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

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Full Length Research Paper

Effect of foliar application of humic acid, zinc and boron on biochemical changes related to productivity of pungent pepper (*Capsicum annuum* L.)

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To study the effect of foliar application of humic acid at 0.05%, zinc at 0.05% and boron at 0.02% on biochemical changes related to productivity of pungent pepper cv. Bullet (*Capsicum annuum* L.), a pot experiment in randomized block design with three replications was conducted in the net house of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia-741252, West Bengal, India. In This experiment, we observed some physiological and biochemical characteristics of pungent pepper. Obtained results revealed that the highest value of height of plant, numbers of leaf plant^{-1}, leaf area, numbers of branch plant^{-1}, numbers of fruit plant^{-1} and fruit length were exhibited by foliar application of HA + Zn + B. The highest value of fruit diameter, weight of 20 fresh fruits, numbers of seed fruit^{-1} and weight of 100 seeds were exhibited by foliar application of Zn + B and HA + Zn + B, respectively. The total chlorophyll content significantly increased in HA + Zn + B followed by HA + B and Zn + B application. The highest value of reducing sugar, total sugar and starch content were obtained by application of HA + Zn + B. The highest content of protein, fat, energy and total phenol were exhibited by foliar application of Zn + B, Zn, HA + Zn + B and HA + Zn + B, respectively. The activity of polyphenol oxidase was significantly 'good performer' in HA + Zn + B followed by HA and HA + B application. There are significant and positive correlation of starch with some physiological characteristics such as leaf area, number of fruits plant^{-1}, fruit diameter and total chlorophyll. The correlation of energy value with carbohydrates, fat, protein, total chlorophyll and leaf area were positive and significant. Based on principle component analysis and considering average values of all variables, our results suggested that the good promising treatment in HA + Zn followed by Zn + B, HA + Zn + B and Zn alone application may bring about the proper value addition in quality as well as productivity of crop by enhancing the some physiological and biochemical characteristics in pungent pepper.

Key words: *Capsicum annuum* L., humic acid, zinc, boron, quality, productivity.

INTRODUCTION

Pungent pepper (*Capsicum annuum* L.) is one of the most important and popular crop grown in India and many countries all over the world. From time immemorial, it has been used as a common vegetable cum spice because of its color, test, pungency, flavor and aroma. Apart from basic quality, productivity of crop depends on

their physical and biochemical properties. Concentrations of these properties in plant may be modified by various factors including foliar nutrition with organic component. Some studies estimates indicate that a large number of diverse materials can serve as sources of plant nutrients. The majority of nutrient input to agriculture comes from commercial mineral fertilizers. Organic manures are considered to play a significant but lesser role in nutrient contribution, leaving aside their beneficial effects on soil physicochemical and biological properties. Foliar feeding is a relatively new and controversial technique of feeding plants by applying liquid fertilizer directly to their leaves.

Most of the used organic-mineral fertilizers are humic substances, since, humic acid is one of the major components of humic substances. Nowadays, the use of humic acid has increased with increase in the agricultural production and the most economical humic acid almost applied directly to the soil and/or as a foliar application to the plants. The mode of action of humic acid on plant growth can be divided into direct and indirect effects as it affects the membranes resulting in improved transport of nutritional elements, enhanced protein synthesis, enhanced photosynthesis, solublization of micronutrients, reduction of active levels of toxic elements, enhancement of microbial population, enhanced soil structure improvement and increased both cation exchange capacity and water retention. MacCarthy et al. (1990). Singaroval et al. (1993) claimed that the increase in dry matter production with humic acid might be due to its direct action on plant growth auxin activity, contributing to increase in the dry matter.

Moreover, application of humic acid also increased the seed weight due to better mobilization of nutrients to seeds. Nardi et al. (1999) found that the biological activity of the humic acid was attributed to their chemical structure and their functional groups, which could interact with harmonic-binding proteins in the membrane system, evoking a hormone-like response.

Micronutrients have received a great deal of importance in crop production during recent years because of the widespread occurrences of their deficiencies from different parts of the country. Researchers from almost all the states in the country have also reported significant responses of many crops to micronutrient fertilization. Zinc (Zn) and boron (B) is an essential trace element for plants, being involved in many enzymatic reactions and is necessary for their good growth and development. The foliar application of micronutrients (Zn and B), increased total sugars (TS), reducing sugar (RS), non-reducing sugar (NRS), ascorbic acid (AA) and TSS/acid ratio of papaya fruit (Sing et al., 2002). The foliar application of micronutrients (Zn and B) also significantly enhanced fruit juice content, TSS, AA and NRS of sweet orange fruits.

Zn is also involved in regulating the protein and carbohydrate metabolism (Swietlik, 1999). Under Zn deficiency, the leaves shoot tips do not elongate fully, resulting in compressed internodes length and a tuft or rosette of leaves at the terminal (Tariq et al., 2007). However, in the light of better fruit quality development, Zn holds more significance besides imparting sustainability in production/productivity by reducing the fruit drop (Malik et al., 1999) and granulation (Kaur et al., 1990). External symptoms of Zn-deficiency in citrus as described by Wutscher (1979) are reduction in leaf size somewhat in proportion to the Zn-concentration in leaf. Zinc rates from 4 to 12 kg ha^{-1} had beneficial effect on physical and chemical characters of fruits, but best results with regard to flavor and contents of juice, vitamin-C, and total sugars were obtained from the lowest Zn-rate (Mdwaradze, 1981). Application of Zn at 0.6% plus 2,4-D at 20 ppm as foliar spray gave the best results with regard to fruit weight, diameter, juice percentage, TSS and ascorbic acid content in Kagzi lime (Batru et al., 1984). Bahadur et al. (1998) claimed that the Zn uptake rate was faster in mango trees when zinc sulfate was foliar applied as compared with its soil application. While, the boron requirement is much higher for reproductive growth than for vegetative growth and increases flower production and retention, pollen tube elongation and germination, and seed and fruit development (Peres and Reyes, 1983). The application of boron as foliar spray also enhanced the fruit set in papaya (Jeyakumar et al., 2001). Furthermore, the supply of boron needed for reproductive growth in many crops is more needed than that needed for vegetative growth (Mengel and Kirkby, 1982; Marschner, 1986; Hanson, 1991) and same may be true in citrus. In citrus B deficiency leads to low sugar content, granulation and excessive fruit abortion (Reuther et al., 1968) as well as rind thickening. There is also concern that B is one of the micronutrient responsible for the changes in concentration and a number of metabolic pathways such as carbohydrate metabolism, nitrogen metabolism, phenol metabolism and ascorbate metabolism in plants (Marschner, 1995; Dordas and Brown, 2005; Lukaszewski and Blevins, 1996).

Therefore, in the present work, we have studied this response in pungent pepper grown at different levels of foliar application of HA and micronutrients (Zn and B), as well as the physiological and biochemical changes that take place during vegetative growth and fruit formation, in order to improve the quality, as well as productivity of crop.

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Table 1. Detail doses of treatments which used as foliar spray for pungent pepper.

Treatment		Humic acid (HA) (%)	Zinc (Zn) (%)	Boron (B) (%)
Control :	$HA_0Zn_0B_0$	0.0	0.0	0.0
HA:	$HA_1Zn_0B_0$	0.05	0.0	0.0
Zn:	$HA_0Zn_1B_0$	0.0	0.05	0.0
B:	$HA_0Zn_0B_1$	0.0	0.0	0.02
HA+Zn:	$HA_1Zn_1B_0$	0.05	0.05	0.0
HA+B:	$HA_1Zn_0B_1$	0.05	0.0	0.02
Zn+B:	$HA_0Zn_1B_1$	0.0	0.05	0.02
HA+ Zn+B:	$HA_1Zn_1B_1$	0.05	0.05	0.02

The source of humic acid was granular form, that of zinc (Zn) was $ZnSO_4.7H_2O$ and boron (B) from $Na_2B_4O_7.10H_2O$. Each spraying was done four times with sticker starting from 25 days after transplanting and subsequent ones at an interval of 10 days during vegetative stage.

MATERIALS AND METHODS

Field experiment

The seedling was grown in nursery beds prepared with a sandy loam soil and were 12 cm tall and 1.0 m wide. Weathered cow dung manure 4 kg/m, was mixed into the beds. Beds were drenched with formaldehyde (4.0%) and covered with polythene sheets for one week to avoid damping off disease. Seedling was treated with Dithane M-45 (2.5 $g.kg^{-1}$ of seed) (Hindustan Pulverizing Mills Industrial Growth Centre, Sumba, Jammu, India) prior to sowing. Fresh seeds of pungent pepper cv. bullet (*C. annuum* L.) collected from AICRP on Vegetable Crops were sown at 10 mm and 5 cm apart. After sowing, beds were covered with straw until seed germination and hand watering was done regularly. Seedlings were hardened by withholding water four days before transplanting.

Pot experiment

A pot experiment was conducted in the net house of Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal and India. The 40 days old seedlings were transplanted in earthen pot of 15 cm size (one $plant.pot^{-1}$) having a central drainage hole. Soil was prepared by mixing appropriate amount of well rotted cow dung and manures (soil 700 $g.pot^{-1}$; cow manure 100 $g.pot^{-1}$; urea 5 $g.pot^{-1}$; single superphosphate 20 $g.pot^{-1}$ and muriate of potash 6 $g.pot^{-1}$) (according to guidance of Department of Spices and Plantation Crops, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal, India). Additional fertilizer 5 $g.pot^{-1}$ was applied 21days after transplanting. The experiment consisting of eight treatments including control (only tap water sprayed) were arranged in a randomized block design with three replications. The detail treatments are summarized as in Table 1.

To prevent blossom and fruit drop, supplementary irrigation was required (hand water was applied at an interval of 1 day with the first being immediately after transplanting). No weeding was required. The insecticide Rogor at 2.5 $ml.L^{-1}$ (Rallis India Ltd., Mumbai, Maharashtra, India) was applied three times beginning just from flowering stage at 15 days intervals to control aphids. The following physiological and biochemical qualities were determined in the present experiment.

Physiological qualities

All the recommended cultural practices for pungent pepper production were followed according to advice of Horticulture Faculty, Department of Spices and Plantation Crops, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia- 741252, West Bengal, India. Data were recorded as follows:

Plant growth characteristics

At the end of the growing season, (~90 days after transplanting) samples of ten selected plants were taken at random from each replication to determine the following characteristics:

1. Average height of plant (cm)
2. Average number of leaves $plant^{-1}$
3. Average leaf area (sq.cm) [Leaf area was determined followed the procedure of McKee (1964)]
4. Average number of branches.$plant^{-1}$

Fresh fruit yield and its components

Fresh fruit yield was started andharvested at 25 days after initiation of flowering. Samples of ten selected plants of each replication were counted until final harvesting to determine the following characteristics:

1. Average number of fresh fruits $plant^{-1}$
2. Average length of fresh fruit (cm)
3. Average diameter of fresh fruit (cm)
4. Weight of 20 fresh fruits (g)

Seed yield and its compositions

Twenty pods were taken at random from each replication to determine the following characteristics:

1. Average number of fresh seeds $fruit^{-1}$
2. Weight of 100 fresh seeds (g)

Biochemical qualities

About 300 g weighed edible pungent pepper from each replication was collected. Fresh materials were chopped with a sharp knife into small pieces before analysis of total phenol and one oxidative enzyme [polyphenol oxidase (PPO)]. Samples were shredded and dried at 40°C for 96 h. This material was prepared for reducing sugar, total sugar, starch, protein and fat analyses by grinding to a fine powder using an electric grinder. A subsample of the dried shredded material was further dried at 100°C to constant dry weight

to determine percent moisture.

Analysis of chlorophyll a, chlorophyll b and total chlorophyll

One gram fresh leaf mass of pungent pepper (*C. annuum* L.) was extracted by macerating with 10 ml of 80% solution of acetone. The procedure was repeated until the leaf mass was completely decolorized. The resulting extracts are kept in the dark in order to prevent the destruction of the chlorophyll molecules. The extracts are spectrophotometrically measured at the wave length of 663 and 645 nm (Arnon, 1956). The concentration (content) of chlorophylls in the solution was calculated with the following formulas:

Chlorophyll a ($mg.g^{-1}$) = $[12.7(A_{663}) - 2.69(A_{645})] \times V/1000 \times W$

Chlorophyll b ($mg.g^{-1}$) = $[22.9(A_{645}) - 4.68(A_{663})] \times V/1000 \times W$

Total chlorophyll ($mg.g^{-1}$) = $[20.2(A_{645}) - 8.02(A_{663})] \times V/1000 \times W$

Where, A = Absorbance of specific wavelength, V = final volume of chlorophyll extract in 80% acetone, W = weight of sample.

Analysis of reducing sugar

Reducing sugar were extracted in 10 ml of 80% alcohol by boiling 0.1 g dry powdered sample for 30 min at 80-90°C followed by centrifugation at 5,000 *g* for 10 min and subsequent analysis was followed using the Nelson-Somogyi's method (Sadasivam and Manickam, 1992). The amount of reducing sugar was determined against a glucose solution (0-500 µg) as standard curve.

Analysis of total sugar and starch

Sugar and starch were extracted in 10 ml of 80% anhydrous alcohol by boiling 0.1 g dry powdered sample for 30 min at 80°C followed by centrifugation at 5,000 *g* for 10 min and subsequent procedures was followed using the Anthrone reagent method (Sen et al., 2005). The amount of concentration was determined against a glucose solution (0-500 µg) as standard curve.

Analysis of total protein

Total nitrogen (N) was analyzed by the Kjeldahl method (Casanas et al., 2002) and used for the calculation of the protein concentration by multiplying with a conversion factor of 6.25.

Analysis of fat

Fat content of dry fruit of pungent pepper was analyzed following extraction with petrol ether in a Soxhlet apparatus and subsequent proceeding was followed by method of AOAC (1984).

Analysis of energy

The energy value was calculated using Atwater factors of 17 kJ/g of protein, 38 kJ/g of fat, 17 kJ/g of starch and 16 kJ/g of sugar (FAO/WHO/UNU, 1985).

Analysis of total phenol

Total phenol was extracted in 50% methanolic 1.2 N HCl by boiling 1 g of finely chopped tissue for 1.5 h at 80-90°C following the method of Vinson et al. (1995) and subsequent analysis was with the Folin-Ciocalteau reagent using gallic acid as standard.

Analysis PPO Activity

One gram of fresh tissue from each treatment was macerated in a pre-chilled pestle and mortar and extracted with 10 ml phosphate buffer (pH 6.0) to determine PPO activity. Triturated samples were centrifuged at 10,000 *g* for 30 min at 0°C and supernatants were assessed for enzyme activity. Estimation of polyphenol oxidase was made by adding 2 ml phosphate buffer and 0.5 ml (0.01 M) catechol solution with 0.5 ml extract. Changes in activities were measured following the procedure of Matto and Diamond (1963).

Statistical analysis

Data were subjected to ANOVA of a randomized block design, simple correlation were calculated and tested for significance and means. Principal component analysis (PCA), as the method of identifying the effect dimension of the data, was used to summarize the treatment information in a reduced number of effects for selection of the best performing treatment. Statistical analyses were done using SPSS Professional Statistics ver. 7.5 (SPSS Inc., Irvine, California).

RESULTS AND DISCUSSION

Physiological qualities

The changes in physiological qualities in response to foliar applications of HA, Zn and B in plants and fresh fruits of pungent pepper (*C. annum* L.), are shown in Tables 2, 3 and 4.

Plant growth qualities

The plant growth quality aspects were determined with regard to average height of plant, average number of leaves $plant^{-1}$, average leaf area and average number of branches $plant^{-1}$ (Table 2). In our present experiment, results revealed that with respect to plant height, there was significantly positive influence in all treatments except Zn and B individual application as compared to that of their corresponding control (only tap water spray) treatment, in which (Zn and B) treatments also positive influenced the height of plant, apparently, though not significantly. The higher performer was observed, when plants are treated with HA + Zn + B followed by HA + Zn and Zn + B.

On the other hand, in respect to number of leaves $plant^{-1}$, there was significantly positive effect in all treatments except B application alone over the control treatment, in which B treatment also positively enhanced, apparently, but not significantly. The maximum value of number of leaves was observed in HA + Zn + B treatment.

Regarding leaf area, significantly positive influence was

Table 2. Effect of foliar application of HA, Zn and B on plant growth quality in pungent pepper.

Treatment	Average height of plant (cm)	Average number of leaves plant^{-1}	Average leaf area (sq.cm)	Average number of branches plant^{-1}
Control	45.60	30.46	1.19	27.31
HA	53.02*	40.85*	1.40^{NS}	45.87*
Zn	51.09^{NS}	46.34**	1.28^{NS}	49.02*
B	48.27^{NS}	39.39^{NS}	1.33^{NS}	35.95^{NS}
HA+Zn	58.00**	56.34**	1.46*	63.04**
HA+B	51.62*	42.63*	1.69**	56.26**
Zn+B	57.00**	53.91**	1.71**	71.17**
HA+Zn+B	64.03**	58.57**	1.80**	75.11**
SE(m)±	1.96	3.21	0.07	5.56
LSD (0.05)	5.89	9.64	0.22	16.68
LSD (0.01)	8.47	13.87	0.30	24.02

*Significant at 5%, **Significant at 1% and ^{NS}Non significant

seen in all combination treatments of HA, Zn and B (HA + Zn, HA + B, Zn + B and HA + Zn + B) as compared to that of their corresponding control treatment. While, individual treatments of HA, Zn and B also exerted positive influence, through not significantly. The best performer was observed, when plants are treated with HA + Zn + B followed by Zn + B and HA + Zn application.

In the case of number of branches plant^{-1}, it was significantly induced in all treatments except B application alone than the control treatment. While, B application alone also induced the number of branches but not significantly. The highest number was recorded from HA + Zn + B followed by Zn + B treatment.

Our results also indicated that, in respect to growth quality, Zn application gave the better flourishing than the HA and B application over the control treatment. Moreover, under HA treatment, the plant height and number of branches plant^{-1} were increased significantly in pea plant, that reported by EI-Hak et al. (2012). Azarpour et al. (2011) also reported that the foliar application of HA gave the best and highest plant height values of cowpea. Kaya et al. (2005) reported that, HA and Zn both alone and/or combination treatments significantly enhanced plant height as compared to control treatment. While, Hatwar et al. (2003) reported that, Zn (0.1%) and B (0.1%) both alone and/or combination treatments exerted significantly positive influence on the plant height and number of branches plant^{-1} as compared to the control. Improvement of plant growth might be due to enhancement in photosynthetic and other metabolic activities which led to an increase in various plant metabolites responsible for cell division and cell elongation. Enhanced photosynthetic reactions in the presence of Zn and B might have increased the plant growth (Rawat and Mathpal, 1984). The further height of plant due to application of Zn explained by Mallick and Muthukrishnan (1979) was due to an active synthesis of tryptophan, an amino acid in the presence of Zn and it is the precursor of IAA which stimulates the growth of plant tissues. Besides, the Zn and B also plays an essential role in the development and growth of new cells in the plat. Plant requires B for synthesis of amino acids and proteins and regulation of carbohydrates metabolism (Dyar and Webb, 1961).

Fresh fruit yield and it's components

The fruit yield aspects were determined with regard to average number of fresh fruits plant^{-1}, average length of fresh fruit, average diameter of fresh fruit and average weight of 20 fresh fruits (Table 3).

Our present experiment indicated that, with respect to number of fresh fruits plant^{-1}, all the treatments except HA application alone exerted significantly positive influence as compared to control. The HA application also exerted positive influence, through not significantly. The maximum number of fruit plant^{-1} was observed by application of HA + Zn + B.

In the case of length of fruit, Zn, B, HA + B, Zn + B and HA + Zn + B application exerted significantly positive influence over the control. The remaining treatments, HA and HA + Zn application also influenced, apparently, though not significantly. The highest values of length of fruit were exhibited by HA + Zn + B followed by Zn + B and HA + B application.

All the treatments except HA alone exerted significantly positive influence on the diameter of fresh fruit as compared to control treatment, in which HA had positive influence but not significantly. The maximum value was recorded by Zn + B application.

Regarding, weight of 20 fresh fruits, HA + Zn + B, Zn + B and HA + Zn application exerted significantly positive effect as compared to the control. The remaining treatments also had positive effect, though not significantly.

The best performer was observed by HA + Zn + B

Table 3. Effect of foliar application of HA, Zn and B on fresh fruit yield and its components in pungent pepper.

Treatment	Average number of fresh fruits plant^{-1}	Average length of fresh fruit (cm)	Average diameter of fresh fruit (cm)	Average weight of 20 fresh fruits (g)
Control	3.37	4.47	3.00	45.67
HA	5.50^{NS}	5.27^{NS}	3.50^{NS}	48.33^{NS}
Zn	6.58*	5.90**	4.10**	52.73^{NS}
B	5.78*	5.57*	4.00**	49.60^{NS}
HA+Zn	8.66**	5.06^{NS}	4.70**	60.33*
HA+B	8.33**	6.20**	4.60**	57.60NS
Zn+B	9.53**	6.90**	4.80**	69.33**
HA+Zn+B	9.80**	7.30**	4.50**	81.60**
SE(m)±	0.75	0.31	0.21	4.05
LSD (0.05)	2.25	0.95	0.63	12.16
LSD (0.01)	3.24	1.34	0.91	17.50

*Significant at 5%, **Significant at 1% and ^{NS}Non significant.

followed by Zn + B application.

Whatsoever, Halwar et al. (2003) reported that, Zn and B both alone and/or combination treatments exerted significantly positive influence on the number of fruit plant^{-1} as compared to control. While Dongre et al. (2000) reported that the application of $ZnSO_4$ (0.10, 0.25 and 0.50%) exerted significantly positive influence on the average length of fruit and average diameter of fruit as compared to control. Concerning the effect of foliar application with HA, number of fruits plant^{-1}, fruit length, fruit diameter and 20 fruits weight were significantly increased during first season, 2009/2010 in pea (El-Hak et al., 2012). Azarpour et al. (2011) reported that HA foliar application gave significant increasing results on number of pods plant^{-1} and pod length of cowpea. Kaya et al. (2005) reported that HA + Zn treatments significantly enhanced number of pods plant^{-1} over the control treatment. Many researchers e.g., Zaky et al. (2006) on beans, Neri et al. (2002) on strawberry and Forgac and Czimbalmos (2011) on pea plants used the HA foliar application in green house and/or open field cultivations and found that number of pods plant^{-1}, total yield plant^{-1} and average pod fresh weight were markedly increased by the treatment at the rate of 1 g.L^{-1} combined with irrigation water.

Fresh seed yield and its compositions

Data regarding number of fresh seeds fruit^{-1} and weight of 100 fresh seeds are presented in Table 4. Analysis of variance revealed that the number of seeds fruit^{-1}, was significantly positive as observed by foliar application of Zn + B, HA + Zn + B, HA + B, HA + Zn and B as compared to control. HA and Zn application also occurred the positive effect, though not significantly. The good promising result was recorded by Zn + B followed by HA + Zn + B, HA + B and HA + Zn application.

On the other hand, in respect to average weight of 100 fresh seeds, all the combination treatments (HA + Zn, HA + B, Zn + B and HA + Zn + B) exerted significantly positive influence as compared to control treatment. While, individual application of HA, Zn and B also had positive influence, though not significantly. The maximum weight of 100 fresh seeds were recorded by application of HA + Zn + B. Moreover, Dongre et al. (2000) reported that the application of $ZnSO_4$ (0.10, 0.25 and 0.50%) exerted significantly positive influence on the average number of seeds fruit^{-1} and weight of 500 seeds as compared to the control. While, number of fresh seeds pod^{-1}, seeds weight and green pods yield significantly increased in pea (El-Hak et al., 2012). Similar results were obtained by Malik and Azam (1986) on wheat; Putintsev and Platonova (1991) on pea; Salib (2002) on peanut; Habashy et al. (2005) on peanut and faba bean and Azarpour et al. (2011) on cowpea plants as they reported that foliar spray with humic acid increased the dry seed yield and its parameters.

Biochemical qualities

Green pigments content

Beneficial effect of Zn on photosynthetic pigments may be due to its role in increasing the rates of photochemical reduction (Kumar et al., 1988), chloroplast structure, photosynthetic electron transfer as well as photosynthesis (Romheld and Marschner, 1991). In our present experiment, results revealed that, all the treatments exerted significantly positive influence on content of chlorophyll a, chlorophyll b and total chlorophyll as compared to control treatment (Table 5). The maximum chlorophylls (chl. a, chl. b and total chl.) content were recorded from HA + Zn + B application. Increased chlorophyll concentration by foliar application of B and Zn were reported in Irish plant over the control treatment (Khalifa et al., 2011). However

Table 4. Effect of foliar application of HA, Zn and B on fresh seed yield and its compositions in pungent pepper.

Treatment	Average number of fresh seeds.fruit^{-1}	Average weight of 100 fresh seeds (g)
Control	56.06	0.51
HA	61.46^{NS}	0.54^{NS}
Zn	59.26^{NS}	0.54^{NS}
B	66.37*	0.53NS
HA+Zn	71.52**	0.57*
HA+B	72.41**	0.58*
Zn+B	78.43**	0.62**
HA+Zn+B	76.54**	0.65**
SE(m)±	2.75	0.02
LSD (0.05)	8.26	0.05
LSD (0.01)	11.88	0.08

*Significant at 5%, **Significant at 1% and ^{NS}Non significant.

Table 5. Effect of foliar application of HA, Zn and B on green pigments content in pungent pepper.

Treatment	Chlorophyll a [mg.g^{-1} (FW)]	Chlorophyll b [mg.g^{-1} (FW)]	Ratio of chlorophyll a:b	Total Chlorophyll [mg.g^{-1} (FW)]
Control	0.91	0.44	2.07	1.32
HA	1.26*	0.71*	1.78*	1.97*
Zn	1.23*	0.70*	1.75**	1.93*
B	1.38**	0.89**	1.56**	2.27**
HA+Zn	1.45**	1.05**	1.46**	2.25**
HA+B	1.56**	1.05**	1.51**	2.72**
Zn+B	1.55**	1.04**	1.49**	2.60**
HA+Zn+B	1.72**	1.15**	1.50**	2.83**
SE(m)±	0.08	0.08	0.07	0.17
LSD (0.05)	0.25	0.24	0.21	0.50
LSD (0.01)	0.36	0.34	0.30	0.73

*Significant at 5%, **Significant at 1% and ^{NS}Non significant.

Farouk et al. (2011) reported that the application of HA also enhanced the chlorophyll concentration in radish plant. Whereas, in the case of values of chlorophyll a:b ratio opposite trend result of total chlorophyll were given, which exerted significantly negative influence in all treatments as compared to that of control (only water sprayed) treatment.

Moisture content

There are differences in moisture content and nutrient contents among the several treatments are presented in Table 6. In our present experiment, foliar applications of HA, Zn and B through different treatments could produce negative significant differences in the content of moisture. Therefore, no positive significant effects could be observed by applications of HA, Zn and B on the content of moisture.

Carbohydrates constituents

The beneficial effect of B may be due to its role in facilitating transport of carbohydrates, that is, starch and sugar (Donald et al., 1998). The obtained results are in conformity with those of Farahat et al. (2007) on *Cupressus sempervirens* and Nahed and Laila (2007) on *Salvia farinacea*. In our present study, results showed that in respect to reducing sugar, significantly enhanced effects were seen in all treatments except HA alone as compared to the control, in which (HA) also positive enhanced, apparently, though not significantly (Table 6). The maximum content of reducing sugar was recorded by HA + Zn + B and HA + B application.

Regarding, total sugar content (Table 6), exerted significantly positive influenced results were observed in HA + Zn + B, Zn + B, HA + B and B applications as compared to control. HA, Zn and HA + Zn also had positive effect but not significantly. The highest concentration of total

Table 6. Effects of HA, Zn and B on biochemical qualities in pungent pepper cv. Bullet (*C. annuum* L.)

Treatment	Moisture content (%)	Reducing sugar [mg.g^{-1} (DW)]	Total sugar [mg.g^{-1} (DW)]	Starch [mg.g^{-1} (DW)]	Protein [mg.g^{-1} (DW)]	Fat [mg.g^{-1} (DW)]	Energy [kJ.g^{-1} (DW)]	Total phenol [mg.g^{-1} (QE) (FW)]	Polyphenol oxidase (ΔA/min/g)
Control	86.02	7.49	26.65	213.80	111.30	71.00	8.65	0.97	0.56
HA	82.30*	8.90^{NS}	33.40^{NS}	270.60**	177.67**	93.00^{NS}	11.69**	1.14^{NS}	1.7**
Zn	83.22*	9.99*	29.21^{NS}	251.60*	200.67**	122.00**	12.79**	1.15^{NS}	0.67^{NS}
B	82.37*	11.61**	39.27*	301.40**	153.67*	60.00^{NS}	10.65*	1.58*	0.66^{NS}
HA+Zn	79.66**	12.21**	35.67^{NS}	291.70**	211.00**	111.00**	13.33**	1.40^{NS}	0.95^{NS}
HA+B	81.01**	13.05**	44.80**	308.23**	203.74**	91.00^{NS}	12.88**	1.86**	1.61**
Zn+B	80.59**	12.21**	47.85**	314.76**	221.11**	115.83**	14.28**	1.99**	1.12*
HA+Zn+B	79.03**	13.05**	52.00**	322.67**	215.32**	121.20**	14.58**	2.25**	1.72**
SE(m)±	0.76	0.68	3.01	12.33	12.56	7.82	0.66	0.15	0.16
LSD (0.05)	2.23	2.05	9.03	36.93	37.68	23.45	1.97	0.46	0.49
LSD (0.01)	3.28	2.94	13.00	53.26	54.26	33.78	2.85	0.65	0.69

*Significant at 5%, **Significant at 1% and ^{NS}Non significant.

sugar was observed from application of HA + Zn + B followed by Zn + B and HA + B.

On the other hand, the starch contents (Table 6), exerted significant positive influence in application of HA, Zn and B alone and/or combination treatments. The highest concentration of starch was observed from HA + Zn + B followed by Zn + B and HA + B application. However Khan et al. (2012) observed a decreased trend in reducing sugar concentration in relation to foliar application of Zn and B in citrus fruit. While, Kalifa et al. (2011) also reported that application of Zn and B increased the carbohydrates percentage in Irish plant. A similar trend of results was found by El-Khayat (1999), Gomaa (2001) and Samia and Mahmoud (2009) showing that Zn increased total carbohydrate in *Antholyza aethiopica* and *Tritonia crocata* plants, respectively. However, under Zn treatment, the values of total and non-reducing sugar increase were observed in citrus fruit (Khan et al., 2012). Increased total sugar concentration by application of HA was reported in radish plant (Farouk et al., 2011).

Protein content

Protein content of groundnut pod was significantly influenced by B application as reported by many workers (Bhuiyan et al., 1997; Murthy, 2006). Application of B stimulated nitrogen content of potato tubers and might have increased protein synthesis and subsequent storage of protein as suggested by Yadav and Manchanda (1979). In our present study, results observed that with respect to protein content, significant increasing trend were observed in all treatments over the control treatment (Table 6). The highest concentration of protein was recorded from Zn + B followed by HA + Zn + B and HA + Zn application. Moreover, increasing the concentration of protein by application of HA was reported in radish plant (Farouk et al., 2011) and in common vetch (*Vicia sativa* L.) (Saruham et al., 2011).

Lipid content

In the case of fat content (Table 6), HA, B and HA+B foliar spray failed to produce any significant effect over the control. The highest value of fat was exhibited by Zn followed by HA + Zn + B and Zn + B application. However, foliar application of Zn and B significantly increased the oil content in flowers of Irish plant over the control as observed by Khalifa et al. (2011).

Energy content

Regarding energy values in pungent pepper fruits (Table 6), all the treatments produced significantly increasing effects over the control treatment. Whereas, plants treated with foliar application of combination treatments gave best results than

individual treatments. The highest value of energy was observed from application of HA + Zn + B followed by Zn + B and HA + Zn.

Total phenol

Phenols are ubiquitous phytochemicals that contributed largely to antioxidant potential of any plant. The B is presumably responsible for the metabolism changes and cell damages in boron deficient tissue (Marschner, 1986) and it is thought that B complexes the phenolic compounds in plant cells, reducing their potential toxicity (Lee and Arnoff, 1967). However, the increase in total phenol effectuated by Zn application in wheat leaves was reported by Vinod et al. (2012). Hamid et al. (2010) found that phenolic content of plant were increasing with increasing level of heavy metal. In our present experiment, results showed that the total phenol content significantly increased in all treatments exception HA, Zn and HA + Zn application as compared to the control, which also followed an increasing trend over the control, apparently, though not significantly (Table 6). The highest concentration of total phenol was observed from HA + Zn + B followed by Zn + B and HA + B application.

Polyphenol oxidase

Polyphenol oxidases (PPO) proteins containing copper catalyze oxidation of hydroxyphenols to their quinine derivatives, which then spontaneously polymerize. Enzymatic browning in fruits and vegetables are due to presence of PPO and is often responsible for unpleasant sensory qualities and losses in nutrient quality (Sanchez-Amat and Solano, 1997). Cakmak et al. (1995) reported that under B-deficiency conditions, sunflower leaves are characterized by a rapid loss of K^+ and a strong discoloration (browning), as occurs with high PPO activity. Our present study showed that the polyphenol oxidase (PPO) activity was significantly increased in all treatments except Zn, B and HA + Zn application as compared to control, where Zn, B and HA+Zn also increased the activity of PPO but not significantly (Table 6). The highest activity of PPO was observed in foliar spray with HA+Zn+B followed by HA and HA + B. While, the PPO activity declined significantly in plant with B deficiency as reported by Cara et al. (2002). However, recently Pfeffer et al. (1998), working with B-deficient sunflower leaves, observed that B does not maintain plasma-membrane integrity by complexing phenols and inhibiting PPO activity to prevent damage by oxygen-free radicals or by regulating ascorbate metabolism. The authors concluded that the resupply of B to deficient leaves might be the result of the inactivation (detoxification) of phenolics by the formation of stable phenol-borate complexes and, thus, repression of phenol oxidation.

