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PLANT SCIENCE

Antioxidative properties of bitter gourd (*Momordica charantia*) and zucchini (*Cucurbita pepo*)

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Abstract

The enzymatic and non-enzymatic antioxidative capacities of bitter gourd (*Momordica charantia*) and zucchini (*Cucurbita pepo*) were investigated in water extracts and chemical buffer extracts. Bitter gourd and zucchini fruits were purchased from a farmer's market and homogenized separately in water and in a native enzyme extraction buffer. Total phenolic compounds, free radical DPPH scavenging activity, SOD activity and β-glucosidase activity were assayed in the extracts. The average total phenolic compounds recorded in bitter gourd were 13.28 GAE/g fresh weights while in zucchini, the average was 8.67GAE/g fresh weight. This study also found that bitter gourd was 82.05% as effective as ascorbic acid in inhibiting the free radical DPPH while zucchini was 12.19% as effective. The results indicated that bitter gourd was significantly higher in antioxidant content and in β-glucosidase activities than zucchini ($P<0.05$). On the other hand, significantly higher SOD activities were recorded in zucchini than in bitter gourd extracts.

Key words: Phenolic compounds, β-glucosidase, Bitter gourd, Zucchini

Introduction

Bitter gourd (*Momordica charantia* L.), also known as bitter melon, is an important vegetable grown in tropical and sub-tropical regions of Africa, India, China, and several Caribbean countries (Aboa et al., 2008; Wu and Ng, 2008). Bitter gourd belongs to the cucumber family (Cucurbitaceae), a group comprising about 130 genera and 800 species (Radford et al., 1968). Bitter gourd is a herbaceous plant that can grow up to 10 meters tall. The plant bears simple, alternate leaves that are 4-5 cm in width with 3-7 deeply separated lobes. The plant bears oblong fruits with a distinct waxy exterior. The interior of the fruit is somewhat hollow, containing white pith and seeds. When ripe, the fruits become yellow and split into segments that curl back to reveal the seeds. The fruits are eaten while still green and unripe. They have been used for generations by indigenous populations in Africa, India, and Latin America for food and folk medicine (Khan and Anderson, 2003; Dey et al., 2006; Lako et al., 2007; Abo et al., 2008).

Bitter gourd has been the subject of intensive investigations for biologically active compounds and for its medicinal properties (Majekodunmi et al., 1990; Begum et al., 1997). Kubola and Siriamornpun (2008) investigated the phenolic contents and antioxidant properties of leaf, stem, and fruit extracts by analyzing the inhibition of the free radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH). They found that the fruit extract had the highest value of antioxidant activity and that gallic acid was the predominant phenolic compound in the fruit extract. Wu and Ng (2008) compared the antioxidant capacity of bitter gourd extracted in water versus ethanolic extraction and found that both water and ethanol extracts were effective in reducing the stable radical DPPH to the yellow diphenylpicryl hydrazine. They also found that both water and ethanol extracts were effective in reducing the stable radical DPPH to the yellow colored diphenylpicryl hydrazine. The folk medicinal properties associated with bitter gourd includes treatment for various chronic and degenerative diseases, including, diabetes mellitus (Nerurkar et al., 2006; Aboa et al., 2008; Nerurkar et al., 2010) coronary heart disease and cancer (Slater, 1984; Cheng et al., 2003). The seeds of bitter gourd are believed to contain anticancer

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agents and remedies for gastrointestinal diseases (Beloin et al., 2005).

Another vegetable food crop believed to have medicinal values is zucchini (*Cucurbita pepo* L.) The genus *Cucurbita* includes pumpkin, acorn squash, crooked neck squash, and straight neck squash, all of which contain significant amounts of cucurbitane glycosides and triterpenoid compounds. Zucchini is a temperate, small plant that can grow up to 2 meters, having leaves 8-30 cm across, with 3-7 dissected lobes that have notches (Radford et al., 1968). The plant is dioecious and bears orange flowers. The fruits of zucchini are cylindrical and have a thin, smooth, and waxy exterior. The color of the fruit ranges from light green to dark green. The interior of the fruit is white and contains small white seeds. Zucchini is consumed either raw in salads or cooked in soups (Stephens, 2009). The folk medicinal properties associated with zucchini are numerous and include treatment for benign prostatic hyperplasia and leprosy (Dhiman et al., 2012).

Both bitter gourd and zucchini are believed to possess enzymatic and non-enzymatic antioxidant activities against the buildup of reactive oxygen species (ROS) in the cells (Dasgupta and De, 2006; Wu and Ng, 2008; Xanthopoulou et al., 2009; Dhiman et al., 2012). ROS include the singlet oxygen $^1O_2$, the superoxide anion O_2^-, the hydroxyl ion OH^-, the peroxide O_2^{2-}, and the hydrogen peroxide H_2O_2. These free radicals cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes. ROS are also cellular signaling agents in the regulation of plant development, stress responses, hormone regulation and programmed cell death (Palma et al., 2002; Breusegem and Dat, 2006). Organisms defend themselves against the destructive actions of ROS by using enzymatic and non-enzymatic mechanisms. The enzymatic mechanism makes use of the enzyme superoxide dismutase (SOD) and the hydrogen peroxidase enzyme (PO) (Bray, 2000; Breusegem and Dat, 2006; Dasgupta and De, 2006) and the non-enzymatic antioxidant pathway includes the actions of vitamins, phenolic compounds, tannins, and Gallic acid (Breusegem and Dat, 2006; Wu and Ng, 2008; Harr and Ishmail, 2012).

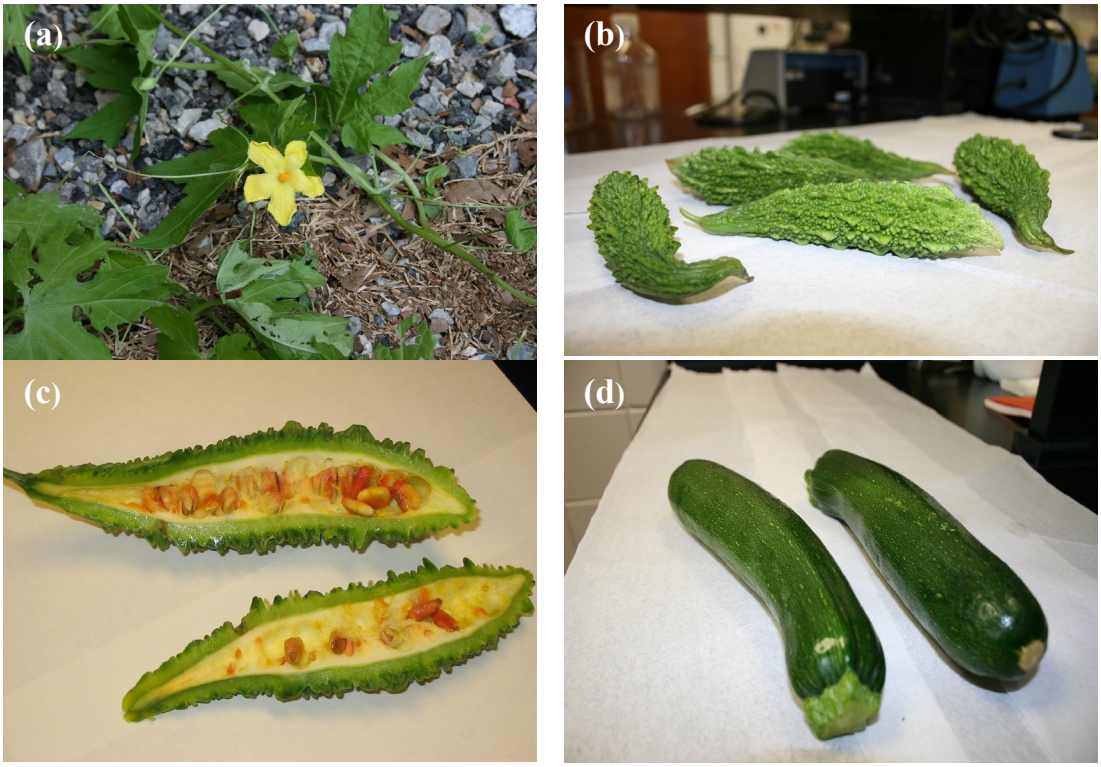

Figure 1. Photographs of bitter gourd (*Momordica charantia*) and zucchini (*Cucurbita pepo*) showing (a) flowering bitter gourd plant, (b) bitter gourd fruit, (c) sliced bitter gourd fruit, and (d) zucchini fruits.

Bitter gourd and zucchini are known to contain a variety of biologically active compounds including the alkaloids momordicine I, momordicine II, and cucurbitacine B (Majekodunmi et al., 1990). Other compounds reported in these vegetables include a cytotoxic ribosome binding terpenoid and several glycosides including charantin, charantoside, and momordicoside (Begum et al., 1997; Harinantenaina et al., 2006; Akihisa et al., 2007; Chang et al., 2008; Dhiman et al., 2012). Glycosides are hydrolyzed by β-glucosidase enzymes (EC 3.2.1.21). β-glucosidase is a member of a large class of enzymes known as glycosidase, which are important for the plant's own defense mechanisms against herbivores, and pathogens. The presence of significant activities of β-glucosidase in plants has been correlated with pharmacological values of the plant (Lako et al., 2007). The USDA Nutrient Database mentioned bitter gourd to be high in carbohydrates and fibers, and to contain large amounts of folate (Vitamin B9) and Vitamin C.

The objectives of this research were to investigate the total phenolic compounds and the effectiveness of bitter gourd and zucchini extracts in inhibiting the free radical DPPH and to investigate the activities of the superoxidase dismutase (SOD) and β-glucosidase enzymes in bitter gourd and zucchini extracts.

Materials and Methods

Plant materials

This research was conducted between 2008 and 2012 in the Department of Biology of Jacksonville State University, Jacksonville, Alabama, USA. Fruits of bitter gourd (*Momordica charantia)* and zucchini (*Cucurbita pepo*) were purchased from the Dekalb Farmer's Market in Atlanta, Georgia (USA) on 2 separate occasions. The fruits were brought to the laboratory where they were weighed and then washed to remove debris as well as residual pesticides. The fruits were separately extracted first in protein extraction buffer for enzymatic analyses and then in distilled water for non-enzymatic antioxidants determination.

Protein extraction

Native enzymes were extracted from each bitter gourd and zucchini fruit as total soluble proteins by grinding the fruits at a ratio of 0.5 g fresh weight per ml extraction buffer (100 mM KPO_4, 1 mM KCl, 1 mM EDTA, 1% PVP, pH 7.5) in a mortar and pestle. The samples were further homogenized in a Teflon glass homogenizer and then sequentially filtered through 1, 2, and finally 4 layers of cheesecloth. The filtrates were centrifuged at 16,000 g for 20 minutes at 4°C. The resulting supernatants were used as total soluble native proteins (enzymes) and used in the determination of superoxide dismutase and β-glucosidase activities. The total protein yield of each extract was determined according to the Bradford (1976) method using bovine serum albumin (BSA) as the standard protein.

Aqueous extraction

For aqueous extraction, the fruits were ground in a Warring blender at a ratio of 0.5 g fresh weight/ml in distilled H_2O. The samples were homogenized in a Teflon glass homogenizer and then sequentially filtered through 1, 2, and finally 4 layers of cheesecloth. The filtrates were centrifuged at 16,000 g for 20 minutes at 4°C and the resulting supernatants were retained as aqueous extracts and used for the non-enzymatic antioxidants determination.

Total phenols assay

The total phenolic compounds of each extract was determined as Gallic acid equivalent (GAE) using the Folin-Ciocalteu method (Zhou and Yu, 2006). The assay consisted of reacting 200 µl of each extract with 1 ml of Folin-Ciocalteu reagent in cuvets and allowing the mixtures to incubate at room temperature for 5 minutes. Following the 5 minute incubation, 1 ml of 0.5 M sodium bicarbonate solution was added to the reaction mixture and incubated at room temperature for an additional 90 minutes. Various concentrations of Gallic acid were made as standards and treated the same way as the experimental cuvets. The absorbances of the cuvets were recorded at 725 nm using a UV/VIS GENESYS spectrophotometer. The absorbances of the samples were compared to those of known concentrations of Gallic acid. The total phenolic content of each extract was expressed as µg of GAE/ml.

DPPH inhibition assay

DPPH free radical scavenging activity was measured spectrophotometrically at 517 nm according to the methods of Cheung et al. (2003) and of Wu and Ng (2008). The procedure consisted of making 3.0 ml dilutions of each sample in spectrophotometer cuvets. The dilutions consisted of 0.1 µg/µl and 0.5 µg/µl total proteins. 3.0 ml extraction buffer was used as a negative control and 3.0 ml of 0.2 µg/µl ascorbic acid was used as a

positive control in 2 separate cuvets. To each cuvet, 3 ml 0.1 mM DPPH were added. The reaction mixtures were incubated at room temperature for 30 minutes and the absorbances were recorded at 517 nm. The percent inhibition of DPPH was calculated as [(Absorbance without extract – Absorbance with extract)/Absorbance without extract] x 100 (Wu and Ng, 2008).

Superoxide dismutase (SOD) activity

The abilities of the extracts to neutralize the superoxide free radical were measured spectrophotometrically according to Beauchamp and Fridovich (1971). Aliquots of 0.1 ml extracts were made in 5 ml cuvets. Three replicates were made for each sample. To each aliquot, 3.0 ml reaction mixture (50 mM sodium phosphate buffer, pH 7.8, 0.2 mM NBT, 10 mM L-methionine, 1 mM EDTA, and 5.0 µM riboflavin) were added. The second set was wrapped in aluminum foil and served as a blank. Both sets were placed in a growth chamber with light intensity of 3,000 mol^{-1} m^{-1} s^{-1} for 10 minutes. The reduction of NBT by the SOD enzymes was measured spectrophotometrically at 560 nm. A standard curve was generated using commercial Chloroplast SOD (Sigma Aldrich, St. Louis, MO, USA) according to Hamissou (2011).

β-Glucosidase assay

β-glucosidase enzyme activity was investigated spectrophotometrically at 420 nm. For each sample, triplicate cuvets containing 2.0 ml each were reacted with 2.0 ml of 8.0 mM p-nitrophenyl glycoside (NPG) in acetate buffer, pH 6.1. β-glucosidase catalyzes the hydrolysis of a wide variety of substrates including p-nitrophenyl- β-glucosides (NPG). This substrate was chosen because one of its reaction products, p-nitrophenol (NP), absorbs strongly in the blue region of the visible spectrum and the reaction can be measured calorimetrically at 420 nm. The reaction mixtures were incubated for 1 hour at room temperature and the reactions were stopped by adding 100 µl of 100 mM sodium bicarbonate to each cuvet. The amount of NP liberated was deduced from a standard curve generated using commercially purchased p-nitrophenol (Sigma Aldrich, St. Louis, MO, USA).

Statistical analyses

The fruits were purchased on two different dates from the Farmers' Markets and subsequently extracted on two different dates. The two different extractions dates were considered blocks. Three bitter gourds and 3 zucchinis were used for each extraction; all biochemical assays were performed 3 times. A two-way ANOVA was performed to compare the data between bitter gourd and zucchini extracts (n = 12).

Results

An average of 4.11 ± 0.67 mg total soluble protein/g fresh weight was obtained in bitter gourd extracts while the average of total soluble protein yield in zucchini extracts was 1.85 ± 0.32 mg/g fresh weight (Table 1).

Table 1. Total soluble proteins recorded in bitter gourd (*Momordica charantia*) and in zucchini (*Cucurbita pepo*) fruits.

	Bitter gourd (mg/g fresh weight)	Zucchini (mg/g fresh weight)
ȳ	4.11^{a}	1.85^{b}
SD	0.67	0.32
SE	0.19	0.09

The means are followed by letters indicating significant differences between bitter gourd and zucchini in total soluble proteins, at 5% probability level (n = 12).

Bitter gourd averaged 13.28 ± 1.71 mg GAE/g fresh weights in total phenolic compounds, while zucchini averaged was 8.67 ± 1.59 mg GAE/g fresh weights (Table 2). On average, bitter gourd was 82.05% ± 7.52 as effective as ascorbic acid (%EAA) in inhibiting the free radical DPPH while zucchini was 12.19% ± 4.20 as effective as ascorbic acid (Table 2).

Table 2. Non-enzymatic antioxidant activities of bitter gourd (*Momordica charantia*) and zucchini (*Cucurbita pepo*) extracts, n = 12.

	Bitter gourd	Zucchini
Total phenolic compounds (GAE/g fresh weight)	13.28^{a} ± 1.71	8.67^{b} ± 1.59
DPPH scavenging (%EAA)	82.05^{a} ± 7.52	12.19^{b} ± 4.20

Numbers in the same row followed by the same letters are not significant at 5% probability level.

The results of the enzymatic antioxidant capacity (SOD) of bitter gourd and zucchini extracts are presented in Table 3. An average of 1.55 ± 0.60 units of SOD activity per µg total proteins (ua/µg total proteins) was recorded for bitter gourd while in zucchini, an average of 1.996 ± 0.88 ua/µg proteins was obtained. The results of β-glucosidase activity are also shown in Table 3. Bitter gourd extracts averaged 0.32 ± 0.14 units of activity per µg total protein (ua/µg total protein while zucchini extracts averaged 0.19 ± 0.02 ua/µg total proteins.

Table 3. Enzymatic antioxidant and β-glucosidase activities of bitter gourd (*Momordica charantia*) and zucchini (*Cucurbita pepo*) extracts, n = 12.

	Bitter gourd	Zucchini
SOD Activities (ua/µg total prot.)	1.55 ± 0.60	2.00 ± 0.88
β-glucosidase activities (ua/µg total prot.)	0.32 ± 0.14	0.19 ±0.02

Discussion

In most plants, total phenolic compounds have been determined to be the main antioxidative compounds. Wu and Ng (2008) and Kubola and Siriamornpun (2008) independently investigated the phenolic compounds of bitter gourd fruits extracted in water and obtained averages of 0.516 mg of GAE/ml per ml and 0.202 mg GAE/ ml respectively. The two independent investigations reported their data in mg GAE/ml extract not specifying the amount of fresh weight in the 1.0 ml extract. In the absence of agreeable expression of units, this study found it more informative to present data in mg of antioxidant/g fresh weight of the fruit or vegetable rather than mg antioxidant/ml extract. Therefore, this research is reporting its findings of total phenolic compounds in mg GAE/g fresh weight. Bitter gourd averaged 13.28 ± 1.71 mg GAE/g fresh weight and zucchini averaged 8.67 ± 1.59 mg GAE/g fresh weight. Although data comparison between the present data and those obtained by Wu and Ng (2008) and by Kubola and Siriamornpun (2008) is not possible, this study found that bitter gourd fruits contain significantly higher amounts of phenolic compounds than zucchini.

Another property useful in determining the non-enzymatic antioxidant values of fruits and vegetable is the capacity of the extracts to inhibit the free radical DPPH. Antioxidants present in the extracts were expressed as the reduction of the purple-colored stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) to the yellow-colored diphenylpicryl hydrazine by donating an electron or a hydrogen. This reduction of the free radical diminishes cellular damage by allowing the neutralization of reactive oxygen species present in the solution (Wu and Ng, 2008). As a result of a buildup of ROS, lipid peroxidation of cell membranes can occur. DPPH scavenging activity is expressed in IC_{50} values or in percent ascorbic acid or Vitamin E equivalents. Ascorbic acid, an antioxidant commonly known as Vitamin C, is known to scavenge 50% of DPPH at 50 µg/ml (Hsu et al., 2007; Arazo et al., 2011; Ramkumar et al., 2012). Wu and Ng (2008) reported IC_{50} values of 129.94 µg/ml in bitter gourd extracted in water. In this research, the DPPH reductions by the zucchini extracts never surpassed the 50% mark, making it impossible to determine IC_{50} values. Since IC_{50} value and percent inhibition indicate the same potential, this research found it necessary to report the present data as percent effectiveness of the extracts to inhibit the free radical DPPH compared to ascorbic acid. Bitter gourd was found to be 82.05% ± 7.52 as effective in scavenging DPPH free radial as ascorbic acid. This is significantly important information to report when considering that zucchini was only 12.19 ± 4.20% as effective as ascorbic acid. Our data are however in agreement with other researchers (Hsu et al., 2007; Wu and Ng, 2008), indicating that bitter gourd is a high antioxidant containing vegetable food.

Enzymatic antioxidants are equally important in protecting organisms against free radical build-up. Enzymatic antioxidants, such as superoxide dismutase, protect cells and tissues from oxidative damage by reactive oxygen species. Superoxide dismutase (SOD), peroxidases (PO) and catalases (Cat) are some of the enzymatic antioxidative defense mechanisms. In this study, only the SOD activity in bitter gourd and zucchini fruits were investigated. In the absence of comparable published data in this area, this research is reporting that zucchini extracts showed higher SOD activity than bitter gourd extracts with values of 1.996 ± 0.30 and 1.55 ± 0.44 u a/ µg total proteins respectively. Although the magnitudes of the values were low, the two-way ANOVA indicated the existence of a significant difference between bitter gourd and zucchini, at 5% probability level. Interestingly, there is evidence suggesting that diabetes and other health problems are complicated by oxidative stress due to generation of free radicals (Garg et al., 1996) and by a decrease in the body's natural antioxidant defenses (Oberly, 1988). In an independent study, Sathishsekar and Subramanian (2005) showed that diabetic rats had lower SOD activity in their kidneys and livers compared to diabetic rats treated with bitter gourd seed extracts.

The presence of β-glucosidase enzyme is another property indicative of potential pharmacological value in plants. The activity of β-glucosidase enzyme does not add to the antioxidant value vegetables and fruits directly, but the products of their reactions may serve as precursors to some non-enzymatic antioxidant compounds (Sanchez-Medina et al., 2001). Zucchini had significantly more β-glucosidase enzyme activity than bitter gourd

with values of 0.19 ± 0.01 units of activity per µg total protein (ua/µg total protein) and 0.32 ± 0.07 ua/µg total proteins, respectively. Plants with high activity of this enzyme have been useful tools in drug discovery due to their importance in generating precursor molecules used in several processes such as hormone regulation, defense mechanisms, and antioxidant production. Some toxic compounds synthesized through the activity of this enzyme include hydrogen cyanide, rotenoids, and quinones. Although minute levels of β-glucosidase activity were detected in both bitter gourd and zucchini extracts, the data establish a foundation for future investigations of the pharmacological properties of bitter gourd and zucchini.

Conclusions

This research found that bitter gourd was 82.05% as effective as ascorbic acid in inhibiting the free radical DPPH while zucchini was 12.19%. Bitter gourd has significantly higher total phenolic compounds than zucchini, indicating that bitter gourd is a high antioxidant containing vegetable food, and that bitter gourd has more β-glucosidase activities than zucchini. On the other hand, zucchini fruits showed higher activities of the enzymes SOD.

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PLANT SCIENCE

Invasive species may offer advanced phytoremediation of endocrine disrupting chemicals in aquatic ecosystems

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Abstract

One of the major areas of advancement in environmental science is bioremediation. Researchers have been using bacteria, fungi, algae and now macrophytes to remove pollutants from aquatic and terrestrial ecosystems. Here we share the results of a study on the macrophyte uptake of xenoestrogens from an urban river. We found that the invasive curly leaf pond weed (*Potamogeton illinoensis*) accumulated an average of 66% higher levels of estrogenic compounds and 94% more Bisphenol-A than the native Illinois pondweed (*Potamogeton crispus*) in an urban river, in the watershed for the greater Chicago, IL area. The invasive species accumulated 76% more estrone, 55% more 17 β-estradiol and 31% more 17 α-ethynylestradiol than the native species. The Non-native plants were also 72% larger than the native Illinois Pondweed. Managers may consider using invasive species to remove pollutants from ecosystems and restore ecosystem biogeochemistry.

Key words: Endocrine Disrupting Chemicals, Environmental Remediation, Invasive Species

Introduction

Examining ecosystem biogeochemistry and the alterations of biogeochemical cycles due to anthropogenic activities has become one of the most important fields within environmental science. The ever-increasing anthropogenic load on environments calls for innovative strategies to remediate and restore ecosystems. Polluted aquatic ecosystems present some of the largest challenges to managers because of the ability of nutrients, organisms and pollutants to vary in concentration at different depths and mediums. Urban aquatic ecosystems also have high rates of flux between the biosphere, hydrosphere and the atmosphere. Many pollutants flow into urban aquatic ecosystems from precipitation, run-off, and point sources. These pollutants may remain suspended in the water column, be taken up by aquatic organisms or accumulate in bottom sediments (i.e. Karickhoff et al., 1979).

Bottom sediments in aquatic ecosystems have the ability to absorb, accumulate, and transform pollutants. However, many lakes and ponds undergo turnover events which may re-suspend pollutants in the water column (Karickhoff and Morris, 1985). It is desirable to remove pollutants from these aquatic ecosystems, since bottom sediments are not a sufficient sequestration pool. Phytoremediation is emerging as the most practical cleanup technology by using certain species of plants to sequester pollutants from sediments and water (Pilon-Smits, 2005; Suresh and Ravishankar, 2004). While much of the research has centered on specific species and their ability to remove particular pollutants from ecosystems (i.e. Kurilenko and Osmolovskaya, 2007), little attention has been paid to the role invasive species may play in regards to phytoremediation.

The utilization of natural biodegradation processes has gained traction in recent years. Their economic and environmental costs are much lower than the conventional incineration of large quantities of soil or the usage of chelating agents (Franks, 2006). Due to anthropogenic activities, pollution is a major driver of imbalance in native ecosystems. Under these adverse conditions, tolerance to these contaminants often leads to a change in the species that dominate ecosystems. Some ecosystems may then be vulnerable to invasion by non-native species. Non-native species

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may act as efficient phytoremediators; however, little research has been completed to assess this. Some studies have deployed non-native species to clean-up environmental pollutants, but this runs the risk of promoting further invasive entry of species (Li et al., 2009).

There is a growing awareness that organic contaminants such as endocrine disrupting chemicals (EDCs) are being found in wastewater, rivers and lakes are very difficult to remove (Farre, et al., 2007). Many of these active chemicals carry unknown risks, and may lead to interference with the endocrine systems of aquatic organisms (US EPA, 2010). EDCs are chemicals that mimic or upset natural hormones and include estrogen derivatives from anthropogenic sources such as treated and untreated sewage effluents, as well as xenoestrogens such as Bisphenol-A. These contaminants often lead to adverse health effects among species in aquatic and terrestrial ecosystems (Barceló and Petrovic, 2007). These effects include feminization, as well as other reproductive and developmental problems (Crain et al., 2007; Kang et al., 2006).

Three of the most common estrogen contaminants are Estrone, 17β-estradiol and 17α-ethynylestradiol. Having an environmental impact at concentrations in the nanograms per liter range, these compounds are hydrophobic, involatile, and practically insoluble in water. Furthermore, sewage treatment plants are unable to effectively reduce these estrogens, leading to adverse health effects amongst populations of aquatic and terrestrial species downstream. Estrone and 17β-estradiol, naturally excreted sex hormones, have been shown to promote the production of vitellogenin (female egg proteins) among fish and brown frog populations (Crain et al., 2007; Rouhani et al., 2005). Furthermore, exposure to the potent synthetic birth control hormone 17α-ethynylestradiol has been linked to decreased fertility of male rainbow trout and shown to alter vitellogenin production at sub-nanogram per liter concentrations (Schultz et al., 2003). Prolonged exposures to these EDCs have a significant effect on biodiversity and food webs (Franks, 2006).

Bisphenol A [BPA, 2, 2-bis (4-hydroxyphenyl) propane] has been found in many water-bodies across the world. The pervasiveness of BPA is due to the fact that it is involved in the production of numerous goods and materials such as epoxyphenolic resins, polycarbonates, polyacrylates and plastics. Many canned foods are also lined with plastics containing BPA (Kang et al., 2006). As the toxicity of BPA has reached the media and the general public, individuals are now becoming aware of the potentially dangerous side effects of BPA and are therefore trying to remove it from human consumption (Takahashi et al., 2001). However, BPA is found in a wide variety of products from pesticides to flame retardants and rubber chemicals (Staples et al., 1998) and it therefore ends up in rivers, lakes and streams. BPA has become a target pollutant for removal due to its acute toxicity towards algae, invertebrates and fish (Alexander et al., 1988).

Several studies have targeted natural bioremediators for BPA. Ligninolytic fungi are known to effectively metabolize EDCs (Loffredo et al., 2012). Their ability to biodegrade lignin depends on extracellular enzymes having low substrate specificity. This property allows Ligninolytic fungi to utilize high concentrations of aromatic compounds, including EDCs, for this process (Loffredo et al., 2012; Tsutsumi et al., 2001). Li et al. (2009), found that the algae *Stephanodiscus hantzschii* could tolerate high concentrations of BPA and serve as a biodegradation agent. The recent work of many others has also determined that endocrine disrupting chemicals may be biologically transformed and/or sequestered by algae, fungi and many bacterial species (North and Halden, 2013). This ability of managers to utilize naturally occurring organisms to biodegrade EDCs from landfill leachates, wastewater, and natural bodies of water is encouraging. Although this research is still in the early stages, using macrophytes for biodegradation and bioremediation may prove to be an effective way to restore balance to polluted ecosystems.

This study examined the phytoremediation potential of two Pondweeds on estrogen derivatives and Bisphenol-A in the Des Plaines River near Chicago, IL. The Curly Leaf Pondweed, native to Eurasia, has been positively identified in North America. It is known to form dense mats displacing native aquatic plants, including the native Illinois Pondweed (Johnson, 2010). Samples of both the native Illinois Pondweed (*Potamogeton illinoensis*) and the invasive Curly Leaf Pondweed (*Potamogeton crispus*) were collected from six sites covering over 8 kilometers of river. Water samples were also collected to determine the current concentrations of estrone, 17 β-estradiol, 17 α-ethynylestradiol and BPA present in the river. Measurements of the accumulation of EDCs in the aquatic and plant samples, may point towards the role invasive species play in phytoremediation.

Materials and Methods

Study site

The Des Plaines River runs from north to south and bisects the Chicagoland area. This river receives large inputs from sewage treatment plants, road run-off and industrial complexes. The river is known to be highly polluted; however, little information has been published on the pollutants found therein. The Des Plaines River watershed covers the greater Chicago area, and about 854,669 acres of Cook, Dupage, Lake, and Will Counties. The majority of the land use in this area is urban (58.7%), followed by agricultural (33.2%), forest (5%), and the remainder being wetlands (Pescitelli and Rung, 2005). The Des Plaines River has at times received both untreated sewage inputs (Hawthorne, 2011) and regularly receives treated sewage inputs. As a river flowing through a major city, it bears a lot of environmental stress created by human activity, and in recent years, infiltration of this river by contaminants such as fire retardants, and oil sludge has been in the news (Dillon, 1999; The Huffington Post, 2009). The city of Chicago has an interest in maintaining forest preserves and wetlands; however, phytoremediation along the river has not yet been attempted. This study aims to test the viability of using phytoremediation on polluted urban river systems. These areas are of great importance because they provide habitat corridors that may allow flora and fauna to flourish in urbanized areas.

Water and plant samples were collected from the Des Plaines River just outside Chicago, IL on August 26-27, 2011. The samples were extracted from the upper Des Plaines River at six sites each approximately 1 km from the other. Sample locations were labeled for sites 1-6 with site 1 being the furthest upstream and approximately 9 km from a known sewage point source. Twenty liters of water samples were obtained from below the river surface about one meter from the shore and near the area where plants would be collected at each site. Water samples were placed into pre-cleaned amber glass bottles and were then stored at approximately 4°C in the absence of light. Six samples of both the native aquatic plant, Illinois pond weed (*Potamogeton illinoensis*), and the invasive, Curly Leaf Pondweed (*Potamogeton crispus*) were collected. Plants were dried in an oven at 80°C for 48 h until a constant dry weight was reached. The dried sample was cooled down to room temperature (ca. 20°C) in a desiccator before weighing each plant sample. Each plant was ground to a fine powder where a homogenized sub-sample of 1 gram of ground material was used for further extractions.

Estrogen and the Estrogen Derivative GC-MS Analyses

Estrogenic compounds and transformation products were identified using gas chromatography coupled with mass spectrometry (GC-MS). To prepare samples for GC-MS identification samples were dissolved in methanol and passed through an Oasis HLB 4 Plus solid phase extraction (SPE) pack (Waters Corp.), which had been preconditioned with 10mL of methanol and Milli-Q water. The SPE packs were eluted with 2 mL methanol over 5 min. The methanol was allowed to evaporate, and the residue was re-dissolved in 50 µL hexane followed by derivatization with 50 µL BSTFA (1% TMCS) at 60°C for 45 min.

GC-MS is a commonly used method for the detection of pharmaceuticals and hormones in water. This process involves the off-line purification of substances using solid phase extraction (SPE) and specific compounds for derivatization, prior to GC-MS analysis (i.e. Kelly, 2000; Kuch and Ballschmiter, 2000; Jeannot et al., 2002). To analyze plant and water samples for estrogenic compounds, off-line sample preparation was necessary. All stock solutions were prepared using HPLC-grade substances and followed the protocol designed by Quintana et al. (2004). Methanol and ethyl acetate were supplied by Sigma Aldrich (St. Louis, USA). Stock solutions of estrone, 17 beta-estradiol and 17 alpha-ethynylestradiol were also prepared using high quality products from Sigma-Aldrich (St. Louis, USA). MTBSTFA, BSTFA, BSTFA containing 1% of TMCS, MSTFA and TMSI, were obtained from Supelco (Bellefonte, PA, USA). We completed off-line preparation of samples using SPE cartridges containing 60 mg of Oasis HLB were obtained from Waters Corporation (Milford, MA, USA) and used as received.

GC–MS analyses were performed on a Varian 220-MS (Agilent, Santa Clara, California) linked to a Varian 431-GC (Agilent, Santa Clara, California) which was equipped with a 30 m x 0.25 mm x 0.25 µm Factor Four VF-5ms capillary column (Agilent, Santa Clara, California). The chromatographic conditions for estrogen and its derivatives are as follows: 5 µL split/splitless injection (injector temperature 250°C). The sample was held at 50°C for 1 min then the temperature was raised to 220°C at 20°C min^{-1} and held for 27 minutes. The oven was then brought to 250°C at 20°C min^{-1} and held for 20 minutes. The ultra-high purity helium carrier

gas was controlled at a velocity of 41.3 cm s^{-1}. The mass spectrometer was operated on auto electron impact ionization mode with an emission current of 10μA, transfer line temperature of 250°C, and an ion trap temperature of 200°C. The mass spectrometer was scanning from 40 to 650 amu with an inter-scan time of 1 s. Compound identification was based on the comparison of mass spectra with the NIST (NIST/EPA/NIH Mass Spectral Library NIST08). We measured standards and blanks periodically throughout the analyses to assure accuracy. Quantitative analyses of target compounds were achieved through the integration of selected gas chromatography chromatograms. Each sample was rerun three times and the detection limit was in the range of 1 ng/L.

BPA Analysis

BPA was extracted from dried plant material by mixing it with ethanol (10 ml for every 1.5 g plant dry weight) for 24 h prior to elution. Our Bisphenol-A standard (99%, solid crystal form) was purchased from Sigma-Aldrich (St. Louis, USA) and the stock solution was prepared in methanol at a concentration of 250 mg L^{-1} and stored in brown bottles at 20°C. Other reagents such as methanol, trichloroethene and carbon tetrachloride was purchased from Sigma-Aldrich (St. Louis, USA). All reagents were of analytical grade or above. Analyses proceeded following the method outlined in Fontana et al. (2011). GC–MS analyses were performed on a Varian 220-MS (Agilent, Santa Clara, California) linked to a Varian 431-GC (Agilent, Santa Clara, California) which was equipped with a 30m x 0.25mm x 0.25 μm Factor Four VF-5ms capillary column (Agilent, Santa Clara, California). The chromatographic conditions are as follows: 100°C; ramping at 10°C min^{-1} to 270°C; ramping at 30°C min^{-1} to a final temperature of 300°C; and held for 2 min. Ultra high purity Helium was used as a carrier gas (purity = 99.999%) with a flow rate of 1.0 mL min^{-1}. The injector temperature was set at 280°C, and the injections were performed in the splitless mode. The mass spectrometer was operated in electron impact ionization mode at -70 eV. The temperatures of the trap, manifold, and transfer line were set at 220, 120, and 280°C, respectively. Compound identification was based on the comparison of mass spectra with the NIST (NIST/EPA/NIH Mass Spectral Library NIST08). Determination of constant error was established through the analysis of a blank sample. Quantitative analysis of target compounds was achieved through the integration of selected gas chromatography chromatograms. Each sample was rerun three times and the detection limit was in the range of 1 ng/L.

