ml of acetone added. Absorbance reading was taken at 414 nm using UV spectrophotometer (Bausch and Lamb Spectronic 21 (PEC Medicals, USA).

Mineral composition analysis

This was done using dry ashing method as described by Pearson (1976).

pH analysis

This was carried out using 20 ml of sample each obtained from the final products. A pH meter was used (pocket-sized pH meter-RI02895, Hanna Instruments Italy).

Total titratable acidity

This was done by mixing 10 ml of distilled water, few drops of Bromothymol blue indicator with 5 ml of emulsion sample. This was then titrated against 0.1 N sodium hydroxide (NaOH) to a blue coloured end point.

Total soluble solids

Five milliliters (5 ml) volume of sample was measured into an already dried, weighed crucible. The crucible was placed in an oven and allowed to dry at 30°C for 2 h. The crucible with the sample was weighed.

$$\text{Total soluble solids} = \frac{\text{Weight (sample + crucible)} - \text{weight of crucible}}{\text{Weight of sample used}}$$

Turbidity

This was carried out using photo-electric colorimeter (Portable photoelectric colorimeter, AP-1000M-Japan) with absorbance reading taken at 530 nm wave length.

Colour

Photo-electric colorimeter was used with transmittance reading at 490 nm (Portable photoelectric colorimeter, AP-1000M-Japan).

Microbial analysis

Total viable and mould counts (CFU/ml) of the "Ncha" products (FCP: filtrate from commercial product; FTPWB: Filtrate from palm