Correlation among variables

Correlation coefficients (Table 7) showed that the number of branches plant^{-1} had positive and significant correlation with length of plant (r^2=0.932), number of leaves plant^{-1} (r^2=0.943) and leaf area (r^2=0.882). While number of fruits plant^{-1} had positive and significant correlation with length of fruit, number of leaves plant^{-1}, leaf area and number of branches plant^{-1}. The correlation of number of seeds fruit^{-1} with fruit length (r^2=0.774) and fruit diameter (r^2=0.882) were positive and significant. There are significant and positive correlation between fruit length and weight of 100 seeds (r^2=0.890), plant height and weight of 100 seeds, total chlorophyll and leaf area (r^2=0.921). The correlation of energy with carbohydrates, fat, protein, total chlorophyll and leaf area were positive and significant. Another, significant and positive correlation was between polyphenol oxidase and leaf area (r^2=0.742). Moreover, there was significant and positive correlation of starch with some physiological characteristics such as leaf area, number of fruits.plant^{-1}, fruit diameter and total chlorophyll. The correlation of total phenol with carbohydrates and chlorophyll were positive and significant. Our results revealed that the positive and significant correlation between number of fruits plant^{-1} and 100 seeds weight (r^2=0.919), agree with that of result by Kaya et al. (2005), they observed positive and non significant correlation. The significant inverse correlation between moisture and starch (r^2=0.901) obtained supports results of Casanas et al. (2002) and Guchhait et al. (2008). Above relationships indicate that improving number of leaves and leaf area as well as increasing number of fruit and chlorophyll contents could accompany improvement in energy value as well as quality of fruits.

Principal component analysis (PCA)

In our present experiment, PCA was used to summarize the treatment information in a reduced number of components, where a total of three components were chosen (PC1, PC2 and PC3) due to their Eigen value being greater than 1.0 and they together explained 93.07% of total variance (Table 8).

The PC1 explained 80.99% of total variance where all the variables other than ratio of chlorophyll a:b and moisture content are positively loaded meaning that all other variables limit ratio of chlorophyll a:b and moisture content, in which the former is not desirable (Table 8). Therefore, on the basis of the first component, the treatments such as HA + Zn + B, Zn + B, HA + Zn and HA + B can be selected as having all desirable traits.

PC2, on the other hand, explained another 7.53% of total variance in which an increase in length of plant, number

Table 7. Pearson's correlation matrix of all variables.

Variable	Plant height	Number of leaves	Leaf area	Number of branches	Number of fruits	Fruit length	fruit diameter	Weight of 20 fruits	Number of seeds
Plant height									
Number of leaves	0.936**								
Leaf area	0.787*	0.711*							
Number of branches	0.932**	0.943**	0.882**						
Number of fruits	0.861**	0.919**	0.892**	0.970**					
Fruit length	0.699	0.681	0.856**	0.801*	0.810*				
Fruit diameter	0.684	0.838**	0.765**	0.856**	0.948**	0.694			
Weight of 20 fruits	0.914**	0.861**	0.874**	0.916**	0.884**	0.855**	0.717*		
Number of seeds	0.767*	0.787*	0.922**	0.875**	0.930**	0.774*	0.882**	0.849**	
Weight of100 seeds	0.900**	0.841**	0.947**	0.945**	0.919**	0.890**	0.759*	0.979**	0.900**
Chlorophyll a	0.803*	0.804*	0.915**	0.869**	0.931**	0.837**	0.874**	0.834*	0.927**
Chlorophyll b	0.780*	0.821*	0.870**	0.855**	0.938**	0.734*	0.922**	0.797*	0.945**
Ratio of chlorophyll a:b	-0.673	-0.784*	-0.747*	-0.775**	-0.886**	-0.648	-0.938**	-0.662	-0.885**
Total chlorophyll	0.714*	0.721*	0.921**	0.820*	0.902**	0.850**	0.865**	0.785*	0.918**
moisture	-0.888**	-0.900**	-0.834**	-0.902**	-0.932**	-0.683	-0.871**	-0.806*	-0.888**
Reducing sugar	0.655	0.736*	0.818*	0.772*	0.897**	0.725*	0.928**	0.732*	0.903**
Total sugar	0.711*	0.649	0.947**	0.783*	0.833*	0.877**	0.732*	0.850**	0.930**
Starch	0.691	0.707	0.856**	0.766*	0.855**	0.772*	0.836**	0.728*	0.921**
Protein	0.797*	0.892**	0.759*	0.924**	0.928**	0.736*	0.898**	0.728*	0.760*
Fat	0.760*	0.819*	0.514	0.813*	0.714*	0.590	0.600	0.680	0.447
Energy	0.875**	0.932**	0.821*	0.969**	0.958**	0.817*	0.883**	0.838**	0.812*
Total phenol	0.702	0.663	0.931**	0.783*	0.845**	0.894**	0.756*	0.873**	0.924**
Polyphenol oxidase	0.605	0.384	0.742*	0.575	0.510	0.528	0.318	0.504	0.502

*Significant at 5%, **Significant at 1%, Student's t-test.

Table 7. Contd.

Variable	Weight of 100 seeds	Chlorophyll a	Chlorophyll b	Ratio of chlorophyll a:b	Total Chlorophyll	Moisture	Reducing sugar	Total sugar	Starch	Protein	Fat	Energy	Total phenol
Plant height													
Number of leaves													
Leaf area													
Number of branches													
Number of fruits													
Fruit length													
Fruit diameter													
Weight of 20 fruits													

Table 7. Contd.

Variable	Weight of 100 seeds	Chlorophyll a	Chlorophyll b	Ratio of chlorophyll a:b	Total chlorophyll	Moisture	Reducing sugar	Total sugar	Starch	Protein	Fat	Energy	Total phenol
Number of seeds													
Weight of 100 seeds													
Chlorophyll a	0.883**												
Chlorophyll b	0.837**	0.977**											
Ratio of chlorophyll a:b	-0.707*	-0.926**	-0.968**										
Total chlorophyll	0.852**	0.988**	0.958**	-0.911**									
moisture	-0.838**	-0.946**	-0.961**	0.924**	-0.893**								
Reducing sugar	0.767*	0.947**	0.975**	-0.959**	0.951**	-0.885**							
Total sugar	0.903**	0.928**	0.881**	-0.784*	0.941**	-0.802*	0.853**						
Starch	0.792*	0.968**	0.957**	-0.941**	0.969**	-0.901**	0.931**	0.923**					
Protein	0.785*	0.828*	0.815*	-0.810*	0.796*	-0.865**	0.766*	0.643	0.734*				
Fat	0.670	0.492	0.457	-0.402	0.423	-0.580	0.381	0.332	0.313	0.832*			
Energy	0.877**	0.869**	0.841**	-0.803*	0.829*	-0.890**	0.781*	0.727*	0.767*	0.983**	0.846**		
Total phenol	0.908**	0.922**	0.880**	-0.781*	0.935**	-0.784*	0.874**	0.990**	0.901**	0.643	0.359	0.732*	
Polyphenol oxidase	0.615	0.630	0.528	-0.397	0.627	-0.608	0.418	0.617	0.565	0.519	0.337	0.546	0.540

*Significant at 5%, **Significant at 1%, Student's t-test.

Table 8. Results of PCA for effect of foliar application of HA, Zn and B on biochemical changes related to productivity of pungent pepper (*C. annuum* L.).

Principle component	Eigen value	Variance	Cumulative variance (%)
Eigen value and variance accounted for (%) by PCA based on correlation matrix			
1	18.63	80.99	80.99
2	1.73	7.53	88.53
3	1.04	4.53	93.07
Variables	**PC1**	**PC2**	**PC3**
Factor loadings due to PCs with Eigen values greater than 1			
Length of plant	0.872	0.324	-0.143
Number of leaves	0.889	0.369	0.139
Leaf area	0.933	-0.118	-0.261
Number of branches	0.953	0.281	-0.049
Number of fruits	0.984	0.111	0.092
Length of fruit	0.857	-0.019	-0.309
Diameter of fruit	0.909	0.012	0.361
Weight of 20 fruits	0.904	0.147	-0.304
Number of seeds	0.950	-0.185	-0.012
Weight of 100 seeds	0.944	0.092	-0.301

Table 8. Contd.

Principle component	Eigen value	Variance	Cumulative variance (%)
Chlorophyll a	0.975	-0.177	0.045
Chlorophyll b	0.960	-0.188	0.186
Ratio of Chl. a/Chl. b	-0.896	0.209	-0.389
Total chlorophyll	0.950	-0.267	0.031
Moisture	-0.948	-0.010	-0.177
Reducing sugar	0.913	-0.279	0.251
Total sugar	0.906	-0.333	-0.252
Starch	0.915	-0.350	0.101
Protein	0.889	0.322	0.225
Fat	0.638	0.754	-0.011
Energy	0.936	0.309	0.082
Total phenol	0.907	-0.311	-0.233
Polyphenol oxidase	0.590	-0.049	-0.358

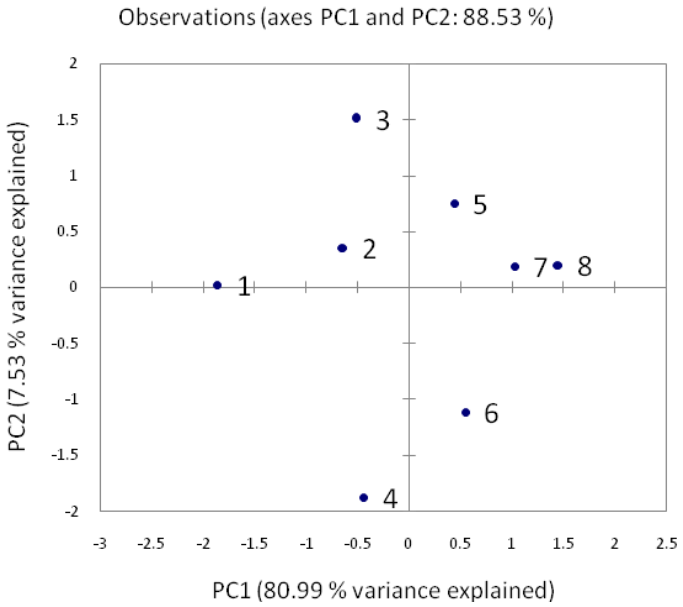

Figure 1. Biplot of regression factor scores for the first and second components produced by PCA: Legend: 1 (Control); 2 (HA); 3 (Zn); 4 (B); 5 (HA+Zn); 6 (HA+B); 7 (Zn+B); 8 (HA+Zn+B).

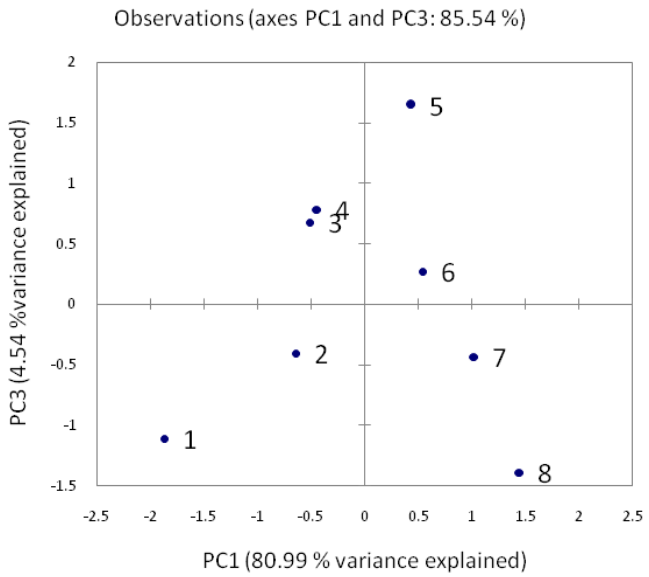

Figure 2. Biplot of regression factor scores for the first and third components produced by PCA: Legend: 1 (Control); 2 (HA); 3 (Zn); 4 (B); 5 (HA+Zn); 6 (HA+B); 7 (Zn+B); 8 (HA+Zn+B)

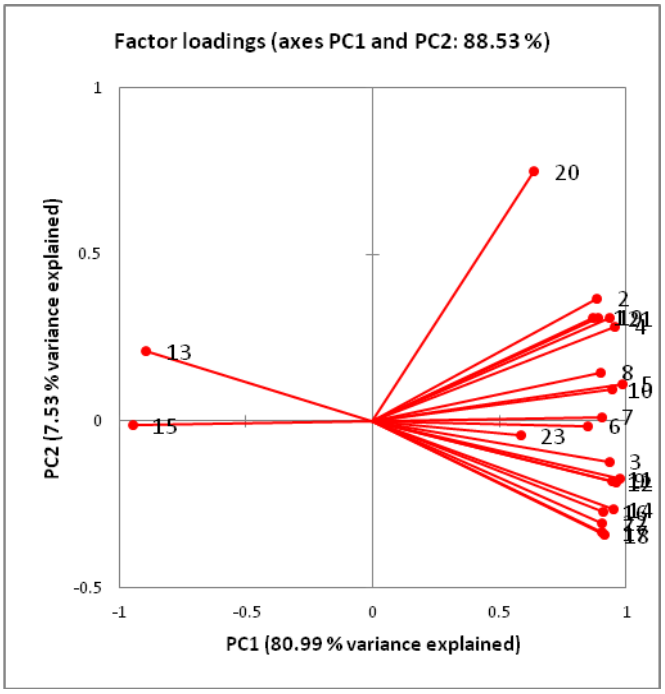

Figure 3. Scatter diagram of correlations between variables and factors loading for the first and second components produced by PCA: Legend: 1 (length of plant); 2 (number of leaves); 3 (Leaf area); 4 (number of branches); 5 (number of fruits); 6 (Length of fruit); 7 (diameter of fruit); 8 (weight of 20 fruits); 9 (Number of seeds); 10 (weight of 100 seeds); 11 (chlorophyll a); 12 (Chlorophyll b); 13 (ratio of Chl. a/Chl. b); 14 (total chlorophyll); 15 (moisture); 16 (reducing sugar); 17 (total sugar); 18 (starch); 19 (protein); 20 (fat); 21 (energy); 22 (total phenol); 23 (polyphenol oxidase).

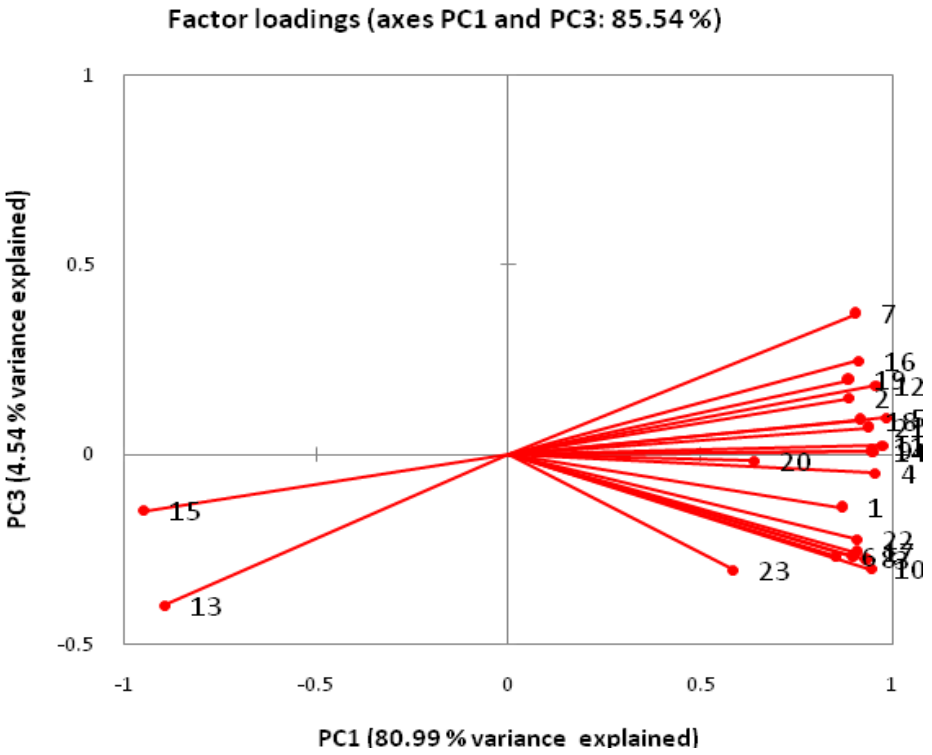

Figure 4. Scatter diagram of correlations between variables and factors loading for the first and third components produced by PCA: Legend: 1 (Length of plant); 2 (Number of leaves); 3 (leaf area); 4 (number of branches); 5 (number of fruits); 6 (Length of fruit); 7 (Diameter of fruit); 8 (weight of 20 fruits); 9 (number of seeds); 10 (weight of 100 seeds); 11 (chlorophyll a); 12 (chlorophyll b); 13 (ratio of Chl. a/Chl. b); 14 (total chlorophyll); 15 (moisture); 16 (reducing sugar); 17 (total sugar); 18 (starch); 19 (protein); 20 (fat); 21 (energy); 22 (total phenol); 23 (polyphenol oxidase).

of leaves, number of branches, number of fruits, diameter of fruit, weight of 20 fruits, weight of 100 seeds, ratio of chlorophyll a:b, protein, fat and energy were associated with a decrease in leaf area, length of fruit, number of seeds, chlorophyll a, chlorophyll b, total chlorophyll, moisture, reducing sugar, total sugar, starch, total phenol and polyphenol oxidase (Table 8 and Figure 3). Based on PC1 and PC2, the treatments such as HA + Zn + B followed by Zn + B and HA + Zn can be selected as performers (Figure 1).

The PC3 explained an additional 4.53% of total variance, in which number of leaves, number of fruits, diameter of fruit, chlorophyll a, chlorophyll b, total chlorophyll, reducing sugar, starch, protein, and energy were positively loaded in contrast to length of plant, leaf area, number of branches, length of fruit, weight of 20 fruits, number of seeds, weight of 100 seeds, ratio of chlorophyll a:b, moisture, total sugar, fat, total phenol and polyphenol oxidase, which were negatively loaded (Table 8 and Figure 4). According to the plot of regression factor scores due to PC1 and PC3, the performing treatment such as HA + Zn followed by Zn can be selected as having all desirable trails (Figure 2).

Conclusions

Based on principle component analysis and considering average values of all variables, our results suggested that the good promising treatment in HA + Zn followed by Zn + B, HA + Zn + B and Zn alone application may bring about the proper value addition in quality as well as productivity of crop by enhancing some physiological and biochemical characteristics in pungent pepper.

Conflict of Interests

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

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Seed germination and abnormality of cotyledon of *Peganum harmala* populations in Mongolia

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The present study assessed whether seed germination of *Peganum harmala* L. is related to population size and whether factors that control three and four cotyledons and difference in cotyledon shape are different and whether abnormality of cotyledons is related to genetic drift. The percentage of final seed germination was highest in Dzungarian Gobian population and followed by Trans-Altai Gobian population and lowest was in East Gobian population (Tukey HSD test, $p<0.01$). Most seedlings of *P. harmala* have two cotyledons in our study areas but there were abnormal cotyledons which are three, four and different shape dicotyledons. Three and four cotyledons showed low frequency in biggest (limited by river basins) and bigger (limited by oasis) populations and high frequency in small population (limited by well) whereas pattern of different shape dicotyledon frequency does not follow difference of population size. The different shape dicotyledons showed higher frequency in region with lower elevation, as compared to regions with higher elevation. The present results suggest that percentage of seed germination of *P. harmala* and frequency of more than two cotyledons might depend on population size, while frequency of different shape dicotyledons might be related to habitat difference which is high soil salinity. Also, our results suppose more than two cotyledons might be related to genetic drift.

Key words: Abnormality of cotyledons, genetic drift, population size, seed germination.

INTRODUCTION

Seed germination is the last stage of embryogenesis and cotyledons are formed during embryogenesis of angiosperms (Goldberg et al., 1994; Gomez et al., 2006; Chandler, 2008). Previous studies described effects of temperature, water supply, light and salinity on seed germination of *Peganum harmala* L. These studies were done in the desert region, east of Cairo and the semi-desert Mediterranean coastal (Hammouda and Bakr, 1969) and Pakistan (Hussain and Nasrin, 1985). Menges (1991) reported that percentage of seed germination is related to population size.

The cotyledon functions primarily during seed germination and senescence shortly after the seedling emerges from the soil (Goldberg et al., 1994). Angiosperms have mono- or dicotyledons but more and less than two cotyledons have been reported in over 15

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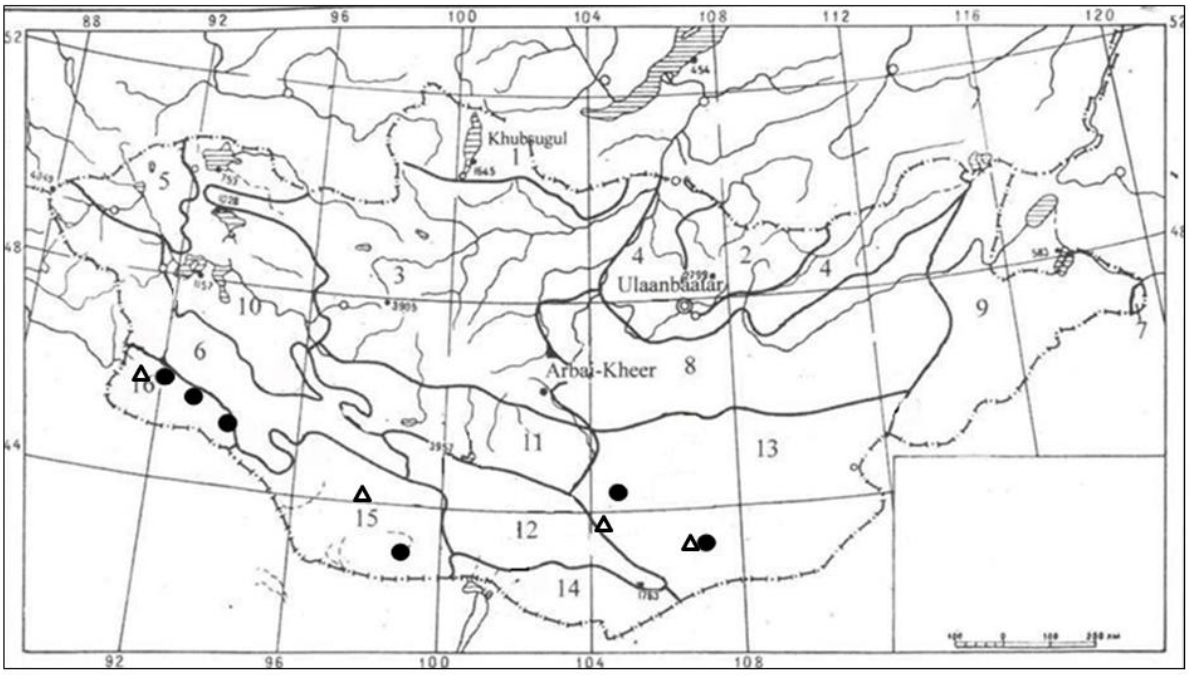

Figure 1. Seed samplng points of *P. harmala* in Mongolia (the botany-geographic region according to Grubov, 2001). ∆- weather station; seed sampling point. 1- Khubsugul, 2- Khentii, 3- Khangai, 4- Mongol-Dahuria, 5- Khovd, 6- Mongolian Altai, 7- Great Khingan, 8- Middle Khalkh, 9- Eastern Mongolia, 10- Great Lake Depression, 11- Valley of Lakes, 12- Gobi-Altai, 13-East Gobi, 14- Alasha Gobi, 15- Trans-Altai Gobi, 16- Dzungarian Gobi.

families (Conner and Agrawal, 2005).

The earliest defects were observed at the transition from the globular to the heart stage of embryogenesis with the formation of more than two cotyledons (Al-Hammadi et al., 2003). Previous studies supposed that cotyledon number range is related to hormonal asymmetric distribution and maternal effects (Liu et al., 1993; Mayer et al., 1993; Hadfi et al., 1998; Al-Hammadi et al., 2003; Orlova et al., 2006; Swarup et al., 2004), some kind of selection (Taylor and Mundell, 1999), lack of genetic variation (Conner and Agrawal, 2005), etc. Experimental studies reported that arabinogalactan proteins (AGPs) play functional roles in embryo development and cotyledon formation on *Nicotiana tabacum* (Qin and Zhao, 2007). Chandler (2008) reported that cotyledon bifurcation and lobing can result in supernumerary cotyledons. External nutrient (organic or mineral) supply during embryogenesis is important for embryo development (Qin and Zhao, 2007). However researchers still argue on the factor that could cause abnormality of cotyledon, most results supposed that genetic reasons might be related to abnormality of cotyledons.

Genetic variation was significantly reduced in endangered species when compared with non endangered species (Frankham, 1996). The effect of genetic drift is larger in small populations and smaller in large populations (Small et al., 2007). Dzungarian Gobi desert isolates distribution of *P. harmala* in Mongolia from a main distribution range in Middle Asia. In Mongolia, its distribution also isolated by deserts and then belongs to endangered species in Mongolia (Shiirevdamba et al., 1997).

The purpose of the present study was to assess whether seed germination of *P. harmala* is related to population size and whether factors that control more than two cotyledons and difference in cotyledon shape are different and whether abnormality of cotyledons is related to genetic drift.

MATERIALS AND METHODS

Seed samples were taken from three sites in the Dzungarian Gobi (46° 06’ 10.33N, 91° 35’14.49E, alt: 1191 m in Khovd river basin; 45° 53’ 03.28N, 91° 47’01.18E, alt: 1163 m in Unch river basin and 45° 34’ 34.89N, 93° 04’33.38E, alt: 1498 m near Shiir us spring) and one in Thras-Altai Gobi (43° 14’ 679N, 099° 00’ 411E, alt: 971 m in Ekhiin gol oasis) and two in East Gobi (44° 46’ 27.12N, 105° 15’32.55E, alt: 1200 m near well of herder camp between the sums Tsogt-Ovoo and Khongor and 43° 10’ 48.11N, 107° 10’29.36E, alt: 1060 m near well in Khanbogd sum, Southgobi aimag).

Climatic data is given based on database of Institute of Metereology and Hydrology of Mongolia. Nearest weather stations with seed sampling points were used for the present study: The nearest one with sampling points was “Baitag” station in Dzungarian Gobi and “Tooroi” station in Trans-Altai Gobi and “Dalanzadgad” and “Hanbogd” stations in East Gobi (Figure 1). Average temperature in July (°C) was 20.9, 23.4 and 21.2-23.8 and annual precipitation (mm) was 71.9, 49.5 and 84.0-127.1 in Dzungarian Gobi, Trans-Altai Gobi and East Gobi. Dzungarian Gobi

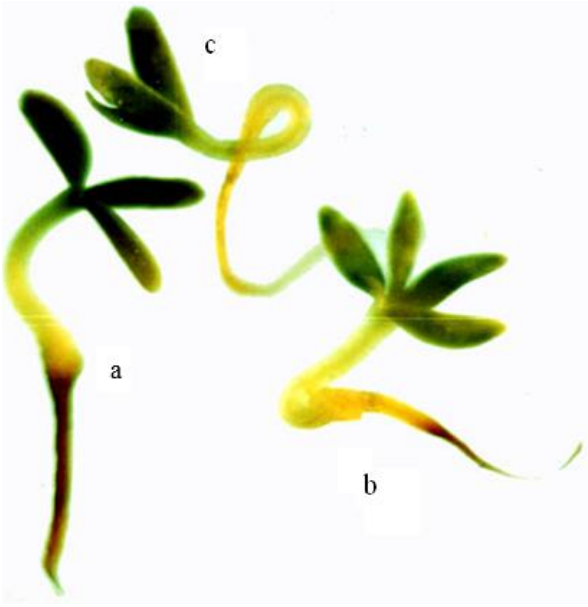

Figure 2. Diversity of *P. harmala* cotyledons in Mongolia. a- three cotyledons, b- four cotyledons, c- different shape dicotyledons.

region belongs to the province Dzungaria, Mts. Tien Shan and East Gobi and Trans-Altai Gobi belong to the province Mongolia, according to botany-geographic divisions (Grubov, 1963; Takhtajan, 1978).

The seed collection at the Institute of Botany was used for seed germination and observation of seedling morphology. In total, 6 seed samples were examined (Figure 1) which was collected between 1983 and 2001, from above-mentioned regions. When plants shed seeds, capsules were harvested then dried them paper bags at room temperature. The seeds were sampled randomly in each population.

Seed maturity of *P. harmala* was evaluated by weight of dry seeds as reported Harrington (1972). Weight of a thousand seeds was measured 10 times, using the analytic scale Shimadzu AY220 (d-0.1 mg). Seed germination was determined at 25±1°C for 10 days in the seed germinator, without dormancy breaking treatments, using Petri dishes and moist blotter by distilled water. Frequency of cotyledon number and shape was counted on 1000 embryos in each seed sample.

Maximum percentage of final seed germination was compared among regions, climatic variability and population sizes, because of only one site in Trans-Altai Gobi.

Population sizes (N) corresponded to the red-listed categories of “critically endangered” and “endangered”, “vulnerable” respectively (Brook et al., 2002). Main distribution range of *P. harmala* is in the Dzungaria, Mts. Tien Shan (Central Asia).

In Mongolia, population of this species in the Dzungaria is isolated from other populations by Gobi-Altai mountain system and Trans-Altai Gobian population is isolated from East Gobian population by Alasha Gobi desert. Distribution of *P. harmala* is limited to river basins, area of oasis and well in the Dzungarian Gobi, Trans-Altai Gobi and East Gobi regions. Hence, population size of *P. harmala* in river basins, oasis and near well was evaluated biggest (in the Dzungarian Gobi), bigger (in Trans-Altai Gobi) and small (in East Gobi), respectively.

Geographic differences of weight of a thousand seeds, final germination, frequency of different shape dicotyledons, three and four cotyledons were estimated by Tukey HSD test.

RESULTS

Mean of a thousand seed weight of *P. harmala* was 2.67, 2.43 and 2.6 g in the regions Dzungarian Gobi, Trans-Altai Gobi and East Gobi. Percentage of final seed germination was 95, 72 and 61% in the Dzungarian Gobi, Trans-Altai Gobi and East Gobi, respectively. To compare percentage of final seed germination with climatic variability, high and low percentage of seed germination was found in region of low and high air temperature in July but regional difference of its percentage does not follow regional difference of annual precipitation. The percentage of final seed germination was highest in Dzungarian population and followed by Trans-Altai Gobian population and lowest in East Gobian population (Tukey HSD test, $p<0.01$).

Most seedlings of *P. harmala* have two cotyledons in our study areas but there were abnormal cotyledons which are three (Figure 2a), four cotyledons (Figure 2b) and different shape dicotyledons (Figure 2c).

In comparison, frequency of abnormal seedlings among populations, 1.6, 2.7 and 6.8% of all seedlings in Dzungarian, Trans-Altai Gobian and East Gobian populations have three cotyledons but 0.5 and 1.5% in Dzungarian and East Gobian populations have four cotyledons. Different shape dicotyledons were found (9.6, 14 and 7.4%) in Dzungarian, Trans-Altai Gobian and East Gobian populations. Frequency of three cotyledons in East Gobian population was significantly higher than in other populations. However, frequency of three cotyledons was not significantly different between Dzungarian and Trans-Altai Gobian populations, Dzungarian population showed low frequency, when compared with Trans-Altai Gobian population. Also, frequency of four cotyledons was not significantly different between Dzungarian and East Gobian populations but its frequency was low in Dzungarian population, when compared with East Gobian one. Frequency of different shape dicotyledons was significantly higher in Trans-Altai Gobian population than in Dzungarian and East Gobian populations. However, frequency of different shape dicotyledons was not significantly different between Dzungarian and East Gobian populations, Dzungarian population showed higher frequency, when compared with East Gobian populations (Figure 3).

Different shape dicotyledons showed low and high frequency in region with high and low altitude but frequency of three and four cotyledons does not follow. To compare frequency of abnormality of cotyledons with climatic variability, high frequency of three and four cotyledons was found in regions with 21.2-23.8°C of average temperature in July while low frequency was found in regions with 20.9-23.4°C. Different shape dicotyledons showed high frequency in region with 49.5 mm of annual precipitation and low frequency in regions with 71.9-127.1 mm whereas frequency of three and four

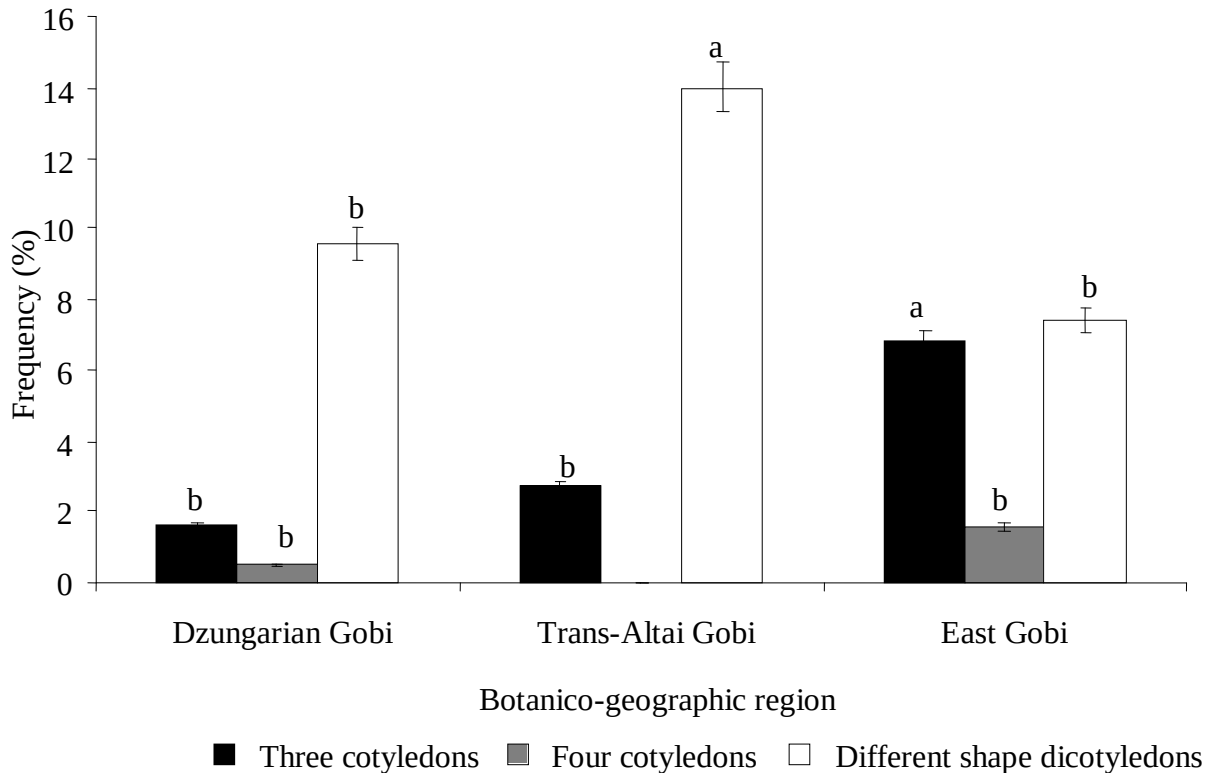

Figure 3. Cotyledon number and shape differences of *P. harmala* among the botany-geographic regions in Mongolia (Tukey HSD test, $p<0.05$).

cotyledons does not follow regional difference of annual precipitation.