Data analysis

Statistical analyses were performed with the software package SigmaPlot for Windows (SPSS Inc., Chicago, IL, USA). All data is expressed as mean ± standard deviation. Prior to all statistical tests, data was examined for normality using a Shapiro–Wilk test and for homogeneity of variances using Levene's test. Depending upon the outcome of these tests, either parametric or non-parametric method was conducted to test for significant differences.

Results

Estrogenic compounds

The concentration of estrogenic compounds present in the water varied from 1.2-20.1 ng/L (Table 1). Estrone concentrations in river water varied from 14.2-20.1 ng/L, 17 β-estradiol concentrations varied from 10.2–15.1 ng/L and concentrations of 17 α-ethynylestradiol were within the range of 1.9–6.2 ng/L (Figure 1). The concentration of estrogenic compounds present in the non-native *P. crispus* and the native *P. illinoensis*, were very low compared to the concentration of estrogenic compounds present in the water (Figure 1). The non-native species, *P. crispus*, contained estrone in concentrations varying from 4.8-9.7 ng/L (Figure 1). The 17 β-estradiol concentrations varied from 3.3–4.9 ng/L, whereas the concentrations of 17 α-ethynylestradiol were very low, 0.39-1.1 ng/L (Figure 1). The native Illinois Pondweed (*P. illinoensis*) contained estrone in concentrations varying from 3.0 -5.9 ng/L (Figure 1). The 17 β-estradiol concentrations varied from 2.1–3.2 ng/L whereas the concentrations of 17 α-ethynylestradiol were very low, 0.3-0.6 ng/L (Figure 1).

Estrogen derivative concentrations were highest closest to the human effluent point source. Sample location 1 was ~9km from the known sewage effluent point source and each successive sampling location was ~1 km from the previous site. As sample locations moved away from the point source the concentration of estrogenic compounds in water, *P. crispus* and *P. illinoensis* decreased (Table 1). The average concentration of estrogenic compounds in Des Plaines River water was 33.1 ± 4.6 ng/L, while the range was between 26-41 ng/L.

Table 1. Estrogenic compounds found in water samples, the non-native species *P. crispus* and the native Pondweed, *P. illinoensis*. The most upstream sample was collected at location 1 while each sample location was ~1km further downstream. Sampling took place ~9km from sewage effluent point sources.

Sample Location	Estrogenic compounds Water ng/L	*P. crispus* ng/L	*P. illinoensis* ng/L
1	41.4 ± 0.6	15.7 ± 0.9	9.7 ± 1.2
2	37.7 ± 0.8	14.7 ± 0.7	7.6 ± 0.6
3	34.0 ± 0.3	12.5 ± 0.4	7.1 ± 0.8
4	30.5 ± 1.1	11.2 ± 0.2	6.0 ± 0.5
5	28.5 ± 0.8	9.1 ± 0.3	6.7 ± 0.8
6	26.3 ± 0.5	8.7 ± 0.7	6.0 ± 0.9

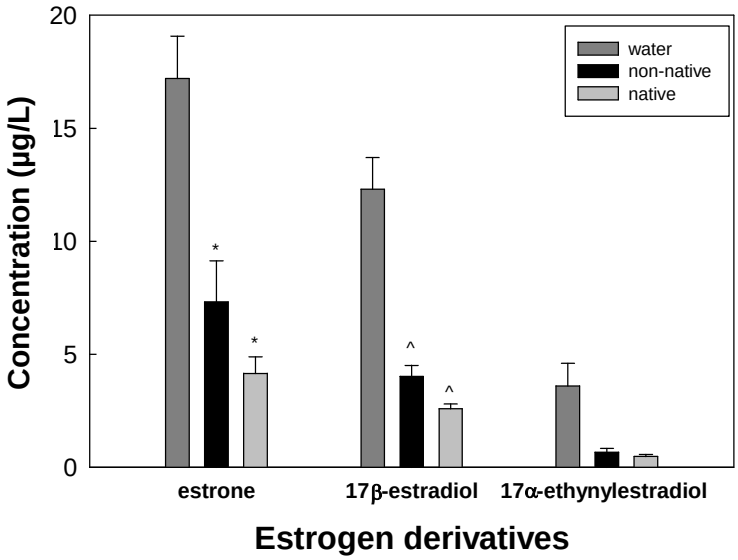

Figure 1. The non-native Pondweed sequesters higher amounts of estrogen derivatives in biomass. The non-native, *P. crispus*, contained significantly more estrone.
* (P=0.009) and 17 β-estradiol^ (P <0.001) than the native *P. illinoensis*.

Bisphenol-A

The concentration of Bisphenol A present in the Des Plaines River water varied from 2.6-3.8 µg/g *P. crispus*, the invasive Pondweed, contained an average of 5.6 µg/g of BPA whereas the native Pondweed contained and average BPA concentration of 2.9 µg/g (Figure 2). At each sampling location 3 full plants were collected. The amount of BPA varied from 3.3-8.5 µg/g and 1.4-5.2 µg/g across sites for *P. crispus* and *P. illinoensis*, respectively (Table 2). We did observe higher levels of BPA in plants closer to the sewage input point source location, with decreasing concentrations down river (Table 2). However, the concentration of BPA in the river water was significantly less (P=0.02) than the concentration of BPA in the invasive Curly-leaf Pondweed (Table 2). The invasive pondweed also held significantly more BPA per gram than the native Illinois Pondweed (P= 0.01; Figure 2). *P. crispus* plants were 72% larger overall (P=0.003) than the native Pondweed, *P. illinoensis* (Figure 3). At all locations, the invasive species were larger than the native species (Figure 3). Total plant biomass dry weight of *P. crispus* was 56%-100% larger than *P. illinoensis* (Figure 3). *P. crispus* possessed a root density ~two times greater than *P. illinoensis*.

As our sample locations moved further away from the point source of the estrogenic contaminations, the accumulation of BPA by these two pondweeds declined (Figure 3). This is likely caused by the decreasing concentrations of BPA in the river water. It is likely that some pulse events, such as the release of fire retardants (The Huffington Post, 2009), accounted for past spikes in BPA concentrations in this aquatic ecosystem.

Table 2. Bisphenol-A concentrations by sampling location in river water, the non-native species *P. crispus* and the native Pondweed, *P. illinoensis*. The most upstream sample was collected at location 1 while each sample location was ~1km further downstream. Sampling took place ~9km from sewage effluent point sources.

Sample Location	Bisphenol A		
	Water µg/g	*P. crispus* µg/g	*P. illinoensis* µg/g
1	3.8 ± 0.5	8.3 ± 0.2	2.9 ± 0.6
2	3.4 ± 0.8	8.1 ± 0.4	3.8 ± 0.9
3	3.2 ± 0.3	4.8 ± 0.5	3.2 ± 0.3
4	2.6 ± 0.3	4.6 ± 0.5	3.0 ± 0.4
5	3.3 ± 0.6	4.0 ± 0.2	2.4 ± 0.2
6	2.9 ± 0.5	3.7 ± 0.3	1.9 ± 0.3

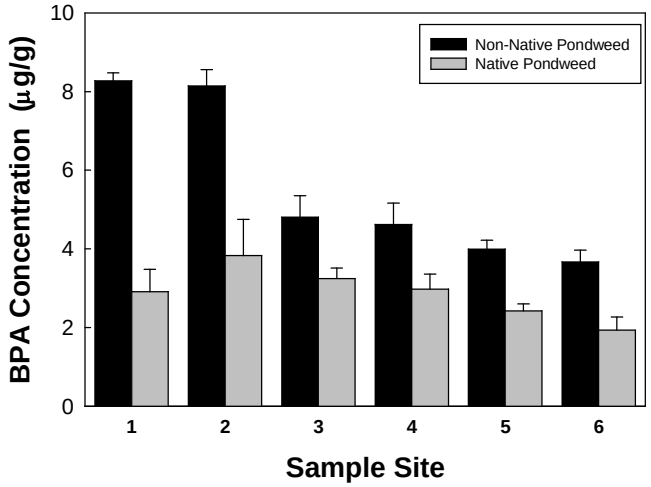

Figure 2. Uptake of Bisphenol-A was greater in the Non-native pondweed. The non-native, *P. crispus*, contained significantly more BPA (P=0.01) than the native *P. illinoensis*. The most upstream sample was collected at location 1 while each sample location was ~1km further downstream. Sampling took place ~9km from sewage effluent point sources.

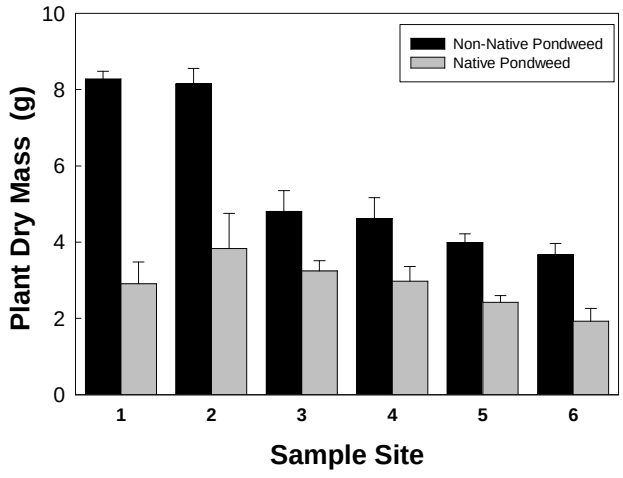

Figure 3. Dry mass of native and non-native Pondweed. The non-native, *P. crispus*, was significantly larger (P=0.003) than the native *P. illinoensis*. The most upstream sample was collected at location 1 while each sample location was ~1km further downstream. Sampling took place ~9km from sewage effluent point sources.

Discussion

Many environmental scientists are using the natural properties of plants to absorb and take up chemicals. Plants have very different strategies when it comes to uptake of nutrients, chemicals and metals (Tsao, 2003). Therefore, it is very important that manager's select the appropriate plants to remove specific pollutants. Certain plant species possess the ability to stabilize chemicals, whereas other plants may transform or concentrate the pollutants in specific plant tissues (Tsao, 2003). We found that the pondweed species did remove estrogen derivatives from the river water, but at very low concentrations. Studies have found that estrogen concentrations in surface waters can vary from 0-100 ng/L (reviewed in Song et al., 2009). The concentrations of estrogenic compounds in the Des Plaines River had an average total concentration of ~33ng/L. Both pondweed species had concentrations of estrogen lower than the river water, suggesting that they were not bioaccumulating any of the estrogen derivatives. The invasive *P. crispus* did sequester and average of 66% more estrogenic compounds than the native *P. iIllinoensis*. The *Potamogeton* species did take up small amounts of estrone 3-10 ng/L, 17 β-estradiol 2-5 ng/L and 17 α- ethynylestradiol 0.3-1 ng/L. However, our species showed relatively low uptake of estrogen derivatives when compared to other studies (i.e. Song et al., 2009) who have successfully removed 40-70% of estrogenic compounds from sewage effluent water using a native reed species. Therefore, we do not recommend the use of *Potamogeton* species for the removal of estrogen derivative compounds from aquatic ecosystems. In contrast, these plants appear to be successful at sequestering the xenoestrogen BPA.

In considering the characteristics necessary for phytoremediation, removal capability plays a significant role. In this study, pondweed was chosen as a model, comparing the ability of the non-native *P. crispus* and native *P. illinoensis* to accumulate EDCs. Comparing the native and non-native pondweed species, the non-native *P. crispus*, had up to three-fold greater accumulation of BPA. This sequestration of BPA is similar to the bioaccumulation potential of *Dracaena sanderiana* (Saiyood, 2010). The mass of these pondweeds and the associated BPA load suggests that these plants may accumulate BPA, a xenoestrogen. When assessing plants to determine their potential as phytoremediators, there are many factors to consider. Particular plant species are better at removing and sequestering specific contaminants to certain tissues. For example, some fruit species have been shown to accumulate BPA and PCBs in their fruit (i.e. Kang et al., 2006; Zeeb et al., 2006). Studies have shown that examining the symbiotic associations of the plants with various bacteria and fungi may be important when determining the uptake capacity of phytoremediators as well.

The increased biomass of *P. crispus* and the larger roots on these plants may increase the surface area necessary for absorbing and assimilating contaminants. Moreover, the roots are sites of microbial attachment, where increased oxygen concentrations and excreted nutrients like carbohydrates symbiotically support microbial populations. In return, these microorganisms are capable of xenoestrogen degradation (Saiyood, 2010). The higher root density found in the *P. crispus* species allows a greater proportion of contact with microbial populations, which may facilitate greater microbial degradation and accumulation of estrogens (Bezbaruah and Zhang, 2004). This indicates that the larger mass of the non-native pondweed may carry an advantage in improved uptake of EDCs and improved recruitment of microbial populations responsible for EDC degradation. Considering its mass and accumulation of EDCs, the non-native pondweed is a significantly better phytoremediator and better adapted to the conditions presented in the Des Plaines River. Further research should be completed to determine how EDC sequestration may be enhanced by specific relationships between plants and their symbiotic bacterial and fungal colonies.

Successful phytoremediation is often the best way to remove nutrients and pollutants from aquatic ecosystems in a safe and cost effective manner. When using macrophytes such as the invasive curly-leaf pondweed or other alien species, it is essential that all of the macrophytes be successfully removed after remediation. Should the plants be left in place, the vast majority of the nutrients and pollutants that were sequestered in plant tissue will be returned to the water as the plants undergo decomposition. This study suggests that utilizing invasive species to remove pollutants may be a viable option, as long as the invasive species is confined to a target area and can be successfully removed after remediation.

Conclusion

Many ecologists recommend removal of exotic and/or invasive species from ecosystems. Our work shows that while non-native species may not be

desirable in a healthy ecosystem, they may serve a purpose in environmental remediation. Many urban aquatic ecosystems are plagued with endocrine disrupting chemicals and pharmaceutical and personal care products which may upset the natural balance of aquatic species. Further research into the effect of these anthropogenic chemicals on native and non-native macrophyte species should be completed to determine which species may help to mitigate and/or remediate anthropogenic pollutants.

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PLANT SCIENCE

Relative importance of the exploitation of medicinal plants in traditional medicine in the Northeastern Sahara

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Abstract

The objective of this study was to highlight the relative importance related to the exploitation of medicinal plants in the South East of Algeria. For this reason, the ethnobotanical survey was conducted in a very systematic way, a record of 10 successive dwellings was collected within rural and urban areas in both Ouargla and Touggourt. This was followed by an analysis of results by the application of some ecological clues. The investigation led to the identification of 65 species covering 36 families that treat several diseases. On one hand, the centesimal frequency of use is the highest in the rural area of Ouargla (QRO) with 95.23% and in descending order for the species *Artemisia herba* alba Asso. (chih), *Mentha spicata* L. (naa naa), *Origanum vulgare* L. (zaatar) and *Juniperus communis* L. (aaraar), on the other hand. The four classes of constancy are present, but the accidental species are very important in the four (04) districts. The type of distribution calculated for all species of medicinal plants in different areas showed a contagious distribution. And the application of automatic classification showed that urban sites are very similar. In the end, this approach has identified the species and has reported the first results obtained by the ecological analysis to better understand the exploitation of species in the studied area.

Key words: Sahara, Medicinal plants, Ethno-botanical, Exploitation, Lifestyle, Ecological indices, Algeria

Introduction

About five hundred species of higher plants inventoried part is nowadays used by people as medicinal plants in the Sahara (Ozenda, 1983). Since herbal medicine offers natural remedies and well accepted by the body, is often associated with conventional treatments. Its exceptional revival, especially for chronic diseases, nowadays, is well marked (OSS " Observatoire du Sahara et du Sahel ", 2010;"Organisation Mondiale de la Santé" O.M.S., 2004; Tabuti et al., 2003). Indeed, the Algerian Sahara has a long tradition and expertise in traditional and herbal medicines. Previous studies were mainly qualitative in nature and related to the correlation between the rural and traditional medicine (Bounaga and Brac De La Perriere, 1988; Benchellahetal, 2004; Maiza, 1993; Ould El Hadj et al., 2003; Chehma, 2006). Nevertheless, during the Anthropocene era, humans have really changed all ecosystems on the planet with an acceleration greater than in any comparable period in human history (Steffen et al., 2007). Indeed, the effects have been observed on the lifestyle of people in the study area, including education, agriculture, industry and especially on the natural environment, including plants. However, the evolution of ethnobotanical research led to the development of the methods of analysis, including various disciplines. Among others, ecological methods for determining the impact on the environment and the level of sustainability - plants - human relations in the past and present were reported (Cotton, 1996). Thus, we believe that the medicinal flora heritage requires monitoring and regular assessment in both qualitative and quantitative ways. That in turn will ensure its preservation and its current medicinal values. Therefore, we focused on the quantitative aspect at the end to identify the importance of the use of medicinal species and was based on a sampling method that seems suitable for our approach. The latter is based on two questions:

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- Is the lifestyles have an impact on the use of medicinal plants?
- What are the plant families and species most involved in regional pharmacopoeia whose use is based on ecological indices and automatic classification?

Materials and Methods

Description of the studied area

The study was conducted in South-eastern Algeria, in the province of Ouargla, which is located 790 km away from Algiers. It is characterized by several oases, the most important are those of Touggourt (X: 33° 07; Y: 6° 04' and A: 70m (Le Houerou et al., 1995) and Ouargla (X: 31° 37 '; Y: 5° 24' and A: 141m (Rouvillois-Brigol, 1975; Cote, 1998) (Figure 1). The aridity is substantially attenuated in Touggourt; however, all the studied area belongs to the hyper-arid bioclimatic Mediterranean-continental (Rivas – martinez, 1996-2009; Chehma et al., 2005).

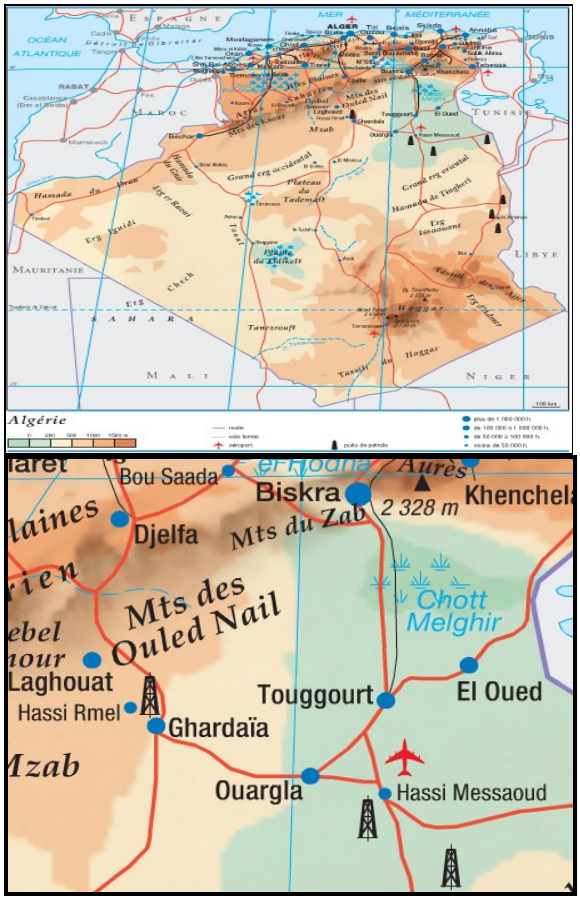

Figure 1. Geographic location of the study area.

In addition to palm cultivation with crops underlying the studied area has a floristic heritage of various strains, Mediterranean and tropical species that have adapted to the desert. The inhabitants are ethnic- multiple, Berber, Arab, African and mestizo came from mixed marriages. Ouargla is as an administrative pole of southeastern Algeria, most commercial activities are concentrated in Touggourt. Also, most young people prefer to leave the oasis and go to the oil fields located nearby. However, small transactions that take a traditional aspect exist in Ksour and in the periphery of the two oases.

Sampling methodology

It aims to evaluate the use of plants by the traditional medical system in its present form and to compare the pharmacological research, agro-ecological and socio-economic development. Overall, our methodology consists of: An exploratory phase from October to December was conducted among the most skilled people in the subject at the studied area to make a first list of species and the development of the survey form. The choice of sites, guided by the characteristics of the studied area, was based on two modes of life, urban and rural areas that are the most representative (Anonym, 2008) (Figure of 2-4). The phase of the ethno-botanical survey itself was conducted during five months. To rationalize the sampling and to facilitate the quantitative study while getting as close as possible to the residents at the district level, we were constrained to be limited to 10% of houses. These were chosen in a systematic or a record of 10 successive dwellings. The duration of each conversation is about 1h30. In each one, any information was gathered on the profile of the questioned people and the medicinal plants they use.

Thus, the profile of everyone includes their age, sex, educational level, their job, their income and the medicinal plants they use. The gathered information on plants includes the scientific and common names, the parts used, the method of preparation and the disease being treated. All species have been authenticated through literature and through comparison with the herbarium specimens produced within the Faculty SNV (Sciences de la Nature et de la Vie) and S.T.U. (Sciences de la Terre et de l'Univers), University Kasdi Merbah, Ouargla. The data recorded on data sheets Gross.

The data recorded on the raw data sheets were analyzed through ecological indices and statistical method:

The frequency of use (Fc), the percentage of individuals of a species considered in relation to the total number of individuals of all species. It can be

calculated for a sample or for all concerned (Dajoz, 1971). It is calculated according to the formula:

$Fc = (ni / N) \times 100$

Where, (ni) - The number of individuals of a species
(N) - The total number of individuals of all species present

The consistency or frequency of occurrence (C) of a given species is the number of times it appears in the sample. It is calculated from the following formula:

$C = (Pi / P) \times 100$

Where, (i) - The number of records where species are present
(P) - The total number of records (P)

Thus, the constancy of a species is called accidental (C <25%), accessory (25% <C <50%), regular (50% <C <75%) or constant (75% <C <100%).

Analysis of Variance (Ó2) (Dagnelie, 1975): is obtained by using the formula:

Ó2=; with the number of squares to be studied (n), the number of individuals in each square (x) and the average individual in the set of squares (m);

-Type of distribution (TR) (Dagnelie, 1975): is given by following the formula: TR= Ó2/ m. The type of distribution is contagious if TR is greater than 1. It will be aleatory if this value is equal to 1, regular if it is less than 1 and uniform with a value equal to zero.

Automatic classification: is performed using the software STATISTICA.

Photo 1. Urban area of Ouargla (QUO): Chourfa.

Photo 2. Rural area of Ouargla (QRO): Hassi Miloud.

Photo 3. Urban district of Touggourt (QUT): Amir Abdelkader.

Photo 4. Rural district Touggourt (QRT): Sidi Mahdi.

Results and Discussion

The ethno-botanical survey

The analysis of 735 ethno-botanical investigations was completed by the distribution shown in Table 1. The latter shows the number of the questioned people by district and by gender. In fact, it is proportional to the number of inhabitants recorded by site.

Table 1. Distribution of data by sex and lifestyle.

Sites	Ouargla		Touggourt		Total interviews
Number of samples	Urbain district (QUO)	Rural district (QRO)	Urbain district (QUT)	Rural district (QRT)	
	Number	Number	Number	Number	
Male	155	8	130	9	302
Female	215	13	190	15	433
Total	370	21	320	24	735

Frequency of medicinal plants according to the profile and lifestyle of the questioned people

At both oases studied, those of age 20-40 have a high frequency of use of herbals, especially in Ouargla (57%). It is in order by age groups 41-60, [under 20] and finally the more than 61 years, these results show that there is a real return to traditional medicine, whatever the lifestyle and age. Among users, there is a preponderance of females where rates are between 62% and 65% compared to men whose rate is not more than 40% for all districts. What seems paradoxical with other ethno-botanical explorations (Hasnaoui et al., 2011; Gbolade, 2009) is that most users are not illiterate. The frequency recorded over 75%. Unemployment is a national problem and the study area is a part, especially in rural sites. In fact, the rate of users exceeds 50% in the districts studied, particularly in the QRO with 65% and rural district of Touggourt (QRT) with 63.2%. In terms, we find that the self-employed with a rate of over 50% remain dominant in spite the fact of what was cited earlier. In this socio-economic analysis plus the regional culture (tradition), health weakened by pollution, the resistance of certain organisms towards the chemical treatment, the ineffectiveness of the public health system that explains the return to phytotherapy. Apparently the knowledge of the users of medicinal plants and their properties grows more and more unlike what has been reported by other studies (Mehdioui and Kahouadji, 2007; Hasnaoui et al., 2011; Gbolade, 2009).

Inventory

In order to record the number of species according to their systematic entity, our results are presented in the Table 1. Upon completion of the investigation 65 plants were identified, distributed between 43 spontaneous and 22 cultivated. Their distribution is unequal between districts, similarly for the genera and families. However, the higher number has characterized the urban lifestyle especially in the urban district of Touggourt QUT, except for the entity of the genus where the urban district of Ouargla QUO dominates with 56 (Table 2). Among the 36 families surveyed, only some are highly exploited in these oases, according to their species richness: Apiaceae (10), Labieae (10), Astéraceae (6), Myrtaceae (4), Fabaceae (3) and Zygophyllaceae (3). They are followed by the Lythraceae, Zingiberaceae, Rutaceae, Lauraceae and Alliaceae with 2 species each. According to many authors, such as Hernandez et al. (2012), the closely related families of Asteraceae, Myrtaceae, Fabaceae are considered among the largely exploited plants in traditional medicine.

Frequency of use of medicinal species identified in the oasis of Ouargla and Touggourt

The results are presented for some of the parameters transcribed in the survey form. From the Table 3, it is clear that the high frequencies involve twelve species whose use is common to all districts. In fact, the most common species is *Artemisia herba alba* Asso, with 16.85% in QRT, 15.74% in QUT, to 14.98% in QUO and 12.50% in QRO. These rates are explained by the therapeutic properties of the plant and also its availability. For the remaining species, the frequencies are relatively low overall. In total, we recorded 39 diseases treated by the listed plants but the most frequent ones were: digestive disorders (8 species, 12, 3%), pneumonia (7 species, 10, 8%,), fever (6 species, 9, 2%) and influenza (5 species, 7, 8%). The recommended methods are the decoction (58.46%), powder (36.92%) and infusion (30.76%). The parts used are leaves (44.61%), seeds (29.23%), stems (27.69%), fruits (21.53%) and roots (1.53%). In literature, there are differences with our results (Maiza et al., 1993; Ould El Hadj et al., 2003).

In addition, the seven identified endemic plants are characterized by a low frequency of use. These plants treat sixteen diseases. They were mentioned by many authors in Traditional Saharian pharmacopoeia (Ozenda, 1983; Maiza et al., 1993; Chehma, 2005) (Table 4). According to these results, we record the use of aerial parts. This preserves the underground organs.

Table 2. Number of Family, Genus and species indicated according to the sites.

Sites	Ouargla		Touggourt		Totals
Systematic entity	QUO	QRO	QUT	QRT	
Family	23	14	25	14	31
Genus	56	28	49	22	60
Species	46	39	53	25	65

QUO: Urban area of Ouargla / QRO : Rural area of Ouargla / QUT: Urban district of Touggourt / QRT: Rural district of Touggourt

Table 3. Centesimal frequency (Fc,) of occurrence (C.) variance (Ó2) and type of distribution (TR) of common medicinal species and diseases treated in the district studied.

Species	QUO		QRO		QUT		QRT		Parameters		Treated diseases
	Fc (%)	C (%)	Fc (%)	C (%)	Fc (%)	C (%)	Fc (%)	C (%)	Ó2	TR	
Artemisia herba alba Asso.	14,98	52,63	12,5	60	15,77	52,40	16,88	78,94	3,41	0,22	Respiratory disease Diarrhea, cough, flu pneumonia, digestive disorders, gastritis
Mentha spicata L.	11,58	40,70	7,29	35	11,77	39,2	10,11	47,36	4,29	0,42	digestive disorders, gastric gas, headaches, hypertension Cough, flu, Menstrual Sore, Asthenia
Origanum vulgare L.	7,99	28,07	8,33	40	9,13	30,4	5,61	26,31	2,28	0,29	Weakness, disorders of the respiratory tract Rheumatism, menstrual disorders, digestive disorders, flu
Juniperus communis L.	6,49	22,8	5,20	25	6,49	21,6	4,49	21,O5	0,98	0,17	Diarrhea, pneumonia Gastritis,Vomiting Digestion trouble
Nigella damscena L.*	3,29	11,57	3,12	15	2,64	8,80	3,37	15,78	0,10	0,03	Hypertension Stomach gas Headache,Asthma Nervous exhaustion
*Trigonilla foenum graecum L.**	5,99	21,05	3,12	15	6	20	4,49	21,05	1,91	0,39	Sexual weakness Weakness, digestive disorders, Anemia, Glomerulonephritis Anorexia,Hypertension
Rosmarinus officinalis L.	4,09	28,07	7,29	35	1,44	4,80	4,49	21,31	5,73	1,32	Dehydration Stomach gas
Cuminus cyminum L.	5,39	18,94	5,20	25	5,16	17,20	1,12	5,26	4,28	1,01	Digestive disorders, Stomach gas, anemia
Eugenia caryophyllata Thumb	0,69	2,45	3,12	15	0,24	0,80	1,12	5,26	1,61	1,24	Headache Sexual weakness Nervous exhaustion
Myrtus communis L. Herm.	-	-	2,08	10	0,48	2,40	-	-	0,97	1,52	Gastritis, Diarrhea Stomach gas, digestive disorders, Heart, menstrual disorders
Lippia citriodora L.	1,79	6,31	3,12	15	1,44	4,80	5,61	26,31	3,58	1,19	Digestive disorders Stomach gas
Zingiber officinales rascoe Var.	5,99	21,05	2,08	10	5,52	18,40	2,24	10,52	4,35	1,09	Hypercholesterolemia, Pneumonia, flu , hypertension, Sexual weakness, The human immunodeficiency, gastric gas, digestive disorders, constipation, diarrhea, cancer, diabetes, anemia

N: Number of species/ C: constancy/ QUO: Urban area of Ouargla / QRO : Rural area of Ouargla / QUT: Urban district of Touggourt / QRT: Rural district of Touggourt/ Centesimal frequency (Fc,) of occurrence (C.) variance (Ó2) and type of distribution (TR)

Table 4. Endemic species used in the east of the northern Sahara.

Families	Scientific names	Names Vernacular	Parts used	Mode of preparation	Diseases treated	Biogéography
Apiaceae	*Ammodaucus leucotrichus* Coss. & Dur	Oum drayga	Leaves and Fruits	Decoction	Asthma Diarrhea, constipation	Endémic
	Ferula Vesceritentis Coss. & Dur.	Habet lehlaoua	Fruits	infusion	Angina,fevers and migraines	Endémic saharan
Asteraceae	*Anvillea radiata* L	Nougd	whole plant	Infusion	Diabetes and indigestion	Endemic Saharan
	Matricaria pubescens (Desf.)Schultz	Garteufa	leaves	Decoction	Pain rules	North African endemic
Brassicaceae	*Oudneya africana* R. Br.	Hannet l'ibel	Leaves and Fruit	Mixture powder	Skin disease	Endémic
Euphorbiacées	*Euphorbia gyuoniana* Boiss. & Reut.	Oum el bina	Leaves	Decoction, mixture, powder	cataplasm Verrus, abscess	Endemic northern Sahara
Liliaceae	*Urginea noctiflora Batt. & Trab.*	Basl elfar	Bulbs	Decoction, Powder and cataplasm wounds	Verrus, Abcès wounds, ulcer, Inflammation of the throat and earaches	Endémic

Table 5. Toxic Species identified in the study area.

Families	Speices	toxic parts	Toxic	Authors
Asteraceae	*Anvillea radiata* L	whole plant	Highly glycosylated flavonol	Dendougui et al., 2006
	Cotula cinerae Del.	whole plant	Flavones	Benayache et al., 2000, Chehma, 2005
Apocynaceae	*Nerium oleander* L.	whole plant, especially the leaves	Glycoside	Bruneton (2001),
Chenopodiaceae	*Hammada scoparia* (Pomel) Iljin	Leaves	alkaloids	Chehma (2005)
Cucurbitaceae	*Colocynthis vulgaris* (L.) Schrader	Fruits	alkaloids	Bruneton (2001)
Euphorbiaceae	*Euphorbia gyoniana* Boiss. & Reut.	Leaves	Lactone euphorbe	The investigation Raouiha (tradipraticien)
Rutaceae	*Ruta tuberculata* Forssk.	Leaves	Essential oil, bitter substances	Passager (1957) Ozenda (1983)
Solanaceae	*Solanum nigrum*	Leaves, fruits	alkaloids	Ozenda (1983), Bruneton (2001), Ducerf (2010)
Liliaceae	*Urginea noctiflora Batt. & Trab.*	Bulbs	Glycoside	Baba aissa (1991)
Zygophyllaceae	*Peganum harmala* L.	whole plant	Alkaloids, harmaline, indole harmalol	UNESCO (1960) Bruneton (2001)
		seeds		
	Zygophyllum album	Fruits	Essential oil, bitter substances	Ozenda (1983)

Table 6. Number and constancy of medicinal species used in the four districts.

District Stances		QUO	QRO	QUT	QRT
Ubiquitous species	N	0	0	0	0
C=100%	%	0	0	0	0
Constant species	N	0	0	0	1
75%< C <100%	%	0	0	0	4
Regular species	N	1	1	1	0
50< C <75%	%	2,22	3,44	1,88	0
Incidental species	N	2	7	2	6
25< C <50%	%	4,44	24,13	3,77	24
Accidental species	N	42	21	50	18
C<25%	%	93,33	72,41	94,33	72
		45	29	53	25

N: Number of species/ C: constancy/ QUO: Urban area of Ouargla / QRO : Rural area of Ouargla / QUT: Urban district of Touggourt / QRT: Rural district of Touggourt

Table 7. Variance of use and type of species distribution in districts.

Settings	QUO	QRO	QUT	QRT
Variance	7,68	6,17	7,93	8,46
Type of distribution	5,02	4,03	5,18	5,53

QUO: Urban area of Ouargla / QRO : Rural area of Ouargla / QUT: Urban district of Touggourt / QRT: Rural district of Touggourt

Table 8. Number of medicinal species by types of distribution

Types of distribution	Contagious TR>1	Aleatory TR=1	Regular TR<1	Uniform TR=0
Number of species	22	0	41	2

TR: Types of distribution; Contagious if TR is greater than 1; Aleatory if this value is equal to 1; Regular if it is less than 1 and uniform with a value equal to zero.

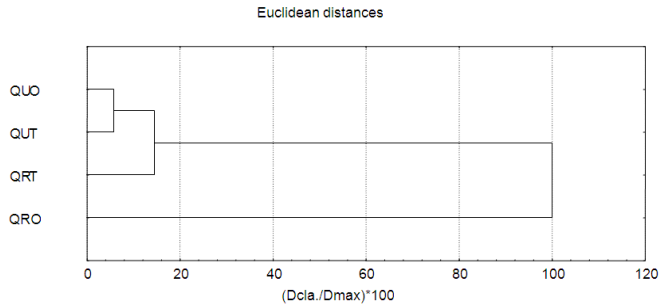

Figure 2. Dendrogram of similarity levels of the exploitation of medicinal species in the four sites.

Given the sample size, the study of the species' toxicity is limited to statements of the interviewed subjects and the necessary documentation. 11 species result from this sampling; they are classified as dangerous species which cover 9 families. They are clearly illustrated in Table 5.

Table 6 shows the prevalence of accidental species, especially in urban areas (93% and 94%). These rates are due to instability probably related to the socio-economic evolution and the revival of the herbal medicine. Incidental species occupy the second position; the constancy in the rural areas (24%) is higher than in urban areas (4%). In this category, we find the species *Origanum vulgare* L. and *Mentha spicata* L. in the two lifestyles. For this, the incidental species *Peganum harmala* L., *Rosmarinus officinalis* L., *Ajuga iva* (L.) Schreb. and *Mentha pelgium* L. are found exclusively in the QRT (Table 4).

The third position is occupied by the regular class with constancy ranging between 2% and 3% in urban and rural areas of Ouargla. *Artemisia herba alba* is the only species where the highest rate is maintained or the QRO (60%). The class of constant species is characterized by a single species which is sagebrush *Artemisia herba alba* with 78.4% in the QRT. It comes in the fourth position (Table 3).