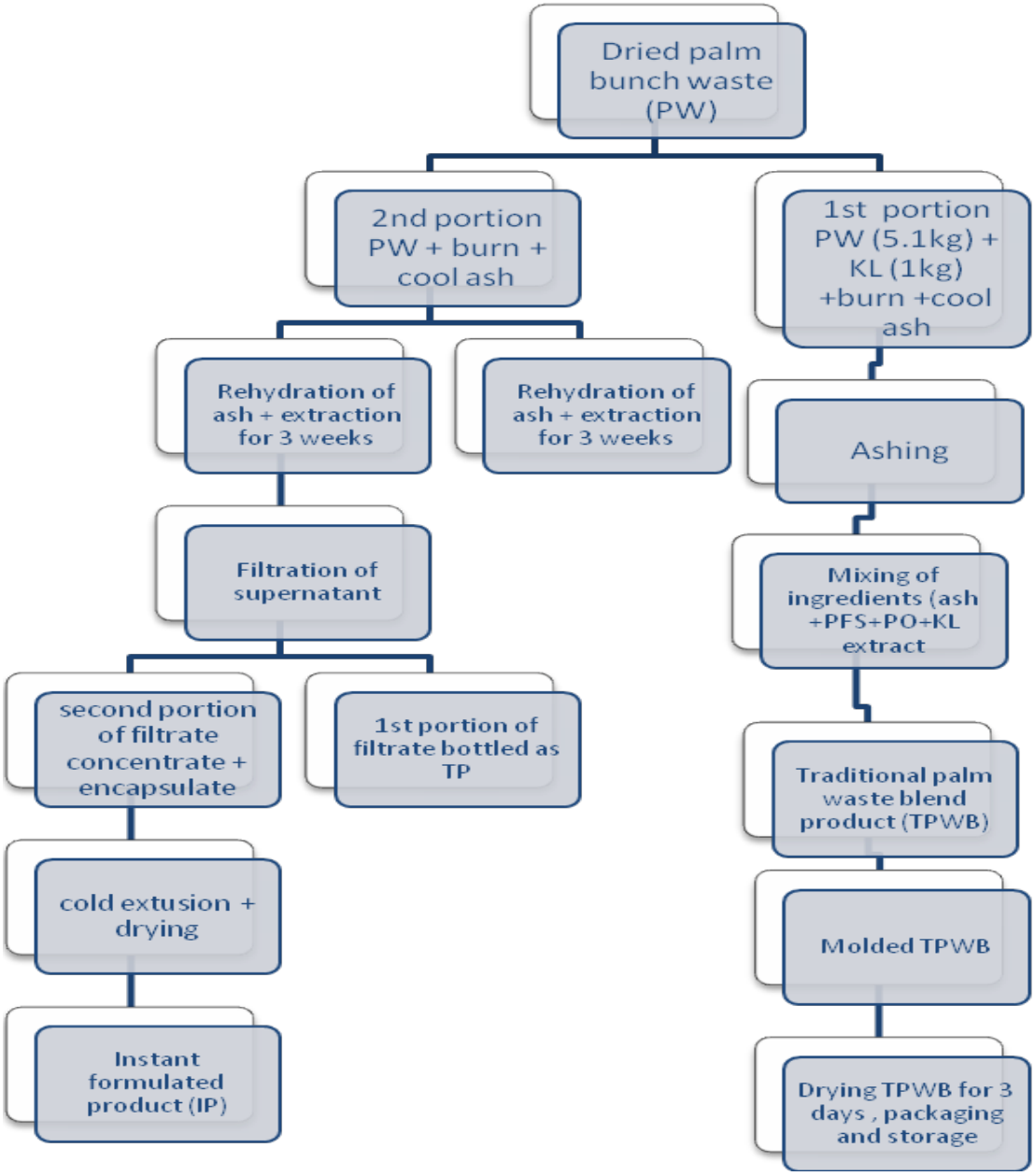

Figure 1. Flow chart for production of emulsion products. PW- palm bunch waste; TP- traditional palm bunch waste product; PFS- palm fruit sludge; PO- red palm oil; KL- kola nut leaves; TPWB- traditional palm bunch waste blend product; IP- instant formulated product.

waste blend product, FIP: filtrate from instant product), were determined using the methods described by Okore (2004).

Sensory evaluation

The emulsion base products (TP: Traditional palm bunch waste product, CP: Commercial product, TPWB: Traditional palm waste blend product, IP: Instant formulated product) were used to prepare "Abacha ncha", the African tapioca salad. The solid base of the final products from CP, TPWB and IP was reconstituted in warm water and filtered. The TP was also re-filtered. Filtrate of each was differently mixed with palm oil in a plastic bowl to form emulsion. Other ingredients such as pepper, salt, crayfish, maggi cubes and "ugba" were added before mixing with the "Abacha".

The prepared"Abacha ncha" samples (TP 1; CP 2; TPWB 3; IP 4) were served to 30 untrained panelists that scored the appearance, colour, taste and smoothness attributes of the tapioca salads on a 9-point Hedonic scale where nine (9) was extremely like and one (1) was extremely dislike (Iwe, 2002). The sensory scores were analyzed statistically using SPSS software package 17.0 version.

Data analysis

The data collected for the experiment were subjected to one way

Table 1. Proximate composition of both raw materials and finished products.

Parameter	PW	KL	FTP	FTPWB	FCP	FIP	CP
Moisture (%)	5.50^{a}±0.141	7.85^{b}±0.071	84.70^{c}±0.283	66.60^{d}±0.424	93.40^{e}±0.566	72.60^{f}±0.424	63.30^{g}± 0.424
Ash (%)	6.0^{a}±0.707	6.35^{ab}0.071	2.40^{c}±0.238	22.40^{d}±0.566	5.20^{ae}±0.283	17.65^{f}±0.071	23.90^{g}±0.141
Protein (%)	4.73^{a}±0.042	17.36^{b}±0.041	1.40^{c}±0.014	0.70^{d}±0.141	1.40^{c}±0.141	0.70^{d}±0.283	2.10^{e}±0.071
Fibre (%)	62.55^{a}±0.707	35.70^{b}±0.014	Trace	Trace	Trace	Trace	Trace
Fat (%)	0.40^{a}±0.283	0.09^{b}±0.014	Trace	0.20^{ab}±0.071	Trace	7.75^{c}±0.071	0.20^{ab}±0.141
Carbohydrate (%)	0.82^{a}±0.028	32.70^{b}±0.283	11.50^{c}±0.141	10.10^{d}±0.028	0.0^{e}	0.0^{e}	10.5^{f} ±0.141

Values are means ± SD for triplicate determinations; n=2. Values with same superscript among rows indicate no significant (p>0.05) difference. CP: Commercial product, PW: palm bunch waste, KL: kola-nut leaves, (TPWB): palm bunch wate blend product, FTP: filtrate from palm bunch waste product; FTPWB: filtrate from palm waste blend, FCP: filtrate from commercial product, FIP: filtrate from instant product.

Table 2. Mineral composition of the ash from palm bunch waste, kola-nut leaves, palm waste blend and the different "ncha" products.