Highest frequency of three and four cotyledons was found in East Gobian population with lowest final seed germination but that was lowest in Dzungarian population with highest final seed germination. The highest frequency of different shape dicotyledons was found in Trans-Altai Gobian population with averaged final seed germination whereas it was significantly low in Dzungarian and East Gobian populations with highest and lowest percentage of final seed germination. These patterns show that frequency of three and four cotyledons increased with decreasing percentage of final seed germination but frequency of different shape dicotyledons was not correlated with percentage of final seed germination.

Three and four cotyledons showed low frequency in biggest (limited by river basins) and bigger (limited by oasis) populations and high frequency in small population (limited by well) whereas pattern of different shape dicotyledon frequency does not follow difference of population size.

DISCUSSION

Seed germination is the most important factor for *P. harmala* growth and distribution. *P. harmala* seeds germinated well at about 10 mm of rainfall (Hammouda and Bakr, 1969). Daily amount of precipitation in our study sites is insufficient for seed germination of this species, resulting in low amount of annual precipitation. But, river, oasis and well water supply could be sufficient for seed germination.

P. harmala exhibits 40 and 80-90% of final seed germination at 25 and 30°C in room temperature (Hussain and Nasrin, 1985). The present experiment indicated that seeds of *P. harmala* germinated 60-95% at 25°C. To compare results of previous and present experiments, seed germination of *P. harmala* is higher in Mongolia than in Cairo, Mediterranean coastal and Pakistan at same temperature. It seems seeds of *P. harmala* in Mongolia could adapt to coldness, better than above mentioned regions. However, negative correspondence between percentage of final seed germination and experimental temperature was found, seeds of *P. harmala* showed highest percentage of germination in Dzungarian Gobi with lowest temperature in July, when compared with other regions. Hence, regional difference of annual precipitation and air temperature in July cannot be main limiting factors for seed germination of this species.

However, ground water supply is sufficient for seed germination of *P. harmala*, water supplying area must be different among river, oasis and well. The results indicate that seed germination of *P. harmala* is low and high in

small and bigger populations, as reported by Menges (1991). Hence seeds of *P. harmala* cannot potentially germinate in Mongolian desert, because of area limitation.

Previous studies reported more and less than two cotyledons in the family Aceraceae, Juglandaceae, Rubiaceae, Pedaliaceae, Protaceae, Ranunculaceae, Papaveraceae, Brassicaceae, Fabaceae, Geraniaceae, Chenopodiaceae, Onagraceae, Solanaceae, Scrophulariaceae, Salicaceae and Euphorbiaceae (Gates, 1910; Went, 1944; Harrison, 1964; Dessureaux, 1967; Magsar and Tsagaanmaam, 1984; Rajora and Zsuffa, 1986; Taylor and Mundell, 1999; Graz, 2001; Conner and Agrawal, 2005; Chandler, 2008). The present results show three, four cotyledons and different shape dicotyledons in *Peganum* genus (Peganaceae), in addition to above mentioned families.

Experimental study on tomato showed that abnormality of cotyledons is formed during embryogenesis (Al-Hammadi et al., 2003). Qin and Zhao (2007) showed that βGlcY affects cotyledon formation of *Nicotiana tabacum* L. Recent studies explain abnormality of cotyledons as a lack of genetic variation (Conner and Agrawal, 2005) but researchers still argue on factors and genetic reasons of abnormality of cotyledons (Chandler, 2008).

Frequency of more than two cotyledons showed same pattern with regional difference of average temperature in July but frequency of different shape dicotyledons showed negative pattern with regional difference of annual precipitation and elevation. The correspondences suppose that frequencies of more than two cotyledons and different shape dicotyledons might be signs of different factors, showing frequency of more than two cotyledons corresponding to heating that cause water evaporation while frequency of different shape dicotyledons was with length of vegetation period that depends on elevation.

Frequency of more than two cotyledons negatively corresponded with percentage of final seed germination. Seed maturation was not different among regions, because of seed weight indifference (Tukey HSD test, $p>0.05$). Correspondence between frequency of more than two cotyledons and population size indicated that frequency of more than two cotyledons decreases towards the main distribution range of *P. harmala*. This results suppose that more than two cotyledon might be related to genetic drift. Several genes such as DRN/ESR1 and DRN-LIKE/ESR2 have a role in cotyledon development, with mutant showing syncotyly and pleiocotyly (Chandler, 2008).

Cytokinin and arabinogalactin proteins (AGPs) play an important role during the stage of embryogenesis at which cotyledon number is determined (Chaudhury et al., 1993; Qin and Zhao, 2007). The present study discuss canalization of cotyledon number and bifurcation or lobbing as cause of more than two cotyledons formation (Conner and Agrawal, 2005; Chandler, 2008).

More than two cotyledons means shape of cotyledon leaves on a seedling of *P. harmala* is similar while different shape dicotyledons means shape of two cotyledon leaves on a seedling is different. The different shape dicotyledons showed higher frequency in region with lower elevation (in oasis), as compared to in regions with higher elevation. High value of average temperature in July and low value of annual precipitation indicated drought condition in Trans-Altai Gobi. Hence, *P. harmala* in oasis used more ground water for growth during drought season. However, soil salinity in oasis is higher than other dry regions, resulting in ground water evaporation (Pankova et al., 2004; Pankova, 2008). Safina (1977) reported that calyx lobe of *P. harmala* was found in high saline habitats (in oasis) but it was complete in low saline habitats and showed it as polymorphism.

Water stress induced by drought affects β-amylase activity of cucumber cotyledons in non-saline soil condition (Todaka et al., 2000) but our results suggest that different shape dicotyledons in *P. harmala* might depends on soil salinity which explains osmotic and toxicity effects, in saline soil condition.

The present results suggest that percentage of seed germination of *P. harmala* and frequency of more than two cotyledons might depend on population size, while frequency of different shape dicotyledons might be related to habitat difference which is high soil salinity. Also, our results suppose more than two cotyledons might be related to genetic drift.

Conflict of Interests

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

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Full Length Research Paper

Germination tests of seeds of argan tree (*Argania spinosa* (l.) skeels) of two sources (Tindouf and Mostaganem) in the semi-arid western Algerian.

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The Argan tree (*Argania spinosa*) is a drought-tolerant, and forest species observed in arid and semi-arid zone in Algeria, has specific ecological characteristics and many interests (forest, field, and fruit). The natural reproduction of the tree has become difficult; we have assessed the propagation method by seedlings. In our experiments, we used two seed sources collected from Tindouf and Mostaganem. Based on the results of the regeneration of the Argan tree seedlings from the laboratory, we report that soaking of seed for at least four days will certainly contribute to the success of germination. Sterilization prevents microbial contamination and improves germination. According to the results, the germination tests revealed a very high rate of germination (95%) for seeds pre-soaked in water for 96 and 120 h at 25 and 30°C. The analysis of morphological characteristics of plants under greenhouse showed that there was growth in the root system of the argan seedlings and the aerial part improved quantitatively and qualitatively. Seedlings that acclimatized are two years and above, and had a well developed and lignified air device with a large leaf, which promotes their growth. The success rate for seedlings of 12 months was very low.

Key words: Argan tree, domestication, germination, pre-soaked, acclimatization, seedlings.

INTRODUCTION

In south Algeria (Tindouf), forest based on the endemic argan tree (*Argania spinosa* L. Skeels), Propagation by seeds is the most common method used to reproduce the argan forest species. All the reforestation projects of argan in Algeria adopt this method. The most successful example of this is the Mostaganem project in coastline, which began to bear fruit after six years, and all nurseries in Tindouf which aim to rehabilitate the argan tree. The sustainability of this agroforestry system is now threatened by over-exploitation and overgrazing in Tindouf (Touaref bouaama, Targanat and Markala) by the local population and the nomads of the Polisario. This

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Figure 1. Components of the argan seed.

results in serious degradation of the soils and a decrease in production of oil (traditional extraction) and food betailles (camels and sheep). It is therefore a national concern for the authorities involved to protect our rare and vulnerable inherited Argan forests against anthropogenic pressure. They should allow the establishment of an executive order by the Wali of Wilaya, Tindouf bearing draft reserve natural creation (Decree No. 04/96 of 1 2/06/2004) on the protection of argan plant species in the Wilaya of Tindouf.

Perfectly suited to its environment, this endemic tree can grow on poor, shallow soils, and owing to its deep rooting system, it is considered as having a strong effect against erosion and desertification, which are the main environmental problems. The argan tree is monoecious and allogamous and exhibits high genetic diversity (Msanda, 1993). This diversity can both be preserved for ecological purposes and used through domestication. Recent initiatives have promoted the domestication of multi-purpose agroforestry species for their ability to alleviate poverty and mitigate environmental degradation (Leakey and Simons, 1998). This approach is relevant to *Argania spinosa* which could be domesticated for oil and fodder production. What is required is a package of technical procedures from the selection of the best genotypes to the production of cultivars in nurseries for successful integration into agroforestry systems. Currently, due to the failure of natural regeneration and reforestation, the only possibility of rejuvenating mature argan forest is through coppicing. Seedling production and use for plant production in nurseries could allow for the conservation of the genetic diversity. However, according to the literature, seed germination is difficult. Through selection and mass production of trees with desirable characteristics, biotechnology could improve argan tree productivity, as well as overall production (Sasson, 1993). Vegetative propagation offers the opportunity to multiply selected genotypes and to provide a significant step towards 'domestication'. But the germination is a very complex biological phenomenon which requires a good understanding and control of the factors behind. For multiplication of argan, it was found that the technique of soaking seeds in water is quite sufficient for good germination (Nouaïm and Chaussod, 1993). To overcome this constraint related to the biological nature of the seed, we performed a pre soaking in water of seeds at varying durations before planting. The purposes of the present study were: to examine the factors affecting variation in the germination of argan seeds, acclimatization of plantlets produced by germination, and to improve the success rates of nursery production and transplantation

MATERIALS AND METHODS

Propagation by seeds

The seeds used in this study came from ripe fruits collected from randomly chosen trees located in Tindouf forest (south of Algeria), and nursery of Mostaganem (coastline). The fruits were dried and their pulp was removed by hand to obtain nuts. Just before germination, they were disinfected with hydrogen peroxide for 20 min (Figure 1).

Tests for germination

The technique used was by soaking argan seeds in water at different times (72, 96 and 120 h), then the seeds were placed in the oven to heat thresholds considered (30, 28, and 25°C). Under these conditions, we want to know the ability of seeds' germination of two sources: Tindouf (natural area of argan) and Mostaganem (introduced species) (Figure 2). We conducted a test with 20 seeds for each treatment.

First germination test with a temperature of 30°C

The argan seeds were previously disinfected with bleach for 20 min; then they were pre-soaked in water and placed on cotton in Petri dishes: Treatment 1: The seeds are soaked in water for 72 h; Treatment 2: the seeds were soaked in water for 96 h; Treatment 3, the seeds were soaked in water for 120 h.

The germination tests with temperatures of 28 and 25°C

The argan seeds were disinfected prior to pure bleaching for 20

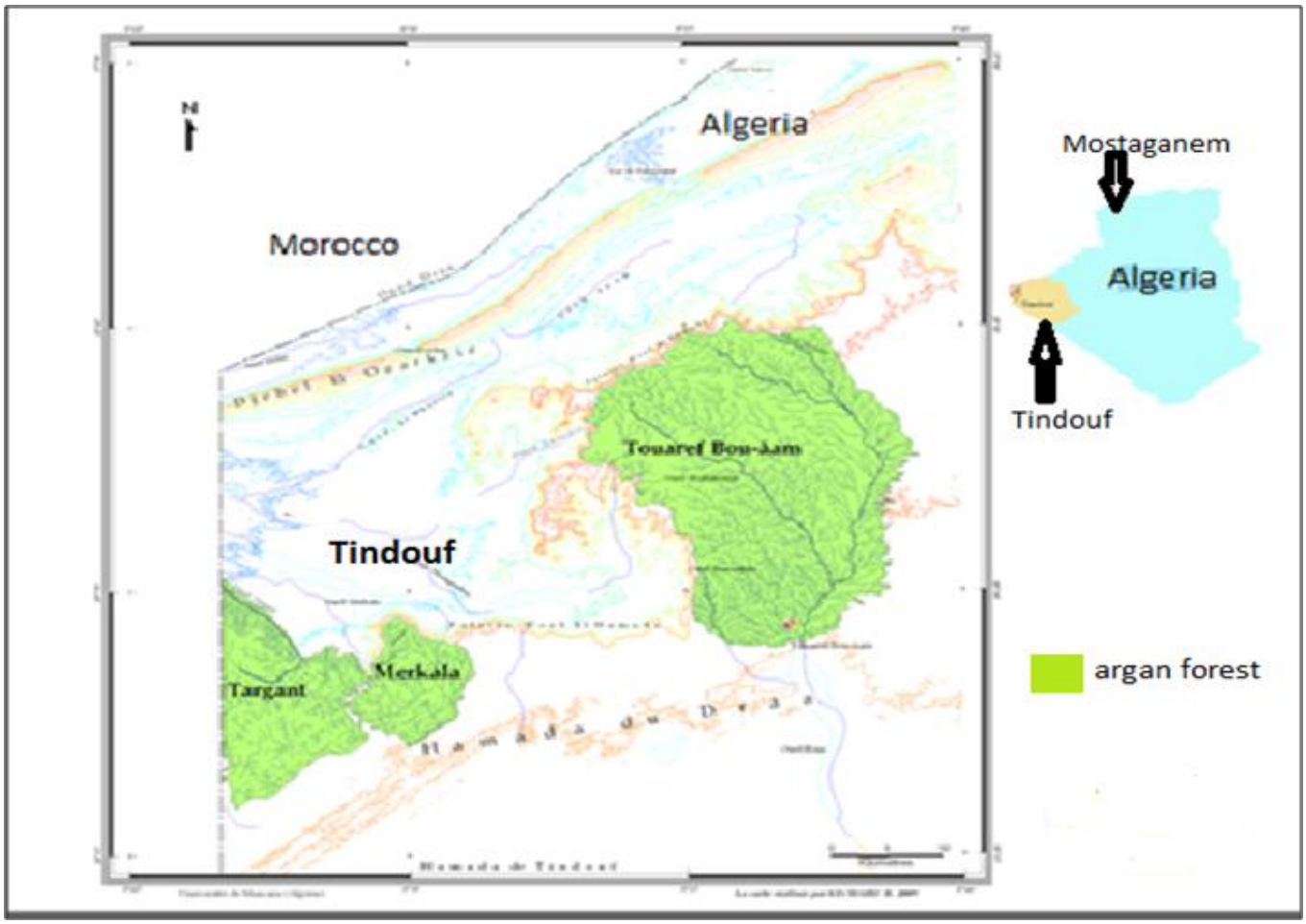

Figure 2. The whereabouts of argan in Algeria.

Figure 3. Reforestation project of argan in Mostaganem.

min, and then they pre-soaked in water treatment for 96 and 120 h only. This is because soaking for 72 h gives us very low germination than when placed on cotton in boxes kneaded (Figure 3).

Figure 4. Seedlings of argan in plastic bag.

Figure 5. Seedlings of argan in pots.

Treatment 1

The seeds were soaked in water for 96 h.

Treatment 2

The seeds were soaked in water for 120 h.

Acclimatization of seedlings

In order to know the behavior of the argan tree under the influence of abiotic conditions, we found it useful to perform planting in two different settings: Under greenhouse and open field. The choice of these two environments was done so that we can follow the growth of seedlings after planting argan and to compare the environmental conditions.

On emissions

Transplanting seedlings argan in plastic bag

In the laboratory we installed in plastic bags peat sand filled to two thirds and one-third. The experimental conditions were characterized in the laboratory by a thermal range of 18 to 24°C, exposure to sunlight and a relative humidity of ambient air of about 65 to 75%. Seedlings of the argan tree were watered every 48 h (Figure 4).

Transplanting seedlings argan in pots

Young seedlings argan obtained under the above conditions (under glass) were removed from the bags with their lumps around the roots, and then they were transplanted into pots. The substrate used consists of peat mixed with sand in equal proportion (1/2 and peat 1/2 sand) (Figure 5).

In the field

The argan tree seedlings were monitored on the ground after planting. They have the following characteristics.

Origin

These two types of argan seedlings that aged between one and two years are from the nursery of Mostaganem (littoral). We also noted that these seedlings had varying dimensions (height, diameter, branch), even those of the same age.

Planting period

Generally, planting periods are chosen depending on the rainfall. In Algeria, plantations are generally done from October to March because the soil is in good condition during that time and the humidity is high (about 90%). In our case, the argan tree seedlings were sown in 28/02/2008.

Planting method

There were 24 argan seedlings of one year and 20 seedlings of 2 years. The length of the seedlings ranged from 06-73 cm, with a number of sheets of about 150 units. A plot at the experimental farm was prepared to serve the planting site. Planting distances were 1 m between seedlings and 2 m between the lines. The first watering was done immediately after planting, then it was repeated once a month for six months after planting (the amount of irrigation water is 3 L per plant).

Statistical analysis

Both parametric (t test) and non-parametric tests (Mann-Whitney-Wilcoxon test, based on sum of ranks) were performed on experimental data for comparison of treatments (Conover, 1980).

RESULTS

Germination rate

Germination is a complex biological phenomenon that requires control and identification of factors causing it. In our case, we are interested in the argan fruit covered with a very hard shell. So we practice several treatments before sowing seeds at different temperatures to find the

Figure 6. Germination of argan seeds

optimal conditions for germination (Figure 6).

Germination tests at a temperature of 30°C

Pre soaking in water for 72 h

The seed germination started on the fourth day at a rate of 10% for those in Mostaganem against those germinated in Tindouf. Raw sproutswe observed as early as 10 days at a rate of 5%. Then, the germination rate increased slowly and reached maximum rates of 95 and 80%, respectively at duration of 20 days (Figure 7).

Pre soaking in water for 96 h

The first sprouts were obtained from the second day at a rate of 10% for both seeds. Then, the germination rate increased to a maximum of 80% on the 12th day (Figure 8).

Pre- soaking in water for 120 h

Seeds pre-soaked in water for 120 h express a relatively high germination rate (15%) from the third day of sowing: 15% for Mostaganem seeds and 10% for those of Tindouf. Germination rate increased rapidly up to 75% in seeds of Mostaganem and 80% for seeds of Tindouf on the 13th day of sowing (Figure 9).

Testing the germination temperature of 28°C

Based on the fact that the best results on germination of argan were obtained from pre soaking in water for 96 h and 120 h, we found it useful to continue the remainder of the tests with the two treatments. Seed treatment consisting of pre-soaking in water for 75 h gave very poor results.

Pre soaking in water for 120 h

Seeds pre-soaked in water for 120 h express variable responses to germination (Figure 10). Indeed for the two batches of seeds, germination begins at the third day of planting at a rate of 15% for seeds of Mostaganem and 10% for those of Tindouf. Thereafter, germination evolves to a maximum of 90% for Mostaganem seeds and 95% for those of Tindouf.

Pre soaking in water for 96 h

There was a significant difference in the behavior of the two types of seeds. Indeed, the seeds of Tindouf germinated on the sixth day and those of Mostaganem, on the fourth day, at 10 and 5% rates, respectively. As a result, germination increased to 95% for seeds of Mostaganem and 90% for those of Tindouf at day 18 (Figure 11).

Test germination temperature of 25°C

Pre soaking in water for 120 h

The first sprouts were obtained from the second day at a rate of 10 to 45% for Mostaganem seeds. Seeds of Tindouf gave a rate of 5% at the 2nd day of planting. Germination evolves rapidly to a maximum of 95% for Mostaganem seeds and 90% for Tindouf seeds on the 18th day (Figure 12).

Pre soaking in water for 96 h

There was an early germination of the second day of planting at a rate of 5% for both types of seed. As a result, the levels increase slowly and reached a maximum of 95% on the 20th day of sowing (Figure 13).

Acclimatization step

Morphological characteristics of the argan tree seedlings

Plants produced from seed had a tap root system with a fast and powerful development under these conditions: 15 days after sowing is enough for the taproot to appear through the hole at the bottom of the bags.

Main characters observations and measurements:

i) Number of leaves and thorns

Young argan seedlings had a strong capacity for growth and development. Therefore, the leaf system was significant in the first month, with an average of 30 leaves per seedling, and about 70 leaves per seedling after 9 months (Figure 14).

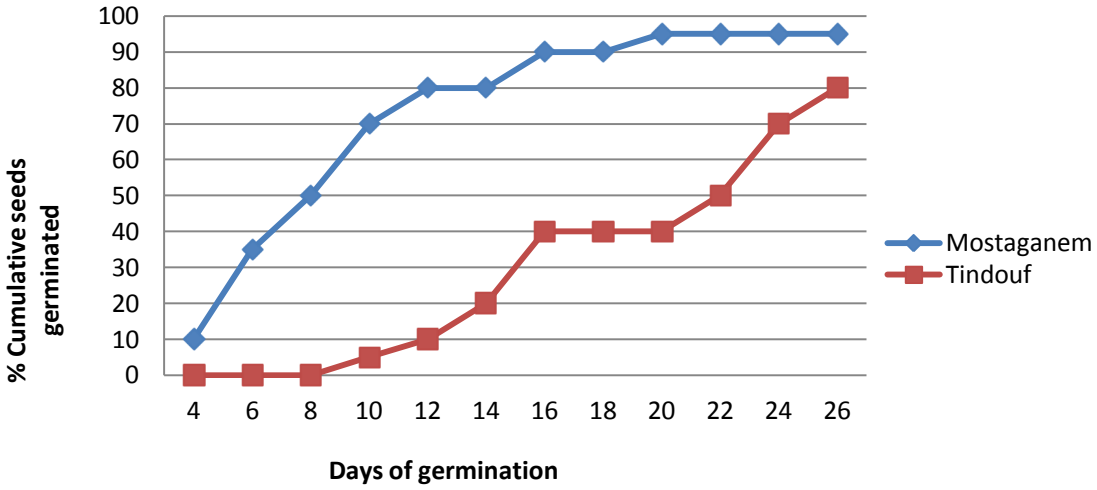

Figure 7. Daily argan seed germination rate pre-soaked for 72 h and subjected to a temperature of 30°C.

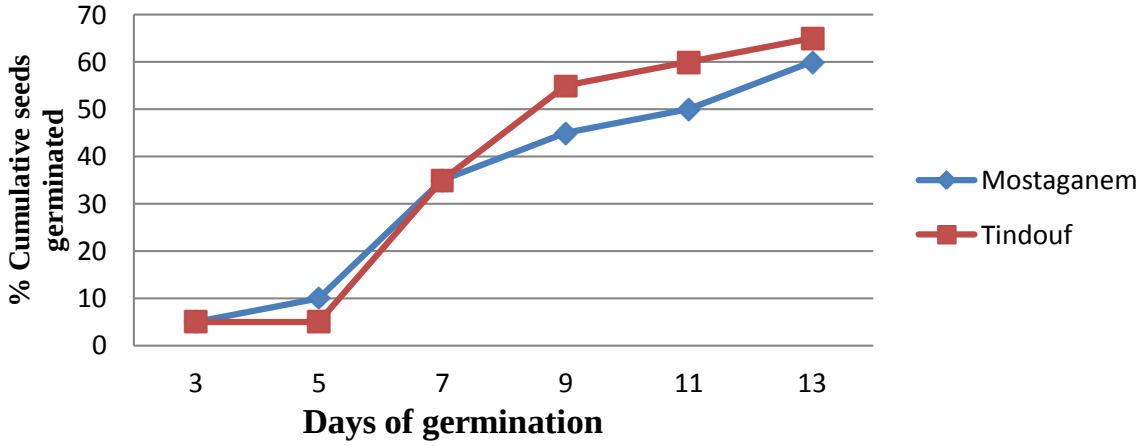

Figure 8. Daily argan seed germination rate, pre-soaked for 96 h and subjected to a temperature of 30°C.

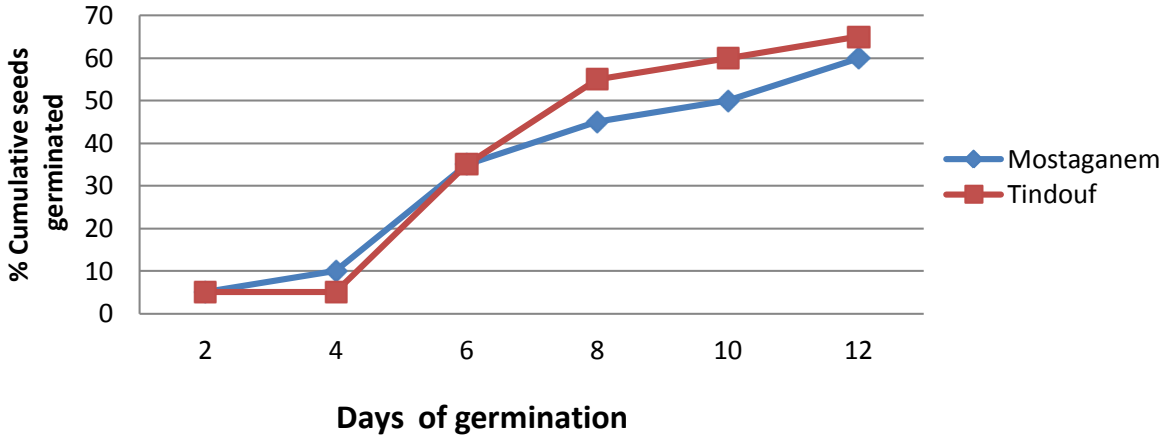

Figure 9. Daily argan seed germination rate, pre-soaked for 120 hours and subjected to a temperature of 30°C.

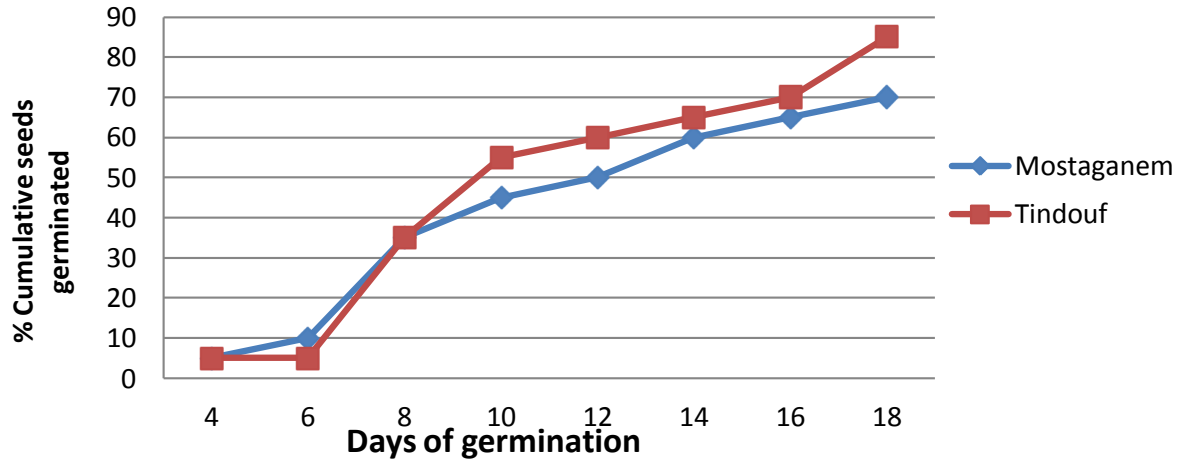

Figure 10. Daily argan seed germination rate, pre-soaked for 120 h and subjected to a temperature of 28°C.

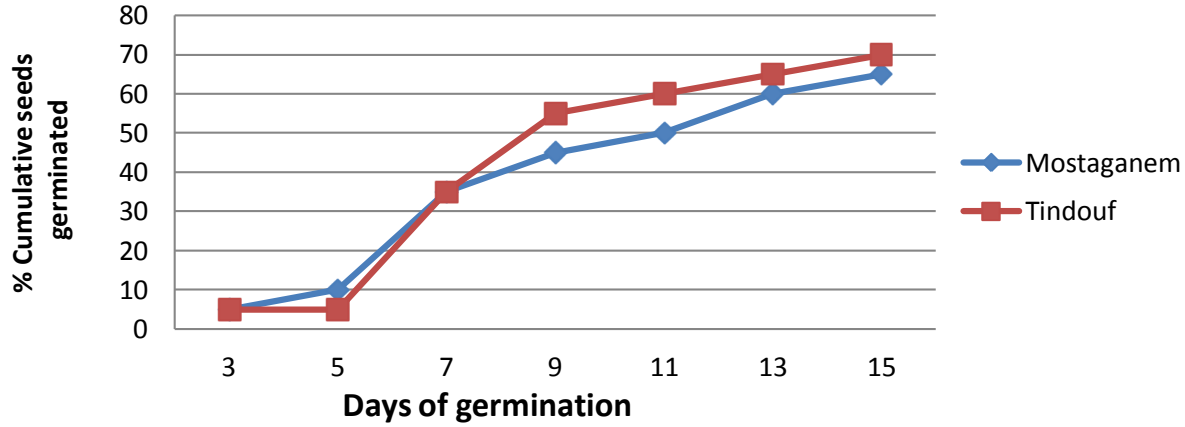

Figure 11. Daily argan seed germination rate, pre-soaked for 96 h and subjected to a temperature of 28°C.

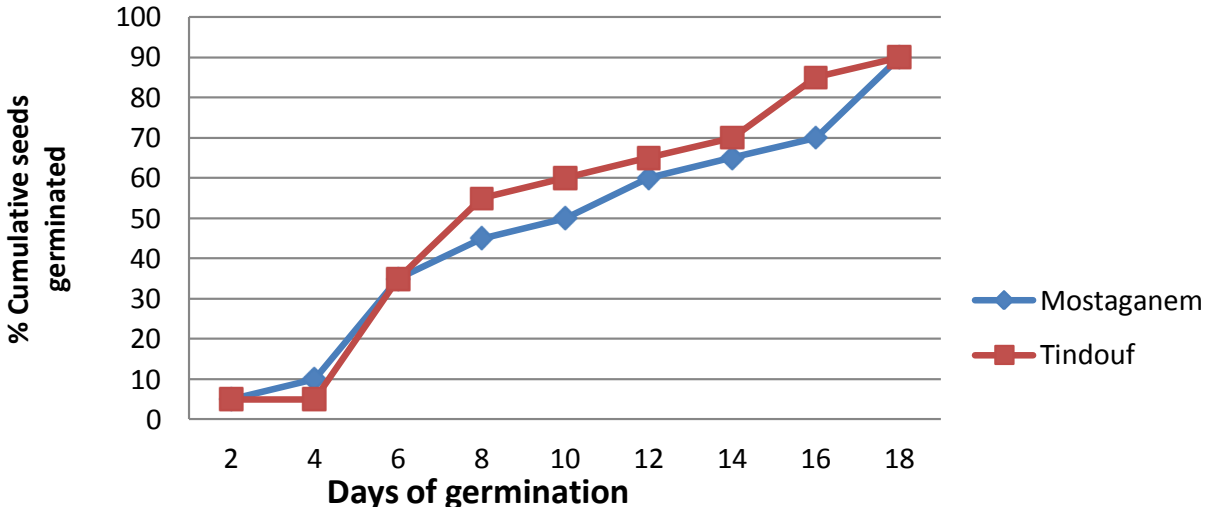

Figure 12. Daily argan seed germination rate, pre-soaked for 120 h and subjected to a temperature of 25°C.

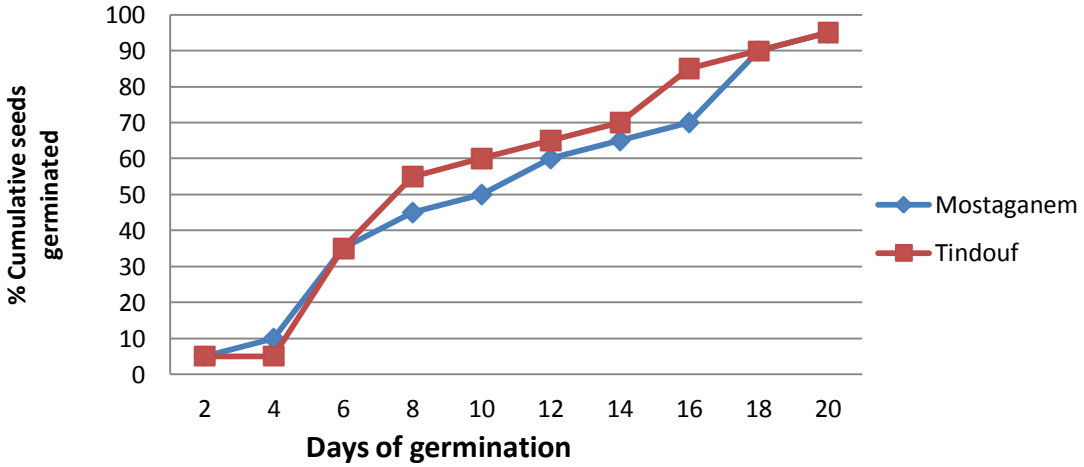

Figure 13. Daily argan seed germination rate, pre-soaked for 96 hours and subjected to a temperature of 25°C.