Analysis of Variance applied to medicinal plants used in the districts

The analysis of variance allows us to know if there is a significant difference between species use

areas (lifestyle) and the difference between the species themselves. In fact, the results of the variance and the type of distribution are illuminated by Table 7.

In Table 7, it is clear that the variance values are quite closer between urban settlements than between rural sites. However, the type of distribution is greater than 1 in all areas, thus the distribution is contagious. In fact, this highlighted the relationship established between users.

Type of distribution of medicinal plants used in the studied area

From the obtained results, we noted that the distribution of medicinal species in common use is variable (Tables 3 and 8). The value of the type of distribution shows that the regular distribution "R" is well represented in the studied area. Thus, this type is used for 41 species. The contagious "C" is illustrated by 22 species. Species representing the uniform distribution "U" are 2, characterizing the QUO. By term, we emphasize the lack of randomization "A". According to ethnobotanical survey, this is due to the rate of mixed use more than that of single use of these species.

Automatic classification applied to medicinal plants used in the studied area

Examining the dendrogram (Figure 6), we distinguished three nodes; the first node is formed between the urban districts of Ouargla (QUO) and Touggourt (QUT). This is equivalent to the number of species (48) in common use which is very important. This was predictable, given the assumptions raised. The second node is located between the first node and the rural district of Touggourt (QRT). The similarity is relatively low because the number (20) of common species decreased. In the end, the third node is between the second node and the rural district of Ouargla (QRO). At this level, the similarity is the lowest, obviously because the number of species (14) in common use is reduced.

Conclusion

This study was focused on the importance of medicinal plants in two oases in northeastern Sahara. It has extended the list of medicinal flora where two- thirds of the species are spontaneous. In fact, it appears that the urban lifestyle is as much involved in herbal medicine as rural lifestyle. However, we noticed that the rural people preserve more of their tradition in using certain plant species. Thereby they escape the pressure of the most acclaimed species of the contemporary period. In summary, the analysis of the constancy showed that the exploitation of medicinal plants was inversely proportional to the number of species transcribed in each lifestyle. The analysis of variance reveals a significant difference in use of species between the two lifestyles. The type of distribution has unveiled two main levels of use. The first group consists of species regularly distributed that express the homogeneous use. Thus, they can be described as the most popular species of the traditional medical system. This might be due to the rate of mixed use that is higher than the rate of single use of these species. In fact, the description of the hierarchy is divided into three classes. The first class is composed of the urban districts reflecting the importance of common species. The second class consists of urban and rural districts where the similarity is relatively low. The third class consists of the rural district of Ouargla that has few medicinal species in common with the other sites. We, therefore conclude that, apart from the interactions (land-use systems, tenure systems, human disturbance etc.,) of local people (ethnic group, language, population size and distribution, migration, social groups, education etc.,) with the natural environment (medicinal species, location, elevation, climate, geology, vegetation, types, species diversity etc.,), we noted a much moderated exploitation of the endemic plants. Yet, the quantitative aspect deserves being dealt with subsequently. Likewise toxicity, doses and methods of the conservation of medicinal plants should make the object of deep researches to supply the ascendant needs of the population.

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PLANT SCIENCE

Organic fertilization: An alternative to produce jalapeño pepper under greenhouse conditions

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Abstract

The experiment was conducted during fall-winter seasons of years 2011-2012 to determine the effects of vermicompost tea (VCT) on growth and productivity of "Hechicero" jalapeño pepper plants grown under organic and synthetic fertilization in greenhouse. Five different fertilization forms were applied to plants [F1 = sand + inorganic nutrient solution (control group); F2 = sand + VCT on concentration of 10%; F3 = mixture of sand + compost (ratio 1:1; v:v) + VCT on concentration of 2.5%; F4 = mixture of sand + vermicompost (ratio 1:1; v:v) + VCT on concentration of 2.5% y F5 = mixture of sand + compost + vermicompost (ratio 2:1:1; v:v:v) + VCT on concentration of 2.5%]. Treatments F4 and F5 showed an increased yield of 70 and 45% with regard to the yield obtained with F1; the F1 and F2 yields were not statistically different at the 0.05 significance level. The fruit length and the pericarp thickness were increased until 7.55 and 7.01% in F5, respectively. These results suggest that, since there were differences in yield when using the organic and inorganic nutrient source, VCT combined with mixtures of sand + compost + vermicompost may be considered a successful alternative fertilizer for organic jalapeño pepper production in greenhouse.

Key words: *Capsicum annuum*, Glasshouse, Vermicompost, Organic farming

Introduction

Manure, crop residues, green manures, dairy industry sludge, herbal pharmaceutical industry waste, biosolids of agribusiness and food processing waste, once they are properly treated through the process of composting and/or vermicomposting are some of the potential sources of nutrients in organic production systems (Ramesh et al., 2005; Alidadi et al., 2007). According to Ramesh et al. (2005), organic production is an alternative for consumers who prefer food free of pesticides and synthetic fertilizers-risk-free, with high nutritional value. Additionally, organic materials are the safer sources of plant nutrient without any detrimental effect to crops and soil (Hasanuzzaman et al., 2010).

Nowadays, it is widely recognized that de compost (C) and vermicompost (VC) are sources of slow-release nutrients, available for plants (Atiyeh et al., 2001; Chaoui et al.,2003; Cruz-Rodrigues et al., 2003; Raviv, 2005) and prevents nutrient losses into the environment (Giuffré et al., 2011). There is evidence that the addition of C and VC to soils and growing media promotes the development and productivity of different horticultural crops such as tomato (*Solanum lycopersicon* L.) (Gutiérrez-Miceli et al., 2007; Moreno-Reséndez et al., 2013), lettuce (*Lactuca sativa* L.) (Steffen et al., 2010), pepper (*Capsicum annuum* L.) (Arancon et al., 2004a), garlic (*Allium sativum* L.) (Argüello et al., 2006), strawberry (*Fragaria vesca* L.) (Arancon et al., 2004b), wetland rice (*Oryza sativa* L.) (Hasanuzzaman et al., 2010), and other species of commercial interest.

On the other hand, when mixing C or VC with inert growth media, such as sand, the physical and chemical characteristics of the latter show an important improvement, and the vegetable

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specimens that grow in it can avoid hypoxia. Also, it has been established that both C and VC can satisfy the nutrient demand of several crops, developed in greenhouse conditions, during the first two months post-transplantation (Márquez-Hernández et al., 2006). However, after this time the crops expressed nutrient deficiencies, especially of N (Rodríguez-Dimas et al., 2007); this may be due to the low rate of N mineralization of both C and VC. Thus, it has been suggested that in production systems under protected conditions, nutritional stress of the culture can be avoided by adding other sources of nutrition, including the vermicompost tea (VCT).

The VCT, a water-based VC extract containing high levels of beneficial microbes and soluble nutrients (Edwards et al., 2010), has attracted the attention of growers and researchers in recent years. The most important reason to apply VCT is to supply microbial biomass, fine particulate organic matter, and soluble chemical components of VC to plant surfaces and soils in a way not possible or achievable with solid VC. However, relatively little work has been done to investigate the effect of VCT on growth and yield of vegetable crops.

We hypothesized that VCT as a fertilizer would positively affect plant growth and productivity of jalapeño pepper. Therefore, the specific objective of this study was to investigate the effects of VCT on growth and productivity of jalapeño pepper in different media under organic and synthetic fertilization.

Materials and Methods

The experiment was conducted during fall-winter seasons of years 2011-2012, in the Comarca Lagunera region (101° 40' and 104° 45' W and 25º 05' and 26º 54' N), in a greenhouse at Universidad Autónoma Agraria Antonio Narro – Unidad Laguna (UAAAN-UL). The greenhouse is semicircular, with acrylic cover and protected reinforced mesh shade during the warmer seasons, gravel floor and automatic cooling system through wet wall and two extractors. It has side windows of 1.20 m high, covered and protected with acrylic roller anti-aphids mesh (Mesh Plas®).

Treatments were distributed according to a completely randomized design; yield of jalapeño (*Capsicum annuum* L.) *cv.* Hechicero (Harris Moran®) with five forms of fertilization was assessed.

The evaluated fertilization forms were: F1 = sand + inorganic nutrient solution (control group); F2 = sand + VCT at 10% of concentration; F3 = mixture of sand + C (ratio 1:1; v:v) + VCT at 2.5% of concentration; F4 = mixture of sand + VC (1:1; v:v) + VCT at 2.5% of concentration, and F5 = mixture of sand + C + VC (ratio 2:1:1; v:v:v) + VCT at 2.5% of concentration.

Seeds were planted on October 1, 2011, in germinating trays of 200 cavities filled with Peat Moss (Premier®). The transplant took place on November 7, 2011, placing one plant per container. These containers consisted of black polythene bags with a capacity of 18 L, filled with volume basis. The population density was 4 pots•m^{-2}. The sand used in the substrates was previously disinfected with a solution of 5% NaOCl.

The manure-based vermicompost was provided by the vermicompost module of UAAAN-UL, and consisted of separated horse and goat solids (mixed in a 1:1 ratio by volume) with alfalfa (*Medicago sativa* L.), processed by earthworms (*Eisenia* spp.) in indoor beds (Atiyeh et al., 2000a) for a period of 90 days (Bansal and Kapoor, 2000). The compost was provided by Max Compost®. The basic chemical properties of the substrates are summarized in Table 1. The nutrient solution used in F1 treatment (Table 2) was recommended by Castellanos-Ramos and Ojodeaagua-Arredondo (2009).

Table 1. Chemical analysis of the materials used during the development of jalapeño pepper under greenhouse conditions (n = 1).

	N	P	K	Ca	Mg	Na	Fe	Zn	Mn	pH	EC
	(%)						(mg•kg^{-1})				(dS•m^{-1})
C	2.41	1.19	3.355	8.76	0.942	0.567	5920	260	160	8.5	6.7
VC	1.27	0.15	0.882	6.92	0.596	0.101	7090	330	210	7.9	2.4
S	0.011	0.0005	0.01	0.004	0.0016	0.007	ND	1.2	2.4	7.5	0.65
VCT	0.83	0.49	0.71	3.23	0.75	0.2	6.4	2.7	4.9	8	2.0

C = compost; VC = vermicompost; S = sand; VCT = vermicompost tea; ND = not detected; EC = electrical conductivity.

Table 2. Concentration of the nutrient solution used to develop greenhouse jalapeño pepper.

Stage/Ion	NO_3^-	NH_4^+	$H_2PO_4^-$	K^+	Ca^{2+}	Mg^{2+}	SO_4^{2-}	HCO_3^-	Na^-	Cl^-	EC
	(mmol•L^{-1})										(dS•m^{-1})
Planting and establishment	6.0	0.5	1.5	4.0	4.0	1.0	1.5	1.0	<5.0	3.0	1.4
Flowering and fruitsetting	8.0	0.5	1.5	6.0	4.0	1.5	3.0	1.0	<5.0	3.0	1.8
Onset of ripening and harvest	10.0	0.05	1.5	7.0	4.0	2.0	3.0	1.0	<5.0	5.0	2.2

EC = electrical conductivity.

The VCT at 10% of concentration was prepared using the method recommended by Edwards et al. (2010), with a variation that consisted in introduce the bag with VC in a container with 20 L of water for 5 min to wash excess salts, before being subjected to oxygenation. Whereas in a container of 60 L of capacity, 45 L of water were oxygenated with an air pump (Biopro: BP9891. Tiray® Technology Co Ltd) for 2 h before inserting the bag with 4.5 kg of VC; oxygenation was performed continuously until completion of the process (24 h). In addition, we added 40 g of piloncillo or panela, a product similar to molasses, made from unrefined sugarcane (*Saccharum officinarum* L.) juice (Solis-Pacheco et al., 2006), as an energy source to promote the growth and development of microorganisms.

The VCT was constantly applied throughout the whole cycle, and to preserve its quality it remained aerated 24 h•day^{-1}. For treatment F2, 0.5 L of VCT at 10% of concentration was poured in each pot; for treatments F3, F4 and F5, the VCT was diluted to a 1:3 ratio using 1 L of VCT per 3 L of water to give a concentration of 2.5%, and each pot received 1 L. The pH of VCT was adjusted to a value of 5.5 with food grade citric acid ($C_6H_8O_7$*H_2O), applied on a concentration of 5 mM (1.2 g•L^{-1}) (Capulín-Grande et al., 2007).

To satisfy the water demand of the crop, a drip irrigation system was used in all treatments, and the amount of water applied, according to the phenological stage of the crop, ranged from 0.35 to 1.9 L•plant^{-1}•day^{-1}. It was classified as low-salinity and low sodium water (C_1S_1, with a sodium absorption ratio of 2.18) (Ayers and Westcot, 1994), it presented an EC of 1.05 dS•m^{-1}, pH 7.8; the cation concentrations were Ca^{2+} = 3.51, Mg^{2+} = 0.48, K^+ = 0.22, Na^+ = 2.71 mmol•L^{-1}, and the anion values were HCO_3^- = 3.12, Cl^- = 2.3, SO_4^{2-} = 2.62 mmol•L^{-1}. During the growing period, which lasted 134 days after transplanting (dat), the temperature in the greenhouse ranged from 17.4 to 36.9°C, while the relative humidity ranged between 20 and 79%.

Plant height and total yield was recorded in each experimental unit. Fruit quality was determined in four plants of each treatment, 15 fruits per plant, considering the following variables: individual weight, pericarp thickness, number of locules in the fruit, fruit length and equatorial diameter.

To analyze the behavior of the plant height over time, we used linear regression analysis, while for yield and fruit quality an analysis of variance was performed among treatments; means were separated using Fisher´s least significant difference (LSD) test in SAS statistical software (SAS, 1999). Statistical significance was obtained at 95% confidence level ($\alpha = 0.05$).

Results and Discussion

The growth dynamics of jalapeño plants, *cv*. Hechicero, are presented in Table 3, the regression line fits the data well. In our experiment, the tallest plants at the end of the crop cycle (134 dat) were in the treatment F5, with a value (1.55 m) that was statistically different from the other plants recorded in the remaining treatments (Table 3); these results suggest that VCT consistently enhanced plant growth, which agrees with the findings of previous studies (Sanwal et al., 2006; Hargreaves et al., 2008; Hargreaves et al., 2009). Also noteworthy is the fact that treatment F4 [mixture of sand + VC (ratio 1:1; v: v) + VCT at concentration of 2.5%] recorded the second highest plant (1.16 m).

Pant et al. (2009) found that the use of VCT had a greater effect on plant height under VC fertilization, and had much smaller effect on plant height under Osmocote fertilization. Additionally, Keeling et al. (2003) and Siddiqui et al. (2008) observed that application of VCT and compost tea on oilseed rape plant and okra plants, increased root development, plant growth and tap root length, respectively; although we did not measure root growth and nutrient uptake, plants grown in F4 and F5 showed better plant height and nutrient uptake compared to control plants, suggesting that these two variables might be part of the mechanisms involved in plant growth stimulation. Our results might be due to the presence of the VC and the VCT, materials that provide higher quality than manure generated by traditional methods of composting (Santamaría-Romero et al., 2001; Panikkar et al., 2004; Lopes-Pereira et al., 2005).

Table 3. Regression equations for fertilization sources regarding plant height in jalapeño pepper under greenhouse conditions.

Treatment	Regression equation*	R^2	Final height (m)
F1	y = 0.7142x – 2.5217	0.95	0.93 c
F2	y = 0.4937x – 4.2867	0.94	0.62 e
F3	y = 0.5757x – 4.5035	0.92	0.73 d
F4	y = 0.8486x + 2.2051	0.91	1.16 b
F5	y = 1.2648x – 14.797	0.97	1.55 a

*y= height; x = das. Values with the same letter in last column are statistically equal, LSD test $P \leq 0.05$.

Treatments F4 and F5 (which included VC and VCT) outperformed the control treatment (sand and nutrient solution), concurring with the results reported by Rodriguez-Ortiz et al. (2010), who concluded that VC fertilization at 1.5 to 3.0 t•ha^{-1} during transplanting of green onions (*Allium cepa* L.) favored a greater height of this species when compared with plants receiving synthetic fertilization. Arancon et al. (2007) reported that humic, fulvic and other organics acids extracted or produced by microorganisms in VCT could induce plant growth. Garcia-Martinez et al. (2002) showed that water extract of compost contained a compound with molecular structure and biological activity analogous to auxins. Leachate from well decomposed compost contains cytokinin-like substance, derived from hydrolysis of cyanogenic glucosides by the enzyme β-glucosidase produced by microbes (Arthur et al., 2001). Although phytohormones or growth regulators in VCT were not measured in the present study, they may play an important role in plant responses.

The F1 and F5 yield, fruit length, pericarp thickness and number of fruits were statistically different at the 0.05 significance level (Table 4). The heaviest fruits were obtained with treatments F1 and F5, in that order, while the lowest weight was recorded in treatment F2. On the other hand, according to the specifications of the official mark "México Calidad Suprema" for chili (SAGARPA-ASERCA, 2011), F4 and F5 treatments correspond to large fruit size.

The difference in yield obtained in the F1 and F5 treatments was due to the number of fruits per plant, not to the weight of fruits. The overall average yield obtained in our experiment was 208.74% higher than the average yield (14.76 Mg•ha^{-1}) obtained by Mexican producers under irrigation and rainfed conditions (SIAP, 2011). The F4 and F5 treatments increased the yield until 70.7 and 45.42% respectively, in relation to the average obtained for the control group F1. While the F5 treatment increased the yield until 17.42% compared with the F4 treatment yield. The F2 and F3 treatments were not statistically different to the control group F1 (Table 4). The results contrast with those reported by Subler et al. (1998), who stated that the best crop development occurs when applying between 10 and 20% VC, although, Atiyeh et al. (2000a, 2000b) indicated that using more than 20% of VC in the growth substrate decreases the yield of the plant. In our experiment, the jalapeño plants in T3, T4 and T5 treatments consumed 92.73, 165.65 and 194.52 kg of N to obtain the respectively yield (Inzunza-Ibarra et al., 2010).

Table 4. Mean values and statistical difference of the variables evaluated in jalapeño pepper grown with organic and synthetic fertilizer under greenhouse conditions.

Treatment	WF (g)	FL (cm)	ED	PT	NF	NL	Yield (Mg•ha^{-1})
F1	38.4 a*	8.21 b	3.18 a	0.57 bc	31 bc	2 b	40.68 b
F2	29.9 b	8.09 b	3.22 a	0.57 bc	30 c	2 b	35.44 b
F3	35.9 a	8.26 b	3.16 a	0.56 c	28 c	3 a	33.12 b
F4	36.3 a	8.41 b	3.36 a	0.59 b	41 ab	3 a	59.16 a
F5	39.4 a	8.83 a	3.34 a	0.61 a	48 a	2 b	69.47 a
mean	36.01	8.36	3.25	0.58	36	2.4	45.57
CV (%)	11	4	7	4	30	9	31

*Values with the same letter within each column are statistically equal, LSD test $P \leq 0.05$. WF = weight of fruit; FL = fruit length; ED = equatorial diameter; PT = pericarp thickness; NF = number of fruits; NL = number of locules; CV = coefficient of variation.

Furthermore, when making the extrapolation of percentage to kilograms of N per hectare for each one of sources of organic fertilizers (Table 1), considering a mineralization of 11% (Eghball, 2000; Adegbidi y Briggs, 2003), the N available needed in the treatments F3, F4 and F5, was 607.3, 320.0 and 463.6 kg•ha^{-1}, respectively. Therefore, we may assume that these three treatments showed adequate levels of N that favored the development of the jalapeño pepper. However, factors as leaching, a lower rate of mineralization, the volatilization and the adsorption process (Sikora and Szmidt, 2001), solely allowed reaching the yields already indicated. Regardless, it is important to note that the obtained yields reflects the high amounts of nutrients contained in C, VC and VCT, as mentioned by Edwards et al. (2010).

Conclusions

The results obtained showed that VCT, prepared from the VC (made with horse and goat manure with alfalfa straw, mixed in a 1:1 ratio by volume), caused positive effects on development indicators of jalapeño pepper. During this study, the organic production of jalapeño pepper under protected conditions and using organic fertilizers resulted in a high yield. The use of VCT, C and VC can be considered as an alternative fertilization method for organic production in greenhouses, since they contain soluble nutrients that can satisfy the nutrient demand of this plant.

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PLANT SCIENCE

Using of Inter Microsatellite Polymorphism to evaluate gamma-irradiated *Amaranth* mutants

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Abstract

Molecular markers ISSR were used to analyse γ-irradiated mutants of amaranth Ficha cultivar and K-433 hybrid; tested six microsatellite - primed PCR markers. First analysis of gamma-irradiated amaranth mutant lines was done using the ISSR. In the study, primers that distinguish the mutant lines from control plant were reported as well as primer with the differentiation ability for all analysed mutant lines. The very specific changes in the mutant lines´ non-coding regions based on inter microsatellite length polymorphism were analysed. Mutant lines of the Ficha cultivar (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0.16 and their ISSR profiles were more similar to the Ficha than those of K-433 hybrid mutant lines. The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0.22 but are more distinct to their control plant as a whole, as those of the Ficha mutant lines. Different ISSR fingerprints patters of the mutant lines when compared to the Ficha cultivar and K-433 hybrid ISSR specific profiles may be part of a consequence of the complex response of the intergenic space of mutant lines to the gamma-radiance.

Key words: Amaranth, Ficha, ISSR, K-433, Mutant lines

Introduction

The genus *Amaranthus* is originated in the Americas, consists of 60–70 species and including 3 cultivated grain species (*A. caudatus*, *A. cruentus*, and *A. hypochondriacus*). Three ancient cereal *Amaranthus* species, *A. caudatus*, *A. cruentus* and *A. hypochondriacus* are nowadays widely studied and cultivated worldwide because of their exceptional nutritional value of both seeds and leaves (Brenner et al., 2000). Molecular genetic cluster analysis could help in defining the genetic similarities among *Amaranthus* species as well as among the lines. Previous molecular genetic analyses of *Amaranthus* species have been designed to determine ancestors of domestic species by using single marker system (Sun et al., 1999; Wetzel et al., 1999a). Using amplified fragment length polymorphism (AFLP) markers, Vos et al. (1995) reported an unweighted pair group method with arithmetic mean analysis (UPGMA) of *Amaranthus* accessions representing agronomically important weedy *Amaranthus* species from a wide geographical area to determine genetic similarity and potential for interspecies hybridization.

Different molecular markers including random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and amplified fragment length polymorphism (ALFP) have been used for more accurate study of genetic diversity and phylogenetic relationships between *Amaranthus* species (Xu and Sun 2001; Štefúnová and Bežo, 2002, 2003; Wassom and Tranel 2005; Lee et al., 2008; Ray et al., 2008; Popa et al., 2010).

ISSR is a source of genetic markers that was previously used for amaranth germplasm assessment (Xu and Sun 2001; Ray and Roy, 2007) and have higher reproducibility than RAPDs (Meyer et al., 1993; Fang and Roose, 1997). The conventional ISSR involves PCR amplification of DNA with a single primer composed of simple sequence repeats (SSRs; or microsatellites). Microsatellites are abundant throughout the eukaryotic genome, evolve rapidly and are widely used in plant germplasm evaluation for well characterized species as well as for underutilized ones (Balážová et al., 2007; Nováková et al., 2010;

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Svobodová et al., 2011). Microsatellite-anchored primer amplifies the region between two closely spaced, oppositely oriented SSRs, and the amplified products are separated by agarose gel electrophoresis. The resultant PCR fragments usually range from 0.3 to 2 kb in size and are often limited in number. Further improvement of the efficiency of this marker system can be achieved by double-primer fluorescent ISSR (Huang and Sun, 2000). Two SSR primers labeled with fluorescein Cy5 are added in each PCR amplification in this approach. Such modified ISSR generates a large number of DNA fragments with only a few pairs of primers and the technique became comparable to the efficiency of AFLP (Huang and Sun, 2000).

In the present study, ISSR technique was used for fingerprints characterization of the amaranth mutant lines. Mutation breeding is still one of the conventional breeding methods for plants. It is relevant with various fields like, morphology, cytogenetics, biotechnology, and molecular biology. Mutation breeding has become increasingly popular in recent times as an effective tool for crop improvement (Acharya et al., 2006) and an efficient means supplementing existing germplasm for cultivar improvement in breeding programs. FAO/IAEA database listed more than 2300 radiation-modified plants including amaranth. Mutation technology was employed as a tool to create genetic variation in *Amaranhtus tricolor* in order to select lines with improved drought tolerance. The results of γ-radiance treatment were manifested in 1 mutant genotype with better growth vigor under the stress conditions and 2 mutant lines which retained more water in leaves under drought conditions compared to the wild type. Another research of mutant lines reported by Kgang (2008) showed increased content of protein concentration. Mutant lines were compared with wild type based on RAPD markers. From 19 arbitrary primers used, only two primer sets showed polymorphisms. The differences observed during the RAPD analyses of the two mutants as compared to the wild type, could be indicative of specific genomic areas possibly involved in drought tolerance (Kgang, 2008). For plant species, γ-radiation is mainly used for breeding or research purposes (Majer and Hidek, 2012; Rebodero and Lidon, 2012). Treatment by γ-radiation was used also for enhancing quality and quantity of amaranth grain of two selected genotypes: *Amaranthus cruentus* genotype 'Ficha' and hybrid K-433. Positive selection was performed from 2nd to 8th mutant generation of several putative mutant lines of *A. cruentus* and hybrid K-433 and characterized which significantly increased weight of thousand seeds (Gajdošová et al., 2008).

Mutant lines and molecular approach selected by Gajdošová et al. (2008) were used in this study. The aim of the study was to perform the ISSR for amaranth mutant lines and their control genotypes. ISSR profiles were compared with the aim to investigate the consistency of the selected markers across individual mutant lines and to analyse changes in the ISSR polymorphism of mutant lines.

Materials and Methods

Selected seeds of *Amaranthus* mutant plants positively selected for the weight of the thousands seeds were used for analyses. Mutant lines together with their control genotypes were obtained from Institute of Plant Genetics and Biotechnology - Slovak Academy of Sciences in Nitra, Slovakia (Table 1, Figure 1). Mutant lines with the statistically confirmed increase of weight of thousand seeds were taken as accessions for ISSR analyses only. The same set of the mutant lines was used in wide biochemical analyses performed by Hricová et al. (2011) and Múdry et al. (2011).

DNA was extracted from leaf tissue of randomly selected six individual plants of each mutant line or control genotype based on the method of Rogers and Bendich (1994). The purity of DNA is one of the major factors affecting the success of genomic or genetic technologies studies. Nucleic acid isolation from polyphenol rich plants fail to produce good quality DNA or RNA as polyphenols adhere and interfere with DNA during isolation (Anuradha et al., 2013). For quantity setting of the extracted DNA, Nanodrop Nanophotometer™ was used. Determination of DNA quality was done by agarose gel electrophoresis on 1.5% agarose gel in 1xTBE buffer coloured by GelRed™.

Table 1. Species and accessions of *Amaranthus* studied.

Species	Accession	Type
Amaranthus cruentus	Ficha cultivar	Control A
	C 15	Mutant lines
	C 26	
	C 27	
	C 82	
	C 236	
A. hypochondriacus x *A. hybridus*	K-433 hybrid	Control B
	D 54	Mutant lines
	D 279	
	D 282	

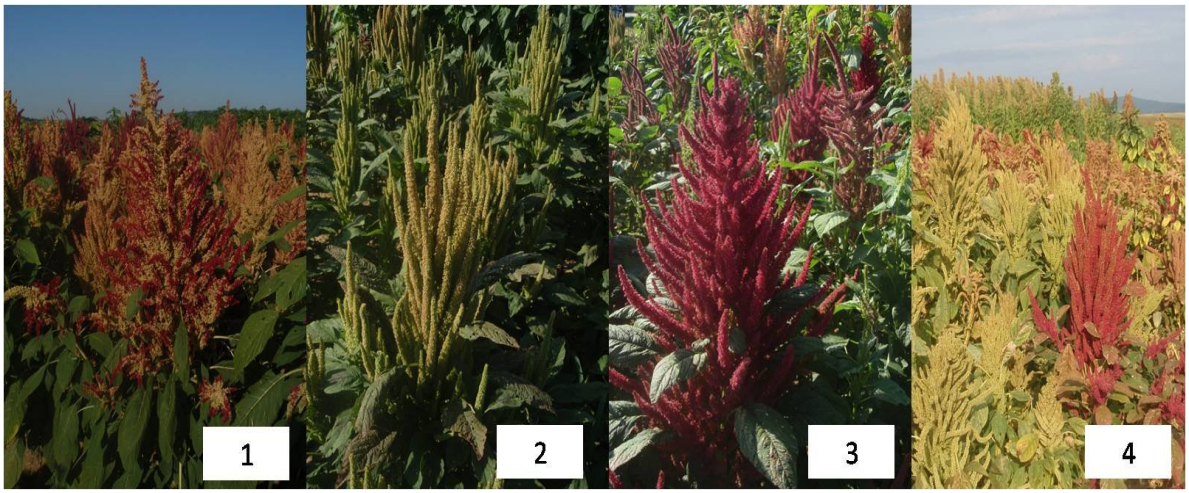

Figure 1. Analyzed specie of amaranth as in field conditions.
1 - Ficha cultivar - ControlA; 2 - C26 mutant line; 3 - K-433 hybrid - Control B; 4 - D282 mutant line.

An analysis of six randomly selected individual plants of each population was conducted with the ten selected ISSR primers. The used ISSR primers were as follows: (GTG)3GC, (CT)10T, (AG)10G, (GATA)2(GACA)2, (CTG)3GC, (GACA)4.

ISSR reactions were done in a volume of 15 µl containing 1.5 mmol × dm^{-3} $MgCl_2$ (Applichem), together with 0.08 mmol × dm^{-3} d NTP (InvitrogenTM) 600-800 nmol × dm^{-3} primer, 1 U Taq polymerase (Applichem) and 50ηg of template DNA.

The repeatability of ISSR banding patterns were examined, and factors affecting the number of band generated per primer were tested, such as DNA concentrations, $MgCl_2$ and primer concentrations.

Amplifications were carried out in a Bio-Rad C1000™ Thermocycler with the following program: an initial denaturation step at 95°C for 4 min followed by 45 cycles at 95°C for 60 s, annealing at 52°C for 60 s, and extension at 72°C for 2 min and a final cycle at 72°C for 10 min. The amplified products were separated using the Experion™ Automated Electrophoresis Station following the manufacturer´s instructions. The band of equal molecular weight and mobility generated by the same ISSR primer were considered to be individual loci. Only consistently reproducible, well resolved fragments were scored.

Amplified products were surveyed for polymorphism and scored in a binary mode (1 as presence of fragment, 0 as absence), assuming that each band represents a single locus. Each experiment was repeated three times. Non-reproducible bands were very rare and were excluded from the analyses along with weak bands.

Jaccard´s (1908) genetic dissimilarity index was used to calculate genetic similarity, employing the software SYN-TAX 2000 HIERCLUS (Podani, 2001). The same software was used for calculating of the cophenetic correlation coefficients for every used primer. Cluster analysis was conducted with UPGMA method in classifying the binary data derived to generate dendrogram to assess the relationships among the mutant lines and their control genotypes.

Results and Discussion

In this study, ISSR markers were applied to distinguish mutant lines of two species of the amarath - *Amaranthus cruentus* and *A. hypochondriacus* x *A. hybridus*. Molecular markers approach was successfully used to understand intra- and inter- specific genetic diversity and/or evolutionary relationships in *Amaranths* (Lanoue et al., 1996; Chan and Sun, 1997; Sun et al., 1999; Xu et al., 2001; Lee et al., 2008, Tony-Odigie et al., 2012; Mladenovic et al., 2012). Lee et al. (2008) reported the potential of microsatellite based markers for the *A. hypochondriacus* assessment; when they successfully amplified in all the tested species 12 loci and demonstrated the applicability of these markers for the study not only of intra-, but for inter-specific genetic diversity of amaranth, too. The same authors have summarized 12 repeat motifs in the amaranth genome with the number of alleles they provided as in the range from 5 to 12 alleles. The CA rich regions were reported, as well as GTG repeats.

Table 2. Individual ISSR primers polymorphism and their average Jaccard similarity indices.

Primer	No. of ISSR markers		Percentage polymorphism of ISSR markers	Cophenetic correlation coefficient	Average Jaccard similarity for Ficha mutant lines	Average Jaccard similarity for K-433 mutant lines
	total	polymorphic				
(GTG)3GC	18	10	56	0.91	0.86	0.74
(CT)10T	18	4	22	1	0.88	0.88
(AG)10G	16	10	63	0.93	0.81	0.91
(GATA)2 (GACA)2	14	3	21	0.83	0.92	0.72
(CTG)3GC	12	12	100	0.96	0.53	0.75
(GACA)4	18	18	100	0.99	0.75	0.75

Both of the above mentioned repeat motifs were used in the primers that were applied in this study. At the individual used primers level, ISSR polymorphism was measured as the proportion of polymorphic loci to the total number of loci scored in all accessions (Table 2). Primers with low percentage of polymorphism distinguish the mutant lines of both, Ficha and K-433 from control genotypes. Primers with higher percentage of polymorphism gave no clear and different ISSR fingerprints pattern for mutant and for control lines. The differentiation ability of the primers for distinguishing Ficha and K-433 based mutant lines were reported previously (Labajová et al., 2011).

In the study of the 20 primers tested, an initial screening resulted in selection of 10 microsatellite primed PCR primers that produced clear and reproducible ISSR profiles in Ficha and K-433. ISSR assays of each primer were performed three times, with only reproducible, amplified fragments being scored. Initially screening of all the used primers showed an identical profile of amplification products in the case of 14 primers.

To yield more accurate and reliable results, profiles for all 6 polymorphic primers were used to generate a UPGMA dendrogram shown in Figure 1. The UPGMA analysis separated mutant lines and their control genotypes into two main clusters.

ISSR is another source of genetic markers that have higher reproducibility than RAPDs (Meyer et al., 1993; Fang and Roose, 1997). Along with the RAPD technique, both of the still preferred method for fast initiating identification of genotypes as they are relatively inexpensive, utilizes arbitrary primers, and randomly samples a potentially large number of loci in a less complex pattern than other PCR based markers (Milella et al., 2005, 2011). Transue et al. (1994) and Chan and Sun (1997) used RAPD markers for the study of evolutionary relationships among grain amaranths and their wild relatives. Popa et al. (2010) studied genetic diversity and phylogenetic relationship among six species of *Amaranthus* by RAPD markers and showed that there is slightly intra and inter species polymorphism. Comparisons between RAPD and ISSR techniques and their effectiveness can be made, first because only a few studies about ISSR as a type of random markers in amaranth exist and second, RAPD products often contain repetitive DNA sequences (Paran and Michelmore, 1993). Both of the techniques are used for minor and underutilized species equally (Milella et al., 2005, 2011; Labajová et al., 2011; Svobodová et al., 2011; Al-Khalifah, 2012).

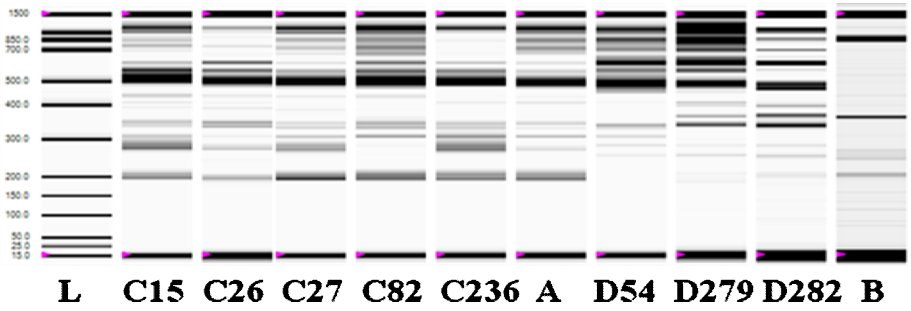

Figure 2. Electrophoreogram of tested Amaranth accessions using the primer $(GATA)_2(GACA)_2$. L - Marker; C -Ficha accession A - Control for Ficha; D - K-433 hybrid accessions: B - Control for K-433.

Table 3. Jaccard dissimilarity indices of analysed mutant lines and their control genotypes.