Parameter (mg/g)	PW	KL	TPWB	CP	FTP	FPWB	FCP	FIP
K	0.21^{a}±0.014	0.22^{ab}±0.028	0.20^{a}±0.028	0.28^{b}±0.042	0.17^{ac}±0.042	0.11^{c}±0.028	0.10^{d}±0.140	0.16a^{cd}±0.028
Na	0.013^{a}±0.004	0.005^{ab}±0.003	0.10^{c}±0.014	0. 00^{b}	0. 00^{b}	0. 00^{b}	0. 00^{b}	0. 00^{b}
Fe	175.46^{a}±0.000	64.01^{b}±0.0141	174.81^{c}±0.000	6.74^{d}±0.057	0.52^{e}±0.028	5.70^{f}±0.283	0.52^{f}±0.014	8. 29^{g}±0.014
Zn	10.75^{a}±0.071	22.39^{b}±0.028	15.40^{c}±0.000	12.89^{d}±0.000	0.72^{e}±0.028	8.96^{f}±0.042	6.09^{g}±0.622	3. 94^{h}±0.042
P	0.70^{a}±0.000	1.21^{b}±0.014	0.91^{c}±0.028	0. 00^{d}	0. 00^{d}	0. 00^{d}	0.00^{d}	0.00^{d}
Mg	25.05^{a}±0.071	51.84^{b}± 0.014	24.91^{c}±0.014	41.68^{d}±0.042	0.028^{e}±0.141	43.61^{f}±0.042	11.87^{g}±0.023	0.83^{h}±0.042
Ca	10.80^{a}±0.141	29.52^{b}±0.028	3.60^{c}±0.028	14.88^{d}±0.028	0.96^{e}±0.000	12.48^{f}±0.000	4.32^{g}±0.014	0.96^{e}±0.028

Values are means ± SD for triplicate determinations; n=2. Values with same superscript among rows indicate no significant (p>0.05) difference. CP: Commercial product, PW: palm bunch waste, KL: kola-nut leaves, TPWB: palm bunch waste blend product, FTP: filtrate from palm bunch waste product; FTPWB: filtrate from palm waste blend, FCP: filtrate from commercial product, FIP: filtrate from instant product.

analysis of variance (ANOVA) in completely randomized design (CRD). The treatment means were separated with Fisher's least significant difference test (FLSD) at 95% confidence limit.

RESULTS AND DISCUSSION

Table 1 displays the result of proximate composition of the raw materials (palm bunch waste- PW and kola nut leaves- KL) and the filtrates (F) obtained from the ashes such as FTP (palm bunch waste product filtrate), FTPWB (palm bunch waste blend filtrate), FCP (commercial product filtrate) and FIP (instant product filtrate). Extract obtained from CP had highest ash content (23.90%) followed by that from TPWB (palm bunch waste blend) and then IP- instant product (Table 1). Consequently, these products may contain a lot of macro and micro minerals (Onwuka, 2005). The protein content of the extract from each "ncha" base product (FTP, FTPWB, FCP and FIP) indicated the presence of nitrogen. This implies that the formulated instant product could make contributions to the nutritional composition as much as the traditional "ncha" products (FTP, FTPWB). Table 2 presents the mineral composition of the ashes obtained from palm bunch waste, kola nut leaves and a blending of the two. Traditionally, kola nut or okra leaves which contain appreciable level of mucilage (Anonymous, 2013b) are used to add binding properties to "ncha" products. Besides, analysis of vitamin C composition of kola nut leaves extract