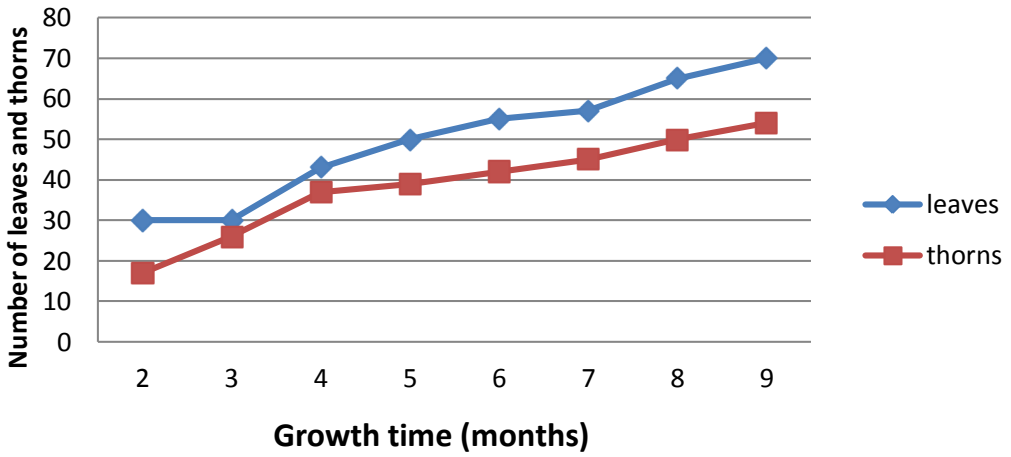

Figure 14. Monitoring the evolution of leaves and thorns of argan seedlings for 9 months.

Thorns appeared from the 20th day of the plantation, and increased with time. Thus, we noted that older seedlings of 2, 7, and 9 months haved a varying number of thorns: 17, 42, and 54, respectively.

ii) Growth in height

Height is the average height of seedlings, measured from collar to the end bud. The results show that height growth was faster in 14 days old seedlings with a height of 0.8 cm and got to 6.5 cm after a month. There is slow height growth in a month or more with two months old seedlings that have height of 18 cm and 9 months old seedlings with a height of 40 cm (Figure 15).

Results on the root system show that the length of the main root was significant; the secondary and tertiary roots were thin and brittle. After 14 days of planting, the length of the main root was 3 cm, then it underwent rapid growth to reach a length of 8 cm in a month. There was continuous growth in the root system with normal development but at a variable rate. For 4 and 5 months, the length of the primary root was respectively 25 and 29 cm. However, we observed a slowdown of root growth after 6 and 9 months; their lengths we slightly higher than the previous (32 and 35 cm). Similarly, we note that the growth in height of stem is small compared to the roots.

In the field

Height of stems

Growth in height of stem varied in the two types of

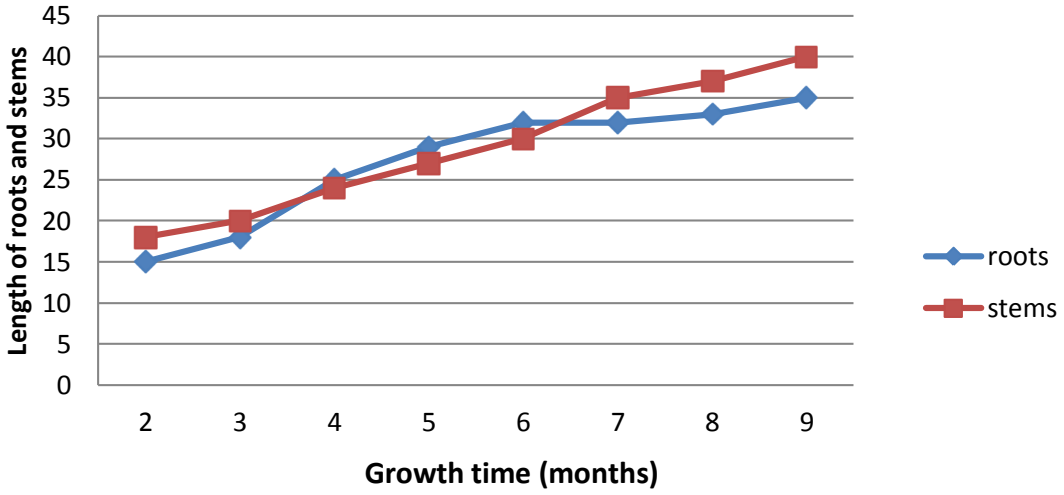

Figure 15. Monitoring the evolution of heights of stems, and roots of argan seedlings of 9 months.

seedlings. After the first week of planting, we observed that stem height has quadrupled in older seedlings of 2 years compared to one year. Then the seedlings were growing two batches of irregular height up to 74 cm (seedlings older than 2 years) and about 12 cm (seedlings older than one year) in the first six months of planting.

At the age of 12 months after planting, shoot growth slowsed down: height reachesd 18 cm respectively in one year seedlings and 32 cm for that of 2 years.

Leaf drying

Leaf desiccation is when the foliage or leaves of few seedlings dry up and become detached from the main stem. After a week when transplantation was done, the rate of drying of the leaves is very low for the two types of seedlings.

Number of thorns

From the observations, we find that the number of thorns increased with the age of seedlings; older seedlings of one year hadve 10-28 thorns each against those of two years, with 40-260 thorns each. So after a month of planting, these have the same values like previous thorns.

Branch of the stems

Young argan seedlings have an axis of stem that is rarely branched. As argan tree is vigorous, it acquires ramifications of the first order. At 2 years, the seedlings have remarkable branching of approximately 2 to 15 branches per seedling up to the 3rd month of the planting.

Rate of recovery after transplantation

The number of successful transplanted seedlings after one year, compared to results of those originally planted is seen in Table 1.

The aim of this study was to highlight the biological conditions related to promoting good regeneration of the argan tree. Our experimental tests on regeneration of seedlings argan of two years have encountered a number of climate and technical field problems, which impact negatively the development of seedlings. Then, the highest success rate was found in 24 months old seedlings (80%) compared to seedlings of 12 months (50%). This high success rate (80%) shows that the age of argan tree seedlings is significant for a good recovery.

DISCUSSION

Many authors have reported the difficulty involved the germination of the seeds of the argan tree and they recommended either scarification or acid treatment. Our experiment showed that in seeds pre-soaked in water for 120 h at 30 and 28°C, raw sprouts were obtained from the third day at a rate of 15%, as against 25 and 20°C, in which germination starts on day 3, but at a rate of 10%. Regarding the rate of germination, it was significant in seeds pre-soaked in water for 96 and 120 h in the thermal range of 30 and 25°C. Under the same conditions, in seeds pre-treated before sowing, there was optimal rate of 85% obtained at 30°C.

Regarding the origin of the seeds, the results show

Table 1. Success rate of transplanted argan seedlings.

Age (month)	Seedlings transplanted	Live seedlings	Success rate (%)
12	24	12	50
24	20	16	80

some difference in the rate and the beginning of the germination. The seeds begin to germinate in Tindouf 2 to four days late compared to those of Mostaganem. The delay in germination may be related to the different morphology of the seeds, compared to those of Mostaganem. We hold that the seeds of Tindouf have a relatively hard and thin shell.

Regarding the relationship between the germination time, temperature, and nature of the pretreated seeds source, the results indicate the existence of a correlation between the duration used for pre-soaking seeds in water and germination temperature. Moreover, the duration used in pre-soaking argan seed is very short when the optimum temperature for germination was high (30°C). The variation in germination parameters of Argan seeds we measured is reported by many authors working in the field. For example, Come (1975) reported that seed will sprout if the embryo has the possibility of imbibing; Mazliak (1982) concluded that temperature remains a limiting factor that affects directly germination by acting on the speed chemical reactions. In addition, Nouaim and Chaussod (1993) argue that the argan tree can regenerate from seeds, but a lot of failures were observed.

These authors report that a simple dipping of argan seeds in water for three or four days encourage a high percentage of germination. In our case, soaking seeds in water for 96 and 120 hbefore sowing has positive effect on early and high rates of germinated seeds (80, 85 and 95%) from 25 to 30°C. Compared to some work done in Algeria on the possibilities of multiplication of argan seeds in a thermal range of 25 to 30°C, we observe that the germination varies with sources of seeds. For example, for seeds from Tindouf, germination rate reached 50% (Slimani, 1996). This rate increaseds to 70% (Kechairi and Lakhdari, 2002) and 80% (Miloudi, 2006) for seed harvested from Oggaz Station, Mascara. For seeds from Mostaganem, germination rate reached 90% (Baoui, 2001) and 55, 70 and 80% in the thermal range of 25 to 30°C (Miloudi, 2006).

Furthermore, Renard (1975) notes that among the seeds used for the test (same experimental conditions), some do not germinate due to the hardness of the integument and others due to the endogenous inhibitors or dormancy. Under these conditions, it is essential to achieve optimal conditions for germination, confirming the general rule on the germination of halophytes (Grouzis et al., 1976), and Glycophytes (Francoit et al., 1986; Belkhodja and Soltani, 1992).

Conclusion

Failure of argan tree regeneration is often attributed to multiplication difficulties. However, our experimental results have shown that this tree can be propagated by seeds. Due to its allogamous reproduction (Msanda, 1993), different multiplication methods could be used, according to the objective. Reforestation by seedlings is the best method because it maintains the great genetic variability of the species and confers ecological resistance. It reduces production costs in forest nurseries. The aim of our work was not to define ideal methods for propagation, but to check the feasibility of this technique. Our results represent regene-ration of argan tree by seeds. This technique will be used for future production of argan trees in agroforestry systems. This points out the imbalance of traditional agroforestry system, which is now clearly threatened. The only way to avoid irreversible damage to the environment and to promote sustainable management of the argan forest is to improve agricultural income. It seems now possible to optimize argan tree based agroforestry systems, through the production of high quality exotic oil, for which there is already a large demand, exceeding present supplying capacities.

'Planting trees that yield a good profit' is the best way to fight against desertification. Leakey and Simons (1998) showed that increasing the quality, number and diversity of domesticated trees could provide a wide array of non-timber forest products (NTFPs). NTFPs could enhance the capacity of agroforestry to fulfill its ultimate potential as a way to alleviate poverty and to mitigate deforestation and land depletion. Domestication of trees for agro-forestry to produce NTFPs can therefore benefit both the farmer and the environment. Vegetative propagation, enabling argan tree domestication, is a real chance for development (Sasson, 1993). We showed that argan tree domestication is technically feasible. However, dissemi-nation of this knowledge is necessary for a true sustainable development.

Conflict of Interest(s)

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

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Full Length Research Paper

Foliar anatomical studies of some taxa of Euphorbiaceae

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Foliar anatomical studies of fifteen taxa belonging to fifteen genera of Euphorbiaceae were done to understand the foliar structural details in unravelling taxonomical disputes if any. There is a range of characters which varies between genera and species. The presence of single, double and multiple layer of epidermis, palisade parenchyma, spongy parenchyma and other special tissues and storage organs are of taxonomic interest. A combination of these characters may be used to identify the specific species.

Key words: Foliar, anatomy, mesophyll, taxonomy, Euphorbiaceae.

INTRODUCTION

An anatomical study was done on fifteen plants belonging to Euphorbiaceae. The family Euphorbiaceae is popularly known as the “Spurge” family. The name “spurge” is derived from Medieval French “epurga” referring to the purgative properties of the seeds of *Euphorbia.* The family consists of mostly monoecious herbs, shrubs, trees and sometimes succulent with about 300 genera and 7,500 species that are further characterized by the occurrence of the milky juice. The leaves are mostly alternate but may be opposite or whorled and they are simple, compound and sometimes highly reduced. Stipules are generally present but may be reduced to hairs, glands or spines. Flowers are unisexual and usually actinomorphic. They may be highly reduced by suppression of parts, in the extreme form consisting of naked stamens as a staminate flower and a naked pistil as a pistillate flower. A specialized type of miniature inflorescence occurs in about 1,500 species comprising the genera *Euphorbia.* It consists of a single naked pistillate flower surrounded by cymes of naked staminate flowers, each consisting of a single stamen. These flowers are all enclosed in cup-like involucre that is provided with peripheral nectarines and petalloid appendages such that the whole aggregations closely resemble a single flower. A comprehensive account of the family was given by Engler and Prantl (1897), Bentham and Hooker (1883), Willis (1966), Webster (1994) and Radcliffe-Smith (2001).

The angiospermic family Euphorbiaceae is one of the most interesting and economically important family. This family is of much importance from the point of view of producing a number of useful products, natural rubber

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Table 1. The list of plants belonging to Euphorbiaceae.

Name of the species	Place of collection	Altitude from M.S.L	Wild or Cultivated
Euphorbia hirta L.	Annamalainagar, South India	50'	Wild
Pedilanthus tithymaloides (L.) Poit.	Sims Park, Ooty, South India	Hills 6000'	Cultivated
Phyllanthus myrtifolius Moon ex Hook.f	Tiruvananthapuram, South India	Hills 3000'	Cultivated
Phyllanthus emblica L.	TNAU, Coimbatore, South India	Plain 1000'	Cultivated
Sauropus androgynus (L.) Merr.	TNAU, Coimbatore, South India	Plain 1000'	Cultivated
Aporosa lindleyana Baill.	Kariavatham, Kerala, South India	Hills 3000'	Wild
Baccaurea courtallensis Arg.	Tiruvananthapuram, South India	Hills 3000'	Wild
Croton sparsiflorus Morong.	Annamalainagar, South India	Plain 50'	Wild
Chrozophora rottleri (Geiseler) A. Juss. ex Spreng.	Annamalainagar, South India	Plain 50'	Wild
Acalypha indica L.	Annamalainagar, South India	Plain 50'	Wild
Ricinus communis L.	Pitchavaram, South India	Plain 50'	Cultivated
Hevea brasiliensis Arg.	Tiruvananthapuram, Kerala, South India	Hills 3000'	Cultivated
Jatropha curcas L.	TNAU, Coimbatore, South India	Plain 1000'	Cultivated
Manihot esculenta Crantz.	Kariavatham, Kerala, South India	Hills 3000'	Cultivated
Excoecaria agallocha L.	Pitchavaram, South India	Plain 50'	Wild

from *Hevea;* biodiesel from *Jatropha;* starch from *Manihot* and castor oil from *Ricinus.* This family possesses number of ornamental plants also. Few of such interesting and familiar plants are *Crotons, Euphorbia* and *Pedilanthus.* These horticulture plants are very useful to plant breeders.

MATERIALS AND METHODS

The materials for the present investigation were obtained from diverse localities of southern part of India (Table 1). Herbarium preparations were made from the collected plant twigs and checked with Standard Floras. Voucher specimens were stored in the herbarium section, Botany Department, Annamalai University, Annamalainagar, Tamilnadu. Collection trips were undertaken to Ooty, Coimbatore, Cuddalore, Pitchavaram, Thiruvananthapuram and Annamalainagar. Plant twigs were collected from the selected species for anatomical studies and stored in 70% ethanol for laboratory studies (triplicate - sample of leaves, from third leaf of a branch twig in each species). Anatomical studies of leaf were done with aid of free hand section and observed under the light microscope with an eye piece lens (12.5x) and an objective of low power lens (10x). The sections were stained with saffranin (1%) (the saffranin was prepared by dissolving 1g of saffranin powder in 100 ml of distilled water and filtered) and mounted in 50% glycerine. All of them were photographed. The plants are listed according to the Gamble's Flora of Presidency of Madras (1956).

RESULTS

The leaf is a variable organ. In Euphorbiaceae leaves are mostly alternate but may be opposite or whorled and they are simple or compound or sometimes highly reduced. Stipules are generally present but may be reduced to hairs, glands or spines. Anatomical work in higher plants has been made by several authors (Cutter, 1971; Ahmad, 1976; Selvaraj and Subramanian, 1979; Ramona Crina Gales and Constaintia Tima, 2006; Essiett et al., 2012; Thakur and Patil, 2011; Martins and Zieri, 2003; Hussein et al., 2012; Idu et al., 2009). The leaf anatomy of Euphorbiaceae is unique in the sense that it is latex yielding. The leaf region was made up of latex cells and laticiferous tissues for translocation of latex. The leaf anatomy showed the following details:

Euphorbia hirta L.

The section shows epidermis, mesophyll cells and vascular traces. The vascular traces are surrounded by photosynthetic tissues. The single layer epidermis with packets of chlorophyll pigments. The region of photosynthetic tissues in leaf area are 90% with the storage tissues (Figure 1a).

Pedilanthus tithymaloides (L.) Poit.

The section shows multilayer epidermis and mesophyll tissues. The mesophyll tissues consist of double layer palisade and spongy parenchyma. The region occupying the palisade layer verses spongy parenchyma is in equal proportion (Figure 1b).

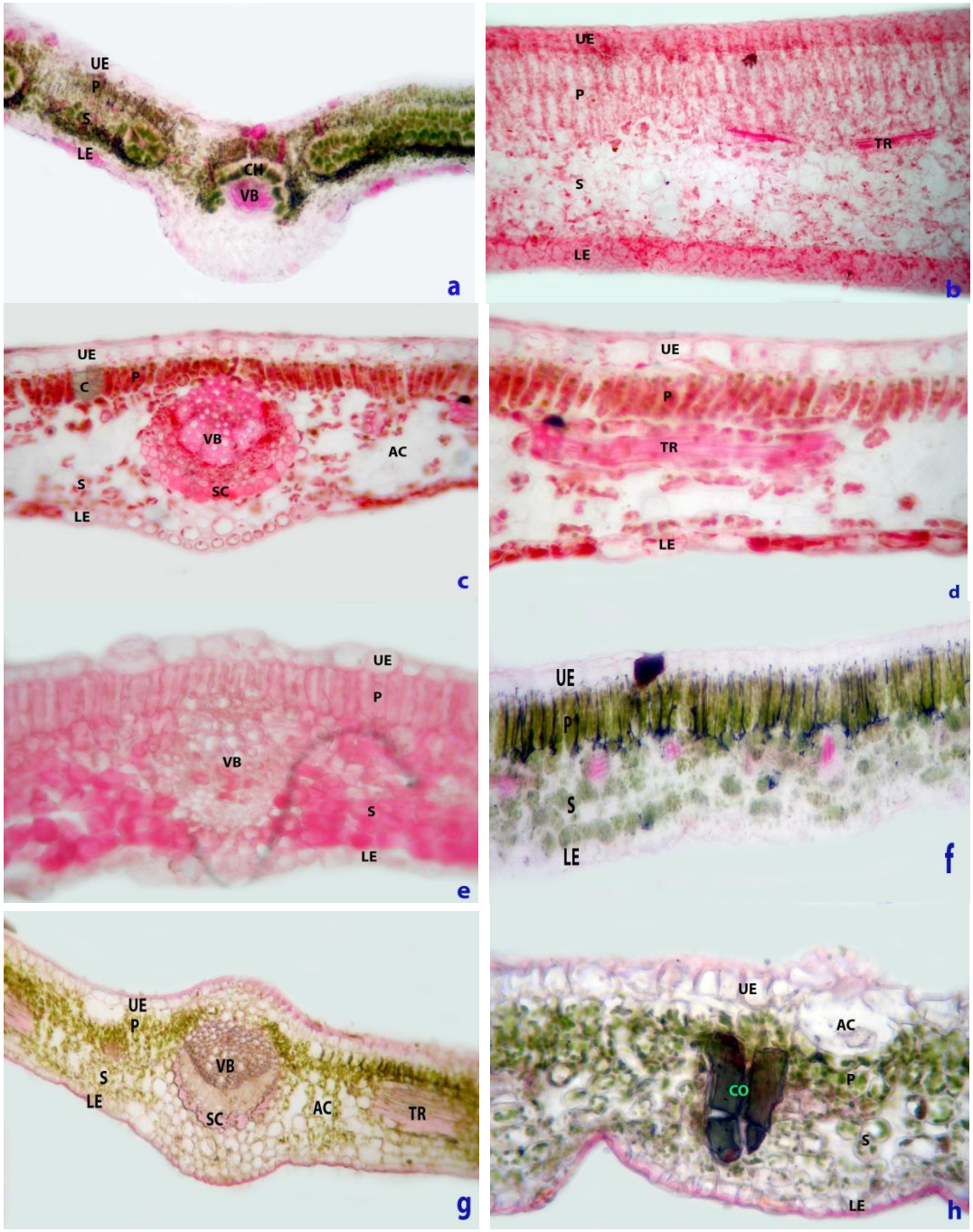

Figure 1. Anatomy of Euphorbiaceae species (a. *Euphorbia hirta*, b. *Pedilanthus tithymaloides*, c and d. *Phyllanthus myrtifolius*, e. *Phyllanthus emblica*, f. *Sauropus androgynus*, g and h. *Aporosa lindleyana*). UE = Upper epidermis; LE = lower epidermis; P = palisade parenchyma; S = spongy parenchyma; VB = vascular bundle; TR = transfusion tissue; AC = air cavities; C = cystolith; SC = sclerenchyma; CH = chlorenchyma; CO = calcium oxalate.

***Phyllanthus myrtifolius* Moon ex. Hook.f**

The section shows a thick walled epidermis, mesophyll tissues, transfusion tissues and vascular bundles. The xylem and phloem are surrounded with sclerenchymatous stone cells. Calcium oxalate cystoliths are distributed in mesophyll tissues (Figure 1c).

Transverse section of leaf of *P. myrtifolius* show enlarged view of mesophyll tissues, where, the palisade cells are single row, long elongated cells with many

nucleate conditions (Figure 1d).

***Phyllanthus emblica* L.**

The section shows epidermis, mesophyll tissues and vascular traces with a single row of palisade cells. Five to seven layer spongy parenchyma are also present (Figure 1e).

***Sauropus androgynus* (L.) Merr.**

The section shows epidermis, equal region of palisade and spongy tissues with dense amount of chlorophyll pigments. The leaves are rich in vitamins and commonly known as multivitamin leaves (edible) (Figure 1f).

***Aporosa lindleyana* Baill.**

The section shows epidermis, mesophyll cells, transfusion tissues, vascular bundle (trace) and storage product of raphids and calcium oxalates in the mesophyll zone. Just below the upper epidermis, cavities are present which are full of air and for water storage purposes, and conductive tissues which connect such cavities. There is no differentiation of mesophyll cells and all are spongy parenchymatous in nature (Figure 1g and h).

***Baccaurea courtallensis* Arg.**

The section shows double layer upper epidermis, mesophyll tissues and transfusion tissues. There is a much different palisade parenchyma and up to 10 layered spongy parenchyma are so prominent (Figure 2a).

***Croton sparsiflorus* Morong.**

The section shows epidermis, mesophyll cells (palisade and spongy parenchyma) and vascular traces. The palisade cells are long with dense chlorophyll pigments. Air cavities seen and transfusion cells are available with spongy parenchyma region (Figure 2b).

Enlarged view of T.S of leaf shows epidermis, long palisade cells, 5 to 8 layer of spongy parenchyma (Figure 2c) and star shaped cystolith (calcium oxalate and calcium carbonate); as storage product (Figure 2d). The lower region of mesophyll cells have black coloured substance known as phlafofeen (an oxidise product of tannin).

***Chrozophora rottleri* (Geiseler) A.Juss. ex Spreng.**

The section shows a vascular bundle, epidermis and mesophyll tissues. In the upper region of the leaf large sized parenchyma cells are present and comparatively small sized palisade parenchyma in the lower region. In between these two palisade parenchyma, spongy parenchyma are prominent full of chlorophyll pigments (Figure 2e).

***Acalypha indica* L.**

T.S of leaf shows vascular bundle, mesophyll tissue and upper and lower epidermis. A single row of thickly packed palisade cells and three to five layer spongy parenchyma are present (Figure 2f).

***Ricinus communis* L.**

The section shows multilayered (two to three layer) epidermis, prominent mesophyll tissue and vascular traces in the midrib. The palisade and spongy parenchyma region are equal in proportion (Figure 2g).

An enlarged view of T.S of leaf shows palisade parenchyma and 5 to 7 layer spongy parenchyma with densely packed chlorophyll pigments (Figure 2h).

***Hevea brasiliensis* Arg.**

The section shows a prominent mid-vein having vascular traces with ground tissues (parenchymatous) a multilayer upper epidermis and a single row of lower epidermis with stomata. Distinct mesophyll cells are present (Figure 3a). A much enlarged view of leaf shows epidermis, palisade parenchyma and 6 to 7 layer spongy parenchyma are present (Figure 3b).

***Jatropha curcas* L.**

The section shows epidermis double layer palisade and a few rows of spongy parenchyma. They are densely packed with chlorophyll pigments (Figure 3c).

***Manihot esculenta Crantz*.**

The section shows epidermis, mesophyll tissue and vascular traces in the ground tissue with cystolith (Figure 3d).

An enlarged view of leaf section shows multilayered epidermis and well developed palisade parenchyma, spongy parenchyma with air spaces (Figure 3e) and cystolith (calcium oxalate) present in leaf area (Figure 3f).

***Excoecaria agallocha* L.**

T.S of leaf shows epidermis, two to three layer palisade

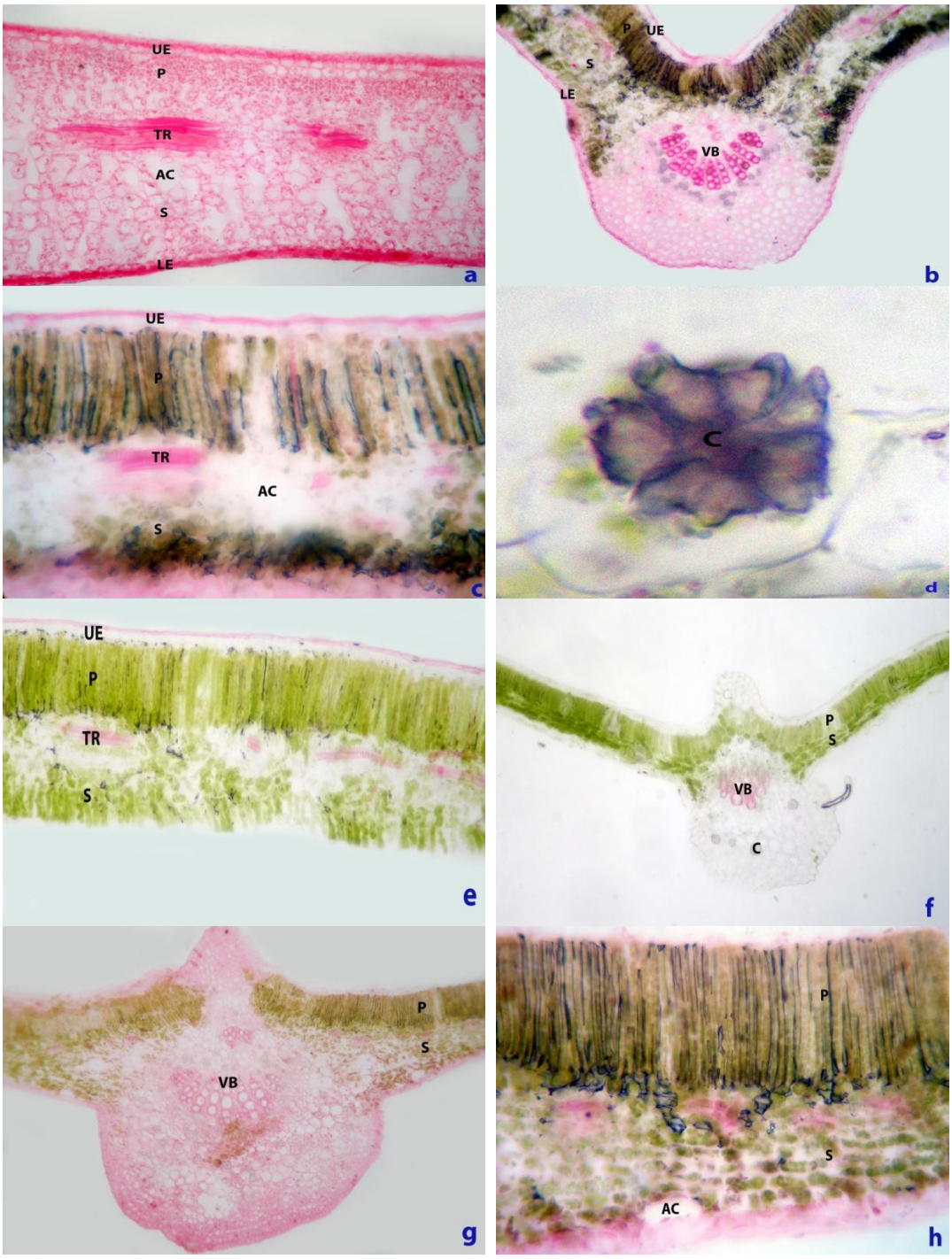

Figure 2. Anatomy of Euphorbiaceae species (a. *Baccaurea courtallensis*, b, c and d. *Croton sparsiflorus*, e. *Chrozophora rottleri*, f. *Acalypha indica*, g and h. *Ricinus communis*). UE = Upper epidermis; LE = lower epidermis; P = palisade parenchyma; S = spongy parenchyma; VB = vascular bundle; TR = transfusion tissue; AC = air cavities; C = cystolith; SC = sclerenchyma; CH = chlorenchyma; CO = calcium oxalate.

parenchyma, 7 rows of spongy parenchyma and air cavities (Figure 3g and h).

There are several elaborate work of leaf anatomical studies including foliar epidermal studies, by different authors. Such studies have been successfully utilized to solve the problems of taxonomical interrelationship of plants. All the features described, were noted in this present study and re-confirmed.

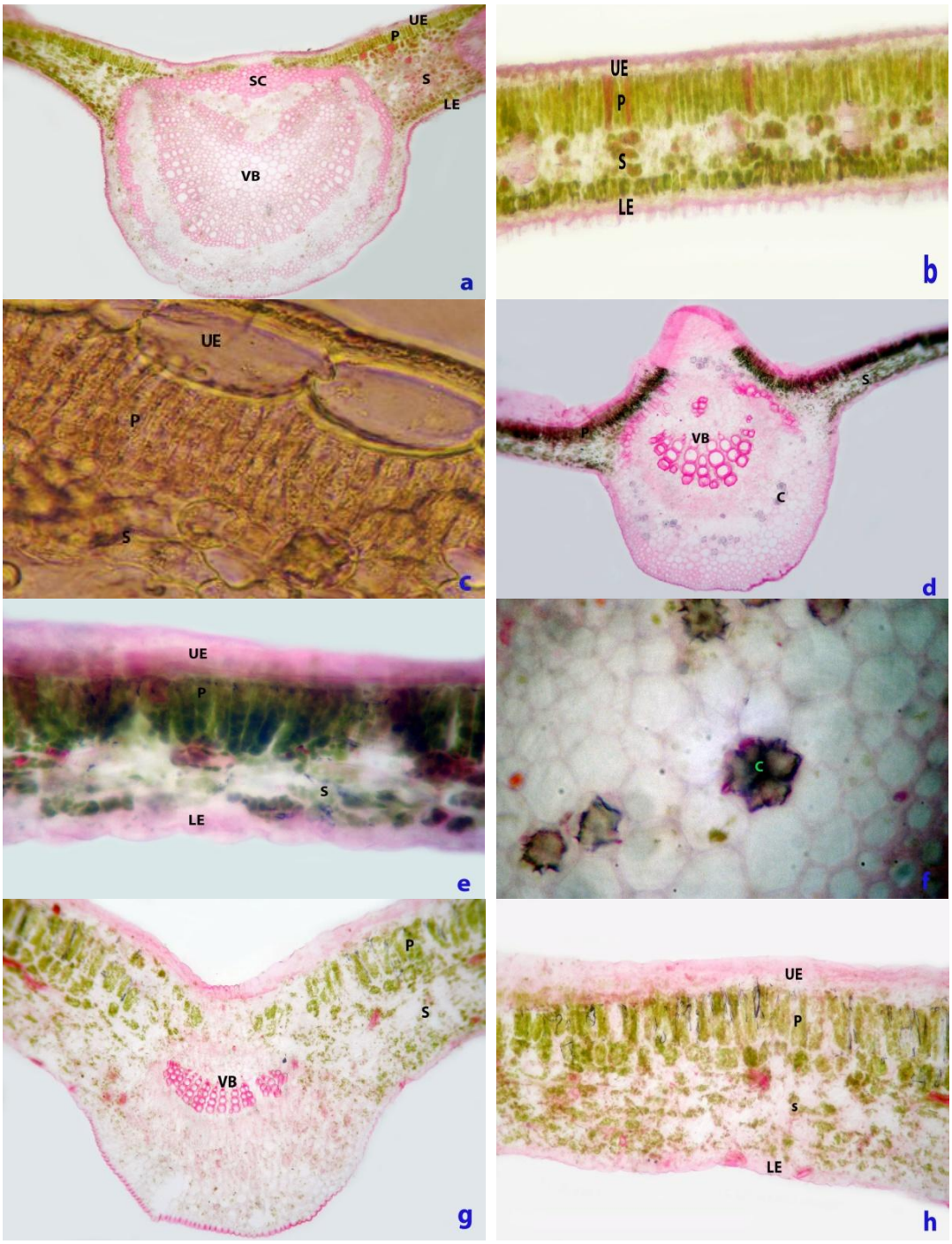

Figure 3. Anatomy of Euphorbiaceae species (a and b. *Hevea brasiliensis*, c. *Jatropha curcas*, d, e and f. *Manihot esculenta*, g and h. *Excoecaria agallocha*). UE = Upper epidermis; LE = lower epidermis; P = palisade parenchyma; S = spongy parenchyma; VB = vascular bundle; TR = transfusion tissue; AC = air cavities; C = cystolith; SC = sclerenchyma; CH = chlorenchyma; CO = calcium oxalate.

DISCUSSION

Foliar anatomical studies with special reference to anatomical characters have been made in fifteen taxa of Euphorbiaceae. In this study, anatomical leaf sections of all the species in Euphorbiaceae revealed unique and species specific. The following characters were observed and may be concluded that the 15 taxa of Euphorbiaceae

studied show the polyphyletic origin and evolution.
The following points are key characters:

1. Thick cuticle were observed in all the species studied.
2. In upper epidermis, single row of epidermal cells (parenchyma) occurred in all the species except *P. tithymaloides*, *R. communis* and *M.utilissima*; where there are two or three rows.
3. The mesophyll tissues are differentiated into palisade and spongy parenchyma in all the species studied except in *A. lindleyana* where the mesophyll tissues are only spongy parenchyma.
4. The occurrence of single row of palisade parenchyma have been observed in all the species, except *P. tithymaloides*; *J. curcas*; and *E. agallocha*. In all the above mentioned species, there are two or rarely three rows of palisade parenchyma cells present, to enhance the photosynthetic rate and efficiency; for quick growth and regeneration.
5. In *C. rottleri*, the occurrence of palisade parenchyma on both sides of the lamina surfaces (upper and lower side) and spongy parenchyma and vascular bundles occur in between them. Hence, *C. rottleri*, is considered as a unique and species specific with regard to the distribution of mesophyll tissue.
6. The vascular bundles in mid-vein, lateral vein in the mesophyll tissues and in the lamina surfaces are uniform throughout the species studied here.
7. The latex, laticiferous tissues, latex vessel and resinous ducts are known in Euphorbiaceae.
8. Thus, this study is an attempt to investigate the 15 taxa of the family Euphorbiaceae which forms an attainment towards an advancement of knowledge.