	C15	C26	C27	C82	C236	Ficha	D54	D279	D282	K-4333
C15	0.000									
C26	0.140	0.000								
C27	0.140	0.073	0.000							
C82	0.141	0.165	0.165	0.000						
C236	0.145	0.169	0.146	0.193	0.000					
Ficha	0.134	0.136	0.136	0.138	0.117	0.000				
D54	0.356	0.378	0.396	0.364	0.391	0.384	0.000			
D279	0.322	0.344	0.363	0.310	0.375	0.349	0.097	0.000		
D282	0.356	0.378	0.396	0.345	0.409	0.384	0.086	0.043	0.000	
K-433	0.422	0.427	0.462	0.414	0.424	0.435	0.222	0.205	0.222	0.000

Ray and Roy (2007) have used both of the techniques - RAPD and ISSR - for the phylogenetic relationships analysis between Amaranthaceae, where RAPD analysis have higher level of polymorphism among leafy amaranths and ISSR shows higher level of polymorphism in grain amaranths. Authors confirmed an effective discriminating power for both of the random primer systems. For the purposes of present study, ISSR markers were chosen rather than RAPD ones, because of the mutation potential of microsatellites in plants (Gao and Xu, 2008).

As ISSR fingerprints results show, all the mutant lines of the Ficha genotype (C15, C26, C27, C82, C236) shared a genetic dissimilarity of 0,16 and their ISSR profiles were more similar to the Ficha than those of K-433 mutant lines. The K-433 mutant lines (D54, D279, D282) shared genetic dissimilarity of 0.22 but as the dendrogram shows, were more distinct to their control genotype as a whole, as those of the Ficha mutant lines. The two major clusters were observed at a dissimilarity coefficient of a 0.38, which means that the used primers had generated two relatively different sets of fingerprints, one for the Ficha and its mutant lines and one for the K-433 and its mutant lines.

Amaranthus cruentus mutant lines were grouped in the first major cluster and the average Jaccard dissimilarity coefficient for all of the Ficha mutant lines was 0.11 (Table 3). Similarly, the second cluster is compounded only from K-433 mutant lines with the average dissimilarity coefficient of 0.07.

As the Table 3 shows, no genetic uniformity was observed among the Ficha mutant lines, nor among the K-433 mutant lines, but low average Jaccard dissimilarity coefficient indicate that their ISSR profiles were similar, but not the same completely.

As the dendrogram (Figure 3), mutant line C82 shared the specific positions to the Ficha control genotype - was the most distinct to the Ficha. The result corresponds to the results of the biochemical analyses of the same mutant lines as in this study, where C82 line is statistically different as the rest of the analysed Ficha lines (Hricová et al., 2011, personal communication; Múdry et al., 2011).

Our data indicates the use of ISSR molecular marker systems in amaranth gamma-rays mutant lines as an advanced technique, because of the possible linkage of changes of the non-coding regions to the specific changes of the genome after the gamma-rays treatment. And, it is significant as already positive selection for the thousand seeds weight was reported from the same by Gajdošová et al. (2005).

Molecular marker analyses have contributed to the elucidation of origin and evolution of cultivated amaranths, and allied wild species. Hauptli and Jain (1984) were among the first to use molecular markers to address evolutionary relationships among the grain amaranths. They observed that with the exception of the *A. caudatus – A. quitensis* pair, grain amaranths are more closely related to each other than either is to their putative wild progenitor. This work was based on isozyme polymorphisms and several authors have since expanded molecular diversity studies in the genus.

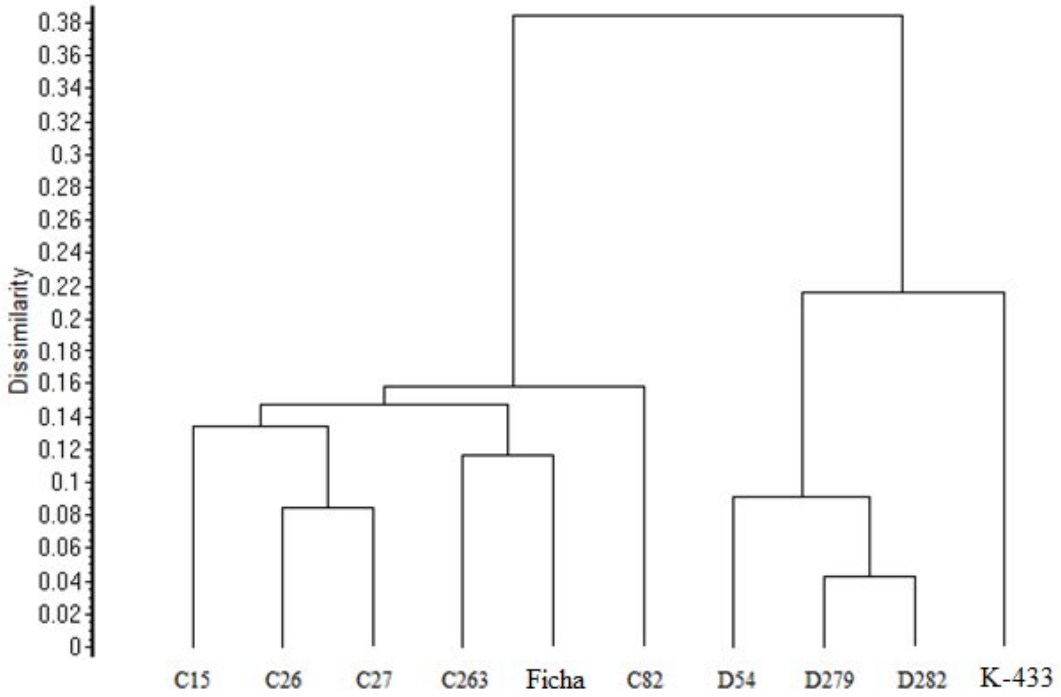

Figure 3. Dendrogram of Ficha and K-433 mutant lines as analysed by ISSR.

In a study including both isozyme and random amplified polymorphic DNA (RAPD) markers, Chan and Sun (1997) reported molecular phylogenies of cultivated and wild amaranths. Authors evaluated 23 different species, including the three cultivated for grain as well as accessions of all species in the *A. hybridus* aggregate. Studies based on restriction site variations in nuclear and cytoplasmic DNA reported that *A. caudatus* and *A. cruentus* are more closely related to each other and to their supposed progenitors than to *A. hypochondriacus* (Lanoue et al., 1996). Isozyme and RAPD markers were used by other authors and findings tends to agree with the different evolutionary hypothesis presented herein (Ranade et al., 1997; Zheleznov et al., 1997).

Xu and Sun (2001) reported that both, AFLP and double-primer ISSR detected high levels of polymorphism among species, averaging 96.7 and 99.5%, respectively. Within species, however, AFLP polymorphism was much lower than double-primer ISSR polymorphism. All these results confirms the reliability of the molecular marker based methods for distinguishing closely related material, such as lines.

Conclusions

Gamma-irradiated amaranth mutants were analysed with ISSR markers. The results showed specific changes in non-coding regions of mutants, based on inter microsatellite length polymorphism. Different ISSR fingerprints patterns of the mutant lines when compared to the Ficha and K-433 ISSR specific profiles may be part of a consequence of the complex response of the intergenic space of mutant lines to the gamma-radiance.

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PLANT SCIENCE

Low cost medium formulation using cow dung ash for the cultivation of Cyanobacterium: *Spirulina* (*Arthrospira*) *platensis*

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Abstract

Spirulina (*Arthroapira*) characterized by helical/spiral shape in open left hand helix is a multicellular, free floating cyanobacterium or blue green alga. Its trichomes are cylindrical arranged in unbranched form and the width ranges from 6 to 12 µm. The basic helical feature of its filaments is unique and can be found only in liquid culture medium. *Spirulina* has been so popular in the world due to its high nutritional contents. It has been used as protein and vitamin supplement to the health food industry, in aquaculture diets and fisheries. Cyanobacterium *Spirulina* is capable to grow in various kinds of culture media. Furthermore, decomposed organic and inorganic nutrients media have been proven to be good source of culture medium for *Spirulina* cultivation by many researchers. Among many strains, *Spirulina platensis* has been cultured in various waste organic matters to evaluate its growth and biochemical components under similar controlled culture conditions. The present investigation was carried out to formulate a low cost medium using cow dung ash for the biomass production of *S. platensis*. Different concentration gradients of cow dung ash medium i.e. 10-60% with standard medium were analyzed for *Spirulina* growth at pH 9.5 ± 2, temperature 28°C ± 2 and light intensity 3 Klux. Success of the experiment was found in 20% concentration level (1.212 g/l dry biomass) having 20% cow dung ash supplemented with 80% prescribed medium i.e. CFTRI medium. The maximum removal efficiency of 20% CDAM at the end of experiment was measured at 98.06%, 51%, 83.64% and 51.01% for TSS, TDS, COD and conductivity respectively. These results indicate the potentiality of cow dung ash to provide the nutrients to the culture medium that reduces its valuable cost and make it a cheaper and economic culture for *S. platensis*. The nutritional status of *S. platensis* cultured in 20% CDAM suggested that the biomass of *Spirulina* is nutritionally rich as per standard.

Key words: Cow dung ash, Cyanobacterium, *Spirulina*, Health food industry

Introduction

Spirulina is most commonly found in natural lakes having high pH value i.e. 8 to 10 all over the earth. *Spirulina* has been consumed from a very long past time in many parts of the world as a food supplement for human as well as animals in various forms like health drink, tablets and powder etc. because of its alimentary value (Ruiz et al., 2003). Now a days, *Spirulina platensis* is gaining great interest for its cellular contents such as vitamins, minerals, polyunsaturated fatty acids, carotenoids and other pigments that have antioxidant activity (Cohen and Vonshak, 1991; Mahajan and Kamat, 1995; Bhat and Madyastha, 2000; Madhava et al., 2000; Lin et al., 2007).

Many factors are important for the production of *Spirulina* at large scale, of which most important factors are nutrient availability, temperature and light. The filamentous cyanobacteria such as *Spirulina* are found to be most compatible microorganisms for the utilization of waste and wastewaters as they are able to produce large quantity of biomass and their harvesting is also relatively easy because of their bigger size and structure. Also these wastes reduce the cost of nutrient medium and act as a source of cheap nutrient medium for cultivation of *Spirulina*. The commercial production of *Spirulina* can be made cost effective by reducing the input cost with cheap and readily available materials without sacrificing the production efficiency. Cow dung is a very common waste material that has been used by many researchers. Cow dung is commonly used as manure or agricultural fertilizer after combining with soiled bedding and urine.

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The aim of the present investigation was to formulate a low cost medium using cow dung ash for *Spirulina* production and to evaluate the nutritional status of *Spirulina* biomass.

Materials and Methods

Procurement of *Spirulina*

S. platensis strain used in the present study was procured from the Centre for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute (IARI), New Delhi, India. The culture was maintained in CFTRI medium by repeated transfer to fresh medium.

Sterilization of culture vessels

The glass wares used in the experiments were first rinsed with water properly and finally rinsed with distilled water. After draining out the water, the glass wares were sterilized in a hot air oven at 160^{o}C for 2-3 hours.

Preparation of culture medium

Cow dung ash was prepared from cow dung cakes. Cow dung cakes were collected from villagers and were dried properly in sunlight for a day or two then burned completely to form ash. After cooling, the ash was collected and filtered 4-5 times with fine mesh to remove the unburned solids and other impurities. Physico-chemical properties of cow dung ash is given in Table 1.

Table 1. Physico-chemical properties of cow dung ash.

Properties	Value
pH	9.21
EC (dS/m) (1:2.5)	0.22
Organic Carbon %	0.17
Available P_2O_5 "Kg/Ha"	60.00
Available K_2O "Kg/Ha"	1613
Zn (ppm)	3.76
Cu (ppm)	1.02
Fe (ppm)	4.88
Mn (ppm)	3.10
Total N%	0.08

Cow Dung Ash Medium (CDAM) was used as growth medium to grow *Spirulina platensis* in pure form (100% CDAM) without any supplementation and in formulated form supplemented with CFTRI medium (prescribed medium) in different concentrations of cow dung ash medium. To prepare cow dung ash medium, 80 gm of cow dung ash was dissolved in 1000 ml of sterilized distilled water (8% concentrated solution; wt/v) and left for 2 hours. After 2 hours, the solution was filtered first with fine mesh to remove the undissolved solid particles and then with filter paper to get a clear solution. This form of solution was called as cow dung ash medium (CDAM). Different concentration levels of CDAM i.e. 10%-60% were prepared using standard CFTRI medium. CFTRI medium was used as control in the experiments.

Standardization of inoculum density and day of harvest

Exponentially growing 14 days old culture with OD 0.8-1.0 at 560 nm was used as inoculum and 10% were inoculated in each flask. The cultures were grown for a period of 24 days and after harvesting growth were measured in the form of dry biomass of all concentration levels of cow dung ash medium.

Experimentation

Experiment I. Cultivation of *S. platensis* in 100% cow dung ash medium (100% CDAM)

An experiment was conducted to evaluate the potentiality of cow dung ash medium in pure form without any supplementation for *Spirulina platensis* cultivation. The medium was taken in duplicate with a control in 500 ml conical glass flasks containing 250 ml medium. For cultivation of *S. platensis*, the pH of CDAM was adjusted to 9.5 ± 2 with the help of 1 N NaOH and temperature 28°C ± 2. The photoperiod was given 12/12 alternative light and dark period with light intensity 3 Klux by white fluorescent tubes. All three flasks were incubated with 10% (v/v) (OD at 0.8-1.0) of inoculum. Medium agitation was carried out by orbital shaker three times a day for two minutes. Experiment was run for 24 days. The results were compared with control.

Experiment II. Cultivation of *S. platensis* in Formulated Cow Dung Ash Medium (Formulated CDAM)

In second experiment, *S. platensis* was cultivated in cow dung ash medium formulated/supplemented with CFTRI medium. Constituents of the basal medium were eliminated in step wise manner and substituted with increasing percentage volume amount of cow dung ash medium (CDAM) in the decreasing percentages volume amount of CFTRI medium i.e. CFTRI%+CDAM% in 100%+0%, 90%+10%, 80%+20%, 70%+30%, 60%+40%, 50%+50%, 40%+60% and named as control, 10%, 20%, 30%, 40%, 50% and 60% respectively. Their effects on growth were studied in order to find out the optimum concentration of CDAM with CFTRI medium. All the culture conditions were kept same as in experiment I. The experiment was conducted in duplicate in a completely randomized design. The results were compared with control.

Estimation of growth performance

Spirulina platensis as an experimental organism was grown in CFTRI medium, 100% CDAM and CFTRI+CDAM in different combinations. Initial growth performance was observed in terms of dry biomass of *S. platensis* and compared with control. After finding the best medium combination of CFTRI+CDAM for *S. platensis* cultivation on the basis of highest amount of dry biomass, the physico-chemical analysis of medium was carried out before inoculation and after harvesting before mass cultivation of *S. platensis.* The nutritional analysis of *S. platensis* was also done.

Physico-chemical analysis

Before the mass culture of *S. platensis* in best medium combination of CFTRI+CDAM; medium was first analyzed before inoculation for its physico-chemical properties viz. pH, Chemical Oxygen Demand (COD), Total Suspended Solids (TSS), Total Dissolved Solids (TDS) and Conductivity following standard methods (Clesceri et al., 1989). Same physico-chemical properties were analyzed again in the filtrate after harvesting the biomass of *S. platensis* at the end of the experiment to assess the efficiency of formulated cow dung ash medium. The results of physico-chemical analysis were statistically analyzed through analysis of variance (ANOVA) at 95% confidence level.

Nutritional analysis

For the study of cellular contents of *S. platensis* in best formulated cow dung ash medium (on the basis of highest amount of dry biomass of *Spirulina*), *Spirulina* was mass cultured. The biochemical contents like protein, carbohydrate, pigments (chlorophyll a, carotenoids, phycocyanin, phycoerythrin and allophycocyanin), minerals and free amino acids of *Spirulina* were estimated by following standard methods. Among pigments, chlorophyll a and carotenoids content of the cells were estimated by the method of Mackinney (1941) using absolute methanol while the phycobiliproteins (phycocyanin, phycoerythrin and allophycocyanin) content was estimated by Benette and Bogourd (1973) method. Total cellular protein content was estimated by the method of Lowry et al. (1951). The method of Duboi's et al. (1956) was employed for determination of total carbohydrate of the cell. This is also known as phenol-sulphuric acid method. Free and bound amino acids were separated by standard methods given by Awapara (1969) and Krishnamurthy and Swaminathan (1955) respectively. Then quantitative estimation of free amino acids was carried out by standard methods of Lee and Takahashi (1966). Among minerals, DDPA extractable exchangeable cations (Zn, Cu, Mn and Fe) were estimated by Atomic absorption spectrophotometer following standard method (Lindsey and Norvell, 1978) while Na% was calculated by standard flame photometer following standard method (Lindsey and Norvell, 1978).

Statistical analysis

Results of the growth evaluation were subjected to analysis of variance (ANOVA) and Tukey's Honest Significance Difference (HSD) test with confidence level of 95 % ($p<0.05$) in order to verify significant difference in different concentration levels and biomass. Also the results of physico–chemical analysis and nutritional analysis were analyzed statistically.

Results

Growth performance of *Spirulina platensis* in cow dung ash medium (CDAM)

When *Spirulina platensis* was grown in 100% CDAM without any supplementation of CFTRI medium, the growth was better, although some small clumps were formed in this medium. The amount of dry biomass of *S. platensis* was found 0.524 g/l. The growth of *S. platensis* was nearly half of the control (CFTRI medium) i.e. 1.112 g/l (Table 2). In order to improve the growth performance of *S. platensis*, CDAM was formulated by supplementation of CFTRI medium at concentration levels from 10% to 60%.

When *S. platensis* was grown in formulated CDAM from concentration level 10% to 60%, growth could found in every medium. Some amount of small clumps also formed in every formulated medium, their quantity was more in higher concentrations. The maximum dry biomass of *S. platensis* found in 20% formulated CDAM was 1.212 g/l that was higher than dry biomass obtained in CFTRI medium (1.112 g/l). It was followed by 10% and 30% formulated CDAM (dry biomass 0.812 g/l and 0.620 g/l respectively). While the minimum amount of dry biomass obtained in 60% formulated CDAM (0.240 g/l) followed by 50% and 40% formulated CDAM (dry biomass 0.300 g/l and 0.380 g/l) (Table 2; Figure 1).

Table 2. Growth Performance of *Spirulina platensis* in 100% Cow Dung Ash Medium (100% CDAM) and in different concentrations of formulated CDAM compared with control (CFTRI Medium).

Medium	Dry Biomass (g/l)
Control	1.112±0.14
100% CDAM	0.524±0.21
10% Formulated CDAM	0.812±0.01
20% Formulated CDAM	1.212±0.40
30% Formulated CDAM	0.620±0.47
40% Formulated CDAM	0.380±1.02
50% Formulated CDAM	0.300±1.31
60% Formulated CDAM	0.240±0.52

*Mean ± Standard Deviation

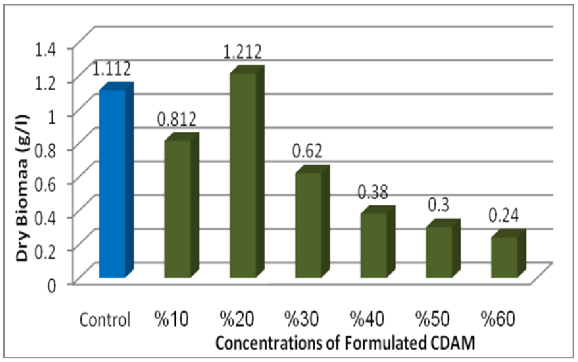

Figure 1. Comparative growth performance of *S. platensis* in formulated Cow Dung Ash Medium (CDAM) at different concentration levels compared with control (CFTRI medium).

Removal efficiency of *Spirulina platensis* in 20% formulated cow dung ash medium (20% formulated CDAM)

20% formulated CDAM was found to be best when *S. platensis* cultivated in different concentrations of formulated CDAM. The results of physico-chemical analysis of 20% formulated CDAM and CFTRI medium are summarized in Table 4.

Before inoculation, the amount of TSS, TDS, COD and conductivity obtained for the 20% formulated CDAM were 13937 mg/l, 8940 mg/l, 1760 mg/l and 14900 mS/m respectively. After the cultivation of microalga *S. platensis*, the solids like TSS, TDS, COD and conductivity were found significantly ($p<0.05$) lower. The TSS, TDS, COD and conductivity values were 270 mg/l, 4380 mg/l, 288 mg/l and 7300 mS/m respectively. In CFTRI medium, before inoculation of *Spirulina* culture; the amount of TSS, TDS, COD and conductivity were 193 mg/l, 4200 mg/l, 592 mg/l and 7000 mS/m respectively. It can be clearly observed from the Table 4, that the amount of TSS was nearly 72 folds higher and COD was 3 folds higher in 20% formulated CDAM as compared to CFTRI medium, while TDS and conductivity values were two times higher in 20% CDAM than control before *Spirulina* cultivation.

The final COD concentration was smaller than before culturing of *S. platensis* evidencing the COD removal during the time of this research study indicating good biomass production of *Spirulina* in 20% formulated CDAM. The removal efficiency of COD in 20% formulated CDAM was very high i.e. 83.64%. The % removal of TSS and TDS was 98.06% and 51% respectively.

After cultivation of *Spirulina*, the decrease in TSS, TDS, COD and conductivity values was found 150 mg/l, 1740 mg/l, 240 mg/l and 2900 mS/m in CFTRI medium. It can be seen that the utilization of these solids was high in 20% formulated cow dung ash medium than CFTRI medium. The % removal of TSS, TDS and COD in CFTRI medium was 22.3%, 58.6% and 59.5% as compared to 98.06%, 51% and 83.64% respectively in 20% formulated CDAM. The pH value increased from 9.5 to 9.78 in control while increment in pH value was high in 20% formulated CDAM i.e. 9.5 to 9.99 (Table 4; Figure 2).

Nutritional Status of Spirulina platensis in 20% formulated cow dung ash medium (20% formulated CDAM)

At constant culture conditions; temperature 28°C-30°C, pH 9.5 and light intensity 3 Klux, the highest mean dry biomass i.e. 1.212 g/l of *Spirulina* was observed in 20% formulated CDAM. In 20% formulated CDAM, the amount of protein, carbohydrate and free amino acids (FAN) was observed 509 µg/ml, 0.196 mg/ml and 0.598 ppm respectively. Minerals such as Zn, Cu, Fe, Mn and Na were present 0.12 ppm, 0.18 ppm, 0.31 ppm, 0.11 ppm and 0.039% respectively in 20% formulated CDAM (Table 3).

The protein content of *S. platensis* in control was found 31% higher as compared to 20% formulated CDAM. The amount of protein in control was 611.6 µg/ml while in 20% formulated CDAM was 509 µg/ml. The carbohydrate content of *S. platensis* was only 16.9% higher while FAN (free amino acids) content was 34.6% higher in control than in 20% formulated CDAM. The amount of carbohydrate and FAN content of *S. platensis* in 20% formulated CDAM was found 0.196 mg/ml and 0.598 ppm respectively while in control was found 0.21 mg/ml and 0.74 ppm respectively.

Table 3. Comparative study of nutritional status of *Spirulina platensis* in 20% formulated cow dung ash medium (20% formulated CDAM) and in CFTRI Medium.

Item	Unit	CFTRI Medium	20% Formulated CDAM
Protein	μgml^{-1}	611.6±0.35	509.0±0.12
Carbohydrate	$mgml^{-1}$	0.21±0.21	0.196±0.01
Free Amino Acids	ppm	0.74±1.02	0.598±0.03
Pigments			
Chlorophyll a	$mgml^{-1}$	0.019±1.03	0.023±0.01
Carotenoids	$mgml^{-1}$	0.0037±0.23	0.0033±0.21
Phycocyanin	$mgml^{-1}$	0.074±0.12	0.052±0.40
Phycoerythrin	$mgml^{-1}$	0.023±1.025	0.022±0.60
Allophycocyanin	$mgml^{-1}$	0.028±0.25	0.034±0.20
Minerals			
Zn	ppm	0.07±0.56	0.12±0.12
Cu	ppm	0.16±0.48	0.18±0.15
Fe	ppm	0.28±1.36	0.31±0.12
Mn	ppm	0.16±1.24	0.11±0.10
Na	%	0.03±1.02	0.039±0.10
Cost of medium	` hl^{-1}	432.0	345.6

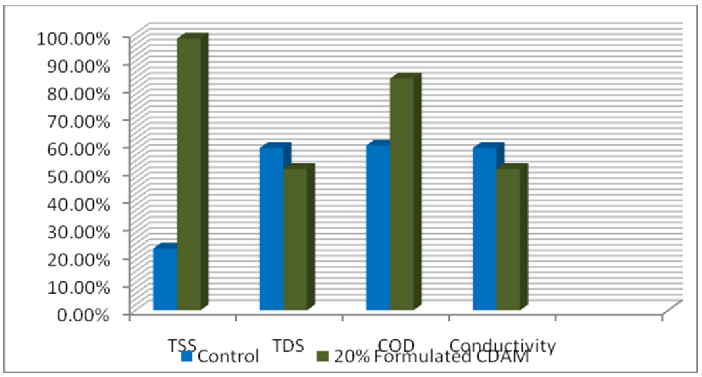

Figure 2. Comparative removal efficiency of *S. platensis* (in terms of % removal) in CFTRI medium and in 20% Formulated Cow Dung Ash Medium (CDAM).

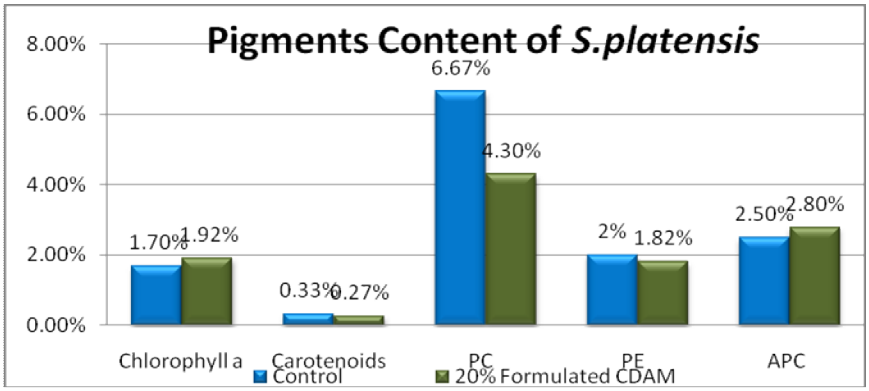

Figure 3. Comparative study of pigments content of *S. platensis* in control and 20% formulated cow dung ash medium (CDAM).

Table 4. Results of physico- chemical analysis and percentage removal in 20% formulated cow dung ash medium compared with control (CFTRI medium).

Physico-chemical Parameter	Unit	20% Formulated CDA Medium			CFTRI Medium		
		Before Culture	After Culture	%Removal	Before Culture	After Culture	%Removal
pH		9.5±2	9.99±2	-	9.5±2	9.78	-
TSS	mg/l	13937±0.01	270±1.04	98.06%±0.02	193±1.60	150±0.38	22.3%±0.05
TDS	mg/l	8940±1.02	4380±1.05	51.0%±0.01	4200±0.06	1740±0.31	58.6%±0.70
COD	mg/l	1760±1.05	288±0.12	83.64%±1.05	592±0.01	240±1.30	59.5%±0.10
Conductivity	mS/m	14900±1.00	7300±0.02	51.01%±1.04	7000±1.02	2900±0.08	58.6%±0.02
Hardness	mg/l	22	Nd	-	nd	nd	-

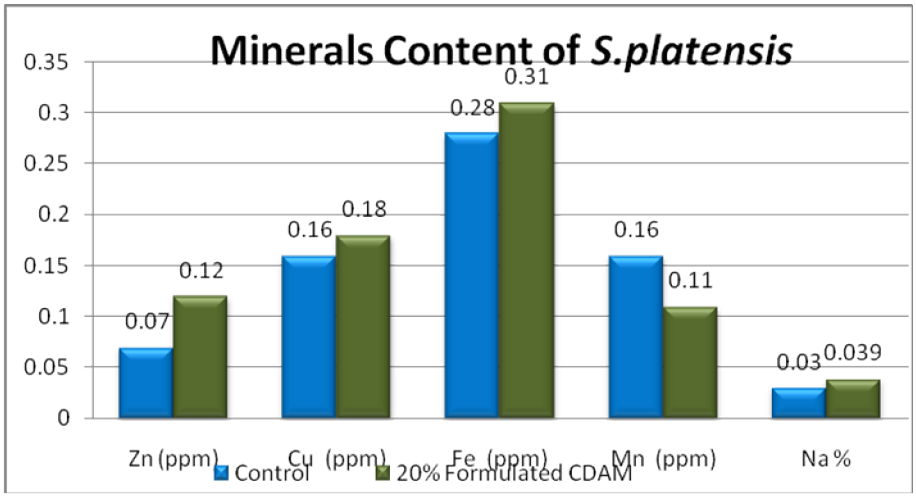

Figure 4. Comparative study of minerals content of *S. platensis* in control and 20% formulated cow dung ash medium (CDAM).

The amount of pigments like chlorophyll a, carotenoids, phycocyanin, phycoerythrin and allophycocyanin of *S. platensis* in 20% formulated CDAM was found 0.023 mg/ml, 0.0033 mg/ml, 0.052 mg/ml, 0.022 mg/ml and 0.034 mg/ml respectively while in control was found 0.019 mg/ml, 0.0037 mg/ml 0.074 mg/ml, 0.023 mg/ml and 0.028 mg/ml respectively. Although the amount of pigments of *Spirulina* like, carotenoids, phycoerythrin and phycocyanin was less in 20% formulated CDAM than the values of control; the pigments like, allophycocyanin and chlorophyll a were higher in *S. platensis* cultured in 20% formulated CDAM (Figure 3). It is clearly indicated from the Figure 4 that the amount of minerals of *S. platensis* grown in 20% formulated CDAM was almost similar to that of *S. platensis* grown in CFTRI medium, even Zn, Cu, Fe and Na content was higher in 20% formulated CDAM that in control.

Assessment of cost effect

The cost of preparation of CFTRI medium and 20% formulated CDAM was calculated. The 20% formulated CDAM, which possessed the minimum cost of Rs. 345.6 hl^{-1}, yielded considerable cost saving (20 %) as compared to the CFTRI medium (Rs. 432 hl^{-1}). 20% formulated CDAM was not only less expensive than CFTRI medium; even it was more effective than CFTRI medium in the biomass production too i.e. 1.212 g/l and 1.112 g/l respectively (Table 3).

Discussion

When cow dung ash medium used as a cheap nutrient growth medium for *S. platensis* biomass production in lab conditions, yield was found better in 100% CDAM (8% wt/v) (used without any supplementation of CFTRI medium). The biomass content of *S. platensis* observed 0.524 g/l (dry basis) in 100% CDAM as compared to 1.112 g/l dry biomass obtained in standard medium. After formulating CDAM with CFTRI medium at

concentration levels 10% to 60%, best biomass production of *S. platensis* obtained in 20% formulated CDAM. Although, blooming of *S. platensis* was successfully carried out in every concentration of formulated CDAM. Biomass in 20% formulated CDAM (1.212 g/l) was significantly higher than that obtained in standard medium (1.112 g/l) (Table 2; Figure 1).

The *S. platensis* biomass obtained in 20% formulated CDAM was nutritionally rich as contained 42% protein, 16.17% carbohydrate and 0.023 mg/ml chlorophyll a content (Table 3). Although, the protein and carbohydrate content of *S. platensis* in 20% formulated CDAM was significantly lower than that obtained in *S. platensis* grown in CFTRI medium (55% protein, 18.9% carbohydrate), was within the range of the value prescribed for *S. platensis* (Vonshak, 1997). Carbohydrate to protein ration (C/P) of *S. platensis* grown in 20% formulated CDAM was significantly higher (0.385) than the ratio observed in standard medium (0.344). The lower value of C/P ratio in standard medium is due to absence of organic source of C in it. Higher and lower C/P ratios are observed in nutrient limited and nutrient sufficient medium respectively (Kaushik et al., 2006).

Among pigments, chlorophyll a (0.023 mg/ml) and allophycocyanin (2.8 mg/ml) contents of *S. platensis* were high in 20% formulated CDAM than that in control (0.019 mg/ml chlorophyll a and 0.028 mg/ml allophycocyanin) (Figure 3). While among minerals content, all minerals (except Mg) were found high in *S. platensis* cultivated in 20% formulated CDAM. All the values were found within the range of the recommended range for *S. platensis* (Vonshak, 1997). However, there was no statistically significance difference among Cu and Fe content of *S. platensis.*

The removal efficiency of COD (83.64%) and TSS (98.06%) was found very high after cultivating *S. platensis* in 20% formulated CDAM as compared to found in CFTRI medium. The % removal of TDS and conductivity was comparable to the values of standard medium (Table 4; Figure 2).

Similar results have been demonstrated by various researchers worked on cattle wastes, swine wastes and poultry wastes (Ungsethaphand et al., 2009; Canizares and Dominguez, 1993; Canizares-Villanueva et al., 1995; Silva et al., 2003; Ayala and Vargas, 1987: Mezzomo et al., 2010; Mitchell and Richmond, 1988 etc.) Mitchell and Richmond (1988) used cattle waste leachate to enhance the power of Zarrouk's medium for the production of *Spirulina*. The results showed that the maximum cell density of *Spirulina* obtained in medium containing 16.3% waste leachate and 83.7% Zarrouk's medium. The effluent of anaerobically digested cow manure was used to supplement the medium by Ayala and Vargas (1987) in experiment for the cultivation of *S. maxima.* The results showed growth rates of 45.3 mg/L /d. These studies supported the present findings.

The present findings were also supported by Oron et al. (1979). They used raw cow manure for the cultivation of *S. maxima* under field conditions. The results showed 3158 mg/L biomass production of *Spirulina*. Lincoln et al. (1996) used anaerobically digested cattle manure in 1:1 ratio for the cultivation of *Spirulina.* The yield of *Spirulina* biomass was found 70 mg/L/ d for laboratory experiments and 24 mg/L/d for outdoor cultivations. $N\text{-}NH_3$ removal was recorded 24 mg/L/ d. At concentration level <75 mg $N\text{-}NH_3$/L, the *Spirulina* growth was smooth while at concentration levels >100 mg $N\text{-}NH_3$/L, *Spirulina* did not grow well.

Churnbarn and Peerapornpisol (2010) cultivated *Spirulina* in a medium containing effluent from anaerobically treated swine wastewater at concentration gradients from 0 to 100%. The results demonstrated best concentration of swine wastewater for *Spirulina* growth was 10%. They declared that 8.0 g/l $NaHCO_3$ and 1.5 g/l $NaNO_3$ are also required along with 10% diluted swine wastewater to obtain highest growth rates of *S. platensis* that support the present findings.

The present results were also confirmed by the work done by Canizares et al. (1993) who found 25% and 50% swine waste best for nutrient removal and biomass production of *S. maxima*. To find out the treatment efficiency of suspension and immobilized systems through the growth response of *S. maxima* towards the waste, different concentration gradients of aeration stabilized swine waste were compared in the experiment. In suspension system, 50% swine waste was found to be best in terms of nitrogen removal and biomass concentration while in immobilized system, 25% and 50% of swine waste were best for nitrogen removal (>90%) and *S. maxima* cultivation.

Canizares and Dominquez (1993) used supernatant of aerated swine wastewater in their study for the biomass production of *S. maxima*. The growth of *Spirulina* was found in every concentration of swine wastewater and also in its pure form. However, 50% dilution of swine wastewater was best for highest growth rate. At this

concentration, maximum 75% N-NH_4^+ removal and 53% total phosphate removal was recorded.

In another study of Canizares-Villanueva et al. (1994) effluent of anaerobically digested pig waste was used to cultivate another microorganism *Phormidium.* The results showed that the dilution levels from 10 to 50% of effluent were suitable for the growth of *Phormidium* sp. Among these dilutions, 25% was found best for maximum removal efficiency of nutrients. The removal of 48%, 100%, 87% and 68% was recorded for Ammonium, P-PO_3^{4-}, N-NO_3^- and total phosphorus respectively. The removal of COD was 91%.

The biochemical components of *S. maxima* (Canizares and Dominquez, 1993; Canizares et al., 1993) and *Phormidium* sp. were found by Caiiizares-Villanueva et al. (1994) to be differing in respect to the growth medium. In a medium with 50% diluted aerated pig wastewater the lipids were 6%, protein content was 36% and carbohydrates 44% in *S. maxima* biomass, whereas in *Phormidium* sp. the lipids were 9.4%, protein content was 53.4%, and carbohydrates 27.5% (Converti et al., 2006).