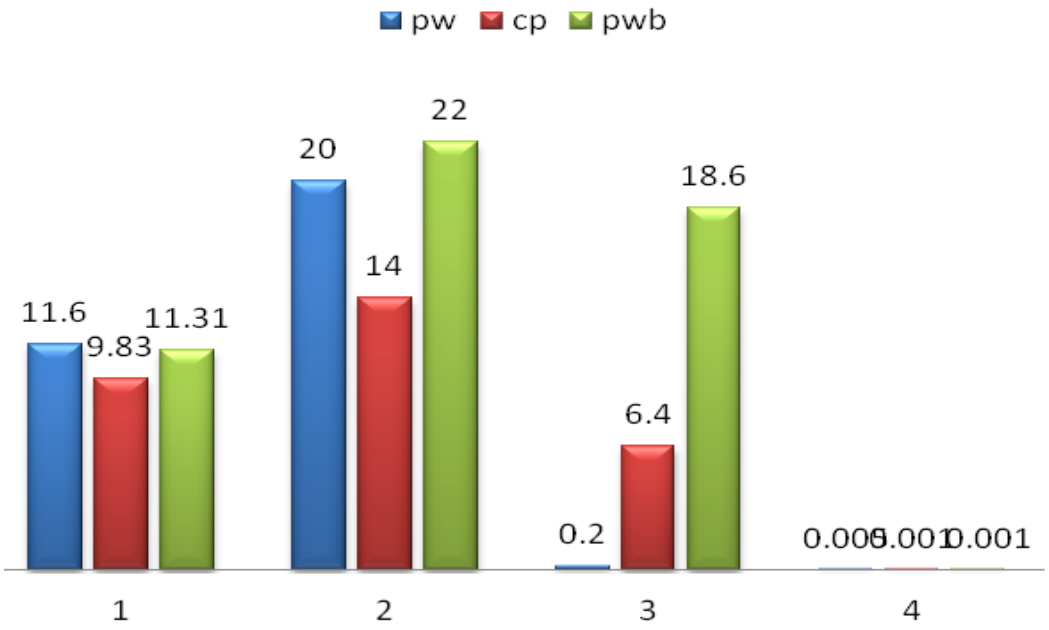

Figure 2. Physico-chemical properties of soaked ashes of "ncha" products**.** 1- pH values; 2- total soluble solids (mg/l); 3- turbidity (TU); 4- colour; pw (TP): Palm bunch waste product, CP: Commercial product; pwb (TPWB): Palm waste blend product.

showed that it contained 15.07 mg per 100 g of the sample. Hence, addition of this extract could be a good source of vitamin C as an antioxidant, to the salad prepared from FTPWB emulsion base. The PW ash was highest in iron (Fe) content, 175.46 mg/g, which is one of the prevalently deficient micronutrients required for the maintenance of health for both adult and children (Wilson, 2010). This is one of the reasons why the extract from the palm bunch waste ash was formulated into instant product. Also, extract from commercial product had very low iron content (0.52 mg/g) as compared to that of formulated instant product (8.29 mg/g). However, the commercial product extract contained significantly ($p<0.05$) higher amount of Mg, Zn and Ca. Generally, the mineral composition of the extract from palm bunch waste ash as TP of Table 2 was low but formulation of part of the extract into instant base product gave rise to improved micronutrient composition. The mineral composition of the extract from TPWB was significantly ($p<0.05$) higher especially for Mg, Ca and Fe micronutrients as compared to the other extracts (FTP, FCP and FIP). Nevertheless, all the extracts including the commercial product, CP, indicated zero phosphorus contents during analysis. However, it is evident from these results that the use of traditional emulsion base products in salad preparations is still highly encouraged as they may contribute these nutrients when utilized in salad preparations. Also, results of selected physical and chemical properties of the ashes from TP, CP and TPWB are shown in Figure 2. The pH of all the extracts was on the high alkaline side and such alkalinity produce very good emulsion base required for the salad preparations. Total soluble solids were very high in TPWB ash extract followed by the TP ash at day 21 (last day of extraction) and suggest that the extraction of soluble constituents from the ash increase with soaking time. This agreed with the report of Irvine (1985) that agricultural waste materials such as palm bunch waste, cocoa pod, plantain peels, banana leaves, maize cob, wood sugar beet waste among others, contain a good percentage of potash. Consequently, when these materials are burnt in air, the resulting ashes contain oxides of potassium and sodium including calcium which can dissolve in water to yield corresponding hydroxides as shown in Equations 1, 2 and 3:

$$Na_2O + H_2O \longrightarrow 2NaOH \quad (1)$$

$$K_2O + H_2O \longrightarrow 2KOH \quad (2)$$

$$2CaO + 2H_2O \longrightarrow 2Ca(OH)_2 \quad (3)$$

Such soluble hydroxides in the emulsion base act as surfactants and combine with palm oil during the preparation of African tapioca salad to form mild soap. Unexpectedly, the turbidity and colour remained unchanged when the total soluble solids changed with soaking/extraction time (3 weeks). This may indicate that these extracts would be stable in terms of these properties under long storage. The results of the turbidity also showed that the extract from TP was clearer than that of CP and TPWB (Figure 2). Again, total microbial