Conflict of Interests

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

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Full Length Research Paper

Utilization of Indian spinach (*Basella* Linn.) in Ondo State, Nigeria

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This paper reports on the survey carried out among consumers on the uses of Indian spinach in Ondo State, Nigeria. Well-structured questionnaires were prepared and one hundred (100) questionnaires were randomly distributed to consumers in each of the 16 local government areas of Ondo State, Nigeria. Result showed that there are more female respondents (59.4 %) than the males (40.6%). This could have resulted from the fact that women make more informed decision on food security in homes. Most consumers are civil servants. Most consumers prefer Indian spinach to other commonly consumed vegetables and their choice is based on availability and taste. *Basella*, could be cooked sole, mixed with grounded seeds of egusi (*Citrillus lanatus*) or other vegetables. Respondents noted that Indian spinach contains carbohydrates, proteins, fats, vitamins and nutrient elements. They also noted that all the parts are useful (leaves, stems and roots) and they are useful in treatment of various ailments in folk medicine. This paper documents consumers' knowledge on *Basella* because information on consumers' knowledge of agriculture products is scarce. The researcher derived information on the genus *Basella*, uses and future prospect of the genus in the study area.

Key words: *Basella*, consumers, ethno-botany, respondents, utilization.

INTRODUCTION

Basella commonly referred to as Indian spinach is one of the traditional leafy vegetables among the Yorubas of the South Western, Nigeria. It is commonly referred to as amunututu in Yoruba language. There are three main types under cultivation (Adeyemi, 2007); *Basella alba*, *Basella rubra* and *Basella cordifolia*. Ozela et al. (2007) noted that *B. rubra* and *B. alba* were the most common species in the family Basellaceae.

Fleshy leaves of *B. alba* and *B. rubra* are used as vegetables (Adeyemi, 2007). The vegetable is rich in vitamins A, E, K, C, B_2, and B_9 (Grubben and Denton, 2004; Mensah et al., 2008). They have micro nutrients and macro nutrients and contain phytochemicals that exhibit antioxidant properties (Shruthi et al., 2012; Olajire and Azeez, 2011). *Basella* is a good source of proteins, calcium, iron and it lacks tannins (Palada and Chang, 2003; Roy et al., 2010). Kayode and Ige (2008) reported that the leaves and shoots of *B. alba* are used to cure boils and hot flushes in Ijesa land in Nigeria. Ethno-botanical uses of *B. alba* are well documented in

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Table 1. Socio-economic profiles of consumers.

Variable	Frequency	Percentage
Sex		
Male	637	40.6
Female	933	59.4
Total	1570	100
Ages in years		
15 - 24	310	19.7
25 -34	465	29.6
35 - 44	532	33.9
45 - 54	212	13.5
55 - 64	34	2.2
64 and above	17	1.1
Total	1570	100
Marital status		
Single	390	25.4
Married	1057	69.3
Divorced	56	2.9
Separated	22	1.4
Widowed	46	2.9
Total	1570	100
Educational attainment		
Primary 6, modern school, no formal education	171	10.9
OL/GCE/Grade 2	337	21.5
NCE/OND	459	29.2
HND/B.A/B.Sc/B.Ed	540	34.4
M.Sc/M.A/M.Ed/Ph.D.	63	4.0
Total	1570	100
House hold size		
1-5	1361	86.7
6-10	184	11.7
Above 10	25	1.6
Total	1570	100
Occupation		
Farming	253	16.1
Artisan	174	11.1
Trading	442	28.2
Civil Servant	548	34.9
Professional	153	9.7
Total	1570	100
Income per month		
< ₦5,000	124	7.9
₦6, 000 - 10,000	188	12.0
₦ 11,000 - 15,000	288	18.0
₦ 16,000 - 20,000	231	14.7
> ₦ 20,000	745	47.5
Total	1570	100

Source: Field survey, 2012.

literatures (Shrutti et al., 2012).

Haghiri et al. (2009) reported on consumers' low levels of awareness of agricultural production services and the need to raise public awareness and understanding of technologies in order to foster consumers' acceptance. Chamberlain et al. (2013) pointed out that consumers are less informed about agricultural products. This paper documents consumers' knowledge on the utility values of *Basella* because information on consumers' knowledge of agriculture products is scarce. The level of knowledge of the ecology and reproductive of edible species combined with their use (the frequency of consumption and market value) and their biological characteristics (life forms, availability periods) assist to shape their management or procurement practices in community, households and among individuals.

MATERIALS AND METHODS

Well-structured questionnaires were prepared and validated at the Department of Sociology and Anthropology, Faculty of Social Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. Sampling was done in Ondo State of Nigeria where it is eaten largely. There are 18 local government areas in the state however sixteen out of the eighteen local government areas were used for this study. The two local government areas that were not used for the ethno-botanical studies are Ilaje and Eseodo. These are located in the riverine areas of the state, where the main occupation is fishing. One hundred (100) questionnaires were randomly distributed to consumers per local government area. Questionnaires were distributed between the hours of 10.00 am - 6.00 pm on working days and 8.00 am and 6.00 pm on weekends. Data collected were analyzed using simple descriptive statistics.

RESULTS AND DISCUSSION

Table 1 shows the sex of consumers. 40.6% were males while 59.4% were females. This shows that there are more female consumers. The table shows that 19.7% the consumers were between the ages of 15-24 years, 29.6% were between the ages of 25-34 years, 33.9% of the respondents were between the ages of the ages of 35-44 years; 13.7% were between the ages of 45-54 years, 2.2% were between the ages of 55-64 years while 1.1 % of consumers were 65 years of age and above. Table 1 also shows that singles constitutes 25.4% of the respondent, 67.3% were married, 2.9% were divorced, 1.4% were separated and 2.9% were widowed. This study indicated that there are more married consumers than the singles. The table reveals that 10.9% of the respondents had either primary 6, modern school certificate or no formal education. 21.5% of respondents had OL/GCE/Grade II, 29.2% had NCE/OND and 34.4% had HND/B.A/B.Ed while 4.0% had M.Sc /M.A M.Ed and Ph.D. The majority of the respondent on this study had HND/B.Sc/B.A/B.Ed; showing that there are more of literate consumers and who are civil servants (34.9%) and earn above ₦20, 000.00 (47.5%). Ahmad et al.

Table 2. Consumers knowledge of *Basella* varieties.

Variety of *Basella* known	Frequency	Percentage
One	55	3.5
Two	1389	88.5
Three	121	7.7
Four	5	0.3
Total	1570	100

Source: Field survey, 2012.

(2007) noted that education is one of the most important factors in acceptance, rejection, adoption and dissemination of useful information to other fellows for their benefits.

The house hold size of majority of the consumers interviewed as shown in Table 1 reveals that most house hold size is between 1-5 (86.7%); 11.7% had house hold size of 6-10, while only 1.6% of the consumers have house hold size greater than 10. This study shows that majority of the respondent have 1-5 house hold size and this within the recommended household size of 4 per family. The small household size identified with the consumers is likely to be related to the literacy level of the respondents.

Table 1 also shows that 16.1% of the respondents were into farming, 11.1% were artisan, 28.2% were traders, 34. 9% were civil servant, 9.8% were professional. This study shows that most consumers of *Basella* were civil servant. Table 1 show that the income of 7.9% of the consumers was below ₦ 5,000, 12.0% of the consumers earned between ₦ 6,000 to ₦ 10.000, 18.0% have income between ₦ 11,000 to ₦ 15,000, 14.7% earn between ₦16,000-₦ 20,000 while 47.5% had above ₦20, 000. Majority of the consumers earned above ₦ 20,000. This may be due to the fact that most of the consumers are civil servants.

Table 2 shows that 3.5% of the consumers indicated that they knew only one variety; 88.5% of consumers know two varieties; 7.7% indicated that they knew three varieties while 0.3% indicated that they knew four. From this study, majority of respondents noted that there are two varieties.

Table 3 shows the ethno botanical uses of *Basella.* 91.0% of the consumers reported that *Basella* is used for fertility enhancement; 94.7% of the consumers indicated that it is used for the treatment of diabetes; 91.8% of the consumers indicated that it is used for the treatment of dysentery. 95.9% of the consumers showed that *Basella* is used in the treatment of constipation while 4.1% of the consumers indicated that it is not used for treating constipation. 86.8% of the consumers interviewed indicated that *Basella* is used for the treatment of rheumatism. 93.7% of the consumers indicate that *Basella* is used for the treatment of cold while 6.3% indicated that it is not used for the treatment of cold. 91.8% of the consumers indicated that *Basella* is used for the treatment of boils and blisters while 8.2% of the consumers noted that it is not used. This study shows that 61.3% of the consumers noted that *Basella* is used for the treatment of gonorrhea while 38.7% indicated that it is not used for the treatment of gonorrhea. 95.8% of the respondents indicated that *Basella* is used for the treatment of hot flushes or internal heat while 4.3% indicated that it is not used for treatment of hot flushes or internal heat.

Table 4 shows consumers preference with regard to the varieties of *Basella* available. 41.7 and 58.3% male and female, respectively showed their preference for the green stemmed form (*B. alba*) 36.3 and 63.7% of males and female respectively showed their preference for red stemmed form (*B. rubra*) while 38.0 and 62.0% male and female, respectively, indicated that they could take any of two varieties. 73.5% of the respondents indicated their preference for *B. alba*, 19.5% indicated their preference for *B. rubra* while 7.0% indicated that any of two varieties can be taken by them.

The Chi square (Table 5) shows that there is no relationship between the choice of *Basella* variety and gender. Table 6 shows that the choice of the variety of *Basella* that is consumed is determined by sweetness, easy digestibility, availability and medicinal importance. 84.8% of the respondents indicated that the variety of *Basella* they eat is determined by its sweetness; 60.3% noted that that their choice is determined based on easy digestibility; 67.8% of respondents indicated that the choice is determined by its medicinal importance and 25% noted that availability is a factor that determines the choice of *Basella*.

B. alba and *B. rubra* are localized vegetables because each locality has a particular type of vegetable her people eat. The forms of *Basella* sown and consumed by consumers have resulted from different agronomic practices, availability of the seeds and market demand for the vegetable. Adeyemi (2007) reported that *B. alba* is preferred to *B. rubra* from survey carried out in Ondo State and the reasons include availability, easy digestibility and attractiveness.

Table 7 shows the price of quantity that will be adequate for a family of four. 28.7% of consumers indicated that ₦50 worth of the vegetable will be adequate, 55.5% of the consumers indicated ₦100 worth, 13.9% indicated ₦150 worth while 1.9% indicated that above ₦150 worth will be available and majority of the respondent noted that ₦100 worth of vegetable is adequate for a family of four. This indicates that the vegetable is a means to ensure food security in homes.

Table 8 shows the relationship between the educational status of consumers and the quantity of *Basella* consumed by a family of four. Among consumers with indicated that ₦50 worth of *Basella* was adequate for a family of four, 46.4% indicated ₦ 100 worth; 7.8% of the

Table 3. Consumers' knowledge of the ethno-botanical uses of *Basella*.

Use	Frequency of yes	Percentage of yes	Frequency of no	Percentage of no
Fertility enhancement	1429	91.0	141	9.0
Treatment of diabetes	1487	94.7	83	5.3
Treatment of dysentery	1442	91.8	128	8.2
Treatment of constipation	1505	95.9	65	4.1
Treatment of rheumatism	1362	86.8	208	13.2
Treatment of cold	1471	93.7	99	6.3
Treatment of boils and blisters	1442	91.8	128	8.2
Treatment of gonorrhea	963	61.3	607	38.7
Treatment of hot flushes or internal heat	1504	95.8	66	4.2

Source: Field survey, 2012; *multiple choices allowed.

Table 4. Consumer's varietal choice.

Forms preferred for consumption	Sex		
	Male	Female	Total
Green stemmed (frequency)	475	665	1140
(Percentage)	41.7	58.3	100
Red stemmed (frequency)	110	103	303
(Percentage)	36.3	63.7	100
Any of the two (frequency)	41	67	108
(Percentage)	38.0	62.0	100
Total (frequency)	626	925	1551
(Percentage)	40.4	59.6	100

Source: Field survey, 2012.

Table 5. Chi-square test to show the relationship between choices of *Basella* forms and gender.

Chi-square test	Value	df	Assump. Sig (2 sided)
Pearson Chi square	3.326[a]	3	0.344
Likely hood ratio	3.361	3	0.339
Linear by linear Association	1.845	1	0.174

Source: Field survey, 2012.

Table 6. Criteria for choosing forms of *Basella*.

Criteria	Yes		No	
	Frequency	Percentage	Frequency	Percentage
Sweetness	1331	84.8	239	15.2
Easy digestibility	947	60.3	623	39.7
Availability	392	25.0	1178	75.0
Medicinal	1065	67.8	505	32.2

Source: Field survey, 2012; *Multiple choices allowed.

Table 7. Price of quantity of *Basella* adequate for a family of four.

Price of quantity	Frequency	Percentage
₦50	444	28.7
₦100	859	55.5
₦150	216	13.9
Above ₦150	30	1.9
Total	1549	100

Source: Field survey, 2012.

Table 8. Relationship between educational status and worth in naira of *Basella* consumed.

Highest education status	Price of quantity adequate for a family of four				
	₦50	₦100	₦150	>₦150	Total
Primary six/modern school certificate/no formal education	70 42.2%	77 46.4%	13 7.8%	06 3.6%	166 100
OL/GCE/GRD 2	85 25.8%	200 60.6%	38 11.5%	07 2.1%	330 100
NCE/OND	129 28.4%	259 57.0%	61 13.4%	05 1.1%	454 100
HND/B.A/B.Sc/ B. Ed	141 26.9%	291 55.5%	84 16.0%	08 1.5%	524 100
M.Sc /M.A/M.Ed/Ph.D	15 23.8%	25 39.7%	20 31.7%	03 4.8%	63 100
Total	440 28.6%	852 55.4%	216 14.1%	29 1.9%	1537 100

Source: Field survey, 2012.

respondent noted that ₦150 worth is okay while 3.6% primary six/modern school/no formal education, 42.2% noted that above ₦150 worth is required.

Among respondents having OL/GCE/Grd 2, 25.8% indicated that N50 worth is adequate, 60.6% noted that ₦100 worth is adequate 11.5% noted that ₦150 worth is adequate and 2.1% noted that above N150 worth of *Basella* is adequate to feed the family of four. Among NCE/OND older, 28.4% indicated that ₦50 worth of *Basella* is adequate for a family of four, 57.0% noted that ₦100 worth is adequate and 13.4% noted that ₦150 worth is adequate and 1.1% noted that above ₦150 worth is adequate for a family of four among the HND/BSC/B.A/B.ED, 26.9% noted that ₦50 worth is adequate for a family of four, 55.5% indicated that ₦100 worth is adequate for consumption,16.0% noted that ₦150 is okay and 1.5% noted that above ₦150 is adequate for a family of four. Among the M.Sc/MA/M.Ed /Ph.d, 23.8% noted that ₦50 worth is adequate for consumption, 39. 7% noted that ₦100 worth is adequate, 31.7% noted that ₦150 worth of *Basella* is adequate for consumption and 48% indicated that above ₦150 is required for a family of four, In this study, majority of the respondent irrespective of their educational status indicated that ₦100 worth of *Basella* is needed for consumption by a family of four. Among respondents having M.Sc\MA\M.Ed\Ph.d.; there was an increase in percentage of respondents (31.7%) who noted that N150 worth was adequate for consumption. There is a sharp increase in the trend among respondents that indicated that ₦150 worth of vegetable is adequate. This may be due to better information that they have on the benefit derived from eating vegetables and better income.

Chi square tests (Table 9) show the relationship

Table 9. Chi square tests to show the relationship between the quantities of *Basella* consumed and educational attainment of consumers.

Chi square test	Value	df	Assump. Sig (2 sided)
Pearson Chi square	48.746[a]	12	0.000
Likely hood ratio	43.951	12	0.000
Linear by linear association	10.971	1	0.001

Source: Field survey, 2012.

Table 10. Ways of preparing *Basella* for consumption.

Way	Frequency	Percentage
Sole	67	4.3
Cooking with melon	1358	86.5
Boil and add stew	122	7.8
Mixed with other vegetables	23	1.5
Total	1570	100

Source: Field survey, 2012.

Table 11. Percentage desirability of *Basella* and other commonly eaten vegetables.

Vegetables	Most desired (%)	Average (%)	Little (%)
Basella	53.1	29.7	5.20
Celosia	3.10	13.1	46.1
T. triangulare	4.00	18.2	25.1
Telfaria occidentalis	39.8	42.5	17.7

Source: Field survey, 2012; *Multiple responses allowed.

Table 12. Reasons for consumers' ranking.

Reason	Frequency	Percentage
Desirability	856	54.5
Medicinal	204	13.0
Nutritional	214	13.6
Availability	296	18.9
Total	1570	100

Source: Field survey, 2012.

between quantity of *Basella* consumed and educational attainment. The quantity consumed is not dependent on the educational attainment.

Table 10 shows the methods of preparing *Basella* for consumption. Forty three percent of the consumers indicated that *Basella* is cooked alone (sole); 86.5% cook *Basella* with melon; 7.8% boil and add stew and 1.5% of the consumers mix *Basella* with other vegetables. The level at which consumers desire *Basella* and other commonly eaten vegetables is shown in Table 11. 53.1% of the respondents indicated that they desire *Basella* most, 29.7% of the respondents indicated average desirability and 5.2% of the respondents showed little desirability for *Basella*. With respect to *Celosia argentea* most desirability was shown by 3.1% of the respondents, 13.1% indicated average desirability and 46.1% indicated little desirability. As for *Talinum triangulare*, 4.0% of the respondents indicated it is their most desired vegetable, 18.2% indicated average desirability, and 25.1% showed little desirability. With respect to *Telfaria occidentalis* most desirability was shown by 39.8% of the respondents, 42.5% indicated average desirability and 17.7% indicated little desirability.

This study shows that majority of consumers desired *Basella* most. Table 12 shows that majority of consumers made the choices between the vegetables based on their desire (54.5%); 13% based their choice on the medicinal value of the vegetable; 13.6% of the respondents based their choice on nutritional value of the vegetable and; 18.9% based their choice on the availability of the vegetable. Table 13 shows the consumers' knowledge of nutrient constituents of *Basella.* 20.3% of the respondents noted that there is trace element in *Basella*. 92.8% of the respondents noted that vitamins are present in *Basella*, 2.2% noted that there is carbohydrate in *Basella* while 88.8% noted that *Basella* contains protein. From this study, majority of the consumers are aware that proteins and vitamins are present in the vegetable. This results shows that the awareness of the presence of micro and macro nutrient element in *Basella* should be created. This will improve the consumption and cultivation of *Basella.*

Conclusion

This study has been able to fill the knowledge gap with regards to documenting the utilization of *Basella* in the study area. Haghiri et al. (2009) reported on consumers' low level of awareness of agricultural production services and the need to raise public awareness and understanding of technologies in order to foster consumers' acceptance. Chamberlain et al. (2013) pointed out that consumers are less informed about agricultural products. The result shows the need to create awareness of utilitarian value of the vegetable. The level of know-

Table 13. Consumers knowledge of nutrient elements in *Basella*.

Nutrient constituent	Frequency of yes	Percentage of yes	Frequency of No	Percentage of No	Total
Trace element	319	20.3	1251	79.7	1570
Vitamins	1457	92.8	113	7.2	1570
Carbohydrates	34	2.2	1536	97.8	1570
Proteins	1394	88.8	176	11.2	1570

Source: Field survey, 2012; *Multiple responses allowed.

ledge of their use (the frequency of consumption and market value) combined with the ecology and reproductive of edible species and their biological characteristics (life forms, availability periods) assist to shape their management or procurement practices at community, households and individual levels.

Conflict of Interests

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

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Full Length Research Paper

Effect of inter and intra row spacing on seed tuber yield and yield components of potato (*Solanum tuberosum* L.) at Ofla Woreda, Northern Ethiopia

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Farmers in southern zone of Tigray are using different spacing below or above the national recommendation depending on the purpose of planting either for seed tuber or consumption due to lack of recommended plant spacing. This study was therefore conducted with the objective of determining the best inter and intra-row spacing for optimum tuber seed yield and quality of potato seed tuber at Ofla Wereda, Northern Ethiopia. Four different intra-row (20, 25, 30 and 35 cm) and inter-row (65, 70, 75 and 80 cm) spacing were used in the experiment. The result reveals that inter and intra-row spacing significantly ($p<0.001$) affected seed tuber yield ha^{-1}, the maximum seed tuber yield (36.89 and 37.54 ton ha^{-1}) was recorded at 65 and 20 cm inter and intra-row spacing, respectively. From this study, it can be concluded that the narrow spacing (20 and 65 cm intra and inter-row spacing) produced higher seed tuber yield per hectare than other spacings. Thus, potato (Jalenie variety) growers in the study area can benefit if they use this narrow spacing (20 and 65 cm intra and inter-row spacing).

Key words: Potato, intra-row spacing, inter-row spacing, seed tuber yield.

INTRODUCTION

Potato (*Solanum tuberosum* L.) originated from the high Andes of South America and was first cultivated in the vicinity of Lake Titicaca near the present border of Peru and Bolivia (Horton, 1987). In terms of quantity produced and consumed worldwide, potato is the most important vegetable crop. It is one of the most important food crops in the world; it produces more energy and protein per unit area and unit of time than most other major food crops.

The potato crop was introduced to Ethiopia around 1858 by Schimper, a German botanist (Pankhurst, 1964). Among African countries, Ethiopia has possibly the greatest potential for potato production; 70% of its arable land mainly in highland areas above 1500 m is believed to be suitable for potato. Since the highlands are also home to almost 90% of Ethiopia's population, the potato could play a key role in ensuring national food security

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(FAO, 2008). However, the current area cropped with potato is about 0.16 million hectares and the national average yield is about 7.2 t/ha, which is very low as compared to the world's average production of 16.8 t/ha (Adane et al., 2010). The crop yield in Ethiopia is lower than that of most potato producing countries in Africa like South Africa and Egypt, which produce 34.0 and 24.8 t/ha, respectively (FAO, 2008).

Many diverse and complex biotic, abiotic and human factors have contributed to the existing low productivity of potato. Some of the production constraints which have contributed to the limited production or expansion of potato in Ethiopia include shortages of good quality seed tubers of improved cultivars, disease and pests, and lack of appropriate agronomic practices including optimum plant density, planting date, soil moisture, row planting, depth of planting, ridging and fertility status (Berga et al., 1994).

The optimization of plant density is one of the most important subjects of potato production management, because it affects seed cost, plant development, yield and quality of the crop (Bussan et al., 2007). The yield of seed potato can be maximized at higher plant population (closer spacing) or by regulating the number of stems per unit area and to certain extent by removing the haulm earlier during the maturity (O'Brien and Allen, 2009). Rahemi et al. (2005) reported that the effect of intra-row spacing on yield of potatoes was significant especially at 20 cm intra-row spacing, which showed 36.85% yield increment as compared to 30 cm intra-row spacing. Intra-row distance of 20 cm increased total tuber number and weight, and tuber weight per plant and the marginal return rate increased by 13% when intra-row distance decreased from 35 to 25 cm. EARO (2004) also determined that there is a little difference in yield between intra-row spacing of 25 and 30 cm for all varieties released so far in Ethiopia and the 30 cm intra-row and 75 cm inter-row spacing accepted as standard.

Farmers in the study area (Sothern zone of Tigray) are using different spacing below or above the national recommendation depending on the purpose of planting either for consumption or for seed tuber due to lack of recommended inter and intra-row spacing. Hence, it is important to maintain appropriate plant population per unit area to have high yield, marketable size and good quality of seed tuber. Even though different research is done in different parts of the country on potato plant density, the condition is not studied in Ofla Woreda, Southern Zone of Tigray. This study was therefore conducted to determine the best inter and intra-row spacing for optimum tuber seed yield and quality of potato seed tuber at Ofla Wereda, Northern Ethiopia.

MATERIALS AND METHODS

The experiment was conducted in 2011/2012 under irrigation condition in Southern Zone of Tigray, Ofla Woreda at Hashenge Kebele, on farmer's field. The experimental site is located at an elevation of 2500 m above sea level. Maximum and minimum temperature ranges from 22.57and 6.8°C, respectively. The mean annual rainfall of the area is 806.5 mm. The major soils include clay (28%), loam (57%) and sandy (15%) with a pH of 6.8 (BoARD, 2009).

The Woreda is classified into three agro-ecological zones, namely, highland, midland and lowland. The midland covers the largest part which accounts about 42% of the total 133, 296 ha while both the highland and lowland covers 29%. The average land holding in the Woreda is about 0.5 ha per household and estimated total population of 132,491 (BoARD, 2009).

Different local and improved potato varieties are being grown in the area. Among the improved variety, Jalenie is growing widely and has got acceptance by farmers due to its high yielding ability and acceptability by consumers.

The experiment was laid out in 4 x 4 factorial arrangements using a Randomized Complete block design (RCBD) with three replications and two factors, which consisted of four different intra-row spacing: 20, 25, 30 and 35 cm, and four different inter-row spacing: 65, 70, 75 and 80 cm. Each plot contain four rows with different plot size of (3.15 x 3.2, 3.15 x 3, 3.15 x 2.8, 3.15 x 2.6) and different number of plants per row which includes 15, 12, 10, 9 plants for 20, 25, 30 and 35 cm intra row spacing, respectively. A foot path of 0.5 and one meter was left between plots and blocks, respectively.

The collected data on different growth stage was analyzed by using SAS Computer software version 9.0 (SAS Institute Inc., 2008).

RESULTS AND DISCUSSION

Leaf area index

Intra-row spacing showed a very highly significant ($P<0.001$) effect on leaf area index. However, the effect of inter-row spacing and interaction showed no significant difference in leaf area index (Figure 1). The result revealed that significantly the highest leaf area index (3.21) was recorded at 20 cm intra-row spacing, and this could be due to high number of haulms per unit area. Whereas the lowest (2.32) leaf area index was recorded from 35 cm intra-row spacing and it is statistically difference from the other three (30, 25 and 20 cm) intra-row spacings.

This result is in agreement with the findings of Ronald (2005) and Tamiru (2005) who reported that the highest density increased leaf area index, possibly indicating potential partitioning of assimilates for vegetative growth.

Total tuber seed yield (t/ha)

The effect of inter-row and intra-row spacing showed a very highly significant ($P<0.001$) differences on total tuber yield ha^{-1} (Table 1). However, the interaction effect was non-significant ($P>0.05$). The highest yield (36.89 t/ha) was obtained from 65 cm inter-row spacing, whereas the lowest (31.87 t/ha) yield was recorded at 80 cm inter-row spacing.

Regarding the intra-row spacing, the higher total yield

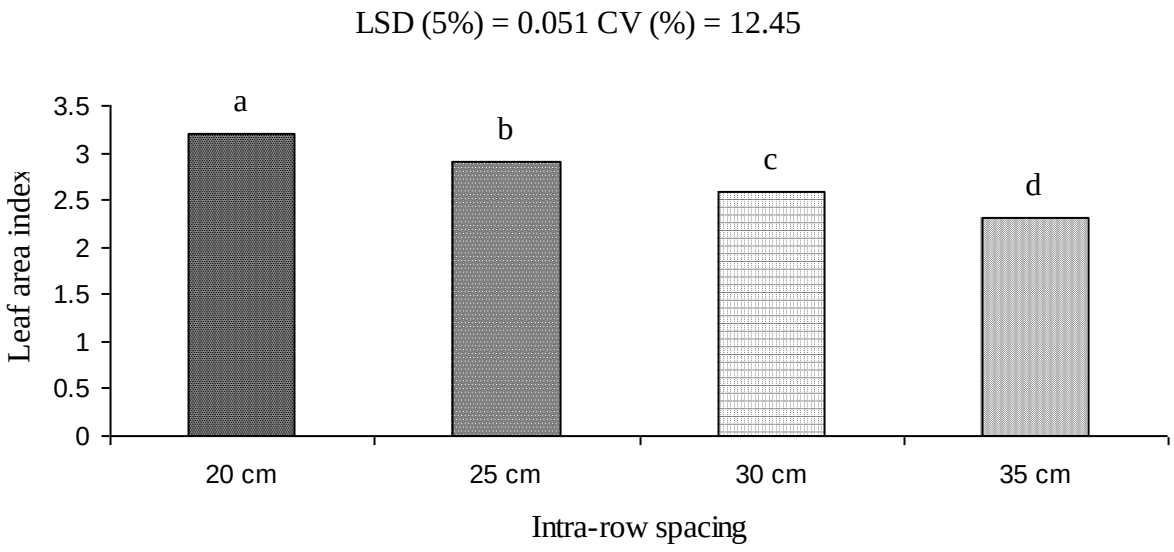

Figure 1. Means for the effect of intra-row spacing on leaf area index.

Table 1. Means for the effect of inter and intra-row spacing on total tuber yield and marketable tuber seed yield per hectare

Treatment	Total tuber seed yield (t/ ha)	Marketable seed tuber yield (t/ ha)
Intra-row spacings (cm)		
20	37.54[a]	35.89[a]
25	35.75[b]	34.49[b]
30	35.61[b]	34.66[b]
35	29.38[c]	28.65[c]
Inter-row spacings (cm)		
65	36.89[a]	35.09[a]
70	35.33[b]	33.86[b]
75	34.18[b]	33.32[b]
80	31.87[c]	31.42[c]
LSD (5%)	1.18	1.18
CV (%)	11.25	10.31

Means followed by the same letter within the same column are not significantly different at 5% level of significance.

per hectare (37.54 t/ha) was obtained from 20 cm intra-row spacing. As intra-row spacing increased from 20 to 35 cm, total tuber yield decreased from 37.54 to 29.38 t/ha. Intra-row spacing of 35 cm showed lower total tuber yield (29.38 t/ha) and it was significantly different from the three levels. It was clearly evident from the results that the yield of seed tuber per hectare was increased with decreasing plant spacing.

The increased yield was attributed to more tubers produced at the higher plant population per hectare although average tuber size was decreased because of increased inter-plant competition at closely spaced plants leading to more unmarketable tuber yield. At closer spacing there is high number of plants per unit area which brings about an increased ground cover that enables more light interception, consequently influencing photosynthesis. It is therefore, very likely that substantial increases in rate of land coverage and thereby tuber yield could be achieved by dramatically increasing the stem density per unit area.

The present result agrees with the findings of Zabihi et al. (2011) who reported that plant density in potato affects some of the important plant traits such as total yield, tuber size distribution and tuber quality. Increase in plant

Table 2. Means for the effect of inter and intra-row spacing on total and marketable seed tuber number of tuber ha^{-1}.

Treatment	Total number of seed tuber per hectare	Number of marketable seed tuber per hectare
Intra-row spacing(cm)		
20	558174^{a}	501651^{a}
25	486858^{b}	445568^{b}
30	455014^{bc}	423513^{bc}
35	430311^{c}	395106^{c}
Inter-row spacing (cm)		
65	532865^{a}	485144^{a}
70	496599^{a}	455026^{a}
75	453307^{b}	411315^{b}
80	447586^{b}	414352^{b}
LSD (5%)	37587.6	37667.7
CV (%)	15.57	15.61

Means followed by the same letter within the same column are not significantly different at 5% level of significance.

density led to decrease in mean tuber weight but number of tubers and yield per unit area were increased. In contrast, Berga et al. (1994) reported that wider row width by wider in-row distance (80 x 40 cm) gave the highest yield (34 t/ha) and the 60 x 20 treatment gave the lowest yield (22.2 t/ha).

Marketable seed tuber yield (t/ha)

The data concerning marketable yield as influenced by planting density is presented in Table 1. Inter and intra-row spacing showed a very highly significant (P<0.001) effect on marketable yield. Significantly maximum marketable yield (35.89 and 35.09 t/ha) was obtained at a 20 and 65 cm intra and inter-row spacing, respectively. While the lowest marketable yield (28.65 and 31.42 t/ha) was obtained at the wider spacing (35 cm intra and 80 cm inter-row spacing, respectively). However the interaction effect did not show significant difference on marketable yield per hectare.

The highest marketable yield recorded at closer spacing is attributed to more tubers produced at the higher plant population per hectare. The present result agreed with the findings of many authors (Stoffella and Bryan, 1988; Khalafalla, 2001) regarding plant density effect on marketability of the crop. Close spacing of 15-25 cm was reported to give better proportion of marketable yield than wider spacing of 35 cm.

Total number of tubers per hectare

The results of total number of tuber (ha^{-1}) as influenced by inter and intra-row spacing is presented in Table 2. Inter and intra-row spacing had very highly significantly (P<0.001) affected total number of tuber per ha. Significantly maximum total number of tuber per hectare (532,865) was recorded at 65 cm inter-row spacing. While the lowest number of tuber per hectare (447,586) was obtained at wider spacing (80 cm) inter-row spacing.

As far as the intra-row spacing is concerned, significantly maximum total number of tuber per hectare (558,174) was obtained from 20 cm spacing. Whereas the lowest total number of tuber per hectare (430,311) was obtained at 35 cm spacing. Total tuber number per hectare was increased with closer spacing. The highest number of tuber at closer spacing is due to high number of plants per unit area. Rahemi et al. (2005) reported that intra-row distance of 20 cm increased total tuber number and weight per unit area.

Marketable seed tuber number per hectare

Marketable tuber number (000's ha^{-1}) as influenced by inter-row and intra-row spacing is presented in Table 2. Inter and Intra-row spacing had very highly significant (P<0.001) effect on marketable tuber number per hectare. However, the interaction effect had no significant (P>0.05) effect on marketable tuber number per hectare.

Maximum marketable tuber number (485,144 and 501,651) was obtained at 65 and 20 cm inter and intra-row spacing respectively, while the result recorded at 20 cm intra-row spacing was significantly different from the other intra-row spacings. The lowest number of marketable tuber per hectare (411,315 and 395,106) was obtained at 80 cm inter and 35 cm intra-row spacing, respectively. Among the inter-row spacings, statistically, the same results were obtained from 65 and 70 cm, which scored the highest marketable tuber number per hectare, 485,144 and 455,026, respectively.