Mezzomo et al. (2010) found 5.0 and 8.5% concentrated swine wastewater best for maximum cellular concentration and maximum specific growth rate of *S. platensis* and 26.5% and 30.0% swine wastewater best for maximum COD removal. In their study, the biological treatment of swine waste water was done by the cultivation of microalgae *Spirulina platensis*. This work was done to study the best dilution of the wastewater for maximum biomass production and for removal of COD, ammonia and phosphorus to the microalgae. The cultivation of *S. platensis* strain Paracas presented maximum cellular concentrations and maximum specific growth rates in the wastewater concentration of 5.0 and 8.5%. The highest COD removal occurred with 26.5 and 30.0% of waste water in the medium. The maximum removal of total phosphorus (41.6%) was with 8.5% of wastewater, which is related to the microalgae growth. These results also support the results of CDAM in present study.

Gantar et al. (1991) studied on the swine wastewater for its nutrient removal in terms of nitrogen and phosphorus in algal suspension during the growth of *Spirulina platensis* and *Scenedesmus quadricauda.* In their study, they found that the growth rate of introduced alga varied with the concentration of waste used and the time of cultivation of alga was also an important factor for its growth. The introduced alga was found growing with autochtonous algae only at 10% to 20% concentration of waste. On the other side, when the concentration of manure was increased, the introduced alga did not compete with autochtonous algae.

Ratana et al. (2010) demonstrated that the use of 0.2 g/L urea and 4.5 g/L $NaHCO_3$ to supplement anaerobically digested pig waste at 20% dilution made the medium cheaper than the modified Zarrouk's medium. They calculated 4.4 times cheaper cost for this medium as compared to modified Zarrouk's medium. The removal rates of nitrogen and phosphorus were recorded 34 mg/L/d and 4 mg/L/d respectively. For pilot scale conditions and for laboratory conditions, the average productivities were found 12 g/m^2/d and 19.9 g/m^2/d respectively. Effluents from chicken and cow sheds and from piggeries were tested for growth of *Spirulina* by Yunus (1990). Half the strength of Zarrouk's medium was used as control and it was observed that effluents from piggery and cowsheds could be used as good substitutes for *Spirulina* cultivation. All these findings are in corroboration with the present results on cow dung ash medium.

20% formulated CDAM yielded considerable cost saving (20%) as compared to the CFTRI medium. 20% formulated CDAM was not only less expensive than CFTRI medium; even it was more effective than CFTRI medium in the biomass production too (1.212 g/l and 1.112 g/l respectively).

Conclusion

When cow dung ash medium used as a cheap nutrient growth medium for *S. platensis* biomass production in lab conditions, the biomass of *S. platensis* in 100% CDAM was lower as compared to control. After formulating CDAM with CFTRI medium at concentration levels 10% to 60%, best biomass production of *S. platensis* obtained in 20% formulated CDAM. The *S. platensis* biomass obtained in 20% formulated CDAM was nutritionally rich as contained 42% protein, 16.17% carbohydrate and 0.023 mg/ml chlorophyll a content. The protein content of *S. platensis* in control was found 31% higher as compared to 20% formulated CDAM. The carbohydrate content of *S. platensis* was only 16.9% higher while FAN (free amino acids) content was 34.6% higher in control than in 20% formulated CDAM. The removal efficiency of COD (83.64%) and TSS (98.06%) was found very high after cultivating *S. platensis* in 20% formulated CDAM as compared to found in CFTRI medium. The % removal of TDS and

conductivity was comparable to the values of standard medium.

The nutritional analysis of *S. platensis* grown in 20% formulated CDAM demonstrated that it is a rich source of protein and carbohydrate. 20% formulated CDAM yielded considerable cost saving (20%) as compared to the CFTRI medium. 20% formulated CDAM was not only less expensive than CFTRI medium; even it was more effective than CFTRI medium in the biomass production too.

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PLANT SCIENCE

Plant growth inhibitory activity of medicinal plant *Hyptis suaveolens*: could allelopathy be a cause?

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Abstract

The present study was conducted to explore the allelopathy of *Hyptis suaveolens* Poit, an important medicinal plant of Lamiaceae family. The aqueous methanol extracts of this plant at four different concentrations (3, 10, 30 and 100 mg dry weight [DW] equivalent extract/mL), were examined on the seedling growth of eight test plant species, cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), rapeseed (*Brassica napus* L.), timothy (*Phleum pratense* L.), crabgrass (*Digitaria sanguinalis* L. scop.), barnyardgrass (*Echinochloa crus-galli* L.) and Italian ryegrass (*Lolium multiflorum* Lam.), and on the germination of cress and Italian ryegrass. The germination of cress, Italian ryegrass and the growth of all eight test species were significantly inhibited by the *H. suaveolens* plant extracts at a concentration greater than 3, 30 and 10 mg DW equivalent extract/mL, respectively. The inhibitory activity of the extracts was concentration dependent. The root growth of all the test plants was more sensitive to the extracts than the hypocotyl/coleoptile growth of those. The concentrations required for 50% inhibition (defined as I_{50}) of the hypocotyl/coleoptile and root growth of the eight test plant species range from 9.3–79.3 and 4.9–29.5 mg DW equivalent extract/mL, respectively. The hypocotyl growth of lettuce and the root growth of crabgrass were most sensitive to the extract, whereas coleoptile growth of barnyardgrass and the root growth of alfalfa were the least sensitive. The inhibitory activities of the *H. suaveolens* on the germination and growth of the test plant species suggest that the plant has allelopathic potentiality and may possess allelochemicals. These allelochemicals might be responsible for the restricted growth of other plant species near their colony in natural ecosystems. However, isolation and identification of these allelochemicals from *H. suaveolens* plant extracts could serve as the lead for new natural herbicides development for sustainable weed management strategies.

Key words: Allelochemicals, Weed management, Sustainable agriculture, Lamiaceae, Natural herbicide

Introduction

The initial use of synthetic herbicides to control weeds in the crop fields superficially increased the crop production by reducing the weed infestation. Eventually the excessive use of synthetic herbicide in the crop fields may obviously lead to a tremendous environmental hazards resulting in degradation of agricultural land by abolishing soil-biota (Pell et al., 1998); ground water contamination (Aktar et al., 2009); reduction of fisheries (Khan and Law, 2005); development of herbicide resistant weeds (Vyvyan, 2002); destruction of beneficial predators of pests and thereby increased the virulence of many species of agricultural pests (Wilson and Tisdell, 2001). To avoid the hurtful effects of synthetic herbicide, research on novel natural plant products have moved from the fringe to the mainstream for the development of ecologically acceptable, environment friendly, cost-effective and relatively safe natural herbicides.

Allelopathy of medicinal plants could play a vital role in identification of new allelochemicals and could accelerate the process of new natural herbicides development. Currently, many researches around the world show their keen interest on medicinal plants for searching new novel compounds and reported that medicinal plants have growth inhibitory effects on different noxious weed species and have the potentiality to use them in the crop fields either directly or as a natural herbicides (Lin et al., 2003, 2004; Han et

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al., 2008; Sodaeizadeh et al., 2009; Li et al., 2009). Moreover, it was reported that screening of allelopathic plant from medicinal plants species is easier than other plants (Fujii et al., 2003) possibly due to their existed certain metabolic compounds which was used for curing many diseases of both animal and human being. On the other hand, there are about 400,000 secondary metabolites in plants with allelopathic activities (Swain, 1977), of which only a few have been examined (Einhelling and Leather, 1988). The rest of the compounds, might contain very promising growth inhibitors are still unknown. Since about 12.5% of the total plants species of the world are considered as medicinal plants (Wakdikar, 2004), therefore, they could be served as important candidates for allelopathic research. Isolation and characterization of that unknown allelochemicals from medicinal plants might provide the chemical basis for new natural herbicides developments.

Hyptis, the genus of Lamiaceae contains approximately 400 species (Willis, 1973) and most of the species are native to the tropical America (Hutchinson and Dalziel, 1963; Hickey and King, 1988). However, one of the species *Hyptis suaveolens* Poit distributed throughout the tropics and subtropics, along the rail tracks, roadsides (Mishra and Verma, 1992), foothills of open forests, forest clearings (Mudgal et al., 1997) and also in the fallow lands. *H. suaveolens* has many medicinal properties and used for several ethnobotanical applications (Adda et al., 2011). For example, leaf decoction is used for the treatment of diabetes (Abdullahi et al., 2003) and cancer (Danmalam et al., 2009), and root decoction is highly valued as appetizer. Moreover, these organs contain urosolic acid, a natural HIV-integrase inhibitor (Chatterjee and Pakrashi, 1997). In addition, the leaves and twigs are considered as antispasmodic, antirheumatic, anti-inflammatory, antifertility agents (Kirtikar and Basu, 1991), and also have antiseptic, mosquitocidal and insecticidal properties (Shenoy et al., 2009; Adda et al., 2011). The leaves of *H. suaveolens* contain alkaloids, terpenes and volatile oils (Gills, 1992). Beside the pharmacological and toxicological properties of *H. suaveolens*, still now very few are known about its allelopathic activities. Therefore, the current research was conducted to explore the allelopathic activity of *H. suaveolens* on different test plant species.

Materials and Methods

Plant materials

Whole plants (leaves, stem and roots) of *H. suaveolens* were collected from Bangladesh during the month of March-April, 2012. After collection, plants were washed with tap water to remove the soil and other debris followed by sun drying. The dried plants were then kept in a refrigerator at 2 ºC temperature until extraction.

Test plant species

Eight test plant species, cress (*Lepidum sativum* L.), lettuce (*Lactuca sativa* L.), alfalfa (*Medicago sativa* L.), rapeseed (*Brassica napus L.*), timothy (*Phleum pratense* L.), crabgrass (*Digitaria sanguinalis* L. scop.), barnyardgrass (*Echinochloa crus-galli* L.) and Italian ryegrass (*Lolium multiflorum* Lam.) were selected for the present research. Among these eight test species, the first four are dicotyledonous and the rest are monocotyledonous. Cress, alfalfa, lettuce, rapeseed and timothy were chosen due to their known seedling growth, whereas crabgrass, barnyard grass and Italian ryegrass were chosen because they are most common weeds in the crop fields and distributed throughout the world.

Extraction procedure

The whole parts (leaves, stem and roots) of dried *H. suaveolens* (30 g) were cut into small pieces and extracted with 300 mL of 70 % (v/v) aqueous methanol for 48 h. After filtration using one layer of filter paper (No. 2; Advantec® Toyo Roshi Kaisha, Ltd., Tokyo, Japan), the residue was re-extracted with 300 mL of 100% methanol for 24 h and filtered. The two filtrates were combined and evaporated with a rotary evaporator at 40ºC.

Germination bioassay

An aliquot of the extract (final assay concentration was 3, 10, 30 and 100 mg DW equivalent extract/mL) was evaporated to dryness at 40ºC in *vacuo* by rotary evaporator, dissolved in methanol and added to a sheet of filter paper (No. 2) in a 2.8 cm Petri dish. The methanol was evaporated in a draft chamber then the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of Tween 20 (polyoxyethylene sorbitan monolaurate; Nacalai Tesque, Inc., Kyoto, Japan) which was used for surfactant and did not cause any toxic effects. Ten seeds of cress or Italian ryegrass were arranged on the filter paper in Petri-dishes. Control seeds were also sown on the filter paper moistened with Tween 20 without plant extracts. Then the Petri dishes were incubated in the dark chamber at 25ºC. The germination bioassay was laid out using a completely randomized design with three replications. Seeds that showed the emergence of the radical by rupturing the seed coat were considered to be germinated as described by

Barrôco et al. (2005) and Faria et al. (2005). Germination of seeds was recorded at every 12 h intervals for four days. The percentage of germination over control in each treatment was calculated using the following equation:

$$\text{Germination (\% of control)} = \frac{G_T}{G_0} \times 100$$

Where,

G_T = average number of germinated seed in the treatment in each time of measurements

G_0= average number of germinated seed in the control at the same time of measurements

Growth bioassay

Test samples of plant extracts were prepared and added to a sheet of filter paper (No. 2) in a 2.8 cm Petri dish, and the filter paper was moistened with 0.6 mL of 0.05% (v/v) aqueous solution of Tween 20 as described above. Then, 10 seeds of cress, lettuce, alfalfa, rapeseed or 10 germinated seeds of timothy (germinated in the darkness at 25ºC for 72 h after overnight soaking), crabgrass (germinated in the darkness at 25ºC for 120 h after 24 h incubation in the light chamber at 25ºC), barnyardgrass or Italian ryegrass (germinated in the darkness at 25ºC for 24 h after overnight soaking) were arranged on the filter paper in Petri-dishes. The hypocotyl/coleoptile and root lengths of the seedlings were measured at 48 h after incubation in darkness at 25ºC. Control seeds were sown on the filter paper moistened with 0.6 mL of 0.05% (v/v) aqueous solution of Tween 20 without plant extracts. The same bioassay experiment was repeated twice with three replications in each case.

The inhibition percentage was calculated using the following equation:

$$\text{Inhibition (\%)} = [1 - \frac{\textit{length with aqueous methanol extract}}{\textit{length of control}}] \times 100$$

The concentrations required for 50% inhibition (express as I_{50}) of the test plant species in the assay was calculated from the regression equation of the concentration response curves.

Statistical analysis

Experimental data were analyzed using statistical software PASW statistics 17.0 (SPSS Inc., Illinois, USA) and GraphPad Prism 6.0 (GraphPad Software, Inc., California, USA).

Results

Effects of *H. suaveolens* plant extracts on the germination of two test plant species

The effects of aqueous methanol extracts of *H. suaveolens* on the germination of cress and Italian ryegrass were determined (Figure 1, 2). The extracts inhibited the germination and the inhibition was concentration dependent. The two-way ANOVA indicates that the four different concentrations, the period of incubations and their interaction have significant effect ($p<0.001$) on the germination of both cress and Italian ryegrass. The germination of cress was significantly inhibited by the *H. suaveolens* plant extracts at all extract concentrations. In contrast, the germination of Italian ryegrass was significantly inhibited at a concentrations ≥ 30 mg DW equivalent extract/mL (Figure 1, 2).

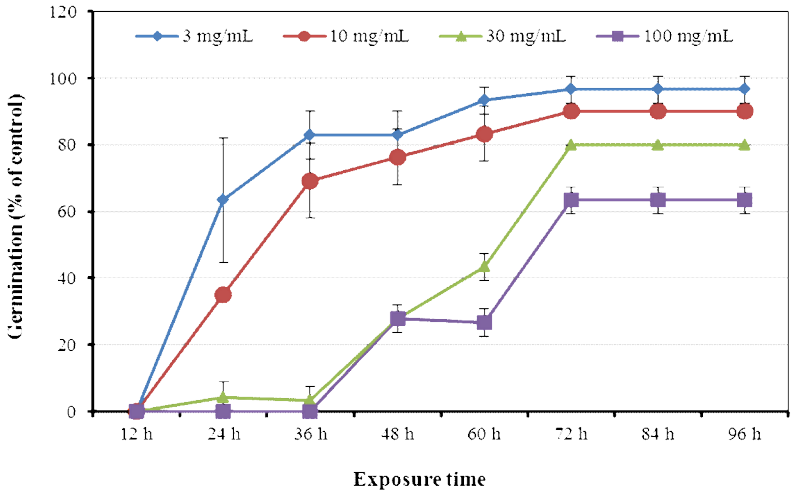

Figure 1. Effects of aqueous methanol extracts of *H. suaveolens* on the germination of cress.
Means±SE from three independent experiments with 10 seeds for each determination are shown. All the values are statistically significant at $p<0.001$.

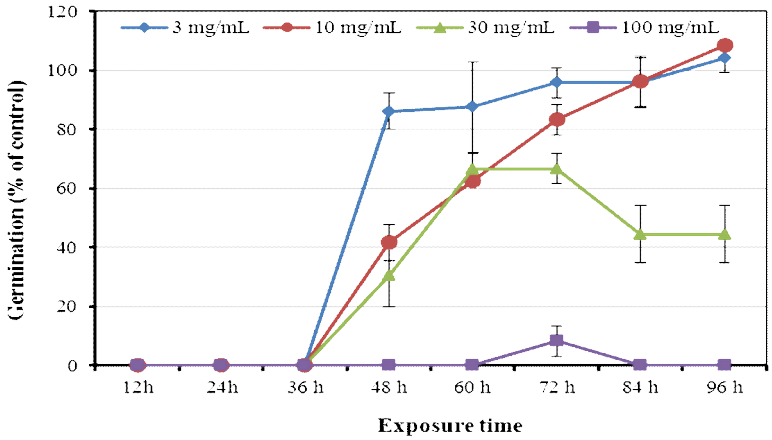

Figure 2. Effects of aqueous methanol extracts of *H. suaveolens* on the germination of Italian ryegrass.
Means±SE from three independent experiments with 10 seeds for each determination are shown. All the values are statistically significant at $p<0.001$.

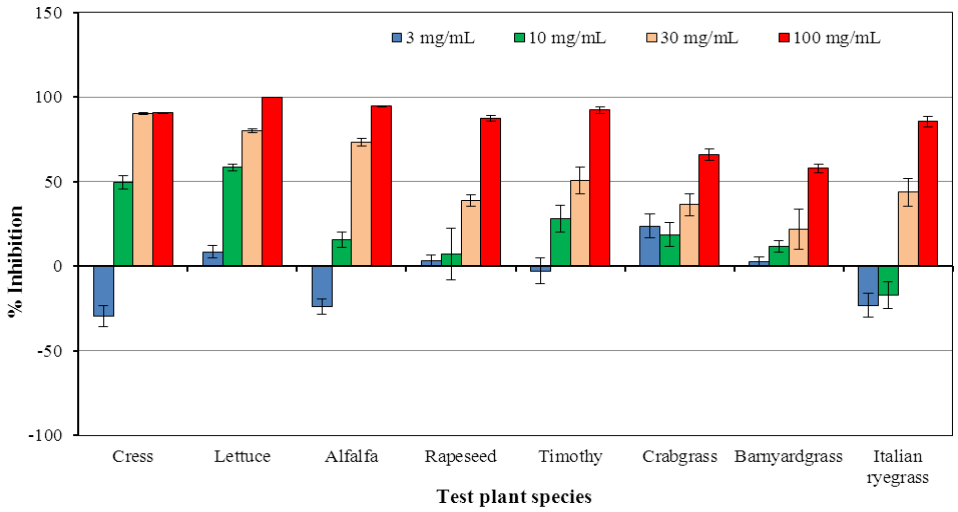

Figure 3. Effects of aqueous methanol extracts of *H. suaveolens* on hypocotyl/coleoptile growth of the test plant species..
Means±SE from three independent experiments with 10 seedlings for each determination are shown. The negative (–) value in the Y axis indicates stimulation and positive (+) value indicates inhibition of the hypocotyl/coleoptile growth of eight test plant species by *H. suaveolens* plant extracts.

Effects of *H. suaveolens* plant extracts on the growth of eight test plant species

The inhibition percent of the aqueous methanol extracts of *H. suaveolens* on the hypocotyl/coleoptile and root growth of cress, lettuce, alfalfa, rapeseed, timothy, crabgrass, barnyardgrass and Italian ryegrass are shown in Figures 3 and 4. The hypocotyl/coleoptile and root growth of all but Italian ryegrass and alfalfa were significantly inhibited by the extracts at concentrations greater than 3 mg DW equivalent extract/mL. Moreover, the effectiveness of *H. suaveolens* plant extracts were different among test species, and the inhibition percent of the extracts was concentration dependent (Figure 3, 4). The two-way ANOVA showed that *H. suaveolens* plant extracts and its interaction with the eight test plant species has a significant ($p<0.001$) effect on the seedling growth of all the test plant species.

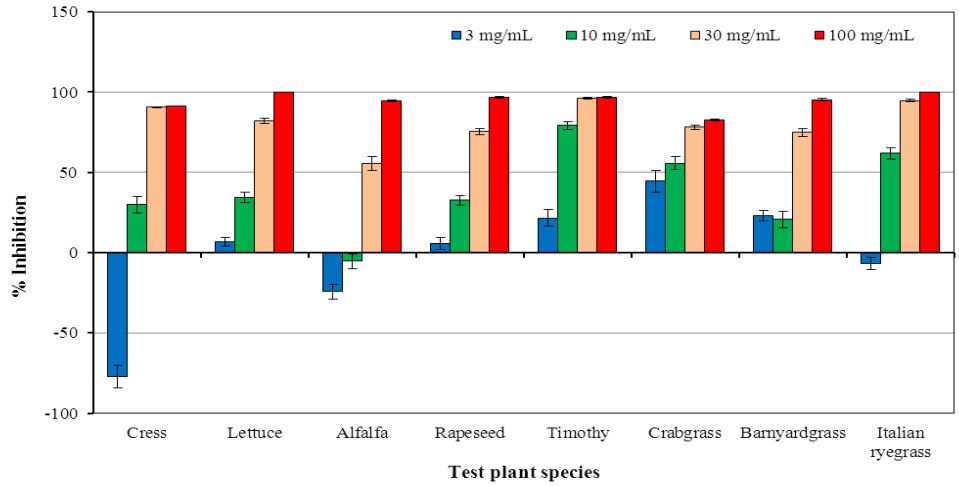

Figure 4. Effects of aqueous methanol extracts of *H. suaveolens* on root growth of the test plant species. Means±SE from three independent experiments with 10 seedlings for each determination are shown. The negative (–) value in the Y axis indicates stimulation and positive (+) value indicates inhibition of the root growth of eight test plant species by *H. suaveolens* plant extracts.

At the concentration of 30 mg DW equivalent extract/mL, the inhibition percent of the aqueous methanol extracts of *H. suaveolens* on hypocotyl/coleoptile growth of cress, lettuce, alfalfa, rapeseed, timothy, crabgrass, barnyardgrass and Italian ryegrass was 90, 80, 73, 39, 51, 36, 22 and 44, respectively (Figure 3). The hypocotyl growth of lettuce seedling was completely inhibited (Figure 5) when the seeds are applied to a concentration of 100 mg DW equivalent extract/mL, followed by alfalfa, timothy, cress, rapeseed, Italian ryegrass, crabgrass and barnyardgrass at 95, 92, 91, 87, 86, 66 and 58% inhibition, respectively. On the other hand, the extracts of *H. suaveolens* stimulated the hypocotyl/coleoptile growth of cress, alfalfa, timothy and Italian ryegrass at a concentration of 3 mg DW equivalent extract/mL. In addition, the coleoptile growth of Italian ryegrass was stimulated by the *H. suaveolens* at a concentration of 10 mg DW equivalent extract/mL (Figure 3). Considering the concentration required for 50% inhibition (defined as I_{50}), it was revealed that the hypocotyl growth of lettuce seedling was most sensitive to the aqueous methanol extracts of *H. suaveolens*, whereas the coleoptile growth of barnyardgrass was least sensitive (Table 1).

At the concentration of 30 mg DW equivalent/mL, the inhibition percent of the root growth of timothy, Italian ryegrass, cress, lettuce, crabgrass, rapeseed, barnyardgrass and alfalfa was 96, 95, 91, 82, 78, 75, 75 and 56, respectively (Figure 4). When the test plant species were exposed to the concentration of 100 mg DW equivalent extract/mL, the root growth of lettuce and Italian ryegrass seedling were completely (100%) inhibited (Figure 5, 6) followed by rapeseed, timothy, barnyardgrass, alfalfa, cress and crabgrass seedlings and the inhibition on their root growth was 97, 97, 95, 95, 91 and 83%, respectively. In contrast, the root growth of cress, alfalfa and Italian ryegrass were significantly stimulated by the *H. suaveolens* plant extracts at the concentration of 3 mg dry weight equivalent extract/mL (Figure 4). Furthermore, the root growth of alfalfa was stimulated by the extracts at a concentration of 10 mg DW equivalent extract/mL (Figure 4). Considering I_{50} value, the root growth of crabgrass was most sensitive to the *H. suaveolens* plant extracts than the other test plant species, whereas alfalfa was least sensitive to the extracts (Table 1).

Discussion

H. suaveolens is a ruderal type of plants that normally grows in a colony. It was reported that the other plant species near their colony is quite restricted (Raizada, 2006), but there have not so much clear evidence of the reasons. However, one of the main reasons for this character of the plant could be due to its allelopathic potentiality. A few evidences are found in the literature about the allelopathic activity of the leaf extracts and dry leaf residue of *H. suaveolens* plants. For example, Chatiyanon et al. (2012) reported that the water and methanol extract of the leaves of *H. suaveolens* has

allelopathic effects on the germination and seedling growth of *Pennisetum setosum* (Swartz.) L.C. Rich and *Mimosa invisa* Mart. Similar findings were also reported by Kapoor (2011) who worked with dry leaf residue of *H. suaveolens* and observed inhibitory activity on the growth and physiological parameters of *Parthenium hysterophorus* L. In the present research the aqueous methanol extracts of *H. suaveolens* significantly inhibited the germination of cress and Italian ryegrass at concentrations greater than 3 and 30 mg DW equivalent extract/mL, respectively. The extracts also significantly inhibited the seedling growth of all test plant species (cress, lettuce, alfalfa, rapeseed, timothy, crabgrass, barnyardgrass and Italian ryegrass) at concentrations greater than 10 mg DW equivalent extract/mL. These results imply that the aqueous methanol extract of *H. suaveolens* may possess allelochemicals which are responsible for their inhibitory activity. Moreover, the inhibitory activity of *H. suaveolens* plant extracts on different test species was concentration dependent. Concentration dependent inhibition on germination and growth by allelopathic plants extracts was also reported by Inderjit and Keating (1999), Kato-Noguchi et al. (2001), An et al. (2005), Bogatek et al. (2006) and Soltys et al. (2012).

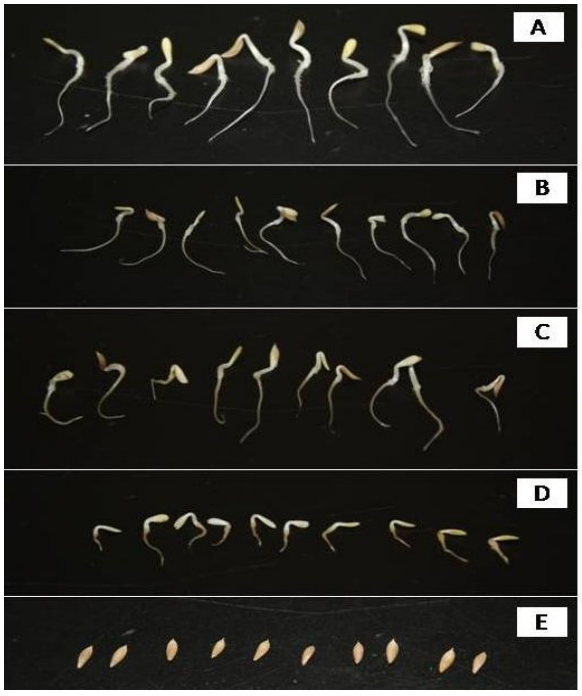

Figure 5. Effects of aqueous methanol extracts of *H. suaveolens* on the seedling growth of lettuce, after 48h incubation in the darkness at different concentration. Here, A, B, C, D and E denotes the concentration of 0, 3, 10, 30 and 100 mg dry weight equivalent extract/mL, respectively.

Table 1. I_{50} values of the aqueous methanol extract of *H. suaveolens* plant on hypocotyl/coleoptile and root growth of eight test plant species.

Test plant species	I_{50} (mg dry weight equivalent extract/mL)	
	Hypocotyl/Coleoptile growth	Root growth
Cress	10.0	11.7
Lettuce	9.3	13.8
Alfalfa	20.3	29.5
Rapeseed	37.7	15.4
Italian ryegrass	30.6	9.5
Barnyardgrass	79.3	13.2
Crabgrass	51.6	4.9
Timothy	25.3	5.4

Note: The values were determine by a logistic regression analysis after bioassays.

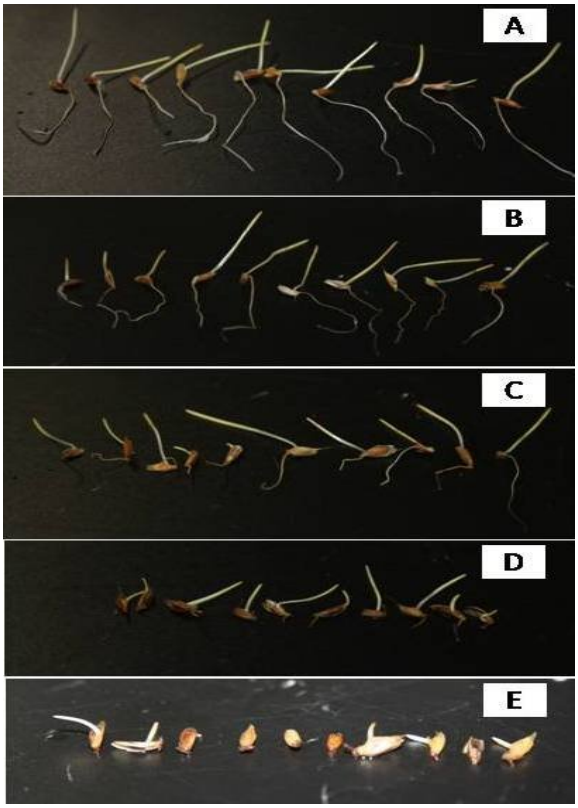

Figure 6. Effects of aqueous methanol extracts of *H. suaveolens* on the seedling growth of Italian ryegrass, after 48 h incubation in the darkness at different concentration.
Here, A, B, C, D and E denotes the concentration of 0, 3, 10, 30 and 100 mg dry weight equivalent extract/mL, respectively.

The inhibitory activity of *H. suaveolens* plant extracts on the germination of cress and Italian ryegrass was congruent with the previous findings of many other researchers. They reported that the decrease of germination ability in presence of allelochemicals could be due to several

abnormalities created by the allelochemicals on seed during germination process. For example, Kato-Noguchi and Macías (2006) reported that allelochemicals like 6-methoxy-benzoxazolin-2(3H)-one (MBOA) inhibit the germination of cress seeds by inhibiting the induction of α-amylase activity, which is very crucial for the conversion of reserve carbohydrate into soluble sugars during seed germination. Oracz et al. (2007) stated that the accumulation of reactive oxygen species caused cellular damage, which resulted in the decrease of germination ability and gradual loss of seed vigour. The decrease in germination ability was also due to enhanced membrane deterioration (Bogatek et al., 2006).

The root growth of all the test plant species was observed to be more sensitive to the *H. suaveolens* plant extracts than the hypocotyl/coleoptile growth. These results are in agreement with the earlier findings of Stachon and Zimdahl (1980) and Aliotta et al. (1993) who reported that allelopathic plant extracts had higher root growth inhibition than the coleoptiles. This phenomenon might be due to the more intensive contact between roots and plant extracts. Salam and Kato-Noguchi (2010) also reported that roots were more sensitive to the allelochemicals than hypocotyls/coleoptiles because the roots are the first organ to absorb allelochemicals from the environment. Whereas, Nishida et al. (2005) stated that the permeability of allelochemicals into root tissue is higher than the shoot tissue. They also explained that the hypocotyl/coleoptile growth of seedlings largely depends on cell expansion which is relatively insensitive to the allelochemicals, whereas root growth requires not only cell expansion, but also cell proliferation which is sensitive to the allelochemicals and therefore, the root growth exerts higher inhibition than the hypocotyl/coleoptile growth.

On the other hand, a number of abnormalities have been found when the test species are subjected to allelochemicals, and is the plausible cause for their growth inhibition. For example, allelochemicals inhibit the process of cell division, elongation and expansion rate, (Rice, 1984; Ortega et al., 1988; Einhellig, 1996; Jacob and Sarada, 2012), respiration process (Inderjit and Keating, 1999), ion absorption process (Qasem and Hill, 1989), enzyme activity (Sato et al., 1982), plant endogenous hormones and protein synthesis (Jacob and Sarada, 2012), alteration of the phytochrome control of germination (Leather and Einhellig, 1988) and consequently, arrested the plant growth. Allelochemicals may produce more than one effect of the above on the cellular processes that could be responsible for the reduced seedling growth of the test plant species. But the details of the biochemical mechanism through which allelochemicals exert a toxic effect on the growth of any plant species are still not well known (Zhou and Yu, 2006). Never the less, the stimulatory activity on the hypocotyl/coleoptiles and root growth of cress, alfalfa, timothy and Italian ryegrass by the aqueous methanol extract of *H. suaveolens* at a concentration less than 10 mg DW equivalent extract/mL are in line with the findings of many other researchers. They reported that allelochemicals can stimulate the seedlings growth at very low concentrations but inhibit the seedlings growth at high concentrations (Rice, 1984; Lovett et al., 1989; Liu and Chen, 2011; Islam and Kato-Noguchi, 2012). The stimulatory activity of any compound at low doses is called hormesis (Southam and Erlich, 1943). In some cases, allelochemicals have also been reported to induce hormesis. The reason behind the hormesis is due to the availability of some chemicals at lower doses which could affect the plant hormones that are responsible for shoot or root elongation, while they might have inhibitory activity on the seedling growth at higher doses due to the same or another mechanism of action (Duke et al., 2006).

Conclusion

The concentration dependent inhibitory activities of the aqueous methanol extracts of *H. suaveolens* on the germination and seedling growth of the test species suggest that the plant has allelopathic potentiality and possess allelochemicals. These allelochemicals could be the main reason for the restricted growth of other plant species near their colony. Isolation and identification of those allelochemicals from *H. suaveolens* could be act as lead for the development of bio-degradable, environment friendly new natural herbicides for sustainable weed management strategies. However, more research is necessary to further confirm the allelopathic potentiality of *H. suaveolens* under field and green house conditions to make our predictions more accurate.

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Availability and fractionation of trace elements in arid calcareous soils

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Abstract

Thirty seven soil samples were collected from seven agricultural regions in Saudi Arabia to investigate trace element availability and fractionation distribution. Di-ethylene tri-amine penta acetic acid extractable micronutrients ranged from 1.1 to 11.5 µg g^{-1} for Fe, 0.2-3.7 µg g^{-1} for Zn, 0.48-13.0 µg g^{-1} for Mn, and 0.2-3.7 µg g^{-1} for Cu. Based on published critical levels, Cu was sufficient for most crops in all soils. Four soil samples were low in Mn, 28 were low in Zn, and 31 were low in Fe. Fractionation of micronutrients (Fe, Cu, Zn, and Mn) in selected seven soil samples revealed that the exchangeable fractions were the smallest, with Zn and Cu below detection limits. Carbonate bound fractions showed the micronutrient concentrations in the order Mn>Fe>Cu>Zn. Oxide and hydroxide bound fraction was higher than the previous two fractions; the order of the metals was Fe>Mn>Zn>Cu. For the organic bound fractions, the order was Fe>Mn>Cu>Zn. The residual fraction was the largest. The order of residual micronutrients was Fe>Mn>Zn>Cu. Overall, and considering the total amount of the four micronutrients within each fraction, the five fractions followed the order: Residual> oxide bound>CO3 bound>organic bound>exchangeable. Based on the data, it is recommended that Fe and Zn applications should be included in balanced fertilization programs. Mn and Cu are sufficiently available in the studied soils, and may not need to be supplied to crops

Key words: Arid, Calcareous, Exchangeable, Fractionation, Micronutrients

Introduction

Saudi Arabia is a vast country with approximately 2 million km^2 of arid land (Saudi Geological Survey, 2012). It represents about 5% of the arid zones of the world (Bashour et al., 1983). During the past three decades about 1.2 million hectares of desert soil have been converted into irrigated farming land, and are being intensively used for growing major field crops, fruit trees, and vegetables (Ministry of Agriculture, 2012). The agricultural soils of Saudi Arabia are in general coarse to medium textured, calcareous, low in organic matter, and moderately alkaline (Bashour et al., 1983; Ministry of Agriculture and Water, 1986; Al Jaloud, 1988). Deficiency of Fe, Zn, Cu, and Mn is common in calcareous soils (Mortvedt et al., 1972; Tisdale et al., 1993). Al Jaloud et al. (1995) studied micronutrients fractions in selected Saudi soils and concluded that available Fe and Zn are low in most analyzed samples while Mn and Cu were present in adequate concentrations. Nevertheless, their study covered selected areas and a relatively small sample size. To our knowledge, there are no comprehensive trace elements availability and fractionation studies covering most agricultural areas of the country. Most of the previous studies looked at di-ethylene tri-amine penta acetic acid (DTPA)-extractable trace elements only, thus ignoring other potential pools of trace elements. In fact, many studies have reported significant correlations between the amounts of trace elements in chemical soil fractions and the amounts taken up in plant tissues (Canet et al., 1998; Qian et al., 1996; Zhang et al., 1998), suggesting the consideration of these fraction related pools in micronutrient research; correlations

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between trace-element soil fractions and trace element concentrations in crop tissue indicate that chemical fractions do relate to phyto-availability.