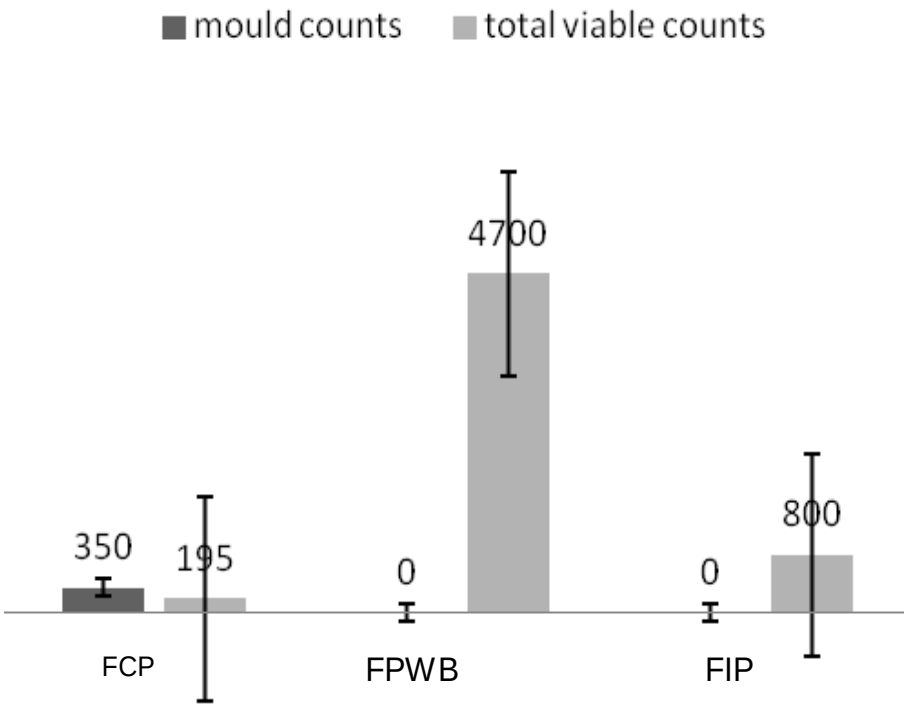

Figure 3. Total viable and mould counts (CFU/ml) of the "ncha" products. FCP: filtrate from commercial product; FPWB (FTPWB): Filtrate from palm waste blend product, FIP: filtrate from instant product; T: error bars with standard error of one (1); sample size, n = 3.

Table 3. Sensory scores of African tapioca salad prepared with the "ncha" samples.

Parameter	TP (1)	CP (2)	TPWB (3)	IP (4)
Appearance	$7.40^{a}\pm1.56$	$8.16^{a}\pm1.17$	$6.17^{a}\pm4.53$	$7.20^{a}\pm0.18$
Colour	$7.10^{a}\pm1.64$	$8.17^{a}\pm1.23$	$7.83^{a}\pm1.29$	$7.60^{a}\pm1.64$
Taste	$7.67^{a}\pm1.44$	$7.70^{a}\pm1.73$	$7.70^{a}\pm0.71$	$7.40^{a}\pm2.69$
Smoothness	$7.37^{a}\pm1.63$	$7.50^{a}\pm1.41$	$8.10^{a}\pm1.26$	$7.17^{a}\pm1.78$
Overall acceptability	$7.30^{a}\pm1.15$	$7.50^{a}\pm1.0$	$8.10^{a}\pm1.0$	$7.17^{a}\pm1.26$

Values are means ± SD for triplicate determinations; n=2. Values with same superscript among rows indicate no significant ($p>0.05$) difference. TP: Palm bunch waste product, CP: commercial product, TPWB: PALM waste blend product, IP: instant product.

load (total viable) and mould counts (Figure 3) for all the products during the extraction period showed zero mould count and reduction in total viable counts as the extraction time increased. However, zero mould count and reduced total viable counts of the instant formulated product as compared to that of commercial and palm waste blends, highlight the reduction of health hazards in using this new product as a salad dressing in Africa tapioca salad preparations. Error bars with standard error of 1 (Figure 3) further showed that the total viable counts obtained for TPWB filtrates was significantly ($p<0.05$) higher than that of FIP with FCP having the lowest value. However, there was no significant difference ($p>0.05$) for the mould counts of FTPWB and FIP while FCP has highest value of this count (Anonymous, 2014). The results of the sensory evaluation of African tapioca salad prepared from the different emulsion base are shown in Table 3. There were no significant differences ($p>0.05$) in appearance, colour, taste and smoothness of the salads prepared from extracts of TP, CP and IP. Overall, acceptability indicated that African salad prepared from the instant product was highly acceptable together with those of traditional products.

Conclusion

This investigation has shown that biological wastes such as palm bunch waste could be processed into edible instant formulated emulsion base product which is very simple, fast and convenient for preparing African tapioca salads. Hence, the local potash popularly known as "ngu"

in the Eastern part of Nigeria can be transformed into convenient form for the benefit of city dwellers and career women. Besides, data base were provided for the traditional emulsion base products.

Conflict of Interests

Authors have declared no conflict of interest

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