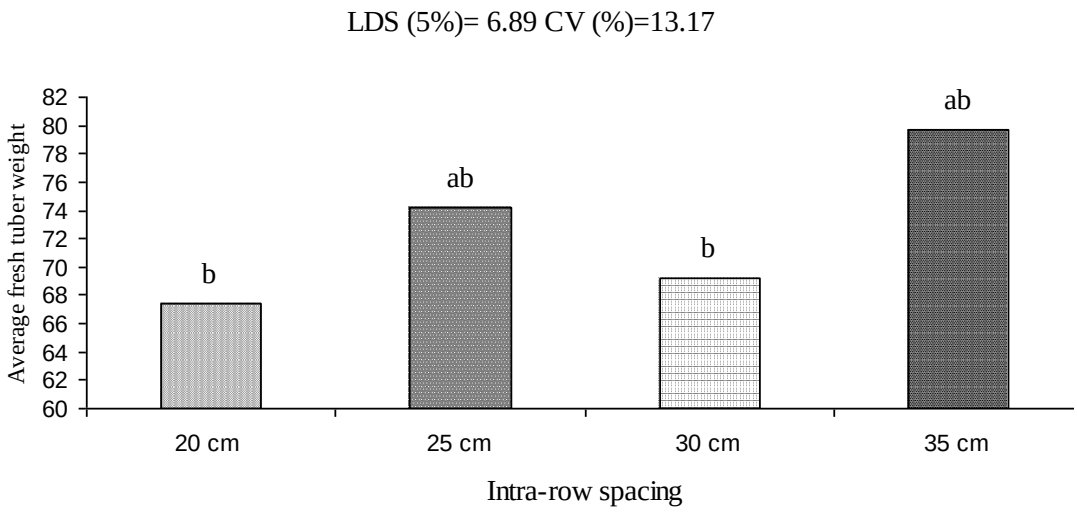

Figure 2. Means for the effect of intra-row spacing on average fresh tuber weight.

Related study was reported by Burton (1989); wider spacing may produce few tubers as it gave rise to few stems that could lead to high number and possibly misshapen tuber while, closer spacing improved quality and saleable yield.

Average fresh tuber weight (g)

Intra-row spacing showed highly significant (P<0.01) difference on average fresh tuber weight per plant (Figure 2). However, the main effects of inter-row spacing and its interaction with intra-row spacing had no significant (P>0.05) difference on average fresh tuber weight. The maximum mean tuber weight (79.68 g) was recorded at 35 cm intra-row spacing but not statistically different with 25 cm intra-row spacing. The smallest average fresh tuber weight (67.3 g) was recorded at 20 cm intra-row spacing. However, it was not significantly different from 25 and 30 cm intra-row spacing for the values of (74.24 and 69.16 g, respectively).

Increase in density probably increased competition between and within plants and hence, leads to decrease in availability of nutrients to each plant and consequently, resulted in decline of mean tuber weight. This result is in line with that of Ali (1997), who found higher average fruit weight at wider spacing as compared to closer spacing. Berga and Caesar (1990) also reported that stem number per plant and tuber number per plant are positively related, however, average tuber weight increased with wider spacing.

Tuber size category

Intra-row spacing had shown highly significant (P<0.01) effect on number of tubers graded less than 20 mm (Table 3). Maximum (9.96%) less than 20 mm number was recorded at intra-row spacing of 20 cm. However, it was not significantly different from 25 cm intra-row spacing. While, the lowest (6.629%) was at 35 cm. Intra-row spacing also showed a very highly significant (P<0.001) effect on weight of tubers graded less than 20 mm. Significantly maximum (0.74%) less than 20 mm weight was recorded at intra-row spacing of 20 cm. It was significantly different from the other intra-row spacings. However, the effect of inter-row spacing and interaction effect had no significant (P>0.05) difference for number and weight of tubers graded less than 20 mm.

Intra-row spacing also showed very highly significant (P<0.001) effect on tubers graded greater than 50 mm in terms of number and weight. Significantly maximum (23.74 number and 52.91 weight percent) greater than 50 mm graded tuber was recorded at 35 cm intra-row spacing. While, the lowest (18.50 number and 42.30 weight percent) was recorded at 20 cm intra-row spacing. Inter-row spacing showed highly significant (P<0.01) effect on tuber graded 30-40 mm weight.

The highest (17.14 percent) tubers graded 30-40 mm weight was recorded at 65 cm inter-row spacing. The results of this investigation clearly indicated that the level of intra-row spacing largely affected potato tuber size distribution. Thus, based on market and consumers' demand, it is possible to produce either seed potato or ware potato of required size through the selection of appropriate planting density (intra-row spacing).

The present result is in agreement with the findings of Wiersema (1987) who reported that at higher stem density, the tuber produced will remain smaller than at lower stem densities. Khajehpour (2006) also reported that increase in plant density decreases mean tuber size probably because of plant nutrient elements reduction, increase in interspecies competition and large number of tubers produced by high numbers of stems. Generally,

Table 3. Means for the effect of intra-row spacing on tuber size category.

Intra-row spacing (cm)	Weight of tubers graded less than 20 mm (%)	Number of tubers graded less than 20 mm (%)	Weight of tubers graded greater than 50 mm (%)	Number of tubers graded greater than 50 mm (%)
20	0.7335[a]	9.961[a]	42.30[c]	18.50[b]
25	0.7066[b]	8.121[ab]	44.21[c]	19.02[b]
30	0.6808[c]	7.485[b]	49.06[b]	22.00[a]
35	0.5005[d]	6.629[b]	52.91[a]	23.74[a]
LSD (5%)	0.005	1.904	3.401	2.419
CV (%)	14.10	25.2	12.12	17.55

Means followed by the same letter within the same column are not significantly different at 5% level of significance.

the result of this study indicates that tuber size category is influenced mainly by intra-row spacing rather than inter-row spacing.

Summary and conclusion

The result of this study demonstrated that yield per unit area is influenced by the different level of inter and intra-row spacing. From this study, it can be concluded that the narrow spacing (20 and 65 cm intra and inter-row spacing) produced higher seed tuber yield and marketable yield per hectare than other spacings. Thus, potato (Jalenie variety) growers in the study area (southern zone of Tigray) can benefit if they use this narrow spacing (20 and 65 cm intra and inter-row spacing).

Conflict of Interests

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

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Full Length Research Paper

Colchicine and duration time on survival rate and micropropagation of *Dionaea muscipula* Ellis

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Young leaf bases and leaf blades about 0.5 cm in height of a carnivorous plant *Dionaea muscipula* were used as explants for determining the callus multiplication. Explants were cultured on 1/2MS medium supplemented with various concentrations of benzyladenine (BA; 0, 0.1, 0.2, 0.5, 1.0 mg/l). 1/2MS medium supplemented with 1.0 mg/l BA gave the highest average diameter of callus about 0.55 cm after culturing for nine weeks. Callus was subcultured into the same medium every three weeks four times. 1/2MS supplemented with 0.5 mg/l BA gave the highest average plant height, number of leaves, number of roots, and root length. When young shoots about 0.5 cm long treated with a combination of different concentrations (0, 5, 10, 15 and 20 mg/l) of colchicine within different incubation times (24, 48 and 72 h), the survival rate was dependent on the concentration of colchicine and incubation time. Their survival rate was the lowest, when young shoots were soaked in 20 mg/l colchicine for 72 h (70%).

Key word: *Dionaea muscipula*, colchicine, incubation time, micropropagation.

INTRODUCTION

Dionaea muscipula Ellis (Venus fly trap) is a carnivorous plant which is endangered and on the brink of extinction, and belongs to the *Droseraceae* family. This plant is native to the eastern coast of the U.S.A. (Slack, 1981). This ornamental plant is attractive and can be used as a medicinal plant. This plant has been used for years as a source for an anticancer drug and various secondary metabolites which have been used as immunomodulator, antileprosy, antifertility, abortifacient and chitin synthetase inhibitor (Finnie and Staden, 1993; Pakulski and Budzianowski, 1996). Due to the insect-trapping characteristics, many people want to grow them as ornamental plants and their demand is increasing. The plant tissue culture technique plays an important role in preservation and micropropagation of this plant. There is a number of reports on the *in vitro* propagation of other carnivorous plants as an effort toward their preservation. Jala (2012) reported micropropagation of the pitcher plant *Nepenthes mirabilis* by using shoot tip. Crouch et al. (1988, 1990) reported *in vitro* propagation of a sundew, *D. rotundifolia* L., by a leaf culture. Jang and Park (1999) reported a method for mass propagation of *D.*

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Table 1. Effect of various concentrations of BA on size and red color of *Dionaea muscipula* Ellis callus after culturing for 9 weeks (±SE).

Concentration of BA (mg/l)	Size of callus (diameter: cm)*	Level of red callus (red)*
0.0	0.0 ± 0.00^{c}	0 ± 0.00^{c}
0.1	0.29 ± 0.28^{ab}	1.2 ± 0.79^{ab}
0.2	0.40 ± 42^{ab}	0.80 ± 0.79^{a}
0.5	0.16 ± 0.18^{a}	0.90 ± 0.99^{a}
1.0	0.55 ± 0.25^{b}	2.20 ± 0.79^{b}

[abc]Values in the same columns not significantly different using the Tukey test at 95% ($p\leq0.05$). Level of the intensity of red color of the callus: 1, light red; 2, red; 3, dark red.

L. through a shoot culture. A few reports on micropropagation of Venus fly traps are available. Teng (1999) used flower stalks as explants. Intact plants can be readily cultured by micropropagation (CZany et al., 1992).

This study examined an effect of colchicine and time for soaking which can change their morphological, mutation characters in suitable formula of medium and plant growth regulator (benzyladenine, BA) for rapid *in vitro* micropropagation of *D. muscipula* Ellis.

MATERIALS AND METHODS

Young leaf bases and leaf blades of *D. muscipula* were used as explants. Explants were surface sterilized by soaking with 70% alcohol for 1 min and soaked with 10% Clorox (NaOCl) for 10 min, followed by 5% clorox and washed with sterilized distilled water 3 times to remove the Clorox. They were cultured on 1/2 MS (Murashige and Skoog, 1962) supplemented with 0, 0.1, 0.2, 0.5, or 1.0 mg/l BA and 3% sucrose, 0.25% gelrite at pH 5.7, which had been autoclaved at 121° C for 20 min. The cultures were maintained at 25 ± 2°C under 16-h photoperiod with illumination provided by cool fluorescent lamps at an intensity of 60 µmol m^{-2} s^{-1} (TLD 36 w/853350 lm Phillips Thailand). These cultures were maintained in a proliferating state by subculturing every three weeks into the same medium 4 times. Callus formation was found at the edge of explants in ½ MS after culturing for four weeks.

A bunch of young leaf bases and leaf blades, about 0.5 cm in height (4-5 leaf bases and leaf blades) were soaked in various concentrations (0, 5, 10, 15 and 20 mg/l) of colchicine for 24, 48 and 72 h. Young leaf bases and leaf blades soaked in combinations of different concentrations of colchicine and incubated for different times were then cultured in 1/2MS medium and subcultured every 3 weeks 4 times. Percentage of leaf bases and leaf blades that survived after culturing in 1/2MS were counted as shown in Table 3. When these leaf bases and leaf blades were cultured longer, it was found that shoots were proliferated and formed; their growth are displayed in Table 4. Mutant characters from plantlets were recorded when these plantlets were cultured for 12 weeks as shown in Figure 2.

Statistical analysis

Experiments were set up in Completely Randomized Design (CRD) with six treatments; each treatment consisted of 20 replicates for the first experiment and 25 replicates for the second experiment. The test of statistical significance was done by applying Tukey's test at 5% confidence level using SAS statistical software, Release 6.03 (SAS Institute Inc., Cary, NC).

RESULTS

Explants of *D. muscipula* approximately 0.5 - 1 cm in size were cultured on half-strength MS (1/2MS) medium supplemented with various concentrations of BA. During the second week, the explants turned brown. During the fourth week, the explants which were cultured in ½ MS medium turned from dark brown to red (Figure 2). It was found that some red callus occurred at the edge of the explants. There was a significant difference ($p<0.05$) in size and red color of callus. The size of callus increased after subculturing every 3 weeks and 3 times and culturing on ½ MS medium supplemented with 0.1, 0.2, 0.5 or 1.0 mg/l of BA. Callus from 1/2MS supplemented with 1.0 mg/l BA gave the highest average diameter of callus, about 0.55 cm, and the level of red color of callus was 2.2 (Table 1).

When the red callus was subcultured into the same medium every 3 weeks 4 times, it was found that red callus was induced to form new young shoots and formed roots at the base. Plant height, number of leaves, number of roots and root length in each concentration of BA are shown in Table 2. No parameter from various concentration of BA between 0.1-1.0 mg/l was significantly different (Table 2). The average plant height with 0.1 and 0.5 mg/l BA was about 0.96 and 0.93 cm, respectively. The values of all parameters at 1.0 mg/l BA were always the lowest (but not significant).

Survival rate

Explants, about 0.5 cm in height, were soaked with combination of different concentrations (5, 10, 15 or 20 mg/l) of colchicine and incubated for various (24, 48, 72 h) time. After being treated, all explants were cultured in 1/2MS supplemented with 0.2 mg/l BA and subcultured every three weeks at two times. After 6 weeks, survival rate was recorded as shown in Table 3.

The results show that the survival rate of *D. muscipula*

Table 2. Effect of various concentrations of BA on growth of *Dionaea muscipula* in terms of plant height, number of leaves, number of roots and root length after being cultured for 12 weeks. (±SE)

Concentration of BA (mg/l)	Plant height (cm)	Number of leaves	Number of roots	Root length (cm)
0.0	0.24±0.23[a]	0.05±0.56[a]	0.0±0.0[a]	0.0±0.0[a]
0.1	0.96±0.36[b]	2.20±0.42[b]	3.60±2.01[b]	0.98±0.57[b]
0.2	0.85±0.42[b]	2.30±1.06[b]	4.40±3.24[b]	0.69±0.55[b]
0.5	0.93±0.36[b]	2.30±0.67[b]	5.90±2.64[b]	0.96±0.50[b]
1.0	0.58±0.47[b]	1.70±1.34[b]	3.20±3.29[b]	0.69±0.53[b]

[abc]Different letters within the same columns indicate statistically significant difference using the Tukey test at 95% ($p \leq 0.05$).

Table 3. Survival rates of *Dionaea muscipula* soaked with a combination of different concentrations of colchicine, incubated for various time and transferred to the culturing medium 1/2 MS supplemented with 0.2 mg/l BA for next 6 weeks.

Colchicine Concentration (mg/l /h)	Number of explants (shoot)	Number of survivors (shoot)	Survival rate (%)
Control	40	40	100
5/24	40	34	85
10/24	40	32	80
15/24	40	34	85
20/24	40	32	80
5/48	40	33	82.5
10/48	40	33	82.5
15/48	40	31	77.5
20/48	40	30	75
5/72	40	34	85
10/72	40	30	75
15/72	40	30	75
20/72	40	28	70

depended on the concentration of colchicine and duration time. Tend of Explants which were soaked at higher concentrations of colchicine and for longer incubation times gave lower survival rate. Concentrations of 5 and 15 mg/l colchicine and 24 h incubation gave the highest average survival rate (85%) and the same as 5 mg/l colchicine for 72 h (85%) as in Table 3. For explants which were soaked with combination of 20 mg/l colchicine for 72 h, the survival rate, had the lowest (70%).

Regeneration

All explants of *D. muscipula* were soaked with a combination of different concentrations of colchicine and incubation times, and subcultured every 2 weeks for 6 times. After 12 weeks, variations occurred in plant traits of colchicines and incubation times were induced. The variations, such as size of callus, level of red callus, plant height, number of leaves, number of roots and root length were obtained by treatment with different concentrations of colchicine and varying incubation times as shown in Table 4. All parameters were significantly different ($p \leq 0.05$). It was shown that low concentrations of colchicine (5 and 10 mg/l) and a short incubation time (24 h) gave the highest result in size of callus, level of red callus, number of leaves, number of roots and root length.

Explants which were soaked with 5 mg/l colchicine for 72 h induced callus and gave the highest average diameter of callus (2.4 cm) as shown in Figure 1a. Explants soaked with 15 mg/l colchicine for 24 h gave the most intensive red callus (2.15cm) as shown in Figure 1b. The highest average plant height was achieved with explants soaked in 5 mg/l colchicine for 24 h, about 2.66 cm (Figure 1c). Explants soaked in 10 mg/l colchicine for 24 h gave the highest average number of leaves approximately 2.85 leaves (Figure 1d). Number of leaves was the highest for explants soaked in 15 mg/l colchicine for 48 h, approximately 13.45 roots (Figure 1e), and the longest average root length occurred with explants soaked with 15 mg/l colchicine for 72 h, approximately 2.15 cm. (Figure 1f). From the experiment, it was shown that explants soaked with higher concentrations of colchicine and for longer incubation times had decreased

Table 4. Effect of colchicine concentrations and incubation time on plant height, number of leaves, number of roots and root length in *Dionaea muscipula* after culturing for 12 weeks (±SE).

Colchicine concentration (mg/l /h)	Size of callus (cm)	Level of red color	Plant height (cm)	Number of leaves	Number of roots	Root length (cm)
Control	1.35±0.67^{a}	1.6±0.75^{ab}	3.58±0.53^{d}	3.40±0.60^{c}	15.3±4.66^{c}	2.80±0.45^{d}
5/24	1.35±0.88^{a}	1.25±0.85^{a}	2.66±0.60^{c}	2.65±0.67^{abc}	9.30±3.39^{ab}	1.66±0.62^{abc}
10/24	1.55±0.76^{ab}	1.85±0.75^{ab}	2.41±0.56^{bc}	2.85±0.59^{bc}	10.00±4.12^{abc}	1.80±0.44^{abc}
15/24	1.90±0.72^{abc}	2.15±0.59^{b}	2.15±0.52^{abc}	2.50±0.69^{ab}	9.40±4.12^{ab}	1.63±0.56^{abc}
20/24	1.50±0.95^{ab}	1.65±0.99^{ab}	1.85±0.84^{ab}	2.40±0.82^{ab}	10.55±5.66^{abc}	1.64±0.79^{abc}
5/48	1.85±0.67^{abc}	2.00±0.79^{ab}	2.26±0.78^{abc}	2.40±0.82^{ab}	13.20±6.21^{bc}	1.78±0.73^{abc}
10/48	1.80±0.77^{abc}	1.80±0.89^{ab}	1.60±0.78^{a}	2.00±0.79^{a}	7.80±4.72^{a}	1.21±0.77^{a}
15/48	2.15±0.75^{bc}	1.50±0.69^{ab}	2.41±0.58^{bc}	2.85±0.67^{bc}	13.45±4.68^{bc}	2.01±0.55^{bc}
20/48	1.95±0.83^{abc}	1.65±0.81^{ab}	2.10±0.70^{abc}	2.40±0.75^{ab}	10.11±6.76^{abc}	1.66±0.86^{abc}
5/72	2.40±0.68^{c}	1.65±0.67^{ab}	1.91±0.69^{ab}	2.35±1.04^{ab}	10.40±6.25^{abc}	1.40±0.71^{ab}
10/72	1.85±0.59^{abc}	1.90±0.55^{ab}	2.31±0.76^{abc}	2.65±0.75^{abc}	13.00±4.15^{abc}	1.76±0.53^{aabc}
15/72	1.55±0.76^{ab}	1.80±0.83^{ab}	2.31±0.73^{abc}	2.65±0.81^{abc}	13.00±4.63^{abc}	2.15±0.76^{cd}
20/72	1.85±0.75^{abc}	1.70±0.73^{ab}	2.19±0.75^{abc}	2.60±0.82^{abc}	13.15±4.75^{bc}	1.80±0.68^{abc}

^{abc}Different letters within the same columns indicate statistically significant difference using the Tukey test at 95% ($p \leq 0.05$).

growth rate.

Explants of *D. muscipula* treated with a combination of different colchicine concentrations and various incubation times were cultured for 90 days. It was found that there are different characteristics which were a change from the control and were counted as mutations. Their mutation rate was recorded in each treatment as shown in Table 5. It was shown that high concentrations of colchicine (10, 15, 20 mg/l) and soaking for long incubation times (48 and 72 h) affected leaf base and leaf blade and gave the highest mutation rate, especially at 20 mg/l colchicine soaked for 72 h which gave 95% mutation (Table 5). But the same concentration of colchicine (20 mg/l) and shorter incubation time (24 h) gave only 55% mutation. The color of the leaf blade and leaf base was still green as only callus phase turned red.

Mutation characteristics of *D. muscipula* that occurred after being soaked with a combination of different concentrations of colchicine and various incubation times and cultured for 12 weeks are shown in Figure 2. The edge of the leaf base changed from smooth to curved edge (Figure 2a), leaf blade doubled (Figure 2b) and size of the leaf blade was bigger with more teeth (Figure 2c) when compared to the control. Their growth rate was decreased also.

DISCUSSION

In this experiment, young leaf base and leaf blade used as explants and cultured on 1/2MS medium supplemented with 0.5 mg/l BA gave the highest average diameter of red callus and red callus regenerated to new shoots within six weeks. This result was the same as that of Crouch et al. (1990) that reported that MS medium was suitable for mass propagation of a sundew, *D. rotundifolia* L. from the young leaf. They cultured the young leaf on MS medium supplemented with 1.0 mg/l BA and got the highest red callus that regenerated to new shoots later. But Jang and Park (1999) used the shoot of *D. rotundifolia* L. cultured on 1/3MS medium supplemented with 0.5 mg/l kinetin for mass propagation. Slack (1981) used a young leaf for micropropagation of *Pinguicula moranensis* (Butterwort) on MS medium. For the Venus fly trap, only a few reports are available. Beebe (1980) and Parliman et al. (1982b) used the leaf for producing adventitious buds on MS medium supplemented with NAA and BA. Parliman et al. (1982a) cultured the rhizome on 1/2MS medium supplemented with 1.9 mg/l NAA and 0.2 mg/l BA and got the best new shoots with

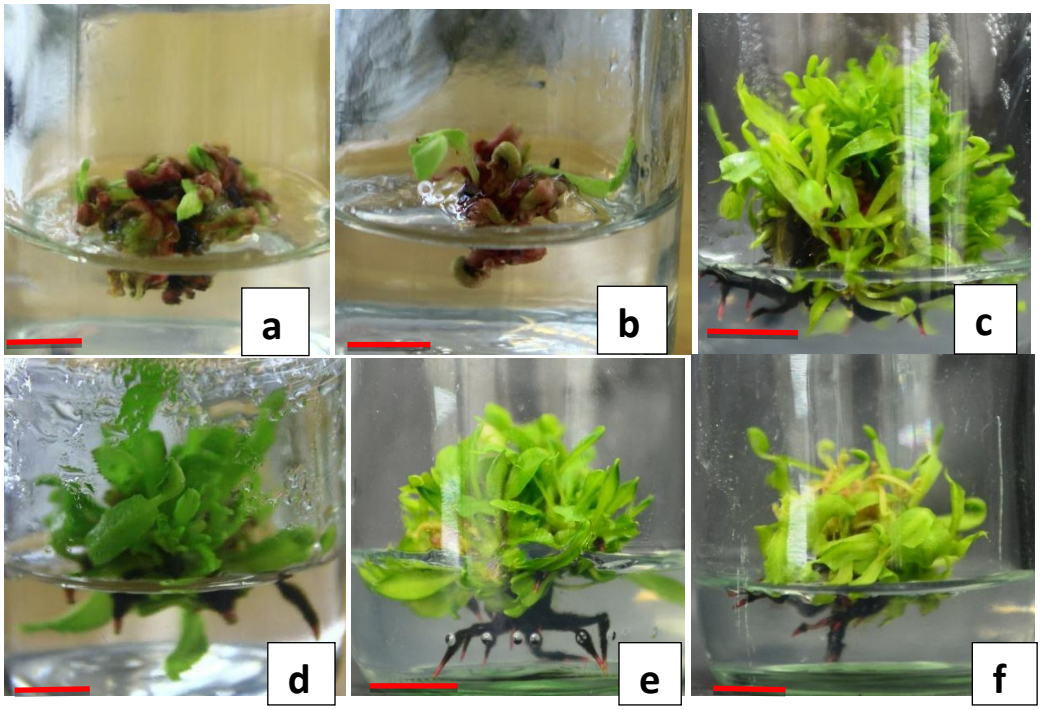

Figure 1. Growth and development of *Dionaea muscipula* after being soaked with a combination of different concentration of colchicine and incubation times, and then cultured on 1/2 MS medium for 30 days: (▬ bar = 1 cm). a) soaked in 5 mg/l colchicine for 72 h; b) soaked in 15 mg/l colchicine for 24 h; c) soaked in 5 mg/l colchicine for 24 h; d) soaked in 10 mg/l colchicine for 24 h; e) soaked in 15 mg/l colchicine for 72 h, f. soaked in 15 mg/l colchicine for 72 h.

Table 5. Mutation rate in explants treated with combination of different concentration colchicine and various incubation times and cultured for 12 weeks.

Colchicine concentration (mg/l /h)	Number of explants (shoot)	Number of mutation (shoot)	Mutation rate (%)
Control	20	0	0
5/24	20	5	25
10/24	20	8	40
15/24	20	8	40
20/24	20	11	55
5/48	20	10	50
10/48	20	11	55
15/48	20	16	80
20/48	20	15	75
5/72	20	12	60
10/72	20	16	80
15/72	20	17	85
20/72	20	19	95

roots. Minocha (1985) used the mature leaf segments of *D. muscipula* for *in vitro* propagation. (Hutchinson and Zimmerman,1984) used the shoot tip to culture and produced plantlets by culturing them on LS medium (Linsmaier and Skoog, 1965) supplemented with 10 µM kinetin and 0.5 µM NAA. Teng (1999) used the flower stalk as explants for *in vitro* culture. Grevenstuk et al. (2010) reported a method for mass propagation of *D. muscipula* by culturing protocorm on 1/4MS supplemented with kinetin.

When explants of *D. muscipula* were treated with a combination of different concentrations of colchicines and

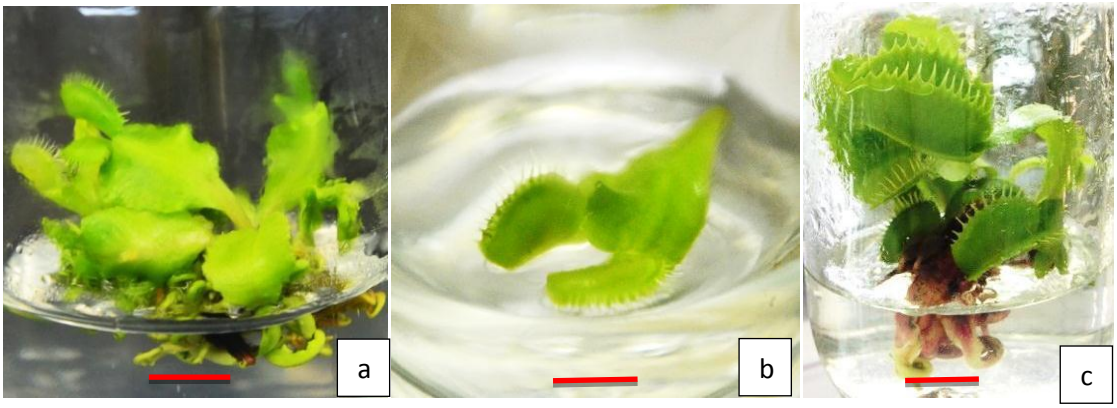

Figure 2. Mutation in *Dionaea muscipula* after being treated with combinations of colchicine And various incubation times and cultured for 90 days in 1/2MS : a) leaf base with curved edge, b) twin leaf blade, c) bigger leaf blade and more teeth(bar = 1 cm).

various incubation times and, then cultured on 1/2MS medium supplemented with 2 mg/l BA for 90 days, it was found that the survival rate depended on the concentration and duration of treatment. The growth rate of explants treated with high concentrations of colchicine (10, 15 and 20 mg/l) and soaked for longer incubation times (48 and 72 h) was very slow. This result corresponded to those of Chaicharone et al. (1995) and Thao et al. (2003). Zhang et al. (2008) reported that induced polypoid in *Phlox subulata* L. by soaking the shoot tip in high concentrations of colchicine (0.005%) for 10 days got the highest growth rate, about 40%. When soaked with 0.4% colchicine for 30 days, their survival and growth rate decreased to zero. (Sun and Hong, 2009) showed that the survival rate depends on the concentration and duration of treatment. Colchicine is a toxic chemical that is often used to induce polyploidy in plants. Sometimes the polyploid plants have larger leaves and flowers (Barry, 2000). *D. muscipula* explants treated with 20 mg/l colchicine for 72 h gave the highest variation which was the same as Gibson (1999) which reported that the *Drosera binata* plants looked larger than the control due to the higher colchicine treatment also and started to generate clearly stunted plantlets. Swanson (1957) explained that reduced growth rate was due to reducing rate of cell division that results from the physiological disturbances caused by colchicine.

Conclusion

Young leaf base and leaf blade were suitable for callus induction on *D. muscipula* when cultured on 1/2MS medium supplemented with 0.1-1.0 mg/l BA. Red callus differentiated to new shoots for micropropagation when cultured callus on 1/2MS supplemented with 0.5 mg/l BA.

The effect of colchicine concentration and duration time for incubation on *D. muscipula* could be observed in morphological change and their growth rate, survival rate and variation which occurred in high concentrations of colchicine and long incubation times as compared to the control.

Conflict of Interests

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

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Full Length Research Paper

A comparative study of antimicrobial and antioxidant activities of garlic (*Allium sativum* L.) extracts in various localities of Pakistan

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The present study was conducted to analyze garlic extracts using compositional analysis and assessment of antibacterial and antioxidant properties against various microbes. It was observed that garlic consist of various bioactive compounds such as alkaloids, phenols, tannins and flavonoids with different concentration levels. Antimicrobial activity of different solvent extracts (n-hexane, chloroform, acetone, butanol and methanol) was tested against *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus epidermidis and Klebsella pneumonia* by using agar well diffusion assay. Results indicate that all the extracts were effective against the microorganism tested but n-hexane extract showed minimum activity against all microbes. Methanol extract was proven to be more effective against the *K. pneumonia*. MIC of these extracts was checked from 1-15 mg/mg concentration levels. Garlic plant extracts have rich contents of alkaloids, flavonoids, tannins and phenols. Various bioactive compounds are responsible for its medicinal properties.

Key words: Antimicrobial activity, antioxidant activity, compositional analysis, flavonoids, alkaloids.

INTRODUCTION

Allium sativum L. commonly known as garlic is among the oldest cultivated plant which is used for therapeutic purposes. Garlic has played one of the most important dietary and medicinal roles in human bodies for centuries and is used as a spice as well as medicinal herb. Garlic is a member of the lily (*Liliaceae*) family. It consists of more than 250 genera and 3700 species. These plants can tolerate unfavorable conditions, that is, winter and dryness due to their resistant structures: bulbs, tubers and rhizomes. The largest and most important representative genus of the *Alliaceae* family is *Allium*. It consists of 450 species, widely distributed in the northern hemisphere. There are over 300 varieties of garlic grown worldwide. In addition to the well known garlic and numerous other species are extensively grown for cooking purpose, such as leek (*Allium porrum* L.), scallion (*Allium fistulosum* L.), shallot (*Allium ascalonicum* Hort.), wild garlic (*Allium ursinum* L.), elephant garlic

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(*Allium ampeloprasum* L. var.*ampeloprasum*), chive (*Allium schoenoprasum* L.) and Chinese chive (*Allium tuberosum* L.) (Nuutila et al., 2002).

The biological and medical functions of members of *Alliaceae* family are mainly due to their high organo-sulphur compound contents. It also contains many other sulfur containing compounds such as alliin, ajoene, diallylsulfide, dithiin, S-allylcysteine, and enzymes, vitamin B, proteins, minerals, saponins, flavonoids, and maillard reaction products, which are non-sulfur containing compounds (Kojuri et al., 2007).

There is a wide range of reported therapeutic effects, such as hypolipidaemic, antiatherosclerotic, hypoglycaemic, anticoagulant, antihypertensive, antimicrobial, antidote (heavy metal poisoning) and hepatoprotective, preventing cold and flu symptoms through immune enhancement and exhibits anticancer and chemopreventive activities (Rivlin, 2001; Banerjee et al., 2003; Thomson and Ali, 2003; Amagase, 2006). The antimicrobial properties of crushed garlic have been known for a long time. A wide range of antimicrobial properties including antibacterial activity has been reported for crushed garlic (Bakri and Douglas, 2005). Recent chemical characterisation of sulphur compounds has shown that they are the main active antimicrobial agents (Rose et al., 2005).

The antioxidant properties of garlic and different garlic preparations are well documented (Cho and Xu, 2000; Nuutila et al., 2003; Saravanan and Prakash, 2004). The potential antioxidant properties of garlic are related to its phenolic and flavonoid fractions (Miller et al., 2000). Garlic has been used as an important flavouring agent, traditional medicine, and functional food to improve physical or mental health (Gedik et al., 2005; Pedraza-Chaverri et al., 2007).

Therefore, keeping in view the importance of garlic as an important medicinal food, the present study was conducted with the following aims and objectives: To evaluate proximate analysis of garlic samples, isolate phenolics, flavonoid, tannins and alkaloids from garlic and to determine antibacterial and antioxident activities of various extracts of garlic.

MATERIALS AND METHODS

Collection and preparation of sample

Garlic samples were collected from local markets of Raja Bazar Rawalpindi and Sihala Bazar, Islamabad in fine plastic bags duly labeled with date of collection and stored at -20°C for further process. Garlic samples were oven dried at 60°C overnight. Samples were crushed into powder form using electric blender and were saved at temperature of 20°C for further use.

Biochemical analysis of *Allium sativum* L.

Determination of moisture contents and protein analysis

Moisture contents of plant samples were analyzed by AOAC (1990) method. Protein content (nitrogen × 6.25) was determined by micro-Kjeldahl nitrogen analysis by using AOAC 979.09 and 920.87 methods (AOAC, 1990) and by Lowry's method (Lowry et al., 1951).

Determination of oil contents

The oil contents were analyzed by AOAC method, 920.85 with Soxhlet apparatus. In the Soxhlet extraction procedure, 5 g of the powdered sample were packed in a thimble and the oil was extracted with diethyl ether for 18 h (AOAC, 1990).

Determination of total minerals (ash)

For total ash contents, 4 g dried plant samples were taken in a pre weighed crucible and then shifted to furnace at 800°C for 1 h. Then samples were cooled in desiccators and weight was taken after cooling. Analysis of total ash was done by the method of AOAC (1990).

Carbohydrate analysis

Carbohydrate contents (%) of the samples were determined by subtracting the total percentages of crude protein, crude lipid, ash and moisture from 100 as outlined by Al-Khalifa (1996).