Several fractionation schemes for micronutrients availability and fractionation in agricultural soils have been reported in literature (McLaren and Crawford, 1973; Wagemann et al., 1977; Shuman, 1979; Shuman, 1985; Shober et al., 2007). Trace elements fractionations were also studied from environmental perspectives (Filgueiras et al., 2002; Ahdy and Youssef, 2011; Okbah et al., 2011). Tessier et al. (1979) developed a procedure for fractionation of micronutrients in calcareous soils, and many scientists adapted it to their specific research needs; this methodology is suitable for the current study because it has a separate step for the CO_3^2 bound fractions which are widely present in calcareous soils. It fractionates soil metals sequentially into five parts: exchangeable, carbonate bound, oxide (and hydroxide) bound, organic bound, and residual. Accurate determination of micronutrient fractions can help in predicting their potential bioavailability to crops under various environmental conditions; in arid environments and calcareous soils, the organic and carbonate fractions play a critical role in micronutrient biodegradation and mobilization (Chanem and Mikkelsen, 1987; Stone and Marsalek, 1996).

The objective of this study is therefore to assess the availability status and fractionation levels of Fe, Zn, Cu, and Mn in calcareous soils representing seven major agricultural regions of the arid environment in Saudi Arabia.

Materials and Methods

A total of thirty seven composite soil samples were collected from farmed lands, covering and representing each of the seven major agricultural areas. The cultivated crops are primarily wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), open field vegetables, and forages; the main forage is alfalfa (*Medicago sativa* L.); for these crops, the majority of mass and effective rooting system develops on the top 20 cm plowable soil layer. Annual irrigation water amounted to 7000 m^3ha^{-1} for wheat and barley, 10000 m^3ha^{-1} for vegetables, and 17000 m^3ha^{-1} for forages. Salinity of irrigation water ranges from a high 3.3 to a low 0.9 dSm^{-1} in Qassim and Hail, respectively (Al Jaloud and Al Rabhi, 2013); Ca, Mg, Na, and Cl are high in Wadi Dawassir and Kharj, but low in Hail; micronutrient concentration in irrigation water was below the detection limits. Fertilizers programs and strategies were adapted to each region, given the differences in soil physical and chemical properties, and environmental conditions. Crops grown on these soils received macro and micro nutrients as per regional recommendations (Al Safi Dairy Est., 2000; Errebhi and Al Jaloud, 2003). The collected samples were air dried, ground to pass through a 2 mm sieve, and stored for subsequent analyses. The $CaCO_3$%, Electrical Conductivity (ECe), and pH were determined as outlined in USDA Salinity Laboratory Handbook 60 (Richards, 1954). The micronutrients were extracted by the DTPA technique, which was reported by Lindsay and Norwell (1978) to be an efficient procedure for simultaneous assessment of Fe, Zn, Cu, and Mn availability in calcareous soils. Extraction of micronutrients with DTPA solution is widely acceptable as it gave beneficial information about the availability of micronutrients in soils. The metal content (Fe, Zn, Cu, Mn) from DTPA-extraction was analyzed by Atomic Absorption Spectrophotometer, Perkin Elmer 503 (Perkinelmer, Inc. 940 Winter St. Waltahm, MA 02451 USA, www.perkinelmer.com).

From each geographic region, one representative composite soil sample (the first row for the region, Table 1) was selected based on prior knowledge of these regions, the commonly dominating soil types and crops, and more importantly, on the surface area they represent for that region. Samples were used to determine soil texture by the hydrometer method (Foth et al., 1977), soil organic matter by the digestion procedure (Nelson and Sommers, 1996), and micronutrient fractionation (Tessier et al., 1979). The fractionation scheme of Tessier et al. (1979) was applied in this case because it involves a separate extraction step for the CO_3 bound fractions; therefore, it is suitable for calcareous soils. Fractionation provides the additional benefit of estimating the other forms in which Fe, Zn, Cu, and Mn are present in various soils components. Sequential chemical fractionation procedures segregate trace elements into soil fractions by subjecting the soil to a series of chemical reagents, each more destructive than the previous one (Zufiaurre et al., 1998; Tessier et al., 1979; Morabito, 1995). The following soil fractions are the most widely used and acceptable among the scientific community: (1) exchangeable ; (2) bound to carbonates; (3) bound oxides and hydroxides (reducible); (4) bound to organic matter and sulfides (oxidizable); and (5) residual: in the mineral lattice of silicates or nonreducible oxides or hydroxides (McLaren and Crawford, 1973; Sims and Kline, 1991; Morabito, 1995; Tessier et al.,

1979; Emmerich et al., 1982). The trace elements in the soil fractions are, ideally, indicative of their potential mobility and bioavailability, in a decreasing parallel to the order of the sequential extraction steps. The fractionation procedure was as follows:

Exchangeable

To 5 g soil sample, 40 mL of 1M magnesium chloride at pH 7.0 was added, and the content was shaken continuously for 1 hour at room temperature. After centrifugation, the supernatant solution was filtered and saved for analysis. The residue was washed with 20 mL water, and the washings were discarded.

Carbonate Bound

The residue from (1) was agitated continuously for 5 hours with 40 mL of 1M sodium acetate (pH 5.0). After agitation, the supernatant solution was filtered and stored for analysis. After extraction, the residue was washed with 20 mL of water, and the washings were discarded.

Iron-Manganese oxide bound

To the residue from (2), 100 mL of 0.04 M hydroxylamine hydrochloride was added in 25% (v/v) acetic acid (HOAc), and the extraction was carried out at 95±2ºC in a water bath, with occasional agitation for 6 hours. After centrifuging, the residue was washed with the required amount of water (20 mL) and the washings were combined with the original extract after filtration to make up a total volume of 100 mL.

Organic Bound

The residue from (3) was first treated with 15 mL of 0.02 M nitric acid (HNO_3) and 25 mL of 30% hydrogen peroxide (pH 2.0) for 5 hours at 85±2ºC in a water bath. A second 15 mL aliquot of 30% H_2O_2 was then added, and the contents were again heated to 85ºC for 3 hours with intermittent agitation. After cooling, 25 mL of 3.2 M ammonium acetate (NH_4OAc) in 20% v/v HNO_3) was added and agitated continuously for 30 minutes. After centrifuging, the residue was washed with 20 mL of water and the washings were added to the extracted solution after filtration to make up a final volume of l00 mL.

Residual

For this study, 1 g of soil sample was taken from (4) and treated with 2 mL of 70% perchloric acid ($HClO_4$) and 10 mL of hydrogen fluoride (70% HF) in a platinum crucible. The contents were digested on a hot plate and when the residue became paste, a second addition of 1 mL of $HClO_4$ and 10 mL of HF was made. Again, the contents were digested to near dryness. Finally 1 mL of $HClO_4$ was added and evaporated again until the appearance of white fumes. The contents were then dissolved in 6N hydrochloric acid (HCl), filtered, and made up to 100 mL, then kept in clean plastic bottles for analytical work.

Metal content from fractionation was also analyzed by Atomic Absorption Spectrophotometry.

Results and Discussion

Soil samples

The regional sources of the soils samples and the analytical results are presented in Tables 1-3. The results show that soils have a wide range of $CaCO_3$ content, a narrow range of pH, different salinity levels, and various micronutrient concentrations.

DTPA-Extractable Micronutrients

Table 1 summarizes some important soil chemical properties of the thirty seven surface soil samples. DTPA-extractable Fe, Zn, Mn, and Cu concentrations are also given. Results of the chemical analyses show that the soil samples are slightly alkaline; pH ranges from 7.3 to 8.2 across all geographic regions, with no major difference among the regions, as the regional mean pH range varies from 7.6 to 7.9 (Table 1). Calcium carbonate data indicates that the soils are calcareous; $CaCO_3$ content ranges from a low of 3.3% to a high of 24.6%, with noticeable regional mean differences varying from 6.87% in Taif to 18.82% in Kharj. Salinity levels, measured by saturated paste extract Electrical Conductivity (ECe), indicate the presence of saline soils in all the regions, except Taif. Across regions, the EC ranges from 1.00 to 11.51 dS m^{-1}. This data agrees with previously reported findings for pH, calcium carbonate, and electrical conductivity in agricultural areas of Saudi Arabia (Al Safi Dairy Est., 2000; Errebhi and Al Jaloud, 2003; Sallam, 2002). The DTPA-extractable Fe, based on collected samples, ranges from 1.1-11.70 µg g^{-1}, but the ranges are narrowed to 2.84-5.65 µg g^{-1} based on regional means (Table 1). DTPA-extractable Zn individual data ranges from 0.2 to 3.7 µg g^{-1}, but on regional average, the interval was 0.3-1.5 µg g^{-1}. Manganese individual data shows a wide range of (0.48-13.00 µg g^{-1}); however, for the averaged numbers across regions, the data narrowed significantly to (0.95-7.93 µg g^{-1}). DTPA-extractable Cu ranges from 0.22 to 2.80 µg g^{-1} with no major change for the regional averaged data.

Table 1. Soil chemical properties and DTPA-extractable Fe, Zn, Mn, and Cu concentrations for thirty seven soil samples from seven different agricultural regions in Saudi Arabia.

Region and Sample ID		pH	$CaCO_3$ [%]	ECe [dS m^{-1}]	Fe	Zn	Mn	Cu
					[µg g^{-1}]			
Kharj	1	7.7	12.2	2.7	2.80	0.20	0.72	0.41
	2	7.5	23.0	5.4	4.00	3.70	4.82	0.52
	3	8.0	16.8	8.3	3.61	0.30	1.27	0.31
	4	7.9	19.9	6.2	2.95	0.52	2.31	0.28
	5	8.1	22.2	3.3	3.40	0.43	2.50	0.22
Mean		7.84	18.82	5.18	3.35	1.03	2.32	0.35
SD		±0.24	±4.41	±2.26	±0.49	±1.50	±1.58	±0.12
Delim	6	8.0	16.8	6.3	3.50	0.30	3.00	0.50
	7	7.8	6.9	2.8	3.66	0.52	3.30	0.24
	8	8.0	17.1	5.3	4.10	0.33	3.50	0.70
	9	7.5	24.6	8.5	6.70	1.41	6.00	0.40
	10	7.4	17.8	4.4	1.10	0.32	2.50	0.34
Mean		7.74	16.64	5.46	3.81	0.58	3.66	0.44
SD		±0.28	±6.32	±2.13	±1.99	±0.47	±1.36	±0.18
Qassim	11	7.6	4.6	6.0	3.00	0.24	4.20	0.38
	12	7.5	14.0	7.5	7.50	0.48	6.30	1.1
	13	7.9	9.7	1.7	3.30	0.50	6.30	0.56
	14	7.9	3.8	1.7	3.30	0.48	1.90	0.22
	15	8.0	12.3	3.5	2.92	0.43	2.2	0.30
Mean		7.78	8.88	4.08	4.00	0.43	4.18	0.51
SD		±0.22	±4.55	±2.60	±1.96	±0.11	±2.13	±0.35
Hail	16	7.8	8.9	1.7	3.00	0.26	3.4	0.26
	17	8.1	12.7	5.2	4.10	0.48	2.98	0.32
	18	7.9	18.3	3.3	2.73	0.33	4.10	0.54
	19	7.8	17.3	2.8	3.42	0.41	2.20	0.44
	20	7.8	17.5	2.9	3.30	0.38	2.18	0.38
Mean		7.9	14.94	3.18	3.30	0.37	2.97	0.40
SD		±0.13	±4.03	±1.28	±0.52	±0.08	±0.82	±0.11
Jouf	21	7.8	15.5	3.8	3.20	0.37	2.82	0.37
	22	7.7	14.3	5.5	4.53	0.29	3.44	0.28
	23	7.7	7.8	2.2	5.22	1.20	5.22	0.79
	24	7.6	8.2	4.1	3.21	0.80	3.85	0.41
	25	7.9	5.7	3.6	2.30	0.66	3.40	0.32
Mean		7.7	10.3	3.84	3.69	0.66	3.75	0.43
SD		±0.11	±4.33	±1.18	±1.17	±0.36	±0.90	±0.21
Wadi Dawasir	26	8.2	10.1	4.2	3.84	0.28	1.55	0.28
	27	7.8	13.8	7.40	3.00	0.32	0.50	1.44
	28	8.1	9.9	8.21	2.90	0.31	0.48	0.42
	29	7.3	8.2	11.51	1.10	0.32	0.60	0.30
	30	7.9	12.4	3.62	3.35	0.27	1.62	0.29
Mean		7.9	10.88	6.99	2.84	0.30	0.95	0.55
SD		±0.35	±2.21	±3.21	±1.04	±0.02	±0.58	±0.50
Taif	31	8.1	7.4	2.30	4.50	1.80	10.0	1.90
	32	7.7	11.5	3.30	3.50	0.86	7.80	1.14
	33	7.8	3.3	3.70	11.7	2.00	8.00	2.80
	34	7.7	4.5	1.00	3.66	0.22	4.70	0.64
	35	7.5	12.5	1.20	3.30	0.44	7.60	1.48
	36	7.4	3.8	1.50	9.10	3.20	13.0	1.82
	37	7.3	5.1	5.80	3.80	1.96	4.40	0.74
Mean		7.6	6.87	2.69	5.65	1.50	7.93	1.5
SD		±0.27	±3.75	±1.72	±3.65	±1.05	±2.97	±0.75
Mean, pooled		7.8	12.17	4.39	3.91	0.74	3.91	0.64
SD, pooled		±0.24	±5.77	±2.41	±1.98	±0.81	±2.74	±0.57

Table 2. Chemical and Physical properties of the soil samples representing all the agricultural regions of Saudi Arabia.

Location	pH	ECe (dSm^{-1})	$CaCO_3$	OC[a]	Sand	Silt	Clay	Textural Class
					(%)			
Kharj	7.7	2.7	12.2	0.10	92	2	6	Sand
Delim	8.0	6.3	16.8	0.10	43	25	32	Clay Loam
Qassim	7.6	6.0	4.6	0.73	50	16	34	Sandy Clay Loam
Hail	7.8	1.7	8.9	0.11	81	8	11	Sandy Loam
Jouf	7.8	3.8	15.5	0.10	79	10	11	Sandy Loam
Wadi D	8.2	4.2	10.1	0.24	87	8	5	Sand
Taif	8.1	2.3	7.4	0.92	50	31	19	Loam

a OC: Organic Carbon

It is interesting to notice that the highest mean concentrations for Fe, Zn, Mn, and Cu are found in Taif region. A probable explanation would be the presence of relatively high organic matter in this region (see Table 2). The relationship between high organic matter and high levels of micronutrients is supported by previous studies from the same and similar regions (Bashour et al., 1983; Al Jaloud et al., 1995). On the other hand, most of the low mean concentrations for Fe, Zn, Mn, and Cu are found in Wadi Dawassir region, most likely due to high pH, high $CaCO_3$ content, and high salinity. Iron is low in 31 of the 37 soil samples, and Zn is low in 28 of 37, when related to the critical levels of Lindsay and Norwell (1978). None of the surface samples is low in Cu, and only 4 samples are low in Mn. Tisdale et al. (1993) reported that calcareous soils, soils low in organic matter, soils with pH higher than 7.0, leached sandy soils, and newly leveled soils are frequently low in Fe and Zn. Most of these conditions appear to exist in nearly all the soils of the present study, and could be the cause for the observed Fe and Zn deficiencies. These findings confirm earlier studies by Ramoliya et al. (2004) who reported that high salinity decreased Zn and Fe accumulation in plants, whereas Cu and Mn remained nearly unchanged.

Hagen and Tucker (1982) reported that Mn deficiency may occur in aerated alkaline calcareous soils irrespective of texture or organic matter content. They found that Mn availability was affected by soil oxidation-reduction conditions. Similar results were reported by EI Fouly (1983) on calcareous soils in Egypt. Generally, the presence of free $CaCO_3$, alkaline pH values, and the medium to coarse texture of the soil samples may account for Fe and Zn deficiencies in the soils (Table 2). These findings could not be supported by the present data; in fact, there is hardly any correlations (Figure 1) between $CaCO_3$ and DTPA-Fe ($y = -0.064x + 4.698$ with $R^2 = 0.035$), and between $CaCO_3$ and DTPA-Zn ($y = -0.011x + 0.871$ with $R^2 = 0.006$). A steeper slope and higher R^2 are expected for this relationship (Figure 1 A and B). Further research and investigation is required for better understanding of this poor relationship. Deficiency of Fe and Zn is common in Saudi Arabia's calcareous soils (AI-Jaloud et al., 1995); Prasad et al., 1984). Similar findings were reported earlier for Fe and Zn deficiency in calcareous soils (Loizides, 1975; Jones and Kelso, 1977).

The present results indicate that the soils are adequate in DTPA-extractable Mn and Cu for crop cultivation, and are generally medium to high in the studied calcareous soils, in all the regions representing nearly the whole country of Saudi Arabia. Fuehring (1973) reported that Mn and Cu are much less susceptible to be deficient in calcareous soils than Fe and Zn. These findings are supported by our data; in fact the lack of correlation between $CaCO_3$ and DTPA-Mn ($y = -0.130x + 5.493$ $R^2 = 0.075$), and DTPA-Cu ($y = -0.033x + 1.045$ $R^2 = 0.110$) prove that $CaCO_3$ content has no effect on extractable Mn and Cu (Figure 1 C, D).

In order to test and validate the present results, we investigated micronutrient availability in a separate project. Two large scale wheat and potato field trials were conducted in Delim and Jouf regions to study crop response to foliar micronutrient applications (Unpublished data). The application of iron as Fe-EDDHA at a rate of 3 kg ha^{-1} gave significant increase in yield for both crops. However, the addition of Zn in the form of Zn-EDTA at a rate of 2.5 kg ha^{-1} increased the yields of both crops, but the increase was not significant. Application of Mn and Cu did not lead to any increase in yield.

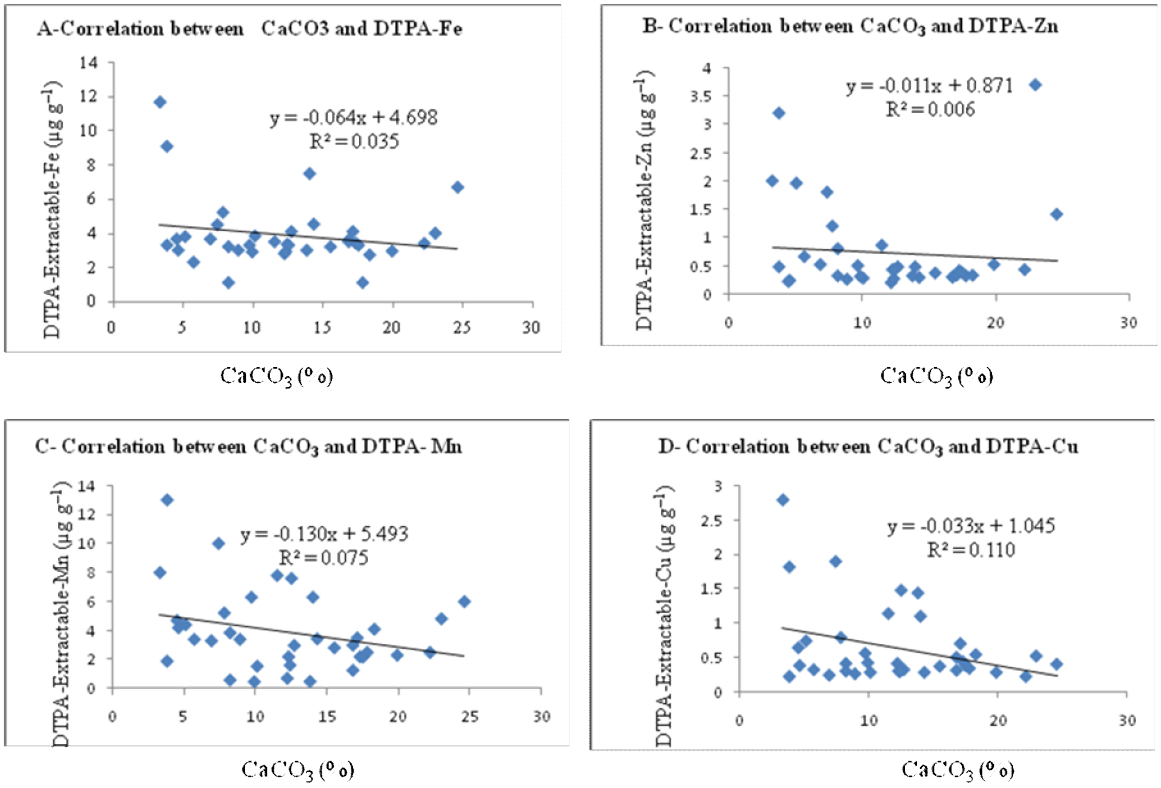

Figure 1. Correlation between $CaCO_3$ and DTPA-extractable iron, zinc, manganese and copper.

Fractionation of micronutrients

The concentrations of Fe, Zn, Mn, and Cu in the different fractions of the soils from all seven agricultural regions are given in Table 3.

Exchangeable fractions

The exchangeable fractions of micronutrients were by far the lowest among all fractions (Table 3). The exchangeable fractions of Zn and Cu in the seven soil samples were below the detection limits of the instrument. Out of the seven soil samples, 4 samples have exchangeable Fe at concentrations range 0.01-0.02 µg g^{-1}, and 5 samples have Mn in exchangeable form at concentration range 0.20-3.91 µg g^{-1}. Ahdy and Youssef (2011), studying the fractionation of heavy metals for environmental purposes, found similar results; in fact, they reported that Cu and Zn ranged from below the detection limit to 1.02 µg g^{-1}. Data from Okbah et al. (2011) confirm these findings; Zn and Mn ranged from 0.29 to 1.13 and 0.8 to 2.0 µg g^{-1}, respectively.

Carbonate-bound fractions

The carbonate bound fraction ranges from 2.2-16.1 µg g^{-1} for Fe, 0.5- l.4 µg g^{-1} for Zn, 12.1-52.30 µg g^{-1} for Mn, and 0.8-2.8 µg g^{-1} for Cu. Shober et al. (2007), studying Zn and Cu and heavy metals, reported similar concentration for Cu, but higher levels for Zn; this is possibly due to the lower pH and $CaCO_3$ values of Pennsylvania soils in the USA. Averaged over the seven geographic regions (see Table 3), the order of the metals in the CO_3 bound fraction is Mn (30.89 µg g^{-1}) > Fe (6.86 µg g^{-1}) > Cu (1.61 µg g^{-1}) > Zn (1.01 µg g^{-1}). Figure 2 shows that there is a very poor correlation between $CaCO_3$ and CO_3-bound Mn (R2 = 0.047). On the other hand, the carbonate contents of the soils are strongly related to the CO_3 bound fractions of Fe (R2 = 0.861), Zn (R2 = 0.685) and Cu (R2 = 0.527). This indicates that $CaCO_3$ content of the studied desert soils has a strong effect on the CO_3 – bound Fe, Zn, and Cu micronutrient fractions. These results agree with the findings of Mustafa et al. (1988) who reported that a strong relationship existed between carbonate-bound Zn contents of soil and the $CaCO_3$ content of soil.

Fe-Mn oxide bound fractions

The oxide bound fraction is by far the second largest among all fractions, after the residual fraction, especially for Fe and Mn. The Fe-Mn oxide bound fractions varies from 190-898 µg g^{-1} with a mean of 573.14 µg g^{-1} for Fe; 1.9-8.5 µg g^{-1} with a mean of 5.43 µg g^{-1} for Zn, 22.0-238.9 µg g^{-1}

with a mean of 109.30 µg g^{-1} for Mn, and 2.1-5.1 µg g^{-1} with a mean of 3.50 µg g^{-1} for Cu. Only Zn data agrees with earlier reports by Shober et al. (2007). These high concentrations are due to the fact that micronutrients are preferentially adsorbed on Fe oxide surfaces in soils (Parizanganeh et al., 2007). Similar results were found by Ahdy and Youssef (2011). Alidoust et al. (2012) reported that concentrations of Mn and Zn in the metal–organic complex in the rhizosphere zone decreased with proximity to the root, indicating that the Fe-Mn oxide fraction is an important source of these 2 trace elements for the plants. The coarse-textured soils have a lower amount of Fe, Zn, Mn and Cu in the Fe-Mn oxide bound fraction than the medium textured soils. It is not clear why there are strong negative correlations (Figure 3) between CaCO3 and Fe-Mn bound Zn (R2 = 0.529), and Mn (R2 = 0.888). The mean concentrations computed over the seven regions indicate that the metals in the Fe-Mn oxide bound fraction follow this order: Fe (573.1 µg g^{-1})> Mn (109.3 µg g^{-1})> Zn (5.4 µg g^{-1})> Cu (3.5 µg g^{-1}) (Table 3). It is possible that a part of the Fe and Mn extracted in this fraction is derived from the parent oxide minerals during extraction with Hydroxylamine hydrochloride (Jenne, 1968).

Table 3. Micronutrient concentration in the different fractions for the soils representing the seven geographic regions.

Element	Location	Exchangeable	CO_3 Bound	Fe-Mn Oxide bond	Organic Bound	Residual
				(µg g^{-1})		
	Kharj	0.01	14.0	698	10.4	14105
	Delim	0.01	5.3	542	8.1	20004
	Qassim	0.02	2.4	585	17.3	24800
Iron	Hail	ND	7.6	898	21.2	8425
	Jouf	ND	16.1	190	12.1	4504
	Wadi Dawassir	ND	5.7	510	9.2	4790
	Taif	0.02	2.2	589	55.5	48780
Mean		NA	7.61	573.14	19.11	17915
Percent of Total (%)			0.04	3.10	0.10	96.76
	Kharj	ND	1.1	4.3	0.01	56.1
	Delim	ND	1.2	5.8	0.01	96.4
	Qassim	ND	0.5	8.5	0.90	92.6
Zinc	Hail	ND	1.2	6.1	0.61	71.6
	Jaof	ND	1.4	1.9	0.01	52.3
	Wadi Dawassir	ND	1.1	4.2	0.5	63.0
	Taif	ND	0.6	7.2	1.3	108.0
Mean		NA	1.01	5.43	0.48	77.14
Percent of Total (%)			1.20	6.46	0.57	91.77
	Kharj	ND	39.0	27.7	3.2	164.2
	Delim	0.2	52.3	137.3	10.1	227.9
	Qassim	3.91	37.7	238.9	36.2	367.4
Manganese	Hail	0.3	12.1	130.4	13.2	215.3
	Jouf	ND	20.0	22.0	3.0	155.7
	Wadi Dawassir	0.64	21.3	76.1	6.7	232.1
	Taif	1.32	33.8	132.7	25.3	698.4
Mean		NA	30.89	109.30	13.96	294.43
Percent of Total (%)			6.88	24.37	3.11	65.64
	Kharj	ND	1.4	2.1	0.01	9.5
	Delim	ND	1.7	5.1	0.01	29.5
	Qassim	ND	0.8	2.4	0.9	40.3
Copper	Hail	ND	1.8	5.1	0.4	26.5
	Jouf	ND	2.8	3.0	0.01	24.6
	Wadi D	ND	1.8	4.3	0.30	20.3
	Taif	ND	1.0	2.5	2.40	58.6
Mean		NA	1.61	3.50	0.58	29.9
Percent of Total (%)			4.54	9.83	1.62	84.01

ND: Not Detected, less than the detection limits of the method of analysis.

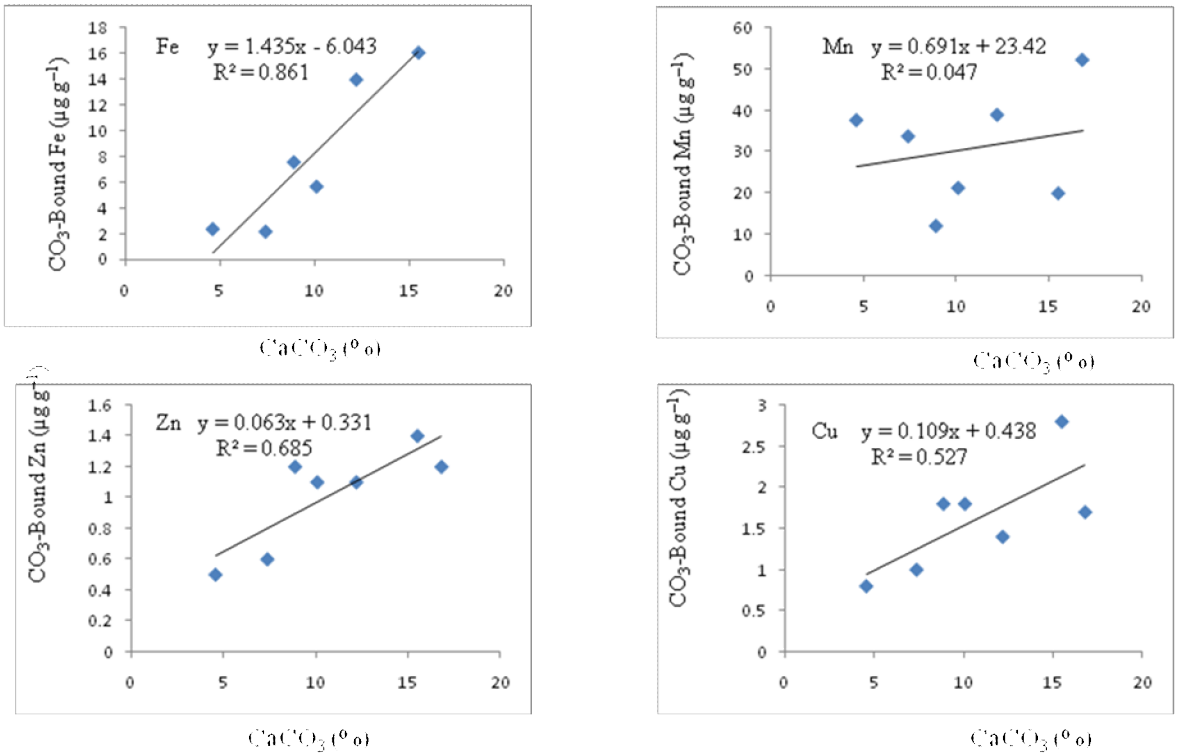

Figure 2. Correlation between CaCO3 and CaCO3 bound iron, zinc, manganese and copper.

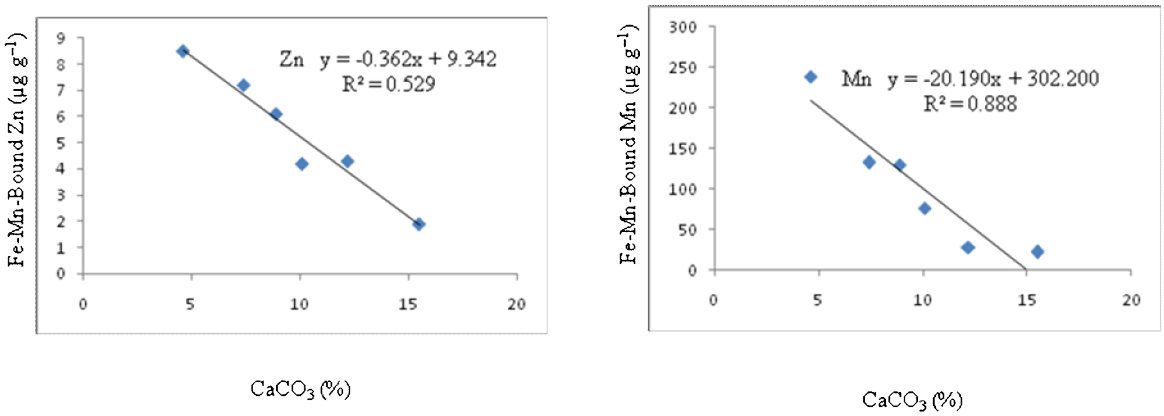

Figure 3. Correlation between CaCO3 and Fe-Mn zinc and manganese.

Organic-bound fractions

The organic bound fractions range is 8.1-55.5 µg g^{-1} with an average of 19.11 µg g^{-1} for Fe, 0.01-1.3 µg g^{-1} with an average of 0.48 µg g^{-1} for Zn, 3.0-36.2 µg g-1 with an average of 13.96 µg g^{-1} for Mn, and 0.01-2.4 µg g^{-1} with an average of 0.58 µg g^{-1} for Cu. The concentrations of metals present in the seven soil samples in organic bound form represent a low proportion of total metal concentrations. The higher percentage of organic carbon is found in soil samples collected from Taif, Qassim, and Hail regions (0.73-1.05%). The same samples contain higher concentrations of all four metals in the organic fraction. This indicates that organic bound micronutrients fraction is positively related to organic carbon in soil samples, as found earlier by Chanem and Mikkelsen (1987). A similar result was reported by Mandal and Mandal (1986). Shober et al. (2007) came to the same conclusion, and when soils were amended with additional organic matter, the organic-bound Cu and Zn concentrations increased significantly. Alidoust et al. (2012) found that the concentrations of organically bound Fe and Mn in soils decreased with the proximity to roots, suggesting that organic fraction is the main source for plant uptake. Averaged over the seven geographic regions (see Table 3), the order of metals in the organic bound

fraction is Fe (19.11 µg g^{-1}>Mn (13.96 µg g^{-1})> Cu (0.58 µg g^{-1}) >Zn (0.48 µg g^{-1}).

Residual fractions

The residual fraction of micronutrients is by far the largest among all fractions. In general, soils with high clay content contained higher amounts of residual micronutrients. Earlier research reported similar findings (Shober et al., 2007; Ahdy and Youssef, 2011; Okbah et al., 2011). The order of residual micronutrients, on average across regions, is in the following sequence: Fe (17915 µg g^{-1}) > Mn (294 µg g^{-1}) > Zn (77 µg g^{-1})> Cu (30 µg g^{-1}). The residual fractions in soil samples collected from Taif region is the highest among all samples. This could be due to the fact that the soil samples in Taif region were derived from a metamorphic origin, while the other soil samples in the study were derived from sedimentary rocks. Residual (non-available) Fe and Zn fractions, on average, constitute 97% and 92% of total soil fractions, respectively. While residual fraction of Mn and Cu constitute lower proportions of total soil fractions, and the means are 66% and 84% for Mn and Cu, respectively. Due to non-availability of the residual fraction to crops, little research and limited publications have dealt with this proportion of soil trace elements. Furthermore, total trace-element concentrations determined by strong acid digestions (residual), have been proven to be a poor indicator of trace-element levels in crop tissue (Qian et al., 1996; Sims and Kline, 1991). The results of the fractionation analysis of the seven soil samples collected from arid calcareous soils in Saudi Arabia indicate that concentrations of micronutrients in the different fractions are: Residual > Fe-Mn oxide bound > CO_3 bound > organic bound > exchangeable (negligible).

Appreciable amounts of trace elements are associated with the various fractions of the soils representing the different agricultural regions. Theses pools constitute important potential reserves of micronutrients; environmental and rhizosphere conditions will determine their mobilization capacity and bioavailability (Karimian and Ahangar, 1998; Ghasemi-Fasaei et al., 2009; Alidoust et al., 2012; Kasemaei and Fekri, 2012).

Combined carbonate-bound and organic-bound fractions of Fe, Zn, Cu, and Mn represent 3%, 6%, 15%, and 31% of total soil trace elements, respectively (Table 3). This important finding is a clear indication that Fe and Zn sources are negligible, whereas Cu and Mn are potentially available to crops in appreciable amounts in soils of the studied regions. These results also explain the absence of Cu and Mn deficiency in all agricultural regions of Saudi Arabia; moreover, they elucidate why Fe and Zn deficiencies are common in crops grown on theses soils; such deficiencies are easily corrected by application of Fe and Zn to these crops (Al Jaloud et al., 1995).

Conclusion

Mean soil pH ranged from 7.6 to 7.9, and $CaCO_3$ data (3.3-24.6%) indicate that the soils are mostly calcareous. Soil ECe data (1.0-11.5 dS m^{-1}) indicate the presence of saline soils in all regions except Taif. Micronutrients DTPA-extractable ranges were 2.8-5.6 µg g^{-1} for Fe, 0.3-1.5 µg g^{-1} for Zn, 0.9-7.9 µg g^{-1} for Mn, and for 0.4-1.5 µg g^{-1} for Cu. High mean concentrations for Fe, Zn, Mn, and Cu are linked to the presence of high organic matter; low mean concentrations for Fe, Zn, Mn, and Cu are associated with high pH and $CaCO_3$ %. Iron and Zn were low in most soils. Few soils were low in Cu and Mn. Thus, it is recommended that Fe and Zn be included in crop fertilization programs. Fractionation of micronutrients reveals that, when averaged across all regions, the order of the metals content in the CO_3 bound fraction was Mn>Fe>Cu>Zn; the oxide- bound fraction followed the order Fe>Mn>Zn>Cu; the organic bound fraction led to the sequence Fe>Mn>Cu>Zn; and the residual fraction was Fe>Mn>Zn>Cu. The fractionation study showed that the residual fraction was the highest, followed by the remaining fractions in this order: oxide bound>CO3 bound>organic bound>exchangeable.

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PLANT SCIENCE

Use of formulated nitrogen, phosphorus, and potassium compound fertilizer using clinoptilolite zeolite in maize (*Zea mays* L.) cultivation

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Abstract

Adoption of new management techniques, such as clinoptilolite zeolite (CZ) utilization has attracted much attention in the fertilizer industry. Accordingly, the aims of this study is to evaluate: if CZ, acting as an inert material, when applied to the soil, might improve the selected soil properties, height, dry matter, nutrient concentration, nutrient uptake, nutrient use efficiency on maize cultivation; the potential for (N:P:K) compound fertilizer when incorporated with CZ to serve same standard as the commercial fertilizer in fertilizer industry. The effect of T_1, T_3 and T_6 on soil total N was found to be significant, when compared with T_7. Treatments with CZ on soil total P, K and available P, K differed significantly relatively to T_1 and T_7. The treatments T_5 and T_6 had the highest accumulation of exchangeable NH_4^+ and available NO_3^- relatively to T_1 and T_7. The significant effect of the treatments having CZ on N concentration, in uptake and use efficiency, suggests that CZ incorporated with fertilizer can reduce NH_3 loss, triggering the formation of NH_4^+ and NO_3^- over ammonia and increase maize uptake. Relatively to P concentration, uptake and use efficiency, it was found that in most treatments having CZ, lower values were obtained, relatively to the commercial fertilizer, although T_3 clearly improved P uptake in roots. Most of the treatments with CZ remained statistically similar in K concentration, uptake and use efficiency compared to commercial fertilizer. It may be concluded that treatments with higher amounts clinoptilolite zeolite ensured good retention of soil exchangeable cations, available P, and NO_3^- within the soil. Treatments with CZ improved N uptake and use efficiency in the maize crop tested.

Key words: Clinoptilolite zeolite (CZ), N:P:K compound fertilizer, Nutrient uptake, Nutrient use efficiency, Selected soil properties.

Abbreviation lists: Atomic absorption spectrophotometry, AAS; Cation exchange capacity, CEC; Day after planting, DAP; Dry weight, DW; Muriate of potash, MOP; Triple Superphosphate, TSP

Introduction

Many studies have been conducted, mainly on nitrogenous fertilizers, to minimize any possible adverse environmental effects (Trenkel, 2010). However, less attention has been devoted to the release of nutrients from N:P:K compounds, in which case the processes are expected to be more complex than with a single nutrient fertilizer. Adoption of new management techniques, such as using clinoptilolite zeolite to control nutrient release from compound fertilizers had in recent times attracted the attention in the fertilization systems. Clinoptilolite zeolite is a porous mineral with high CEC up to 3000 mol cm^{-3} and with great affinity for NH_4^+ (Ming and Mumpton, 1989), yet it can physically protect NH_4^+ against microbial nitrification (Ferguson and Pepper, 1987). Clinoptilolite zeolite, which maximizes N use efficiency and water use efficiency, may decrease environmental degradation through the use of balanced fertilizers in agriculture (Ramesh and Reddy, 2011). Slow release of N fertilizers can be produced by amending N fertilizers, such as urea with clinoptilolite zeolite (Omar et al., 2011; Ahmed et al., 2008). The mechanism for slow release of K may be similar to the reactions shown for PO_3^- and NH_4^+. This is possible because of the sequestering effect of the exchanger of clinoptilolite zeolite (Payra and Dutta, 2003; Lai and Eberl, 1986). According to Gholizadeh (2008), clinoptilolite

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zeolite when saturated with monovalent nutrient cations, such as NH_4^+ and K^+, increases the solubility of phosphate rock. Besides, clinoptilolite zeolite acts as carriers of nutrients for cations and as a medium with free exchangeable nutrient (Perez et al., 2008). Clinoptilolite zeolite has also been reported to have a relatively high cation adsorption capacity (Oste et al., 2001; Li et al., 2001; Mineyev et al., 1990). Accordingly, the aims of this study is to evaluate: if clinoptilolite zeolite, acting as an inert material, when applied to the soil, might improve the selected soil properties, height, dry matter, nutrient concentration, nutrient uptake, nutrient use efficiency on maize cultivation; the potential for (N:P:K) compound fertilizer when incorporated with clinoptilolite zeolite to serve same standard as the commercial fertilizer in fertilizer industry.

Materials and Methods

A pot trial study was conducted to test the selected N:P:K compound fertilizers in a completely randomized block design with 3 replications. The soil used in this study was a *Tipik Tualemkuts* (Bekenu Series). Soil samples were taken at 0-15 cm depth using an auger, air dried and ground to pass a 5 mm sieve. The treatments evaluated were:

i. 14.88 g commercial compound fertilizer with ratio 15:15:15 (control) (T1)
ii. Compound fertilizer with ratio 15:15:15 (4.85 g urea + 4.85 g TSP + 3.72 g MOP) mixed with 1.46 g clinoptilolite zeolite (T2)
iii. Compound fertilizer with ratio 13:15:13 (4.85 g urea + 5.61 g TSP + 3.73 g MOP) mixed with 3.01 g clinoptilolite zeolite (T3)
iv. Compound fertilizer with ratio 10:10:10 (4.85 g urea + 4.85 g TSP + 3.73 g MOP) mixed with 8.92 g clinoptilolite zeolite (T4)
v. Compound fertilizer with ratio 8:8:8 (4.85 g urea + 4.85 g TSP + 3.71 g MOP) mixed with 14.46 g clinoptilolite zeolite (T5)
vi. Compound fertilizer with ratio 5.5:5.5:5.5 (4.85 g urea + 4.85 g TSP + 3.72 g MOP) mixed with 27.04 g clinoptilolite zeolite (T6)
vii. Soil alone (control) (T7)

The amounts of the compound fertilizers applied were based on N standard requirements for mature Masmadu Maize (Malaysian Agricultural Research and Development Institute, 1990). The fertilizers were surface applied at the 9th DAP and 27th DAP. Two out of eight seedlings were maintained for observation before first fertilization in pots (25 cm of top diameter x 21 cm of height).

The clinoptilolite zeolite was powder applied. The pH of the soil and clinoptilolite zeolite were determined in a ratio of 1:2.5 soil:distilled water suspension and 1M KCl solution using a glass electrode (Tan, 2005). Soil organic carbon was determined using Loss on Ignition (Piccolo, 1996). Soil total N was determined by the Kjeldahl method (Tan, 2005). Soil total P and K were extract using aqua regia (Tan, 2005). Filtrates were analyzed for K by AAS and UV-spectrometer for P. Soil CEC was determined by leaching with ammonium acetate buffer adjusted to pH 7.0 followed by steam distillation (Bremmer, 1965) and exchangeable K using AAS. The CEC of clinoptilolite zeolite was determined using the CsCl method (Ming and Dixon, 1986). Exchangeable K of the clinoptilolite zeolite was extracted using the method of Ming and Dixon (1986) and the concentration determined as previously outlined. The soil texture followed the hydrometer method (Tan, 2005). The exchangeable NH_4^+ and available NO_3^- were extracted from the soil by the method of Keeney and Nelson (1982), being the amounts determined using the steam distillation method (Tan, 2005).

The plants were monitored for 65 days. At the 65th DAP, the plants were harvested and partitioned into leaves, roots and stems. These parts were oven-dried until a constant weight at 60˚C and recorded for their mass of dry weight. At the 65th DAP, soil samples were taken and analyzed for total and exchangeable N, P and K, exchangeable NH_4^+, available NO_3^- and pH as previously outlined. Nitrogen concentrations in the selected plant parts were determined by the Kjeldahl (Bremmer, 1965), while the single dry ashing method (Cottenie, 1980), for the extraction of P and K in the plant tissues. The filtrates were analyzed for K by AAS and UV-spectrometer for P. The concentrations of N, P and K in the plant parts were multiplied by their dry matter to obtain the amount of N, P and K taken up by the plant parts. Nitrogen, P, and K use efficiency were calculated using the subtraction method (Pomares-Gracia and Pratt, 1987). Analysis of variance (ANOVA) was used to test the treatment effects and means of treatments were compared using Tukey's test.

Results

Selected soil physico-chemical properties before planting

The selected physio-chemical properties of *Tipik Tualemkuts* (Bekenu Series) and clinoptilolite zeolite are presented in Table 1. The soil pH (water), exchangeable K and texture were consistent with the report of Paramananthan (2000). Total organic carbon and CEC contents were higher than the standard range probably due to previous cultivation.

Table 1. Selected chemical and physical properties of Bekenu Series and clinoptilolite zeolite.

Property	Soil (0-15cm)		Clinoptilolite zeolite
	Value obtained	Standard data range	
pH(water)	4.70	4.6-4.9	7.03
pH(0.01M KCl)	3.43	3.8-4.0	6.37
Total organic carbon (%)	3.6	0.57-2.51	Nd
CEC(cmol kg^{-1})	12.8	3.86-8.46	75.4
Exchangeable K (cmol kg^{-1})	0.174	0.05-0.19	22.29
Texture	SL	SL	Nd
Bulk density(gm^{-3})	1.01	Nd	Nd

CEC, Cation exchange capacity; SL, Sandy Loam; nd, not determined; standard data range (Paramananthan, 2000).

Table 2. Effect of treatments on selected soil physico-chemical properties at 65 DAP.

Treatment	pH (water)	Total			Available	Exchangeable
		N (%)	P	K ($mgkg^{-1}$)	P	K
T1	4.30^{d}	0.15^{a}	342.5^{c}	110.6^{d}	188.8^{c}	82.0^{cd}
T2	4.70^{c}	0.15^{a}	1412.5^{ab}	679.6^{c}	606.4^{a}	412.6^{c}
T3	4.69^{c}	0.11^{ab}	969.8^{b}	599.6^{c}	487.4^{ab}	346.5^{cd}
T4	4.92^{bc}	0.11^{ab}	1519.8^{a}	1132.1^{b}	423.0^{b}	835.7^{b}
T5	5.09^{b}	0.08^{ab}	1587.5^{a}	1407.5^{b}	495.0^{ab}	1144.7^{ab}
T6	5.50^{a}	0.13^{a}	1707.8^{a}	1886.9^{a}	463.3^{b}	1293.6^{a}
T7	4.65^{dc}	0.05^{b}	65.3^{c}	53.8^{d}	22.13^{d}	5.2^{d}

Different letters (with column) indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

Soil pH, total N, P and K and available P and K at 65 DAP

The soil pH (water), total N, P and K and available P and K after harvest under fertilized and unfertilized condition are summarized in Table 2. At harvest, the soil pH (water) for T1 (commercial fertilizer) and T7 (soil alone) were 4.30 and 4.65. The soil pH (water) was significantly affected in the treatments with higher amounts of clinoptilolite zeolite contents (T5 and T6). The effect of T1, T3 and T6 on the soil total N was significant (when compared with T7). The effects of the treatments with clinoptilolite zeolite on soil total P and available P differed significantly relatively to T1 and T7. Soil total K of all treatments with clinoptilolite zeolite significantly increased compared to T1 and T7. T6, showing the treatment with the highest content of clinoptilolite zeolite the highest soil K content (1886.9 mg kg^{-1}). T6, T5 and T4, treatments with higher clinoptilolite zeolite significantly increased soil exchangeable K content, relatively to the other treatments.

Exchangeable NH_4^+ and available NO_3^- accumulation at 65 DAP

There were significant differences in the accumulation of exchangeable NH_4^+ and available NO_3^- for all treatments (Table 3). In the case of soil exchangeable NH_4^+, T6 showed a significant effect (when compared to the other treatments, except for T5). T5 and T6 showed the highest accumulation of available NO_3^-.

Plant height at 65 DAP

The height of maize at 65 DAP is presented in Figure 1. T6, treatment with the highest clinoptilolite zeolite significantly increased the height of maize plants compared to T2, T4 and T7, but its effect was statistically similar to those of T5, T3 and T1.

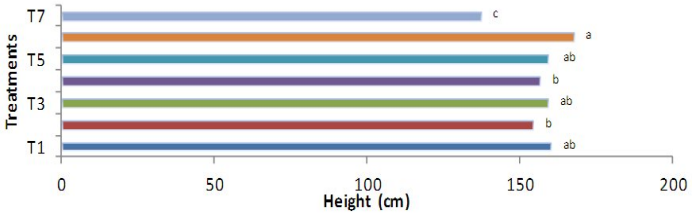

Figure 1. Height of maize plant at the 65th DAP. Different letters indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

Table 3. Effect of treatments on exchangeable NH_4^+ and available NO_3^- accumulation at 65 DAP.

Treatment	T1	T2	T3	T4	T5	T6	T7
NH_4-N (%)	0.07^{cd}	0.07^{cd}	0.06^{cd}	0.12^{bc}	0.18^{ab}	0.21^{a}	0.04^{d}
NO_3-N (%)	0.007^{c}	0.007^{c}	0.007^{c}	0.014^{b}	0.021^{a}	0.021^{a}	0.007^{c}

Different letters (with row) indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

Table 4. DW of maize at the 65th DAP

Dry weight (g plant^{-1})	Stem	Leaf	Root	Total
T1	11.13^{ab}	18.60^{a}	13.22^{a}	42.95^{a}
T2	10.35^{b}	17.89^{a}	13.29^{a}	41.52^{a}
T3	14.03^{a}	17.76^{a}	16.10^{a}	47.89^{a}
T4	10.94^{ab}	19.15^{a}	14.53^{a}	44.61^{a}
T5	13.65^{a}	17.69^{a}	12.62^{a}	43.95^{a}
T6	12.47^{ab}	17.41^{a}	11.20^{a}	41.09^{a}
T7	6.22^{c}	10.00^{b}	10.74^{a}	28.91^{b}

Different letters (with column) indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

Dry weight at 65 DAP

The DW of maize stems, leaves and roots and total DW are presented in Table 4. In the case of dry matter production (stem, leaves and total DW), T1 to T6 caused significant effect compared to T7. Regardless all the treatment, roots dry matter production was not statistically different.

N, P, and K concentrations

Nitrogen, P, and K concentrations in stems, leaves and roots are presented in Table 5. Except for root, N concentration for all treatments differed statistically. In the case of stem, T1, T2 and T4 caused the highest concentrations of N (when compared to the T7). In the leaves, N concentrations for T1, T5 and T6 were statistically higher, relatively to that of T7. The differences in P and K concentrations in the stems, leaves and roots for all the treatments were significant. For P concentrations in the stems, T1, T2 and T3 caused significantly higher amounts compared to T7. Among these treatments, T1 caused the highest P accumulation (when compared to the other treatments). Except for T1, P concentration in roots, were statistically similar to that of T6. T1, T2, T3, T4, T5 and T6 improved K concentrations in all the parts of maize compared with T7. T1 and treatments with clinoptilolite zeolite did not statistically differ in K concentrations for all stems and leaves. T1 caused the highest K accumulation in the roots, when compared to the other treatments. Overall, all treatments with clinoptilolite zeolite significantly improved N and K concentrations in all plant parts relatively to T7. Only T1 clearly improved P concentrations in all plant parts.

Table 5. Effect of treatments on N, P, and K concentrations in maize plant parts at 65 DAP.

Treatment	N	P (%)	K
Stems			
T1	1.16^{a}	0.130^{a}	1.65^{ab}
T2	1.04^{a}	0.110^{ab}	1.95^{a}
T3	0.62^{c}	0.100^{bc}	1.41^{ab}
T4	1.00^{ab}	0.080^{bcd}	1.51^{ab}
T5	0.65^{bc}	0.070^{d}	1.12^{bc}
T6	0.86^{abc}	0.077^{cd}	1.80^{ab}
T7	0.56^{c}	0.063^{d}	0.40^{c}
Leaves			
T1	1.42^{ab}	0.17^{a}	1.53^{a}
T2	1.66^{a}	0.11^{b}	1.47^{a}
T3	0.60^{c}	0.11^{b}	1.43^{a}
T4	1.49^{ab}	0.11^{b}	1.40^{a}
T5	1.66^{a}	0.09^{b}	1.30^{a}
T6	1.76^{a}	0.09^{b}	1.35^{a}
T7	0.73^{bc}	0.07^{b}	0.54^{b}
Roots			
T1	1.36^{a}	0.13^{a}	1.15^{a}
T2	1.20^{a}	0.09^{ab}	0.70^{b}
T3	1.28^{a}	0.10^{ab}	0.79^{b}
T4	1.45^{a}	0.08^{ab}	0.62^{b}
T5	1.30^{a}	0.09^{ab}	0.61^{b}
T6	1.32^{a}	0.07^{b}	0.64^{b}
T7	1.07^{a}	0.09^{ab}	0.19^{c}

Different letters indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

N, P, and K uptake

The influence of treatments on N, P and K uptake in plant stems, leaves and roots are summarized in Figure 2. In the case of N uptake in stem, all treatments showed no significant difference. However, treatments with clinoptilolite zeolite caused significantly higher effect compared to T7, but its effect was statistically similar to that T1 (in terms of N uptake in leaves). Only T3

statistically improved N uptake in roots (as compared to T7). All treatments enhanced P and K uptake in the stem, leaves and roots (Figures 3, 4). Treatments with clinoptilolite zeolite significantly increased P and K uptake, relatively to T7 in all plant parts. It was observed the effect of T1 was statistically similar with those of T2 and T3 for P uptake in stem. T1 significantly improved P uptake in leaves (when compared to T7). Only T3 statistically improved P uptake in roots, as compared with T7. Higher P uptake in roots was attained because T3 had higher rock phosphate content (13:15:13 ratio of N:P:K), when compared to the other treatments with clinoptilolite zeolite. The effect of T1 on K uptake in stem and leaves was statistically similar to those of T2, T3, T4, T5 and T6. However, only T1 significantly improved K uptake in the roots (as compared to T7). In Figure 5, T2, T3, T4, T5 and T6 significantly enhanced total N, P and K uptake (when compared to T7) in stem, leaves and roots (when compared with T7). However, total N and K uptake triggered by T2, T3, T4, T5 and T6 were not statistically different from T1. T1 caused the highest P uptake compared to other treatments. A similar trend was observed for N and K uptake whereby all treatments with clinoptilolite zeolite significantly differed in the effect on N and K uptake.

Table 6. Nitrogen, K and P use efficiency of maize plant parts after 65 DAP.

Treatments	N	P	K
		%	
Stems			
T1	4.19^{a}	1.08^{a}	8.31^{ab}
T2	3.18^{a}	0.73^{ab}	9.19^{ab}
T3	2.29^{a}	0.90^{ab}	7.68^{ab}
T4	3.23^{a}	0.48^{b}	7.37^{ab}
T5	2.82^{a}	0.58^{ab}	7.21^{b}
T6	4.00^{a}	0.62^{ab}	11.74^{a}
Leaves			
T1	8.68^{a}	3.02^{a}	10.91^{a}
T2	6.08^{ab}	1.44^{b}	11.01^{a}
T3	1.54^{b}	1.45^{b}	8.71^{b}
T4	10.23^{a}	1.50^{b}	10.67^{a}
T5	9.99^{a}	1.05^{b}	7.44^{b}
T6	9.34^{a}	0.86^{b}	8.85^{b}
Roots			
T1	2.14^{b}	1.04^{ab}	7.50^{a}
T2	2.67^{ab}	0.53^{b}	3.93^{b}
T3	6.21^{a}	1.10^{a}	3.70^{b}
T4	5.63^{ab}	0.50^{b}	4.28^{b}
T5	3.87^{ab}	0.21^{b}	2.94^{c}
T6	1.95^{b}	0.27^{b}	2.33^{c}

Different letters indicate significant different between means using Tukey's test at P ≤ 0.05. For key to treatments see Materials and methods.

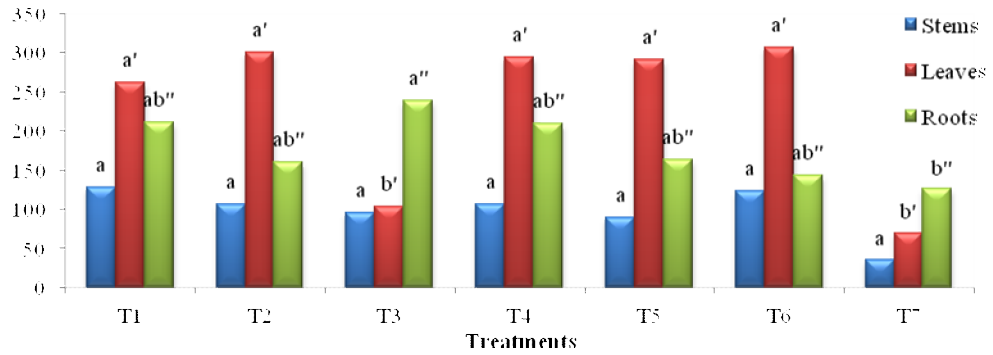

Figure 2. Effect of treatments on N uptake of maize (stems, leaves and roots) at 65 DAP. Different letters indicate significant difference between means using Tukey's test at P ≤ 0.05. For key to treatments see Materials and methods. Note: Letters without prime represent stems; single prime superscript is for leaves and double prime superscript is roots.

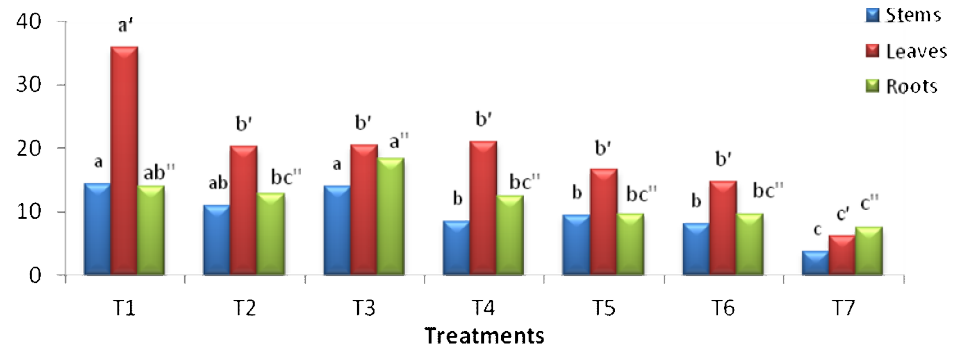

Figure 3. Effect of treatments on P uptake of maize (stems, leaves and roots) at 65 DAP. Different letters indicate significant difference between means using Tukey's test at P ≤ 0.05. For key to treatments see Materials and methods. Note: Letters without prime represent stems; single prime superscript is for leaves and double prime superscript is roots.

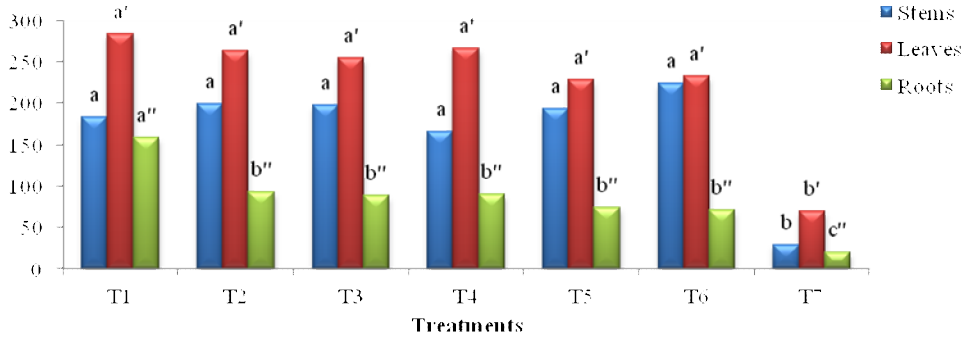

Figure 4. Effect of treatments on K uptake of maize (stems, leaves and roots) at 65 DAP. Different letters indicate significant different between means using Tukey's test at P ≤ 0.05. For key to treatments see Materials and methods. Note: Letters without prime represent stems; single prime superscript is for leaves and double prime superscript is roots.

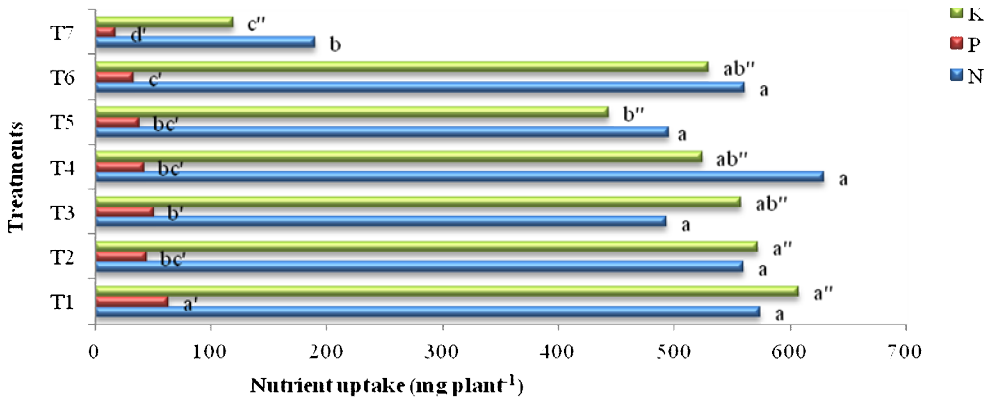

Figure 5. Effect of treatments on total N, P, and K uptake of maize at 65 DAP. Different letters indicate significant different between means using Tukey's test at P≤ 0.05. For key to treatments see Materials and methods. Note: Letters without prime represent Nitrogen; single prime superscript is for Phosphorus and double prime superscript is Potassium.

N, P, and K use efficiency

The effect of treatments on N, P and K use efficiency in stems, leaves and roots are shown in Table 6. Except for stems, there were significant differences for leaves and roots in N use efficiency for all treatments. T1, T4, T5 and T6 caused higher

N use efficiency in the leaves (when compared to T3). However, T3 caused higher N use efficiency in roots, relatively to the T1 and T6. The effect of T1, T2, T3, T5 and T6 were statistically similar in P use efficiency for stems. T1 had the highest effect on P use efficiency in the leaves, relatively to the treatments with clinoptilolite zeolite. T3 caused higher P use efficiency (when compared to T2, T4, T5 and T6). However, its effect was similar to that of T1. In the case of K use efficiency, T6 caused higher effect in stems, when compared to T5. However, T6 was statistically similar to other treatments. T1, T2 and T4 significantly improved K use efficiency in the leaves compared to T3, T5 and T6. In the roots, K use efficiency significantly improved in T1 (relatively to the treatments with clinoptilolite zeolite). However, T2, T3 and T4 significantly affected total K use efficiency in roots compared to T5 and T6. From Figure 6, T1, T2, T4 and T6 showed similar total N use efficiency. In the case of P use efficiency (Figure. 7), T1 showed the best total P use efficiency compared to treatments with clinoptilolite zeolite but that of T3 was higher compared to T6. The effect of T3 was similar to those of T2, T4 and T5. Total K use efficiency for T1, T2 and T3 were significantly higher compared with that of T6 (Figure. 8). Overall, with the exception of T1, treatments with higher clinoptilolite zeolite (T4, T5 and T6) significantly improved N use efficiency (when compared to the other treatments). In the case of the overall P use efficiency, treatments with lower clinoptilolite zeolite content (T2 and T3) significantly enhanced it compared to T4, T5 and T6 but lower than that of T1.

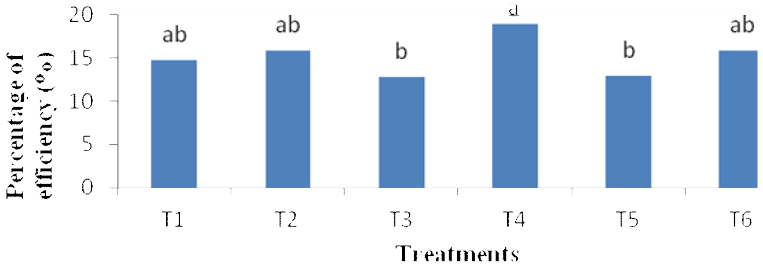

Figure 6. Effect of treatments on total N use efficiency at 65 DAP. Different letters indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

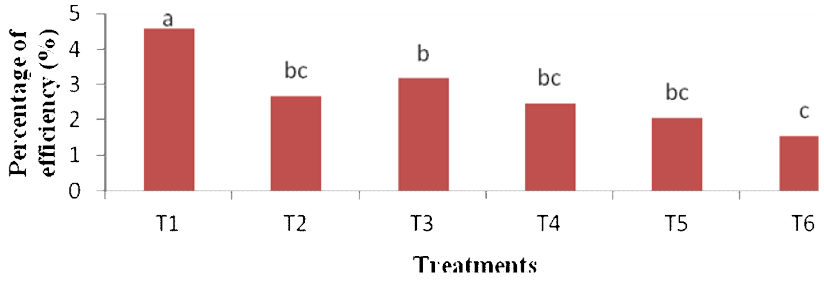

Figure 7. Effect of treatments on total P use efficiency at 65 DAP. Different letters indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

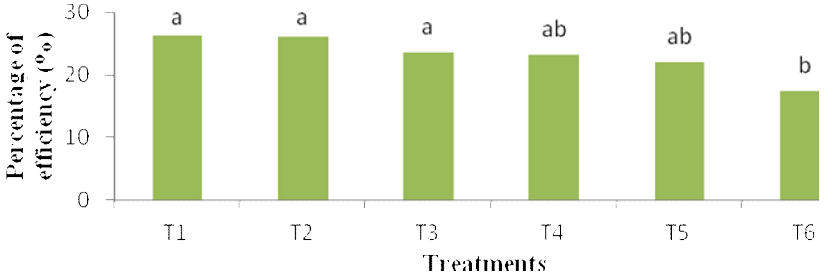

Figure 8. Effect of treatments on K use efficiency at 65 DAP. Different letters indicate significant different between means using Tukey's test at $P \leq 0.05$. For key to treatments see Materials and methods.

Discussion

Soil pH, total and available N, P, and K at 65 DAP

There were significant effects of the treatments with clinoptilolite zeolite on soil pH, total N, P and K and available P and K. This was because of the compatibility of the clinoptilolite zeolite with mineral fertilizers to attain soil buffering and indirectly regulate the soil pH (Polat et al., 2004; Mumpton, 1999). This observation agrees with the reports of Ahmed et al. (2009) and Junrungreang et al. (2002), who also reported significant improvement of in soil chemical properties upon the application of clinoptilolite zeolite. The large internal porosity of clinoptilolite zeolite allows high cation exchange capacity for nutrients retention especially selective retention of NH_4^+ and K^+. Clinoptilolite zeolite also might favoured the release of soluble P when Ca^{2+} ions are exchanged with NH_4^+ or K^+ ions.

Exchangeable NH_4^+ and available NO_3^- accumulation at DAP

The significant effects of the treatments with clinoptilolite zeolite on exchangeable NH_4^+ and available NO_3^- accumulation suggest that clinoptilolite zeolite incorporated in fertilizer could be because of the high CEC of clinoptilolite zeolite (75.4 cmol kg^{-1}). Clinoptilolite zeolite can store NH_4^+ and NO_3^- temporally in the internal before it is slowly released and becomes readily for plant uptake (Mumpton, 1999).

Plant height at 65 DAP

A similar finding was reported by Junrungreang et al. (2002), stating that the chemical fertilizer with the highest content of clinoptilolite zeolite improved the height, diameter, and yield of sugarcane. However, in another study, the highest increase of weight or length ratio of maize primary root was obtained for treatment with micronized clinoptilolite at the lower dose of 0.1% compared to higher dose of 3.0% (Trinchera et al., 2010).

Dry weight at 65 DAP

In several studies, the dry matter production of test crops, such as silage corn, strawberries, and swan significantly improved when fertilizers were enriched with clinoptilolite zeolite (Bernardi et al., 2010a; Ahmed et al., 2009; Rehakova et al., 2004). In the findings of Bernardi et al. (2010b), urea coated with inhibitor, produced a statistically similar rygrass yield (when compared to the urea coated with clinoptilolite zeolite). This observation is in agreement with the findings of this study, where the effect of the commercial fertilizer (T1) was statistically similar compared to treatments with clinoptilolite zeolite (T2, T3, T4, T5 and T6). According to Mishra and Shrivastava (1985), availability of NH_4^+ and NO_3^- in soils leads to increase in dry matter production of maize primary leaves.

N, P, K concentrations, uptake, and use efficiency

The significant effects of the treatments with clinoptilolite zeolite on N concentration, uptake, and use efficiency suggest that clinoptilolite zeolite incorporated in fertilizer can reduce NH_3 loss by encouraging formation of NH_4^+ and NO_3^- over NH_3 (Ahmed et al., 2008). NH_3 loss reduces N losses and increases its uptake in maize. Higher amount of clinoptilolite zeolite may have retained higher exchangeable NH_4^+ and available NO_3^- hence the higher N concentration, uptake, and use efficiency in the maize dry matter production. In terms of P concentration, uptake, and use efficiency, most of the treatments with clinoptilolite zeolites showed lower P content than the commercial fertilizer but T3 clearly improved P uptake in roots. This may due to the higher TSP content in T3. This might have increased the solubility of the phosphate in the TSP. According to Mumptom (1999), the equilibrium of soil solution is disturb when small amount of Ca^{2+} in the soil solution with the apatite exchanges onto the zeolite forces more Ca^{2+} into solution. The apatite is ultimately destroyed, releasing P into the soil solution. The zeolite with rock phosphate combination possibly acted as an exchange fertilizer, with Ca^{2+} exchanging onto the zeolite in response to plant uptake of nutrient cations enhancing the dissolution of the rock phosphate (Ramesh and Reddy, 2011; Lai and Eberl, 1986). Most of the treatments with clinoptilolite zeolite had similar effect on K concentration, uptake, and use efficiency compared to T1. This suggests that clinoptilolite zeolite incorporated in the fertilizers enhanced maize growth. Most cations such as K^+, Ca^{2+} and Mg^{2+} neutralize the negative charges in the zeolite tunnels. According to Gholizadeh (2008), some natural zeolites contain considerable amounts of exchangeable K^+ that can improve plant growth in potting media. This suggests that clinoptilolite zeolite does play an important role as nutrients carriers and ensuring good retention of soil exchangeable cations within the soil.

Conclusion

Treatments with higher amounts clinoptilolite zeolite showed the highest accumulation of exchangeable NH_4^+ and available NO_3^- compared to the commercial fertilizer used in this study. Most of the treatments with clinoptilolite zeolite had similar effect on plant height, dry weight, N, P, and K concentrations, uptake, and use efficiency compared to commercial fertilizer used. It may also be concluded that treatments with higher amounts clinoptilolite zeolite ensured good retention of soil exchangeable cations within the soil.

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ANIMAL SCIENCE

Differentiation between three Saudi goat types using Size-free Canonical Discriminant Analysis

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Abstract

Body weight and 16 body measurements were utilized to discriminate between 188 animals randomly selected from three Saudi goat types, namely Ardi, Line1 and Line2. A size-free canonical discriminant analysis was conducted. Results indicated that Ardi breed is separated from the other two lines. The Mahalanobis distance of 0.55 was obtained between Line1 and Line2, while the corresponding distances between Ardi and each of Line1 and Line2 were 25.03 and 21.45, respectively. These values indicate that Line1 and Line2 are more closely related to each other than the relation between Ardi and each of both, which they are far apart. The three distances were significantly ($P<0.01$) different from each other. The cross-validation procedure assigned 100% of Ardi animals into their genetic group, while percentages of animals assigned in Line1 and line2 were 86.10 and 42.55, respectively. The size-free canonical approach is proved useful and informative in differentiating between the three breeds. Application of selective breeding to genetic improvement would benefit from the detected phenotypic differentiation.