Determination of metal ions

Garlic samples underwent dry digestion as described by Solvak et al. *(*2006). One gram was placed in porcelain crucible and turned into ashes at 450°C for 18-20 h. The ash was then dissolved in 1 ml concentrated nitric acid (HNO_3) and was evaporated to dryness. Then it was heated again at 450°C for 4 h, treated with 1 ml concentrated H_2SO_4, 1 ml HNO_3 and 1 ml H_2O_2 and finally diluted with deionized water up to volume of 10 ml. Blank sample was also treated in the same way. Metal ions including Mg, Zn and Cu in the sample were determined by using flame atomic absorption spectrophotometry.

Analysis of phytochemicals

Determination of flavonoid

Flavonoid contents were determined by dissolving 5 g of sample in 50 ml of 80% aqueous ethanol and the whole mixture was left in shaker incubator for 24 hThe extract was then centrifuged at 10,000 rpm, at 25°C for 15 min. Pellet was discarded and supernatant containing flavonoid was stored at 4°C. Estimation was carried out by a calorimetric assay as described by Lillian et al. (2007a). The flavonoid extract (250 µl) was mixed with 1.25 ml of distilled water and 75 µl of 5% $NaNO_2$ solution. After 5 min, 150 µl of a 10% $AlCl_3.H_2O$ was added and after 6 min 500 µl of 1 M NaOH and 275 µl of distilled water were also added to the mixture. The solution was mixed well and absorbance was measured at 415 nm. Different concentrations of quercetin (50 to 250 µg) were used as standard to draw the standard curve (Lillian et al., 2007a).

Determination of phenolic compounds

Total phenols were extracted by boiling 2 g of defatted sample with 50 ml of diethyl ether in water bath for 15 min. The estimation was carried out by a calorimetric assay as described by Lillian et al. (2007b). 1 ml of sample was mixed with 1 ml of Folin Ciocalteu

reagent and after 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and volume was adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was measured at 725 nm. Different concentrations of phenol (50 to 500 mg) were used as standard to generate the standard curve and concentrations of garlic phenolics were measured (Lillian et al., 2007b).

Determination of tannins

Tannins were extracted by dissolving 0.5 g of sample in 100 ml of 70% acetone. Estimation of tannins was carried out by a calorimetric assay as described by Akindahunsi and Oyetayo (2006). Different concentrations of tannic acid (6.25 to 50 mg) were prepared by serial dilution from stock solution (50 mg/100 ml of 70% acetone). The absorbance was measured at 725 nm after the addition of 0.5 ml of folin phenol reagent and 2.5 ml of Na_2CO_3 (Akindahunsi and Oyetayo, 2006).

Determination of alkaloids

According to the method, dried sample was dissolved in ethanol (1:10) and was left on shaking for 24 h. Extract was concentrated near to dryness in oven and was re-dissolved in ethanol with addition of 1% HCl. The mixture was placed in refrigerator for three days. The solution was filtered and pH was maintained 8-10 and was extracted with chloroform by using separating funnel. Chloroform layer was recovered and ethanol layer was discarded whereas the solution was heated in hot water bath for evaporation. After that the sample was dried in oven to constant weight. Alkaloid contents were calculated on the basis of weight obtained and weight used (Gulfraz et al., 2011).

Bioassays

Preparation of extract for antibacterial activity

The powder form (80 mashes) of sample was extracted with different solvents (n-hexane, acetone, ethyl acetate, chloroform, butanol, ethanol and methanol) on the basis of their polarity. Initially the sample was extracted with n-hexane (1:10) by shaking for 24 h followed by centrifugation at 10,000 rpm for 15 min. Supernatant was then transferred to a pre-weighed falcon tube and residue was re-extracted with next solvent which was slightly polar then n-hexane. The same procedure was repeated with all solvents and all extracts were allowed to dryness in incubator. The dried extracts were dissolved in dimethylsulfoxide (DMSO) for antimicrobial assay.

Microorganisms tested

Antibacterial activity was tested against *Escherichia coli, Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus epidermidis and Klebsella pneumonia* by using agar well diffusion method. Inoculums of all microbes were prepared in sterilized Lauria-Bertini media gL^{-1} (10 g tryptophan, 10 g NaCl, 5 g yeast extract and distilled water) in separate test tubes which were then placed in shaker incubator at 37°C for 24 h to contain approximately 10^8 cfu/ml.

Antibacterial activity

Antibacterial activity was tested by agar well diffusion method. Lauria-Bertini agar media was prepared and autoclaved at 121°C for 15 min which was then cooled and poured in Petri plates under sterilized condition of laminar flow hood. The wells of 6 mm were bored in each plate and the plates were inoculated with 30 µl of inoculum. Then 1000 µg/75 µl of each of sample was pippetted in each well and plates were incubated at 37°C for 24 h. After 24 h, zones of inhibition were measured in millimeter (mm).

Minimum inhibitory concentration (MIC)

Various concentrations of the garlic extracts were prepared: 1, 5, 10 and 15 mg/ml. The cultured plates were again seeded with test bacterial organism and allowed to solidify and thereafter punched with a sterile cork borer (5.0 mm diameter) to cut uniform wells. The open wells were filled with 0.05 ml of the extract. The plates then incubated at 37°C for 24 h. The lowest concentration of extract that showed inhibition of growth of the test organism was taken as minimum inhibitory concentration (MIC) as described by Ettebong and Nwafor (2009).

Antioxidant activity

The extracts (750 µl) of each sample were mixed with an equal amount of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide (a source of ferric ions). The mixture was incubated at 50°C for 20 min followed by addition of an equal amount of trichloroacetic acid (10%) to stop the reaction and was then centrifuged at 3000 rpm for 10 min. Upper layer (1.5 ml) was separated and mixed with an equal amount of distilled water and 0.1 ml $FeCl_3$ solution (0.1%). A blank was also prepared by using same procedure and the absorbance was measured at 700 nm as the reducing power (Gulfraz et al., 2011).

Statistical analysis

All experimental data was given as mean ± SD (Standard Deviation). Statistical analysis was conducted by using *t*-test.

RESULTS AND DISCUSSION

Garlic samples were analyzed for chemical composition like total protein, oil content, and total carbohydrate and for phytochemicals. Whereas antibacterial and antioxidant activities of different garlic extracts were also assessed and results are presented in Figure 1.

Biochemical analysis of garlic

Chemical composition of garlic shows higher concentration of carbohydrate (67.5-68.5%) and low concentration of oil (3.6-3.8 %) in samples (Figure 1). It was assumed that prolonged heating affected the oil quantity and resulted in the degradation of polyunsaturated fatty acids (Yunusova et al., 1998). The concentration level of protein (17.5-17.6%), moisture (9.6 to 10.2%) and ash (0.81-0.9%) were found in garlic samples (Figure 1). The values of different parameters analyzed from garlic were almost similar and comparable to values reported by other researchers (Haciseferogullar et al., 2005;

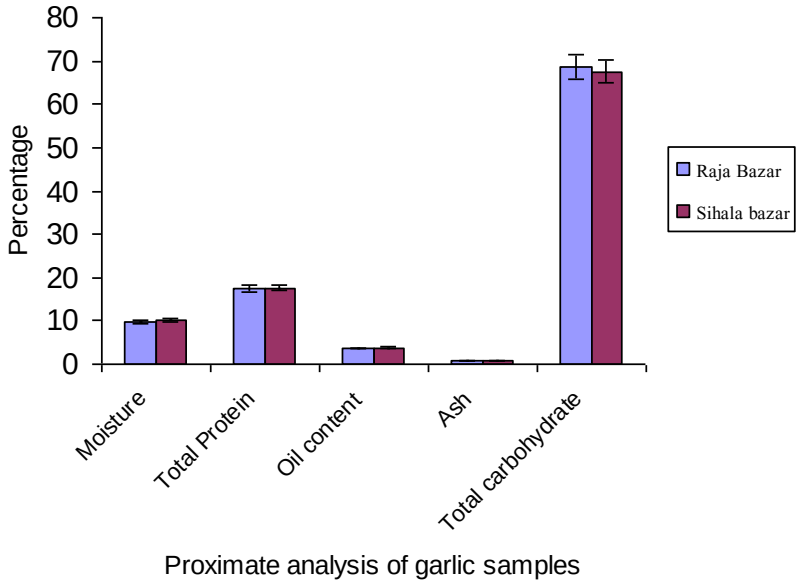

Figure 1. Comparison of proximate analysis (%) of garlic samples from Raja bazaar and Sihala bazaar.

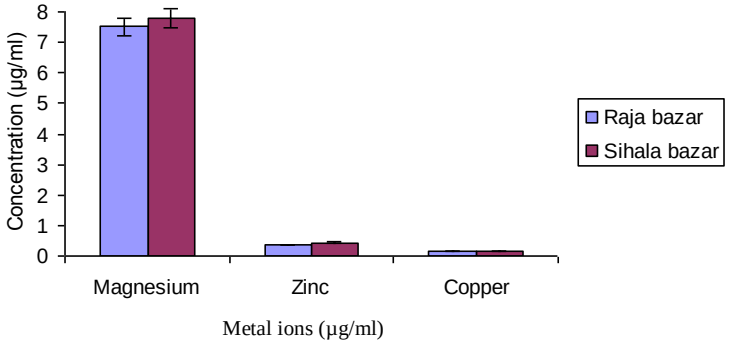

Figure 2. Comparison of metal ions (µg/ml) of garlic samples from different areas.

Nwinuka et al., 2005) especially the value for total protein which were similar to the study (17.5%) whereas literature showed that ash content in garlic is 4.06% but in the present study both garlic samples showed low ash contents (0.81-0.9%), whereas moisture contents (66.32%) obtained in literature was higher than that in this study (9.6-10.2%). Garlic samples were found to be good sources of carbohydrates but poor sources of ash, protein and fat. On the basis of protein and less oil content in garlic, it is a useful diet and could be used as a good medicinal food. Protein content was found to be considerably higher than concentrations in other vegetables such as bean and pea but crude oil contents were considerably lower in quantity.

Comparison of chemical composition of garlic from two localities is given in Figure 1. The results show that percentage of carbohydrate is higher in both samples whereas the concentration of moisture, protein, oil content and ash was found to be higher in sample collected from Sihala as compared to the other sample. The values obtained for moisture, oil, ash and total carbohydrate contents were significantly different with each other except the value for total protein which is non significant. The higher concentration of moisture, protein, oil contents and ash in Sihala samples might be due to better environmental conditions in this area.

Metal ion detection

Metal ions such as magnesium, zinc and copper from garlic samples were analyzed by Flame Atomic Absorption Spectroscopy (FAAS) and results are given in Figure 2. Macro elements including Mg (7.504 ± 0.1 and

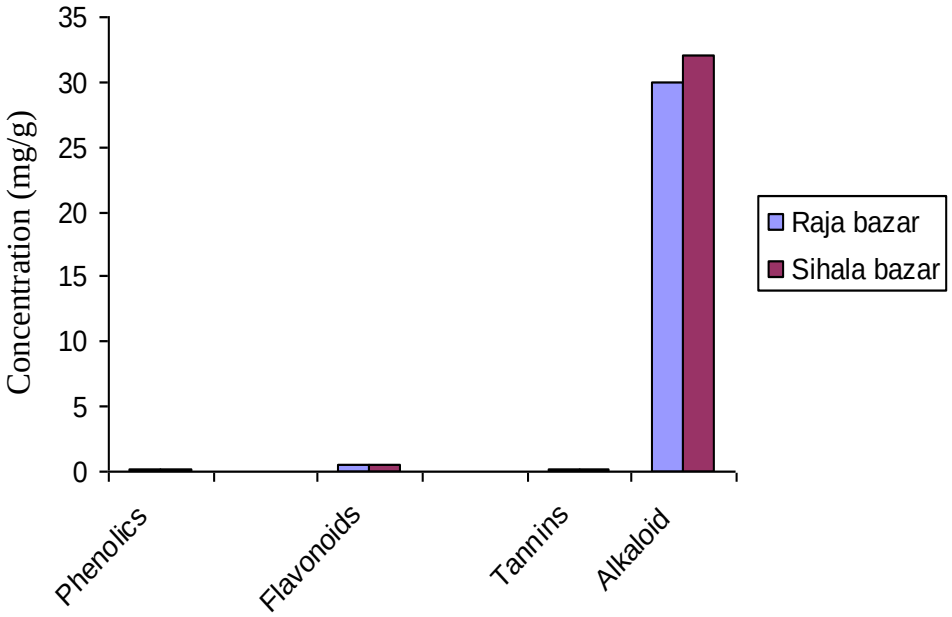

Figure 3. Comparison of phytochemical analysis of garlic samples from different areas.

7.77± 0.1 µg/ml) were found in high concentrations in samples from Raja bazaar Rawalpindi and Sihala bazaar Islamabad, respectively while other micro elements including Zn (0.370 ± 0.01 and 0.428 ± 0.07 µg/ml) and Cu (0.147 ± 0.01 and 0.16 ± 0.01 µg/ml) are found in lesser amounts. All the values obtained are significantly different from each other. Many of these metal ions act as cofactor for different enzymes. Higher concentration of Mg (7.504 ± 0.1 and 7.77± 0.1 µg/ml) are present in garlic whereas reported Mg, Cu and Zn concentrations in garlic were 1.056 ± 0.025 , 0.0912 ± 0.07 and 0.0274 ± 0.01 µg/ml, respectively (Haciseferogullar et al., 2005). Presence of all these metal ions increases the nutritional value of garlic (Metwally, 2009). The metal ion composition of garlic is mainly dependent on their ecosystem and soil composition. Furthermore garlic has higher capability to extract metal ions from other substrate. Therefore, metal ion composition of garlic is mainly dependent on their ecosystem and substrate composition (Richa et al., 2005). Zn and Mg activate DNA polymerase, which is necessary for synthesis and transcription of DNA. Magnesium is also cofactor for enzymes that play very important role in glycolysis to control sugar metabolism in the body by converting glucose to pyruvate. Comparatively, metal ions concentration increases for Sihala bazaar Islamabad as shown in Figure 2. These increases may be due to variations in soil condition of these areas.

Analysis of phytochemicals

The results of organic compounds such as phenolics, flavonoid and tannin of two garlic samples collected from different areas are shown in Figure 3. The data shows that phenolics (0.152 ± 0.05 and 0.162 ± 0.01 mg/g), flavonoid (0.451 ± 0.03 and 0.498 ± 0.01 mg/g), tannins (0.167 ± 0.05 and 0.123± 0.048 mg/g) and alkaloid (30.2 ±0.5 and 32.1 ± 2.0 mg/g) were present in garlic samples (Figure 3). All the values obtained after analysis were significantly different from each other (Gulfraz et al., 2011).

The results showed that phenolics (0.152 ± 0.05 mg/g), flavonoids (0.451 ± 0.03 mg/g), tannins (0.167 ± 0.048 mg/g) and alkaloid (30.2 ± 0.5 mg/g) were found in garlic sample from Raja bazaar Rawalpindi whereas values of phenolics (0.162 ± 0.01mg/g), flavonoids (0.498 ± 0.01 mg/g), tannins (0.123 ± 0.01 mg/g) and alkaloids (32.1 ± 2.0 mg/g) obtained from garlic sample Sihala bazaar Islamabad are significantly different from each other (Figure 3). Further, the figure showed that there is significant difference among all the samples with respect to concentrations of chemical compounds in the samples collected from different areas. This data also shows gradual increase in values for chemical compound composition of Sihala bazaar Islamabad. These increases may be due to variations in environmental conditions as well as soil of that area.

Antibacterial activity of garlic extracts

Historically, garlic has been used worldwide against high bacterial infections. Garlic exhibits broad spectrum anti-bacterial activity against both Gram-positive and negative

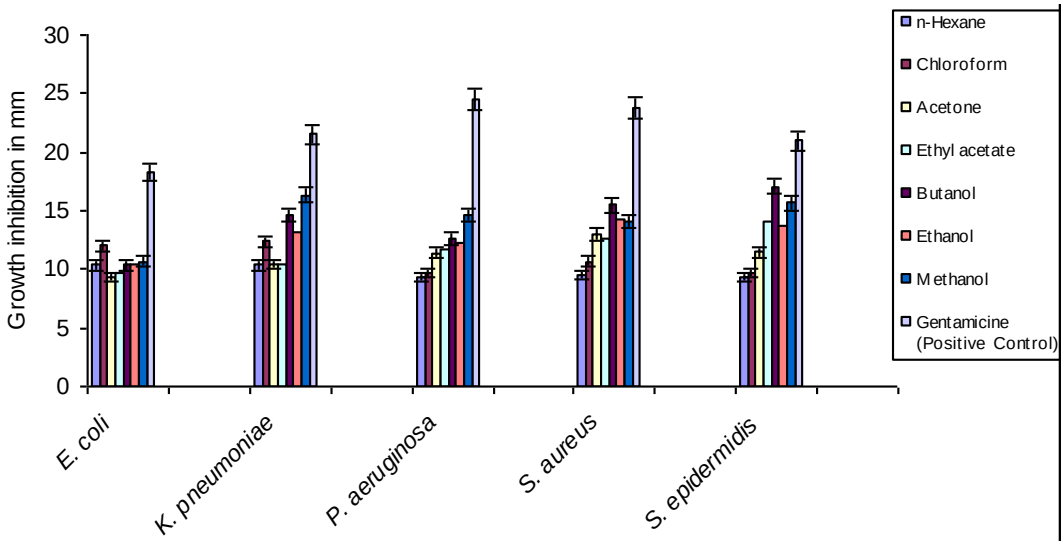

Figure 4. Antibacterial activity of garlic extracts.

Figure 5. Antimicrobial activity of different garlic extracts against *K. pneumoniae.*

Figure 6. Antimicrobial activity of different garlic extracts against *S. epidermidis.*

bacterial strains. The growing concern about food has just led to the progress of antimicrobial agents to manage food borne microorganisms (Nevas et al., 2004). Spices are the most frequently used normal remedial agents in foods and has been used traditionally for preserving foods and to enhance flavor of food (Souza et al., 2005).

Antibacterial activity of different extracts (n-hexane, chloroform, acetone, butanol, ethanol and methanol) of garlic samples were tested against *E. coli, S. aureus*, *P. aeruginosa*, *S. epidermidis and K. pneumonia* (Figure 4). According to our results, n-hexane, chloroform, acetone, butanol, ethanol and methanol medium have shown inhibitory effects against the tested microorganisms (Ali and Blunden, 2003). It was observed that n-hexane extract of garlic showed activity only in the the range of 9.3±0.5-10.3±0.5 mm against all microorganisms. Acetone extracts showed less activity against *E. coli* but was active against all other microbes. The ethanol extract was proved to be effective against *S. aureus, K. pneumineae* and *S. epidermidis* but was not much effect on *E. coli*. Methanol extracts were proven to be more effective against all the microbes (Gulfraz et al., 2011).

Higher inhibition zone of butanol extract of garlic have been shown against *S. epidermidis* (16±1.0 mm) followed by methanol extract against *K*. *pneumineae* (15±1.0 mm). The results are almost similar to those reported by Pundir et al. (2010) when they studied the antibacterial activity of garlic extracts (Figures 5 to 6). In the present study, the ethanolic extracts of garlic showed inhibitory activity against all the five bacterial strains in which the diameter of zone of inhibition was 10.3±0.5 mm to 14.3±0.5 (Figure 4). The butanol extract revealed maximum zone of inhibition (16±1.0 mm) against *S. epidermidis* followed by *S. aureus* (15±1.0 mm) and *K. pneumineae* (14.6±0.5 mm) (Sulieman et al., 2007).

Table 1. Minimum inhibitory concentration (mg/ml) of various extracts of garlic sample (diameter of zone inhibition in mm).

Microorganism	Diameter of zone of inhibition			
	Extract concentration (mg/ml)	Growth inhibition zone diameter		
		Butanol	Ethanol	Methanol
E. coli	15	-	6±0.5	8±1.0
	10	-	5±0.5	8±1.0
	5	-	-	-
	1	-	-	-
K. pneumoniae	15	8±1.0	10±1.0	17±1.0
	10	5±0.8	4±1.0	14±1.0
	5	-	-	11±0.5
	1	-	-	-
S. aureus	15	10±1.0	18±1.7	9±1.0
	10	4±0.5	12±1.0	9±1.0
	5	-	-	-
	1	-	-	-
P. aeruginosa	15	11±1.0	16±1.0	13±1.0
	10	8±1.0	8±1.3	7±1.0
	5	-	-	-
	1	-	-	-
S. epidermidis	15	14±1.0	5±0.9	11±1.0
	10	6±1.0	-	8±1.0
	5	3±0.5	-	8±1.0
	1	-	-	-

- No inhibition. Values are Mean ± SD after triplicate analysis.

Values in terms of garlic extracts against *E. coli*, *S. aureus* revealed the highest antibacterial activity was observed against *E. coli*. Antimicrobial activity of all extracts for *E. coli* was in the range of (9.3±0.5-11±1.0 mm), *K. pneumineae* (10.3±0.5-15±1.0 mm), *P. aeruginosa* (9.3±0.5 -14.6±0.5 mm), *S. aureus* (9.3±0.5-15±0.5 mm) and *S. epidermidis* (9.3±0.5 -16±1.0 mm) (Figure 4).

The antibacterial activity of garlic has been reported by many workers against *S. aureus, E. coli* and *K. pneumoniae* (Jabar and Mossani, 2007) and *E. coli* and *S. aureus* (Vuddhakul et al., 2007). Shelef (1983) reported that allicin (an essential oil) isolated from garlic inhibited bacterial growth and it was shown that most of the antimicrobial activity was due to phenolic compounds like eugenol, thymol and carvacol found in garlic (Gulfraz et al., 2011).

Minimum inhibitory concentration (MIC) of garlic extracts

Garlic extracts in methanol, ethanol and butanol showed better inhibition against the bacterial strains used, as compared to other extracts, so different concentrations of these extracts were subjected to determination of MIC. The concentrations of the extracts used for MIC determination were 15, 10, 5 and 1 mg/ml and the results are given in the Table 1. It was observed that the most susceptible bacterial strain was *S. epidermidis* against butanol extract (3±0.5 mm) at concentration of 5 mg/ ml and it was followed by *K. pneumonea* against methanolic garlic extract (11±0.5 mm) at concentration of 5 mg/ml. Whereas *S. aureus* showed (12±1.0 mm) inhibition when 5 mg/ml of ethanol extract was applied. All bacterial strains showed higher susceptibility against methanol extracts, moderate activity against ethanol and less activity against butanol extract. The large size of zones of inhibitions indicates the increase in the concentration of extracts and the inhibition activity of extracts. Furthermore, gentamicine a standard antimicrobial agent had shown zones of inhibition ranging from 18±0.5 - 24±0.5 mm, and highest value for *P. aeruginosa* (24±0.5 mm). The zones of inhibition of effective extracts were close to that of the drug. It seems that the organisms may

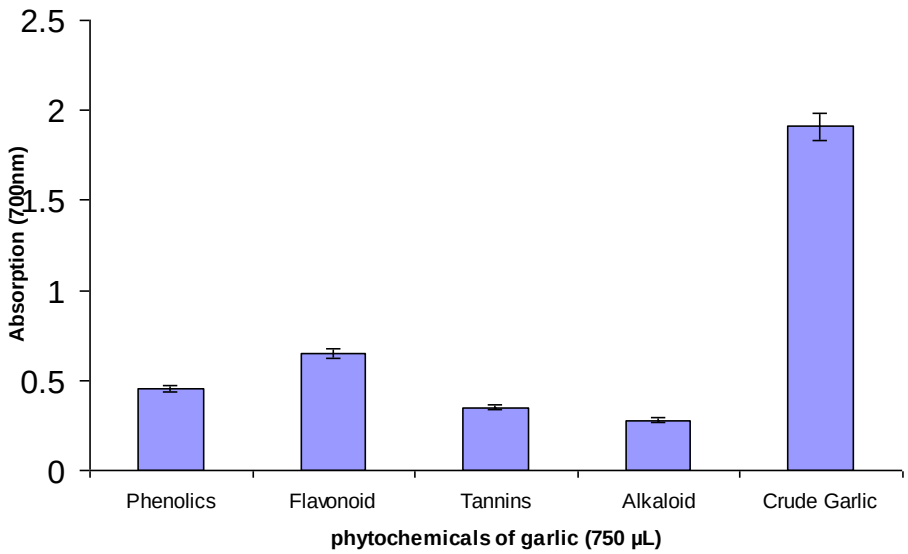

Figure 7. Antioxidant activity of phytochemicals found in garlic samples. Mean ± SD after triplicate analysis.

need higher concentrations of extracts to kill them, due to their cell wall components (Gulfraz et al., 2011). Antimicrobial activities for butanol extract values ranges from (3±0.5 - 14±1.0 mm), ethanol extract (4±1.0 - 18±1.7 mm) and methanol extract (6±1.0 - 17±1.0 mm) for all microorganism tested (Table 1). The results showed that the MIC values range between 3±0.5 - 18±1.7 mm.

Antioxidant activity of phytochemicals

Antioxidant activity of different phytochemicals, that is, phenols and tannins were determined and it was found that antioxidant activities of phenolics (0.45 ± 0.01 mg/g), flavonoid (0.65 ± 0.01 mg/g), tannins (0.35 ± 0.01 mg/g), alkaloid (0.28 ±0.01 mg/g) and crude garlic (1.91 ± 0.12 mg/g) were present. Results of antioxidant activity are shown in Figure 7. It shows that crude garlic (1.91 ± 0.12 mg/g) has higher antioxidant activity than all other phytochemicals and alkaloid has less antioxidant activity than other phytochemicals. Among all phytochemicals, total phenols, flavonoids and tannins had shown antioxidant activity (Figure 7). Flavonoids and alkaloids of garlic extract which was evaluated using ascorbic acid as a standard. Assay involved the use of $FeCl_3$/ $K_3Fe(CN)_6$ complex as a source of ferric ions which may reduce to ferrous ion in the presence of extracts containing active flavonoid and alkaloids and was confirmed by production of green colour complex which intensity was measured spectrophotometrically. Thus, increase in absorbance of experiment was due to increased antioxidant activity (Nethravathi et al., 2006). Flavonoid and tannins are polyphenolic compounds which donate their hydrogen atom to ferric ions and convert them to their reduced form resulting in the production of intense green colour and greater absorbance. These polyphenolic compounds act as antioxidant agent and may protect the body from oxidative damage.

The present study indicates the presence of different concentrations of polyphenolic compounds in garlic which may protect the human body from its damaging effects. Therefore, utilization of garlic in diet may become useful in preventing number of diseases like carcinogenesis and cardiovascular diseases in human as reported by Gezer et al. (2005).

Conflict of Interests

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

ACKNOWLEDGEMENT

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Full Length Research Paper

Salt spray as a micro-environmental factor affecting the growth of *Commelina maritima* L. at Lekki Beach, Nigeria

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***Commelina maritima* L. is a dominant perennial halophyte that is restricted to sandy beaches where it plays major ecological roles in Southern Nigeria. This study examines the response of this plant to saltwater sprays, a factor that affects the growth of coastal plants. Plants were sprayed with seawater twice per week (2/week), four times per week (4/week) or six times per week (6/week) while control was sprayed without seawater (de-mineralized water) six times per week (0/wk). Survival, growth and biomass allocation of the plants were determined. Salt spray did not affect plant survival but significantly ($p \leq 0.05$) decreased number of leaves, shoot length, stem girth, leaf area and root growth. Relative growth rate and number of branches were not significantly ($p \geq 0.05$) affected by salt spray. Sea spray significantly ($p \leq 0.05$) reduced fresh and dry mass of plant parts, total biomass and leaf total chlorophyll when compared with the control. Root : shoot ratio increased significantly ($p \leq 0.05$) under seawater treatment as the shoot growth was more negatively affected than root growth. Relatively more biomass was allocated to the root than shoot in seawater-treated plants. Salt spray increased shoot ash content and negatively affected plant organic content. *C. maritima* can be classified as a salt spray-sensitive plant. Salt spray is a micro-environmental factor affecting its survival and growth, thus influencing its distribution in strandline.**

Key words: Strandline, survival, growth, biomass allocation, Commelinaceae, distribution.

INTRODUCTION

Strandlines are created along the high water mark on a range of habitats including coastal vegetated shingle, coastal sand dunes and coastal salt marshes (Lee and Ignaciuk, 1985). Several natural and anthropogenic factors affect the vegetation of strandlines (Owen et al., 2001; Peter et al., 2003; Pichler and Oberhuber, 2007). Studies on coastal plant communities in many parts of the world have found that air-borne saltwater spray is an important natural selective abiotic factor (Boyce, 1954; Cartica and Quinn, 1980; Sykes and Wilson, 1988; Hesp,

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1991). Salt-sensitive species can be eliminated entirely from areas with high salt spray so that plants adapted to salt spray grow close to the ocean and are replaced by less salt-resistant plants farther inland (Cheplick and White, 2002). It has been found that natural salt spray accumulation on plants in the field can cause water stress, leaf necrosis, growth reduction and death depending on the frequency and severity of storms and winds that carry aerial salt drift inland (Griffiths and Orians, 2004; Griffiths, 2006).

Strandline community composition is structured by the effects of salt spray, and plants typical of coastal beaches are salt spray tolerant, which gives them a competitive advantage over others (Tominaga and Ueki, 1991; Greaver and Sternberg, 2007; De Vos et al., 2010). To date, the possible influence of natural processes on coastal vegetation has largely focused on soil salinity while air-borne salinity has been largely ignored.

Commelina maritima L. commonly called white mouth dayflower belongs to the family Commelinaceae. It is a fleshy, creeping, almost glabrous herb with blue flowers, in coastal sand bar vegetation. It is perennial and monocotyledonous with succulent stem and swollen nodes often mucilaginous (Hutchinson et al., 1986). This plant provides essential habitat, spawning sites and food for many diverse species of terrestrial and marine wildlife. The plant is able to withstand periodic coastal disturbances and it is particularly important on exposed shores, where its population can act as precursors to sand dunes. It helps in storm protection and controls shoreline erosion. It helps in shoreline stabilization by enhancing organic and moisture content; pioneer plants can then become established and sand dune formation is initiated (Williamson, 2005). *C. maritima* is widely distributed in West Tropical Africa where it is found a short distance from sea on the sandy beaches (Hutchinson et al., 1968). This plant species is dominant, wide spread and restricted to strandline habitat in Nigeria. We hypothesize that salt spray is a micro-environmental factor affecting its growth. This is because strand vegetation is affected by environmental factors such as soil salinity, sand burial, drought, waterlogging, nutrient deficiency, trampling and other anthropogenic activities. Each factor is usually considered as a micro-environmental factor among the various environmental conditions that affect strand vegetation (Barbour, 1978). It was reported that the death of *Impereta cylindrica* and *Miscanthus sinensis* transplanted to beach was as a result of salt spray, inhibiting them from becoming established on the front dunes. They concluded that salt spray is among the non-negligible factors controlling the distribution of plants in sand dune vegetation (Ogura and Yura, 2007). Plant's ability to survive in the strandline is largely influenced by salt spray. Since *C. maritima* thrives exclusively in the environment, salt spray is a natural abiotic factor that influences its growth in the strandline (Barbour, 1978). We experimentally manipulated salt spray in laboratory studies and examined its influence on the survival, growth and biomass allocation of the plant species. The results are used to discuss its tolerance and some adaptations for survival.

MATERIALS AND METHODS

Stem cuttings of *C. maritima* collected from Lekki Beach in Lagos, Nigeria were used to raise uniform plants in perforated plastic pots filled with 2:1 ratio of washed river sand to top soil (Khan et al., 2000a, b). Seawater was collected off the shore, the source of the air-borne salts and stored in a plastic keg at room temperature. Salinity of the seawater was 30.00 ppt and later measurements with conductivity meter did not change with short time storage. Plants were randomly assigned to either salt-spray treatments or control. On Mondays and Thursdays, plants were sprayed with seawater twice per week (one seawater spray on each of the two days), four times per week (two seawater sprays on each of the two days) or six times per week (three seawater sprays on each of the two days) while control were sprayed without seawater six times per week (three de-ionized water prays on each of the two days) following the methods of Cheplick and Demetri (1999), Cheplick and White (2002) and De Vos et al. (2010). Individual plant was sprayed to run-off with all the aerial parts equally exposed. The accumulated salt onto the shoot for 1, 2 and 3 sprays was estimated following the method described by Cheplick and Demetri (1999), which equaled on average 4, 8 and 12 mg NaCl dm^{-2} leaf area day^{-1}. These fall within the levels found in the natural habitat of beach plants (Barbour et al., 1985; Griffiths, 2006). A plant mist bottle held about 20 cm from the shoot was used for spraying. Pots were arranged on the Greenhouse bench of the Plant Science and Biotechnology Department, Adekunle Ajasin University, Akungba Akoko, Ondo State Nigeria (Lat. 7° 28^{1}N, Long. 5° 44^{1} E). Seawater was applied as a fine mist from a spray bottle held 20 cm from the side of each shoot. Plants in all treatments were repositioned after each saltwater treatment. Salt that might possibly deposit on soil during misting was flushed out weekly with water. There were 6 single-plant replicates per treatment in a completely randomized experimental design. Prior to beginning the salt spray treatments, five plants were harvested to determine the initial growth parameters and successive measurements were taken at an interval of two weeks.

Percentage survival and growth parameters were recorded. Shoot length was measured from the soil level to the terminal bud and leaf area was obtained with a leaf area meter. Stem girth was measured at about 10 cm from stem base using model 0-200 mm digital caliper. Number of leaves and lateral branches of individual plants were counted manually. The experiment was terminated at 12 weeks after initiation of treatment. Plants were harvest, major roots were counted and their lengths measured. The harvested plants were separated into leaves, stems and roots and their fresh mass determined. Dry mass was obtained after oven-drying to constant mass at 80°C. Root dry mass was divided by that of the shoot to obtain root : shoot ratio while total biomass of all plant parts was weighed. Relative growth rate was calculated using the formula, RGR (relative growth rate) = (ln mass2-ln mass1)/time.

Total chlorophyll was extracted in 80% (v/v) aqueous acetone and absorption was read in a spectrophotometer at 645 and 663 nm (Arnon, 1949). Each sample of the dried plant materials was ground to fine powder using Philips model blender. Percentage ash content was determined by weighing the plant sample (powder) in a Pyrex beaker and placed in muffle furnace set at 500°C. Ash and organic mass components were also determined. The soil physico-chemical properties were analyzed in the Central Laboratory of The National Institute for Oil Palm Research (NIFOR), Nigeria, following the standard methods of Association of Official Analytical Chemists

Table 1. The physico-chemical properties of soil used for planting.