Key words: Saudi goats, Distance, Discriminant analysis, Body measurements

Introduction

Canonical discriminant analysis (CDA) is a multivariate statistical technique used to discriminate two or more naturally occurring groups based on a suite of continuous or discriminating variables. The technique consists of two closely related procedures that allow determining underlying, dominant gradients of variation among groups of sample entities from a set of multivariate observations, to elucidate how variation among groups is maximized and variation within groups is minimized along a gradient (McGarigal et al., 2000). Thus, its usefulness resides in its mathematical deduction which permits comparison of the amount of variation existing among populations to that present within populations (Morrison, 1976; Chatfield and Collins, 1980).

Discriminant function analysis can be used not only as a means to explain differences among groups, but also to predict group membership for sampling entities of unknown membership. In general, populations or species under study are considered to be groups defined a priori and linear measurements of morphological characters are obtained from the specimens within each sample. When applying this procedure, it is important to control within sample sources of variation in such a way that the possible variation among samples will not be masked or result from sampling errors. Thus, factors such as sex dimorphism in size, different developmental stages and indeterminate growth should be considered before different samples are submitted to a discriminant study for the analysis of species differentiation (Thorpe, 1983, 1987). The application of discriminant methods without controlling these factors may lead to spurious results, since discrimination among populations may represent a mere sampling artifact concerning the groups under study.

Discriminant analysis has been used for differentiating goat populations utilizing various morphological measurements simultaneously (Jordana et al., 1993; Herrera et al., 1996; Capote et al., 1998; Zaitoun et al., 2005; Dossa et al., 2007). In an attempt to distinguish between brown and

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gray Bengal goats, Mukeherjee et al. (1979) reported significant difference between both breeds due to body length and chest circumference. Later, Herrera et al. (1996) employed discriminant analysis on several body measurements such as, shin circumference, chest girth, chest depth, rump length and width, and shoulder height to differentiate among five Spanish goat breeds. In Jordan, Zaitoun et al. (2005) applied discriminant analysis on 20 metrical variables to discriminate among different goat genetic groups. In these studies, step-wise discriminant analysis was first applied to select the most important discriminant variables used for differentiation among breeds under study. Then, the canonical discriminant analysis was conducted based on the selected variables.

In organisms such as goats, individuals within breeds show different developmental stages, and the samples utilized often incorporate a sampling error reflecting the presence of different age classes in the groups under study. Thus, size frequency distribution of individuals in the different populations will be a function of the ontogenetic development of individuals present in the different samples. A way of correcting the problem of the existence of variation in size due to ontogeny or to other causes would be to remove statistically the effect of size present within the samples of each group and then apply CDA to the samples already corrected for within group differences in size, (Strauss, 1985).

Saudi Arabia possesses about 2.2 million head of goats (FAOSTAT, 2007). Their share in the total annual production of meat and milk is approximately 14% and 6%, respectively. This low productivity is due to the lack of sound breeding programs for genetic improvement, operating nationwide. The population belongs to several breeds and their crosses. Unfortunately, these breeds are not completely exploited and their main characteristics are not well documented. Nevertheless, the breeds vary in color, body weight, size, morphostructural characteristics such as shape and presence or absence of horns and wattles. These differences have not been previously investigated and knowledge of such diversity is important.

In this study, three Saudi goat types were considered for investigation. They represent an important genetic resource in their environment and make up an integral part of the livestock population in the country. Our main purpose is to achieve uniformity within these three types, and develop them into three definite breeds in terms of morphological and production characteristics, since the breed is the operational unit for the assessment of livestock diversity (Simon, 1999; Duchev and Groeneveld, 2006; Duchev et al., 2006). For this reason, characterization of local domestic animal populations is of major importance. The first step of the characterization process of any unexploited genetic resources falls on the knowledge of the morphological trait variation (Azor et al., 2008; Delgado et al., 2001). The foregoing justifies the main objective on which the present study was based, i.e. differentiation between these types based on some morphological characteristics, using size-free canonical discriminant analysis. Results obtained from this study would provide information useful to contribute to the establishment of standard for breeds both in magnitude and variability.

Materials and Methods

A total of 188 animals belonging to three different goat types were used in this study. The first type, called Ardi, is considered a definite breed, while the other two (Line1 and Line2) have been developed from unknown origins. All goats are predominantly horned. Ardi breed is black in color, and has very long dropping ear, while the other two lines are white, with a short, erected ears with different colors inside. Animals of Line1 have short hair, while those of Line2 are covered with a medium hair length. Line1 and Line2 differ in the shape of the horns and the color of the muzzle, as it is black in the former and red in the later. Age of animals and their pedigree are not known.

Data were collected on body weight (BW) and 16 body measurements including Ear length (EL), Head length (HL), Jaw length (JL), Head width (HW), Canon length (CL), Canon circumference (CC), Body length (BL), Rump width (RW), Heart girth (HG), Chest width (CW), Height at withers (WH), Tail length (TL), Fumer length (FL), Rump length (RL), Neck length (NL) and Neck circumference (NC). Weight was recorded to the nearest 100 gram using an electronic scale. After weighing, body measurements were taken, to the nearest 0.5 cm. All measurements were taken while the animal standing on a flat surface with the head held up, while restricting the animal by holding. A graduated measuring stick was used for taking the height measurements, while the length and circumference measurements were taken using a flexible tape; and a special wooden caliper was used for the width measurements. All measurements were taken by the same person.

Statistical analysis

The statistical analyses were performed using

various procedures of SAS package (SAS, 2000). Basic statistics for body weight and measurements were obtained using the PROC UNIVARIATE and PROC FREQ of SAS (2000). Variance components were also estimated to partition variation in body weight and measurements into intra-breed (residual) and inter-breed variance components, considering breed effect as random. The maximum likelihood technique (ML), as described by Van Vleck and Searle (1979), was adopted and the analysis was conducted using the VARCOMP procedure of SAS (SAS, 2000). The ML procedure was preferred because its estimators maximize the likelihood of the parameters and are consistent, asymptotically normal and efficient, (Harville, 1977). Then, the data were subjected to the logarithmic transformation, as the scatter plot of each two trait measurements followed a curved line. This relationship usually becomes linear if both measurements are transformed to logarithms (Klingenberg, 1996). In practice, log transformation often renders relations among variables more linear and also can make variances more homogeneous. Furthermore, log-transformed data are independent of measurement units, but retain the information about scale.

The method of size-free CDA consists of several steps, starting from removing the within-breed variation by regressing each character separately on the first pooled within-breed principal component. Then, CDA is applied to residues obtained from the regressions (Strauss, 1985). The analyses were performed sequentially, according to the following order:

1. The transformed data were first standardized using the STANDARD procedure of SAS (2000) to center the log transformed values of each character by the breed mean. Data were standardized to zero mean and a unit standard deviation.

2. The first eigenvector was then used as a multivariate size estimate, because the following two conditions were met:

a. All coefficients of the first eigenvector were positive

b. These coefficients were positively and significantly correlated with the values of body weight and measurements, (Strauss, 1985).

3. The standardized values were then used to estimate the first principal component, using PRINCOMP procedure of SAS (2000). The first principal component is the linear combination that accounts for the maximum variance.

4. The values of each trait were then regressed on the first principal components (PC-1), to remove the effect of size on each character and the residues resulted from the regression analysis were obtained. The residues express variation after the removal of the within-breed size effect. This step was carried out using a combination of REGRESSION and SCORE procedures of SAS (2000), because both procedures do not standardize the regression residuals by the mean, i.e. they do not prevent the demonstration of inter-breed differences since each breed would have a mean equal to zero for each character (dos Reis, et al. 1990).

5. Canonical discriminant analysis was then performed on the residuals obtained from regression of each character on PC-1 (Strauss, 1985), using CANDISC procedure of SAS (2000). In this case, using size-free CDA would remove the effect of within-breed size variation by regressing each character separately on the first pooled within-breed principal component (a multivariate size estimate). Size-free CDA computation was then normally continued by multiplying the inter-breed covariance matrix by the inverse of the within-breed covariance matrix and extracting the eigenvalues and eigenvectors from the resulting matrix (Morrison, 1976). These eigenvectors, which express the axes of the greatest variation, are then linearly combined with the values of body weight and measurements to compose the canonical variables that produce the individual scores. The ability of this function to identify the three genetic groups was indicated as the percentage of individuals correctly classified from the sample that generated the function.

Mahalanobis distances were also calculated using the CANDSIC procedure. The accuracy of the classification was evaluated using split-sample validation (cross-validation) of the DISCRIM procedure of SAS (2000). In cross-validation, one individual is removed from the original matrix and the discriminant analysis is then performed from the remaining observations and used to classify the omitted individual. The proportion of individuals correctly re-allocated is taken as a measure of the morphological distinctness of the population. The individual scores for each breed were plotted in the space of the canonical variables that determine the patterns of discrimination among breeds, using the GPLOT procedure of SAS (2000). Phylogenetic tree of the three goat breeds was constructed based on the calculated Mahalanobis distances, using the Neighbor program of the PHYLIP (Phylogeny Inference Package) (Felsenstein, 2001).

The KDE procedure of SAS (2000) was used to

perform the estimation, through approximating a hypothesized probability density function from the observed data. The Kernel density estimation is a nonparametric technique for density estimation in which a known density function (the kernel) is averaged across the observed data points (the canonical scores) to create a smooth approximation, (Silverman, 1986). The KDE provides a very useful means of investigating the entire populations. Then, the three dimensional plotting procedure (PROC G3D) of SAS (2000) was conducted on the KDE to gain more insight into the structure of the data.

Results

Means, standard errors and coefficients of variation (CV) of the morphometric characteristics of the three goat breeds are presented in Table (1). Mean of body weight of Ardi goats (4.98) was lower than the corresponding values observed for the other two breeds; coincided with the lowest coefficient of variation (%41.97). Ardi goats were younger at the time of measurements than animals from the other two lines. Line1 had higher mean of body weight than that of Line2, while the corresponding CV was lower. Generally, the coefficients of variation of BW were large, reflecting great differences in size among the studied animals. On the other hand, Ardi goats had longer ear than those of the other two breeds, while its CV value was the lowest. An obvious characteristic of Ardi goats is their dropping ears. In general, means of the other body measurements were higher in Line1 compared to the corresponding values observed for the other two breeds, i.e. Ardi and Line2. The CV values coincided with these body measurements ranged between 7.60 (CC) and 18.53 (CW), between 12.84 (CC) and 26.53 (HL), and between 9.16 (CC) and 28.02 (NL) for Ardi, Line1 and Line2, respectively, reflecting differences among breeds, uncontrollable environmental circumstances such as nutrition and size as well as personal errors during taking the measurements. The high variability, as indicated by the CV values of the body weight and measurements, may also be a reflection of the wide variation observed among the ages of the studied animals. Generally, little differences between Line1 and Line2 in terms of body weight and measurements were observed in comparison with those observed between Ardi breed and each of both

Table 1. Means and their standard errors (SE) of body weight (kg) and measurements (cm) for the three breeds.

Trait	Ardi		Line 1		Line 2	
	Mean± SD N=88	CV(%)	Mean± SD N=72	CV(%)	Mean± SD N=28	CV(%)
BW	4.98±0.22	41.97	15.15±0.99	55.39	12.07±1.34	58.71
EL	18.77±0.25	12.29	14.13±0.29	17.56	13.71±0.42	16.35
HL	5.00±0.07	12.87	6.67±0.21	26.53	6.00±0.31	27.22
JL	7.11±0.07	9.33	9.48±0.27	24.27	8.65±0.36	21.67
HW	6.77±0.07	10.39	7.72±0.17	19.21	7.77±0.25	17.03
CL	9.28±0.10	9.77	11.00±0.21	16.40	10.61±0.29	14.70
CC	7.02±0.06	7.60	6.83±0.10	12.84	6.70±0.12	9.16
BL	28.44±0.44	14.67	41.05±1.15	23.77	37.13±1.57	22.37
PW	8.72±0.21	13.01	11.33±0.36	26.72	10.68±0.44	21.63
HG	38.81±0.50	12.46	55.01±1.45	22.32	50.43±2.30	24.16
CW	9.91±0.20	18.53	14.77±0.47	26.91	13.89±0.61	23.31
WH	37.93±0.43	10.56	50.84±1.13	18.81	46.61±1.74	19.72
TL	9.46±0.51	14.74	12.06±0.25	17.69	11.45±0.41	18.89
FL	18.76±0.21	10.74	25.63±0.68	22.38	23.16±1.00	22.84
RL	8.82±0.12	13.21	11.83±0.29	20.94	11.27±0.46	21.79
NL	16.78±0.26	14.36	22.80±0.64	23.63	21.02±1.11	28.02
NC	17.64±0.19	10.20	23.50±0.54	19.40	21.77±0.77	18.68

N: Number of observations

CV: Coefficient of variation

Body weight (BW), Ear length (EL), Head length (HL), Jaw length (JL), Head width (HW), Canon length (CL), Canon circumference (CC), Body length (BL), Rump width (RW), Heart girth (HG), Chest width (CW), Height at withers (WH), Tail length (TL), Fumer length (FL), Rump length (RL), Neck length (NL) and Neck circumference (NC).

Table 2. Intra-breed and inter-breed variance components and their percentages out of the total.

Trait	Inter-breed (%)	Intra-breed (residual) (%)
BW	17.79 (33)	36.09 (67)
EL	5.18 (48)	5.60 (52)
HL	0.46 (21)	1.78 (79)
JL	0.89 (25)	2.68 (75)
HW	0.20 (13)	1.30 (87)
CL	0.49(20)	1.96 (80)
CC	0.01 (1)	0.48 (99)
BL	27.25 (34)	54.00 (66)
PW	1.19 (20)	4.84 (80)
HG	45.69(34)	89.82(66)
CW	4.38 (32)	9.15 (68)
WH	28.45 (34)	54.80 (66)
TL	1.20 (26)	3.33 (74)
FL	7.93 (30)	18.44 (70)
RL	1.65(30)	3.82(70)
NL	6.22(25)	18.79(75)
NC	6.01 (34)	11.83 (66)
Average of percentages	27	73

Body weight (BW), Ear length (EL), Head length (HL), Jaw length (JL), Head width (HW), Canon length (CL), Canon circumference (CC), Body length (BL), Rump width (RW), Heart girth (HG), Chest width (CW), Height at withers (WH), Tail length (TL), Fumer length (FL), Rump length (RL), Neck length (NL) and Neck circumference (NC).

Variance components are routinely estimated in quantitative genetics and animal breeding (Hofer, 1998; Ferreira et al., 1999), using several calculation methods. Maximum likelihood method was used in this study to partition the variability in body weight and measurements into intra-breed (residual) and interbreed variance components and to assess the morphological divergence between breeds. Table (2) shows the intra-breed and inter-breed variance components and their percentages out of the total. As shown from this table, percentages of the intra-breed variance (residual) were larger than those of the interbreed variance, ranging between 52% and 99% for EL and CC, with an average equal to 73%. Percentage of the inter-breed variation for CC was the lowest, accounting for 1%, confirming the lowest values of CV observed for this trait for the three breeds (7.60, 12.84 and 9.16 for Ardi, Line1 and Line2, respectively) (Table 2). This showed that most of the variability was due to within breed differences. This residual variance may be attributed to random, environmental or ontogenetic factors. According to Klingenberg (1996), discrimination between groups is often difficult because of allometric variation within groups. He further noted that the amount of within group variation may far exceed between-group differences, which in agreement with our findings. Actually, any of these causes may confuse the study of discrimination among these breeds since they generate a variability component within the breeds. Thus, it is necessary to apply a procedure that will remove the effect of character size variation within breeds to permit the study of differentiation among them.

The first principal component, the correlation coefficients between the first eigenvector (PC-1) and body weight and measurements and the correlation coefficients between body weight and measurements and scores obtained by canonical analysis are presented in Table (3). Eigenvalue of the first principal component was 0.211, accounting for 89% of the total variation existing in the covariance matrix among body weight and measurements within the three breeds, and could be considered a measure of the general body size. Values of the first principal component ranged between 0.02 (EL) and 0.66 (BW). Highly significant correlation coefficients were observed between PC-1 and body weight and measurements, ranging between 0.67, between PC-1 and BW and 0.99, between PC-1 and NC. The first principal component of within-group character covariance matrix may be interpreted as a general size variable since all characters presented positive and statistically significant coefficients ($P<0.0001$). It is important to note that the size estimate should be a non- measurable variable (Bookstein, 1982; Bookstein et al., 1985), a linear combination like the first principal component, and not a separate morphometric character, since size is, by definition, a multi-dimensional quantity (Wright, 1954; Rohlf and Bookstein, 1987). The first eigenvector can be utilized in size-free CDA as a generalized size estimate. Canonical discriminant analysis indicates the characters that most contribute to discrimination among populations (Pimental, 1979; Neff and Marcus, 1980). The criterion commonly used for character selection is based on the relative magnitude of the eigenvector coefficients (Neff and Marcus, 1980; Campbell and Atchley, 1981). However, these coefficients are difficult to interpret in biological terms since, if two characters are correlated, the canonical coefficient of the character having the lower F value will be close to zero (Morrison, 1976), even though each character may be as important as the other. The importance of each character for discriminating among breeds may be better evaluated by transforming the

coefficients into correlation vectors, which may be calculated from the correlation between individual scores for the canonical variables and the values of the characters for each individual (Strauss, 1985). Size-free CDA was then applied to the three breeds on the basis of the residuals of each character. The correlation coefficients between the linear combination of the morphostructural variables of the native goat breeds and the scores obtained by canonical analysis are presented in Table (4). Variances of the two canonical variates (CAN1 and CAN2) were 7.02 and 0.15, representing 0.98 and 0.02 of the total variation. A highly significant canonical correlation (0.93, P<0.01) for the first canonical derived function was observed and allowed a perfect identification of individuals (Wilks lambda = 0.107; P<0.0001), while the correlation of the second correlation canonical function (0.36) was not significant. Using canonical discriminant analysis for differentiating among Creole goat breed and their F1 and F¬2 crosses with Nubian, Vargas et al. (2007) showed that percentage classification was higher in F2 (94.4%) and Creole (90.6%), than F1 (72.9%) does. They noted that the morphological traits of the Creole and F2 crosses were more stable than those of the F1. They further indicated that height at rump, rump width, rump length, head length, and chest depth were the most discriminative variables. In the same year, Dossa et al. (2007), working on morphological characterization of goats from Benin, reported that variance of CAN1 and CAN2 accounted for 92% of the total variation. When discriminating among several goat breeds of Burkina Faso, Traore et al. (2008) reported percentages of 94.5 and 5.5 for CAN1 and CAN2, respectively. Ebegbulem et al. (2011) reported an eigenvalue of 3.113, explaining 97.3% of the total variance of the morphological variables utilized for discriminating among three groups of West African Dwarf goats in Nigeria.

Correlation coefficients between body weight and measurements and the first canonical scores (CV-1) were all negative, except those of EL and CC, being the most discriminative variables. The coefficients were all significant (P<0.01), ranging between -0.71 (BW) and 0.76 (EL). The first canonical means were 2.80, -2.53 and -2.28 for Ardi, Line1 and Line2, respectively. These values indicate that Ardi breed was best identified, i.e. discriminated, by CAN1 and differed markedly in body weight and measurements from Line1 and Line 2. Correlation coefficients between body weight and measurements and the second canonical scores (CV-2) were all positive, except that of HW. The values ranged between -0.10 (HW) and 0.35 (HL), and they were significant, except that between CV-2 and each of HW and CW measurements. These values were lower in magnitude than the corresponding values obtained for CV-1. Means of the second canonical scores were 0.01, 0.32 and -0.86 for Ardi, Line1 and Line2, respectively, indicating that Line1 and Line2 were closer and best identified by this function. Generally, the correlations between the length measurements and the canonical scores were stronger than those between width and circumference measurements and the canonical scores.

Table 3. The first principal component (PC-1), the correlation coefficients (r) between the first eigenvector and body weight and measurements and the canonical discriminant analysis coefficients (CV-1 and CV-2) expressed as Pearson correlation coefficients between body weight and body measurements and scores obtained by canonical analysis (CV-1 and CV-2).

Trait	PC-1	r	CV-1	CV-2
BW	0.66	0.67**	-0.71**	0.25**
EL	0.02	0.76**	0.76**	0.18*
HL	0.22	0.78**	-0.51**	0.35**
JL	0.19	0.82**	-0.59**	0.30**
HW	0.12	0.89**	-0.40**	-0.10ns
CL	0.13	0.90**	-0.53**	0.15*
CC	0.04	0.90**	0.19**	0.15*
BL	0.24	0.90**	-0.66**	0.27**
PW	0.20	0.92**	-0.52**	0.14*
HG	0.23	0.93**	-0.66**	0.27**
CW	0.26	0.93**	-0.66**	0.10ns
WH	0.19	0.94**	-0.67**	0.33**
TL	0.17	0.95**	-0.59**	0.19**
FL	0.21	0.95**	-0.62**	0.34**
RL	0.20	0.97**	-0.63**	0.14*
NL	0.22	0.98**	-0.57**	0.29**
NC	0.19	0.99**	-0.66**	0.27**

*Significant at P<0.05

** Significant at P<0.01

NS not significant

Body weight (BW), Ear length (EL), Head length (HL), Jaw length (JL), Head width (HW), Canon length (CL), Canon circumference (CC), Body length (BL), Rump width (RW), Heart girth (HG), Chest width (CW), Height at withers (WH), Tail length (TL), Fumer length (FL), Rump length (RL), Neck length (NL) and Neck circumference (NC).

Regression residuals are an appropriate way of correcting for size if the relationship of a dependent variable to a size variable is known a priori and measured independently. The residuals in this case are the deviations of the actual measurements from the expected value for the average specimen of that particular size. These deviations are computed in the direction of the dependent variable by subtracting their values estimated by regression from the observed values. In this case, the first PC-1 is a good

choice for such an estimate. The "residuals" are subsequent PCs, which are perpendicular to the PC-1 (Strauss, 1995). Table (4) shows the regression coefficients of body weight and each body measurement on the first principal component scores, their standard errors and the corresponding "t" values. All coefficients were significant (P<0.0001), indicating the presence of differences in size among breeds. The coefficients ranged between 0.09 (CC) and 0.62 (BW). The regression coefficient of BW was the highest indicating that differentiation among the three breeds is largely dependent on the size of the animal. The lowest coefficient was observed for CC (0.09), followed by that of EL (0.12), coincided with the lowest PC-1 values of 0.04 and 0.02, respectively, Table (4).

The plot of the canonical variables CAN1 and CAN2 showed two well defined and non-overlapping phenetic groupings, one corresponded to the Ardi breed that was clearly isolated from the other two overlapping lines, (Figure 1). This finding was confirmed by the three dimensional plot, shown in Figure (2). There are two clear and isolated modes observed in the diagram, corresponding to the two clusters observed in Figure (1). The highest one is coincided with Ardi breed, while the lowest coincided with the two lines. This indicates that Ardi breed is more homogenous and greatly differs from the other two breeds. This was supported by the values of the Mahalanobis distance estimated between the three breeds based on the 17 variables studied (Table 5). Distances between all pair-wise comparisons were significant (P<0.001). The distance between Line1 and Line2 was the closest (0.55), i.e. they were poorly differentiated from each other, while distances between Ardi and each of Line1 and Line2 were larger, accounting for 25.03 and 21.44 between Arid and each of Line1 and Line2, respectively. The dendogram (Figure 3) showed two clusters: cluster one included Line1 breed as a large group and one sub cluster representing Line2, while cluster two included Ardi breed which is clearly separated from cluster one. The distribution of the three breeds shown in the three figures agrees with the results of the Mahalanobis distances (Table 5). The two discriminant functions accurately classified the individual goats into their respective breeds (Table 6). Although the cross-validation process with split-sample method correctly assigned 100% of Ardi animals into their distinct genetic groups, it assigned only 86.10% and 42.55% of animals belonging to Line1 and Line2 into their original distinct genetic groups. The results of Table (6) indicated one animal from each of Line1 and Line2 was misclassified under Ardi breed with percentages accounting for 1.39 and 3.57, respectively. On the other hand, 9 goats were misclassified from each line, i.e. Line1 and Line2 with percentages accounting for 12.50 and 32.14 for both lines, respectively. The reason that could be adduced for these misclassifications is the high degree of intermingling between the two lines. Working on three groups of West African Dwarf goats, Ebegbulem et al. (2011) indicated that 83.5%, 82.8% and 85.2% of the original cases of the three groups were correctly classified, while 16.7% of individuals belonging to class 1 was classified as belonging to class 2; 10.3% and 6.9% individuals in class 2 were wrongly classified as belonging to classes 1 and 3 respectively. They further indicated that 14.8% of individuals belonging to class 3 were wrongly classified as belonging to class 2. They concluded that the cross-validation analysis upheld the results of the original classification in classes 1 and 3, but shows that 77.6% of individuals in class 2 were correctly classified and that 12.1% of class 2 individuals were wrongly classified as belonging to class 3.

Table 4. The regression coefficients of body weight and each body measurement on the first principal component scores, their standard errors (SE) and the corresponding t values.

	Coefficient±SE	t-value
BW	0.62±0.01	102.30**
EL	0.12±0.01	12.37**
HL	0.24±0.01	36.96**
JL	0.20±0.01	27.60**
HW	0.14±0.01	16.71**
CL	0.14±0.01	25.73**
CC	0.09±0.01	15.62**
BL	0.23±0.01	40.48**
PW	0.22±0.01	31.49**
HG	0.23±0.01	59.56**
CW	0.25±0.01	28.71**
WH	0.19±0.01	52.93**
TL	0.16±0.01	19.22**
FL	0.21±0.01	39.53**
RL	0.21±0.01	34.49**
NL	0.22±0.01	27.78**
NC	0.18±0.01	34.72**

Body weight (BW), Ear length (EL), Head length (HL), Jaw length (JL), Head width (HW), Canon length (CL), Canon circumference (CC), Body length (BL), Rump width (RW), Heart girth (HG), Chest width (CW), Height at withers (WH), Tail length (TL), Fumer length (FL), Rump length (RL), Neck length (NL) and Neck circumference (NC).

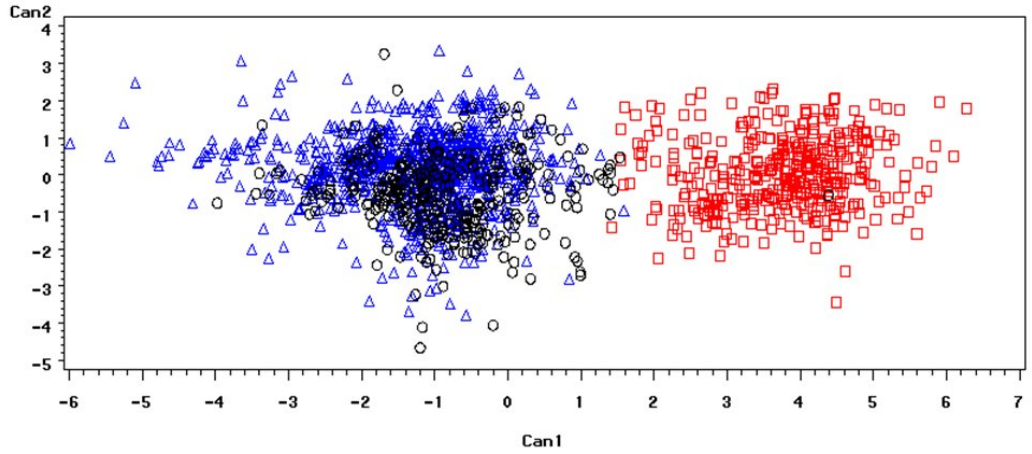

Figure 1. Canonical representation of the three native Saudi goat breeds using the morphological variables.

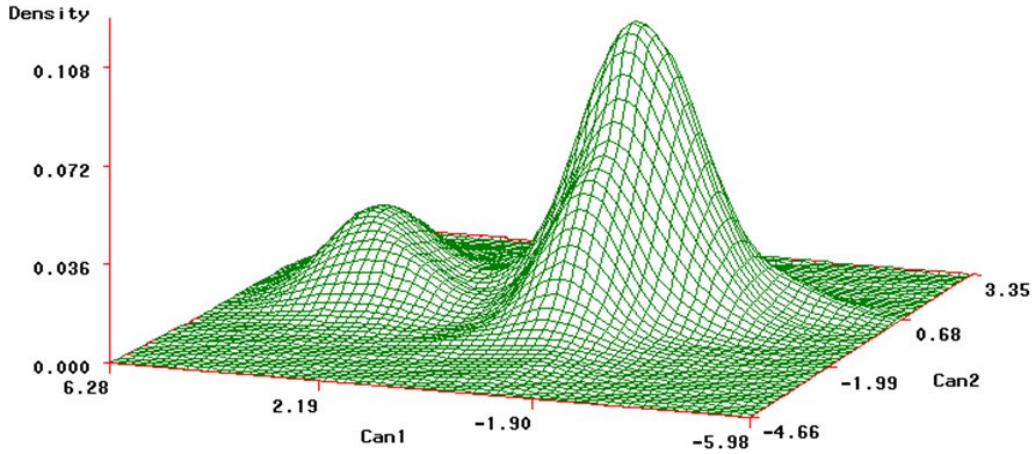

Figure 2. The bivariate kernel density of the two canonical discriminant scores of the three goat types.

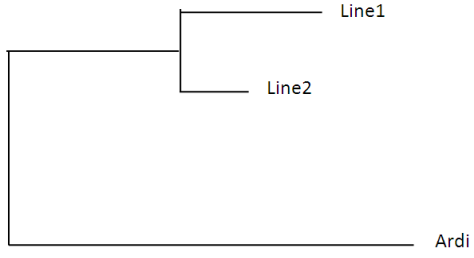

Figure 3. Representation of the similarity among the three goat types.

Table 5. The Mahalanobis distances (above the diagonal) and their F values (below the diagonal) estimated between the three breeds according to body weight and measurements.

	Ardi	Line1	Line2
Ardi		5.03	21.45
Line1	411.73**		0.55
Line2	230.57**	8.86**	

Table 6. Actual and cross-validated numbers of goats and their percentages classified in the three breeds.

	Breed	Ardi	Line1	Line2	Total
Actual	Arid	88 (100)	0	0	88
Count	Line1	0	72	0	72
	Line2	0	0	28	28
Cross-validated Count	Arid	88 (100)	0	0	88
	Line1	1(1.39)	62(86.10)	9(12.50)	72
	Line2	1 (3.57)	18(42.55)	9(32.14)	28

Discussion

Morphometric measurements have been found useful in contrasting size and shape of animals (Mckracken et al., 2000; Latshaw and Bishop, 2001; Afolayan et al., 2006; Ajayi et al., 2008). These measurements can be utilized as criteria for describing breeds and differentiating between them. Further, they can be used for distinguishing groups of animals and establishing breeds (Sierra, 2009). Herrera (2007) stated that morphometric variables could be used to explore breed structure and variability between various breeds. Later, Parés i Casanova (2009) mentioned that these measurements could be considered a proxy for describing a breed. On the other hand, canonical discriminant analysis has usually been used to discriminate goat populations based on morphological characters measured from adequately large sample sizes (Jordana et al., 1993, Herrera et al., 1996; Zaitoun et al., 2005). However, the application of CDA to study organisms varies within samples due to sampling bias, may result in artifactual discrimination due to shifts in mean character values. In such cases, data must be corrected for within-group size differences before conducting the canonical discriminant analysis. Separation of size-related variation within groups from between-group differences has been a traditional topic in several morphometrics studies (Bookstein et al., 1985; Rohlf and Bookstein, 1987; Marcus, 1990; Reyment, 1991). In the present study, several morphometric measurements were utilized to differentiate among three Saudi goat breeds using a size-free canonical discriminant analysis. Data were corrected for within-breed size differences, which found to be larger than between breed variance, before conducting the canonical discriminant analysis. The method did effectively discriminate among the three breeds despite the variation in size existing within each population.

Mahalanobis distance between Line1 and Line2 suggest that both lines are more closely related to each other than their relation with Ardi breed. In related studies, Herrera et al. (1996) and Luque et al. (2005) used classical discriminant function analysis to differentiate between various Andalusian goat breeds. In all these cases, the method was found to be relatively efficient and allowed differences between breeds and subpopulations to be detected, as well as the relative distance between them to be assessed. Zaitoun et al. (2005) used different discriminant analysis methods to discriminate among different genetic groups based on 20 metrical variables. Their results identified four genetic groups: Damascus, Mountain, Dhaiwi and Desert in addition to a population of crossbred goats. All pair-wise Mahalanobis distances were significant ($P < 0.001$). In their study, the canonical discriminant analysis and the stepwise discriminant analysis revealed that nose shape was the most discriminating variable among different pair-wise breeds' comparisons, followed by withers height then body weight, ear type, color and teat placement. Chest width, withers depth (WD) and rump width showed small discriminatory power. Yakubu et al. (2010) used the discriminant analysis for discriminating between West African Dwarf and Red Sokoto goats based on several body measurements and indicated that rump height, body length, horn length, face length, chest girth, neck circumference and head width were the most important morphometric traits permitting discrimination between both breeds. Later, Yakubu, et al. (2011) estimated Mahalanobis distance of 72.28 between West African Dwarf and Red Sokoto goats in Nigeria, indicating that there is considerable genetic variation between both breeds. Our results indicate that ear length and canon circumference were the most discriminative variables among the three breeds. Head length was found to be strongly related o breed differentiation among Andalusian White, Florida, Granadina, Malaga and Andalusian Black goat populations in Spain (Herrera et al., 1996), and to Creole goats in Mexico (Hernández, 2000). Bouchel et al. (1997) and Zeuh et al. (1997) also proposed chest depth as

a good discriminant variable on Rove breed and Chad's Creole goats. Vargas et al. (2007) indicated that height at rump, rump width, rump length, head length, and chest depth were the most discriminative variables among Creole breed and their F1 and F2 crossbreds sired by Nubian bucks.

The proportion of individuals correctly re-allocated is taken as a measure of the morphological distinctness of the population. In this study, the cross-validation process assigned 100% of Ardi animals into their distinct genetics group, while percentages of animals assigned in Line1 and line2 were 86.10 and 42.55, respectively. In related investigation, Dossa et al. (2007) was able to correctly allocate more than 70% of individual goats into their different groups. Similarly, Traore et al. (2008) used discriminant analysis to correctly classify most Sudan and Sudan-Sahel goat populations of Burkina Faso into their source population (79.3% and 82.7%, respectively).The observed morphological differences among the studied breeds may support the hypothesis that much of the morphological variation is under genetic control, indicating that they can be objectively improved under proper management practices. The phenotypic characterization in previous studies aimed to identify and differentiate goat breeds in different countries and to determine which variables, among several zoometric variables, were most effective for this purpose. The statistical methods used were mainly discriminant analysis, canonical discriminant analysis and step-wise discriminant analysis. The conclusion differed for each objective, highlighting the need to standardize the statistical methods according to the objective. It was necessary to explore new classification methods that may be more accurate for distinguishing between breeds and allow a better understanding of the involvement of each variable in the process of classification. In the present study, size-free CDA is applied to differentiate among three Saudi goat types. The approach has not been previously applied on livestock species, although it was employed in evolutionary biology and systematic (Campbell and Atchley, 1981; Neff and Marcus, 1980). It has also been used in studies of geographic differentiation and in the analysis of species differentiation and macro-evolution (Thorpe, 1983; Lessa and Patton, 1989; Patton and Smith, 1989). The approach is proved useful and informative in differentiating between the three breeds under study, but it needs further investigation regarding its reliability.

Conclusion

The results indicated that there was clear separation between Ardi breed and the other two lines that were closely related to each other. Therefore, the technique used in this study could be utilized in field assessment, management and conservation of different goat populations, where the goal is to distinguish among them and to obtain phenotypically pure local genetic resources for future selection and breeding improvement strategies. In this regard, there is a need to exploit traits peculiar to each breed for establishing sustainable genetic improvement programs under on-farm conditions. However, an investigation on the genetic characterization of Saudi goats utilizing the tools of molecular genetics will complement the results obtained from morphometric differentiation. Molecular genetic analysis together with participatory breeding programs involving local breeders is needed to validate the present results. This could permit rapid field assessment and subsequent conservation of the goat genetic resources in the country.

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