Physico-chemical parameter	Value
pH	5.48
% C	3.67
N (ppm)	20.42
P (ppm)	3.56
K (meg/100 g)	3.56
Ca (meg/100 g)	2.32
Mg (meg/100 g)	2.60
CEC (meg/100 g)	8.2
Sand (%)	80.68
Silt (%)	12.06
Clay (%)	8.36

(AOAC, 1985). Data were subjected to single factor analysis of variance and means were separated with Duncan's MRT using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) at $P \leq 0.05$.

RESULTS

Table 1 shows the physico-chemical properties of the soil used for planting. The ratio 2:1 of washed river sand to top soil is typical of sandy beach soil in strandline. The sandy soil supported the plant growth while the top soil was for nutrient supply. The soil was low in nutrients in comparison with nutrient composition of agricultural soils. Damage caused by salt spray include leaf browning, chlorosis and necrosis. Salt spray led to leaf firing and leaf injury which resulted in early leaf senescence and defoliation. Leaf death and defoliation in plants sprayed with seawater resulted in leaf number reduction. Leaf number decreased with increase in level of seawater (Figure 1). One-way ANOVA however revealed that reduction in leaf number became significant ($p \leq 0.05$) only at 4/week and 6/week when compared with the control treatment.

Table 2 shows the influence of salt spray on the growth parameters of *C. maritime*. Generally, plant growth was inhibited by salt spray but there was no plant mortality. Leaf area decreased significantly ($p \leq 0.05$) in plants sprayed with seawater relative to the control. At the highest level of salt application, leaf area was reduced by as high as 53.45%. In plants exposed to salt spray, root growth was negatively affected. This is evidenced by a significantly ($p \leq 0.05$) reduced root length and root number under seawater treatment as compared to the control. There was however no statistical difference ($p \geq 0.05$) when different levels of salt treatments were compared. Plants sprayed with seawater exhibited a significantly reduced ($p \leq 0.05$) stem girth in comparison with those exposed to de-ionized water. Relative growth rate likewise decreased with increase in salt spray but there was no significant difference ($p \geq 0.05$) between control and seawater-treated plants. Total biomass and chlorophyll content of plants subjected to salt treatment were reduced significantly ($p \leq 0.05$) as compared to the control. Root : shoot ratio increased significantly ($p \leq 0.05$) under seawater treatments relative to de-mineralized water treatment. This revealed that shoot growth was more negatively affected by salt spray than root growth. Shoot length of *C. maritima* treated with seawater decreased progressively with increasing salt level. This became significant ($p \leq 0.05$) from eight weeks after treatment. At the end of the investigation, shoot length was reduced by 37.14% at 6/week relative to the 0/week treatment (Figure 2). However, plants sprayed with seawater had reduced number of branches but there was but there was no significant difference ($p \geq 0.05$) between the control and those treated with salt spray (Table 2).

Fresh and dry mass of plant parts declined progressively with increasing salt spray levels. One-way ANOVA showed that fresh and dry mass were significantly lower ($p \leq 0.05$) under salt sprays than in the control treatment (Table 3). Figure 3 shows the biomass allocation pattern of *C. maritime* under different spray treatments. Relatively more organic biomass was allocated to the root than the shoot in plants subjected to salt spray. This was due to salt accumulation on the shoot following foliar spray with seawater. This led to an increase in the shoot ash content thus having a negative effect on percentage organic content. Organic biomass allocation was not affected by salt treatment in roots since they were not in direct contact with salt. When compared with the control, shoot ash mass was significantly increased ($p \leq 0.05$) by salt sprays. However, there was no significant difference ($p \geq 0.05$) when root ash mass was compared between control and seawater treatments (Figure 4). Organic mass was significantly ($p \leq 0.05$) reduced both in the shoot and root due to the influence of salt deposition on shoot surfaces (Figure 5).

DISCUSSION

The ratio 2:1 washed river sand to top soil has been reported to have properties of beach soil where strand plants naturally grow (Khan et al., 2000a, b). Although, the soil was low in nutrients, strand plants have low nutrient requirements for growth (Lee and Ignaciuk, 1985). Since no negative effect was observed in control plants, the observed effect on the plant was not due to nutrient unavailability but salt spray. The changes in plant morphology due to salt sprays are consistent with the previous studies demonstrating damage to plants as a result of salt spray accumulation (Griffiths, 2006). Necrosis on *Myrica pensylvanica* leaves decreased as distance from the dune crest increased (Griffiths and Orians, 2003). Sea spray likewise resulted in leaf necrotic

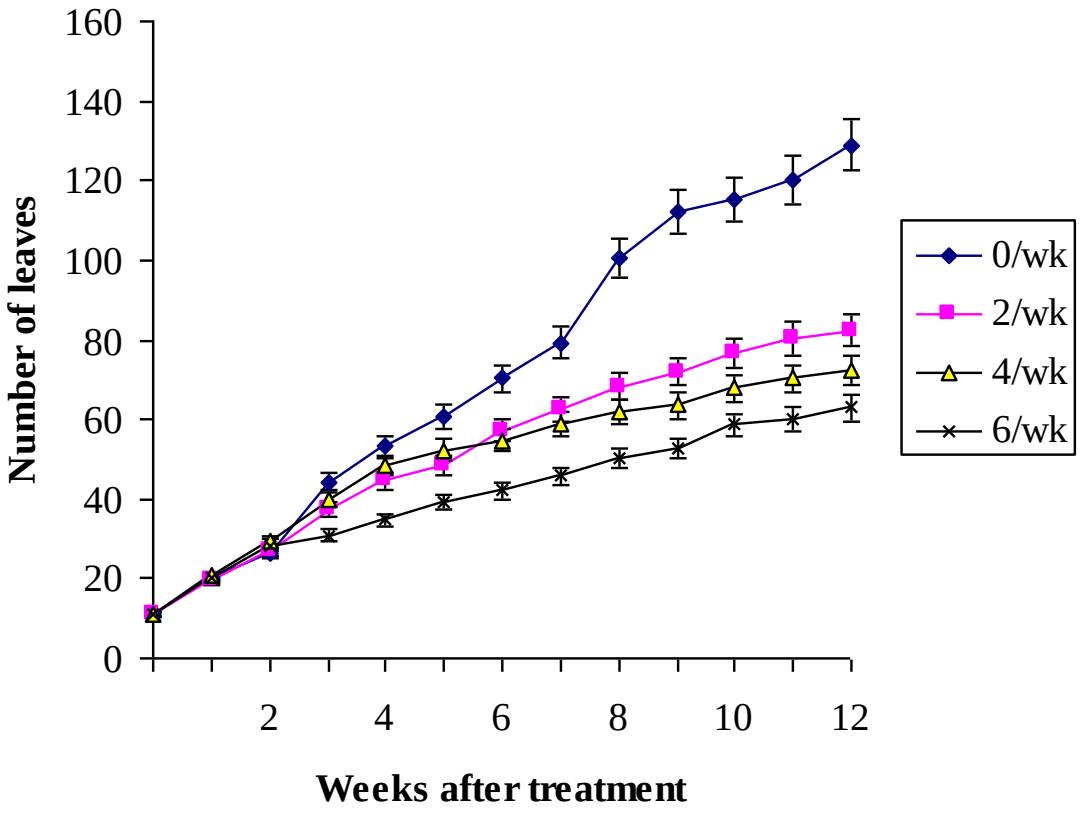

Figure 1. Effect of salt sprays on the leaf number of *C. maritima*, taken at 2-week interval for 12 weeks. Each value is a mean ± standard error of 6 determinations. 0/week = sprayed without seawater (de-mineralized water) six times per week, 2/week = sprayed with seawater twice per week, 4/week = sprayed with seawater four times per week, 6/week = sprayed with seawater six times per week.

damage in seedlings of *Pinus rigida* (Griffiths and Orians, 2004), *Solidago puberula*, *Solidago rugosa*, *Gaylussacia baccata* and *Quercus ilicifolia* (Griffiths and Orians, 2003) and in some coastal plant species (Griffiths et al., 2006). Kim et al. (2004) also observed browning symptoms followed by defoliation after the foliar spray of 3% NaCl in apple, pear, peach and grape trees. These could result in a decrease in net photosynthesis that might be expressed in reduced growth (Griffiths and Orians, 2004).

In coastal areas, salt spray often influences the distributions of species (Cheplick and Demetri, 1999; Griffiths and Orians, 2003; Ogura and Yura, 2007). Complete survival of plants under salt sprays conforms to the findings of Gagne and Houle (2002) on *Leymus mollis* and De Vos et al. (2010) on *Crambe maritima*. It is however in contrast with that of Gagne and Houle (2002) who reported a significantly reduced seedling survival in *Honckenya peploides*.

Growth reduction in *C. maritima* in this investigation confirms the report that salt spray usually leads to growth reduction (Tominaga and Ueki, 1991; Griffiths, 2006). Salt spray caused growth reduction in *Leymus mollis* (Gagne and Houle, 2002) and *M. pensylvanica* (Griffiths and Orians, 2003). Reduction in root elongation has been reported on many coastal plant species (Griffiths and Orians, 2004; Griffiths et al., 2006). The reduction in plant shoot length as obtained in this study affirms the report that salt spray inhibits the shoot elongation in coastal plant species (Griffiths et al., 2006). This could be one mechanism through which the characteristic dwarf stature of strand vegetation is maintained (Griffiths and Orians, 2003).

Reduction in number of branches agrees with the previous work of Cheplick and Demetri (1999) who recorded reduced number of tillers in *Triplasis purpurea* sprayed with salt relative to control plants. Reduction in leaf number in plants sprayed with seawater likewise supports the earlier report on *Scaevola sericea* seedlings by Goldstein et al. (1996) and De Vos et al. (2010) on *Crambe maritima*. Reduced leaf number in this study was due to leaf firing leading to leaf death and defoliation. Leaf area reduction is in conformity with that of Ogura and Yura (2007) on *Miscanthus sinensis* and *Imperata cylindrica* and in *Pinus rigida* (Griffiths and Orians, 2004). Leaf length was also reduced in *Crambe maritima* seedlings 13 weeks after spraying with salt water. Reduced leaf size was as a result of reduction in leaf area expansion and hence reduction of light interception. Reduction in leaf area also provided reduced area for water loss through transpiration and changes in

Table 2. Effect of salt sprays on leaf total chlorophyll and growth parameters of *C. maritima*.

No. of spray (s) per week	Survival (%)	Stem Girth (cm)	Leaf area (cm^2)	Number of branches	Total biomass ($gplant^{-1}$)	Root: Shoot	RGR ($gg^{-1}d^{-1}$)	Total chlorophyll (mgg^{-1})	Root number	Root length (cm)
0	100	0.40^{a}	5.04^{a}	22.72^{a}	20.67^{a}	1.34	0.08^{a}	3.24^{a}	67.43^{a}	44.71^{a}
2	100	0.31^{b}	2.41^{b}	19.55^{a}	13.45^{b}	1.95	0.07^{a}	1.43^{b}	49.87^{b}	32.04^{b}
4	100	0.31^{b}	2.36^{b}	16.89^{a}	11.50^{b}	2.15	0.07^{a}	1.71^{b}	47.65^{b}	34.11^{b}
6	100	0.28^{b}	2.54^{b}	16.87^{ab}	8.47^{bc}	1.87	0.07^{a}	1.68^{b}	48.55^{b}	34.67^{b}

RGR = Relative growth rate. Each value is a mean of 6 replicates taken at 12 weeks after initiation of treatments. Means with the same letter (in superscript) in the same column are not significantly different at P ≥ 0.05.

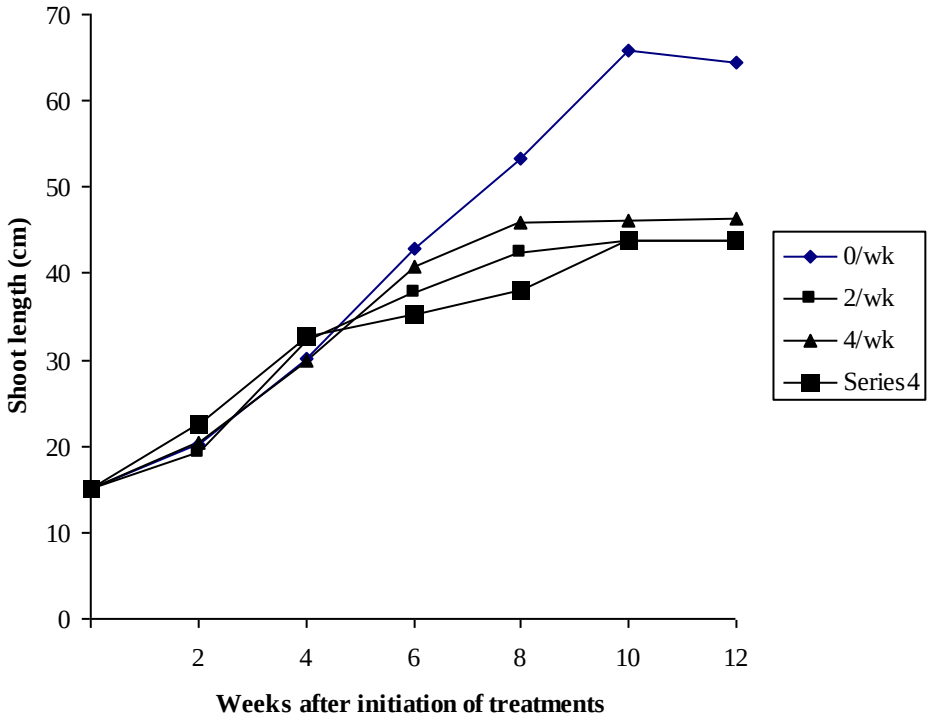

Figure 2. Effect of salt sprays on the shoot length (cm) of *C. maritima*, taken at 2-week interval for 12 weeks. Each value is a mean of 6 replicates. 0/week = sprayed without seawater (de-mineralized water) six times per week, 2/week = sprayed with seawater twice per week, 4/week = sprayed with seawater four times per week, 6/week = sprayed with seawater six times per week.

Table 3. Effect of salt sprays on the fresh and dry mass of plant parts in *C. maritima*.

Parameter	Plant part	No of spray(s)per week			
		0	2	4	6
Fresh mass (g)	Leaf	5.10[a]	2.56[b]	2.11[b]	1.25[bc]
	Stem	10.03[a]	5.94[b]	4.70b	5.16[b]
	Root	14.87[a]	11.14[a]	9.76[a]	7.38[c]
Dry mass (g)	Leaf	3.77	2.04	1.67	1.01
	Stem	5.07	2.53	1.97	1.96
	Root	11.88	8.91	7.81	5.56

Each value is a mean of 6 replicates, taken at 12 weeks after initiation of treatments. Means with the same letter (in superscript) in the same row are not significantly different at P ≥ 0.05. 0/week = sprayed without seawater (de-mineralized water) six times per week, 2/week = sprayed with seawater twice per week, 4/week = sprayed with seawater four times per week, 6/week = sprayed with seawater six times per week.

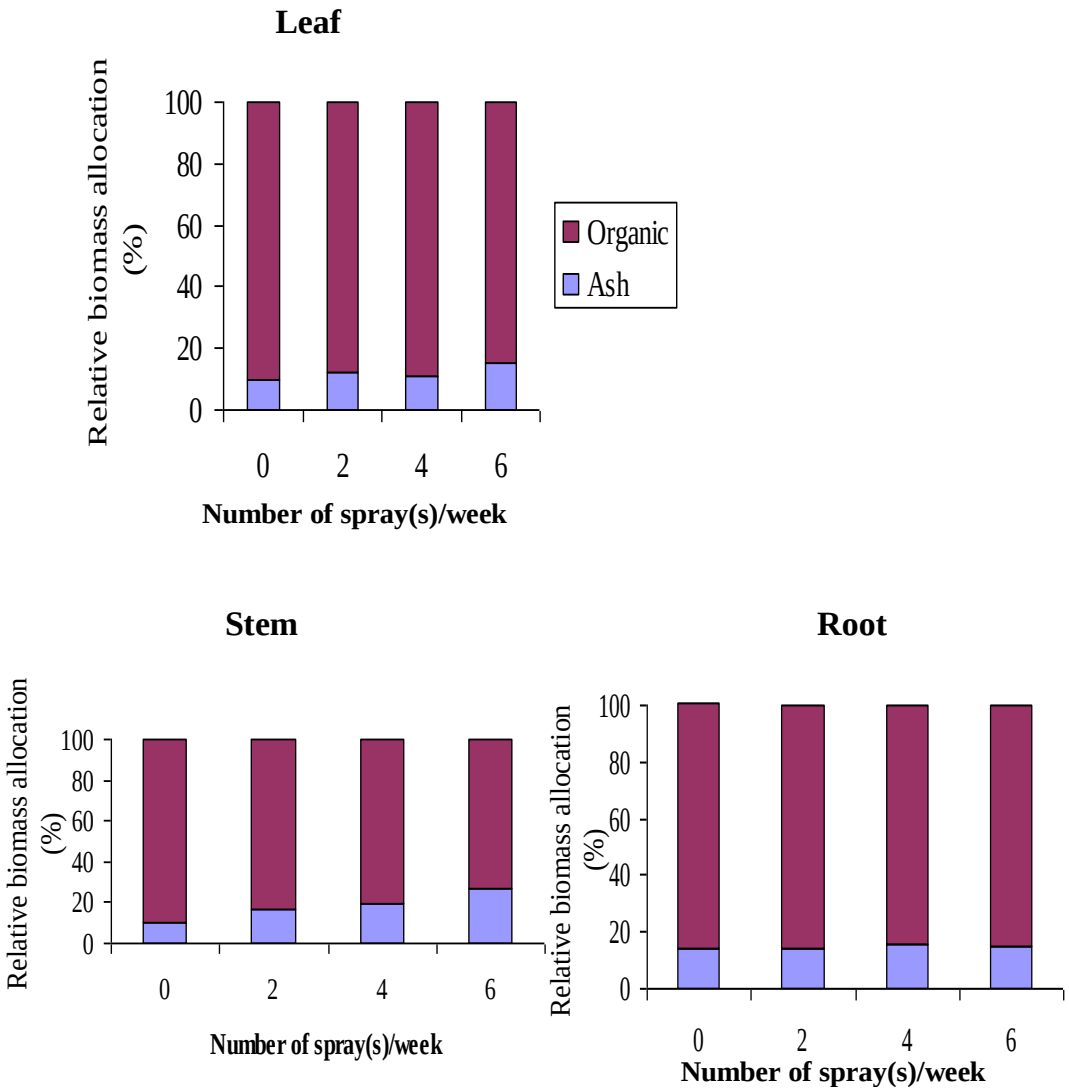

Figure 3. Effect of salt sprays on the relative biomass allocation (%) of *C. maritima.* Each bar represents mean of six determinations, taken at 12 weeks after initiation of treatments.

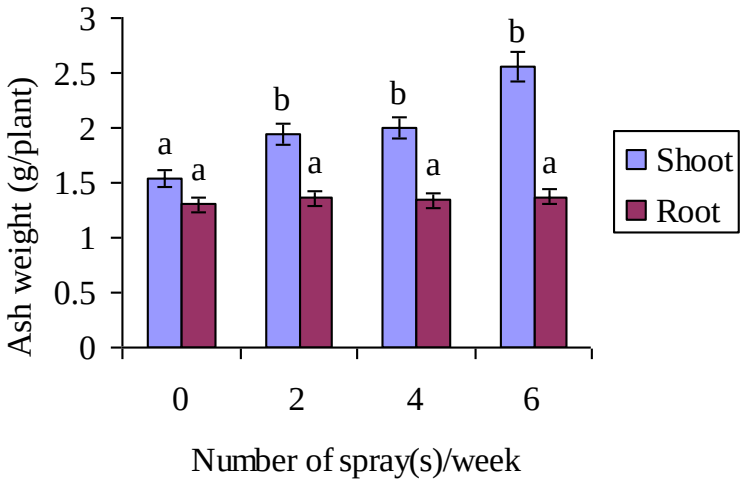

Figure 4. Effect of salt sprays on the ash mass (g plant^{-1}) of *C. maritima*. Each bar represents mean ± standard error of 6 determinations. Bars with the same letter are not significantly different at P ≥ 0.05.

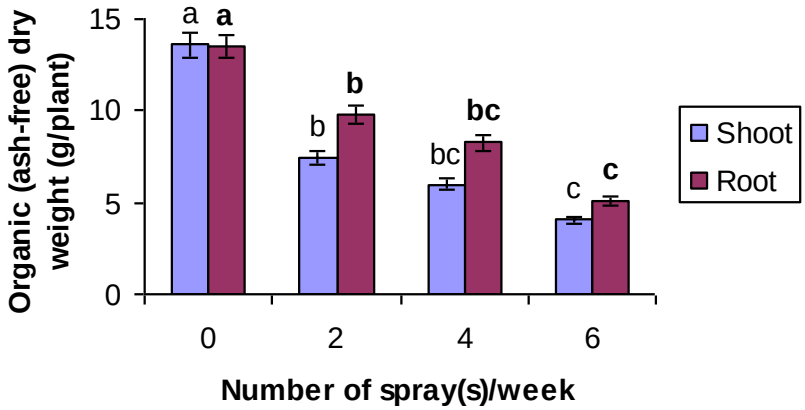

Figure 5. Effect of salt sprays on the organic mass (g plant^{-1}) of *C. maritima*. Each bar represents mean ± standard error of 6 determinations, taken at 12 weeks after initiation of treatments. Bars with the same letter are not significantly different at P ≥ 0.05.

water use efficiency in plants, which have been identified to be adaptive mechanisms under water stress (Morant-Manceau et al., 2004). Reduced photosynthetic leaf area was due primarily to chloride and sodium ions from the salt spray (Cartica and Quinn, 1980). However, leaf area reduction is in contrast with that of Touchette et al. (2009) on *Spartina alterniflora*. It has been reported that salt penetrates leaves through lesions or via stomata and is consequently translocated to other plant parts (Boyce, 1954; Barbour et al., 1985) which could affect root development.

Reduction in chlorophyll content was due to leaf damage resulting in increased necrotic spots. Necrotic damage by salt spray has been reported by many authors (Sykes and Wilson, 1988; Griffiths and Orians, 2004). This usually results in a decreased net photo-synthesis that might be expressed in reduced growth and long-term survivorship (Griffiths and Orians, 2004). Chlorophyll reduction can also be due to ion deficiency or ion toxicity.

Factors such as soil salinity, salt spray, sand burial, water deficit, flooding and nutrient deficiency are the natural factors that determine the distribution of strand vegetation (Lee and Ignaciuk, 1985). Strand plants vary considerably in the factors that affect their survival and growth, with each considered as a micro-environmental factor among the various environmental conditions including soil salinity, sand burial, drought, waterlogging and nutrient deficiency that affect strand vegetation (Barbour, 1978). This experiment has shown that *C. maritima* growth was negatively affected by salt spray. Therefore, salt spray is a micro-environmental factor

affecting *C. maritima* growth, which determines its presence and distribution in the strandline.

Conclusions

Strand plants vary considerably in the specific factor(s) among the various abiotic factors that affect them in the strandline, hence the question: which of the factors determine the distribution of every plant species that grow naturally in the environment? This research has revealed that salt spray affects the growth of *C. maritima*, and it can be categorized as a salt spray-sensitive plant. Salt spray is therefore a micro-environmental factor (among the various abiotic factors) that contributes to the survival, growth and distribution of *C. maritima* in the strandline.

Conflict of Interests

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

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Full Length Research Paper

Anticancer activity of n-hexane extract of *Cichorium intybus* on lymphoblastic leukemia cells (Jurkat cells)

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***Cichorium intybus* commonly known as chicory is a member of the family Compositeae. It has been known for various pharmacological activities and anticancer effects against various cell lines including breast cancer MCF-7, prostate cancer LNCaP, amelanotic melanoma C32 and renal adenocarcinoma ACHN. In the present study, aerial parts of *C. intybus* were collected, identified, soaked in n-hexane non polar solvent, filtered and evaporated till the extract residue was isolated, dried and stored at 4°C and then checked for cytotoxic activities. Lymphoblastic leukemia cells (Jurkat cells) were used to evaluate the cytotoxic effects of n-hexane extract of *C. intybus*. Extracts were added to the cultured cells of selected cell line in various concentrations (10, 25, 50, 75 and 100 µg/ml) and incubated for 24 h. Trypan blue exclusion assay, MTS assay and FACS analysis were carried out to analyze cell viability, cell proliferation and apoptosis, respectively. The non polar n-hexane extract significantly reduced the number of viable cells and cell proliferation percentage but induced the apoptosis haphazardly. Results of this study demonstrate that n-hexane extract of *C. intybus* has potent anti-proliferative and cytotoxic activity against Jurkat cells, a human leukemia cell line.**

Key words: *Cichorium intybus*, Jurkat cells, lymphoblastic leukemia cells, MTS, trypan blue assay, apoptotic analysis.

INTRODUCTION

Cichorium intybus is a small herb (Keshri et al., 1998) also known as chicory belonging to the family Compositeae (Pushparaj et al., 2007) or Asteraceae (Shaikh et al., 2012). Its common name is Kasni. It widely exists in Mediterranean region, Iran, Europe and Northern Asia (Amirghofran et al., 2000) and also present in Punjab, New Frontier and Hyderabad (Keshri et al., 1998). *C. intybus* is one of the plants cited by Hadith (Wani et al., 2011) and traditionally used in many indications. Its roots and leaves are used as bitter tonic cholagogue, cardio protective, diuretic, laxative, anti-hypertensive, anti-rheumatic and anti-diabetic agent

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(Pushparaj et al., 2007). Seeds are used for the treatment of ulcer, skin and microbial diseases (Daniela et al., 2009). Its ethanolic extract has antitumor effects (Shaikh et al., 2012) and aqueous extracts act against mitogen induced lymphocytic proliferation (Amirghofran et al., 2000) and hepatic inflammation (Gadgoli et al., 1997). It has contraceptive activity in female rats (Keshri et al., 1998) and analgesic and sedative activity as well (Wesolowska et al., 2006).

Phytochemical studies shows that it contains polyphenols including flavonoids such as kaempferol, lutedin, epigenins and quercetin (Heimler et al., 2009), sesquiterpene lactones, coumarins and vitamins (Varotto et al., 2000). The phenolic compound traz\ns-3-p-coumaroylquinic acid contains hydroxycinnamic acid and coumaroyl groups isolated from this herb causes caspase 3 mediated apoptosis in gastric tumor cells (Hsieh et al., 2010). Other constituents are alkaloids, carbohydrates, triterpenoids, tannins, volatile oils and fatty acids in minute quantities. Aqueous extract of chicory contains reducing sugar including vanilic acid, syringic acid and butelic acid. Other water soluble contents are rutin and inulin which replace fat or sugar and reduces the food calories (Niness, 1999). Lacteucin and lactopicrin, two isolated compounds show activity against the malarial parasite, bacteria (Bischoff et al., 2004; Shaikh et al., 2012) and fungi (Monde et al., 1990; Yusuf et al., 2002.). The objective of the present study was to evaluate the cytotoxic effect of n-hexane extracts of whole plant of *C. intybus* on lymphoblastic leukemia Jurkat cells.

MATERIALS AND METHODS

Plant material

Whole plant of *C. intybus* was collected from the fields of Punjab, Pakistan. The plant was identified by the Department of Botany, GC University Faisalabad Pakistan. A voucher specimen was kept in the herbarium as future reference. The plant was thoroughly washed with distilled water then shade dried, grinded into powder and stored at room temperature.

Extraction and isolation

The plant was put in macerating flask with ample non polar solvent n-hexane for 5 days with occasional shaking within mixture. The macerate was filtered with filter paper and the extract was obtained by evaporating the solvent in rotary evaporator at reduced pressure, until the semisolid mass was obtained. The extract was stored at 4°C until further use on selected cell line. To prepare the stock solution, 10 mg of solid residue was dissolved in 1 ml (500 µl ethanol + 500 µl water). Serial dilutions of various concentrations 10, 25, 50, 75 and 100 µg/ml were freshly prepared from stock solution before use.

Cell line and culture conditions

The Jurkat cell line was incubated after obtaining in the incubator provided with 5% CO_2 at 37°C. The cell culture was grown in RPMI-1640 along with 10% (v/v) fetal bovine serum (Biowhitaker, Lonza, Belgium), 100 Units/ml of penicillin, 2 Mm L-glutamine and 100 µg/ml of streptomycin (Sigma St. Louis, MO).

Trypan blue exclusion assay

The effect of n-hexane extract of *C. intybus* on cell viability of the Jurkat cell line has been determined by the trypan blue exclusion assay. The Jurkat cell line was implanted in 6 well plates at the concentration of 10^5 cells/well. The cells were incubated for 24 h before the addition of extract or vehicle. After 24 h, cells were collected, suspended in 0.4% trypan blue (Sigma-Aldrich, St-Quentin Fallavier, France) and number of cells were counted by haemocytometer.

MTS assay

The determination of the effect of n-hexane extract of *C. intybus* on cell proliferation of Jurkat cells was done by MTS assay. The Jurkat cell line was cultured in RPMI-1640 medium in the presence of vehicle and extract for 24 h in 96 well plates. After this, 20 µl/well of MTS reagent (Aqueous One® Reagent, Promega, United States of America) was applied and cells were incubated again for 2 h. The numbers of living cells were calculated by using multiwell ELISA plate reader by observing absorbance at 490 nm wavelengths.

Apoptosis analysis

For the analysis of late and early apoptosis, the Annexin V-FITC apoptosis kit (BD Pharmingen, USA) was used. The phosphatidyl serine is externalized in apoptotic cells for what the Annexin V has strong affinity. In short, the cells were exposed both to vehicle and n-Hexane extract for 24 h then washed with BPS. The cells were resuspended in the binding buffer, 5 µl of Annexin V-FITC and 10 µl propidium iodide (final concentration of 50 µg/ml) were added to each sample and cells were incubated for 20 min. Then 10,000 events for FACS analysis were considered and expressed as dots.

Statistical analysis

The data was expressed in terms of bar graph along with means ± SEM for at least three individual experimental trials, applying one way ANOVA. The data were subjected to statistical analysis. The level of significance was established according to standard notations.

RESULTS

n-Hexane extract of *C. intybus* decreased cell viability of Jurkat cell line

Trypan blue exclusion assay was used to evaluate the cell viability of Jurkat cells. n-hexane extract of *C. intybus* reduced the cell viability significantly, after 24 h of the addition of the extract of vehicle. The reduction happened in a concentration dependent manner and observed at a concentration greater than 10 µg/ml as shown in Figure 1. Cell viability determination indicated that *C. intybus* n-hexane extract at various concentrations: 10, 25, 50, 75 and 100 µg/ml reduced cell viability by 85, 75, 70, 58 and 50.3%, respectively, as compared to the control group

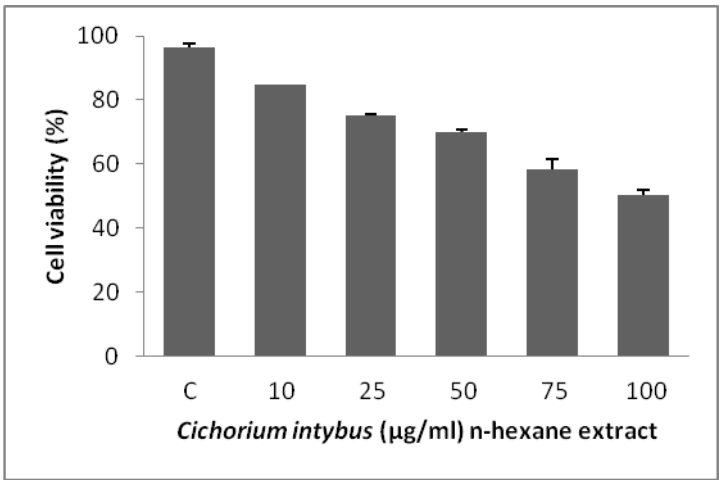

Figure 1. n-Hexane extract of *C. intybus* decreased cell viability of Jurkat cells in concentration dependent manner. The cells were exposed to either vehicle or different concentration of n-hexane extract of *C. intybus*. Trypan blue assay was used to determine the concentration of living cells. Values are shown as means ± SEM; n = 3. P< 0.05 versus control.

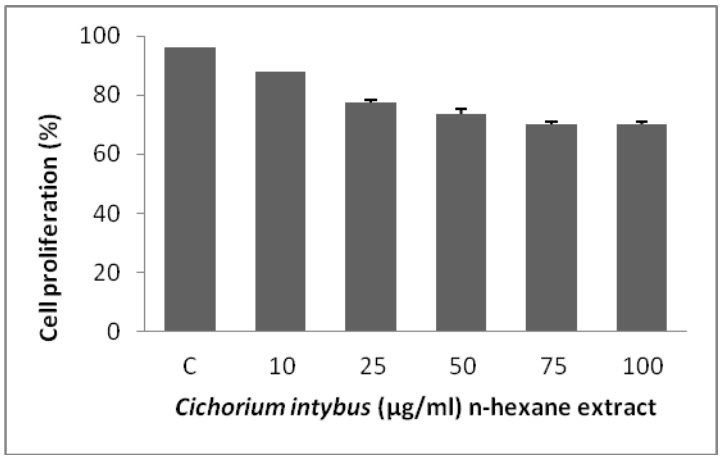

Figure 2. n-Hexane extract of *C. intybus* decreased cell proliferation of Jurkat cells in concentration dependent manner. The cells were exposed to either control or different concentration of n-hexane extract of *C. intybus*. MTS assay was used to determine the concentration of living cells. Values are shown as means ± SEM; n = 3. P< 0.05 versus control.

having value of 96.3%.

n-Hexane extract of *C. intybus* decreased cell proliferation of Jurkat cell line

The cytotoxic effect of *C. intybus* n-hexane extract was further assessed by using the MTS assay for cell proliferation. This extract significantly decreased the percentage of living cells in a concentration-dependent manner.

For example in controlled group cell proliferation is 96.3% but at max concentration of extract 100 µg/ml, the cell proliferation declined at 70% as shown in Figure 2. This result had shown that as the concentration of extract increases, the percentage of living cells significantly decreases.

n-Hexane extract of *C. intybus* enhanced apoptosis

n-Hexane extract of *C. intybus* affect apoptosis in Jurkat

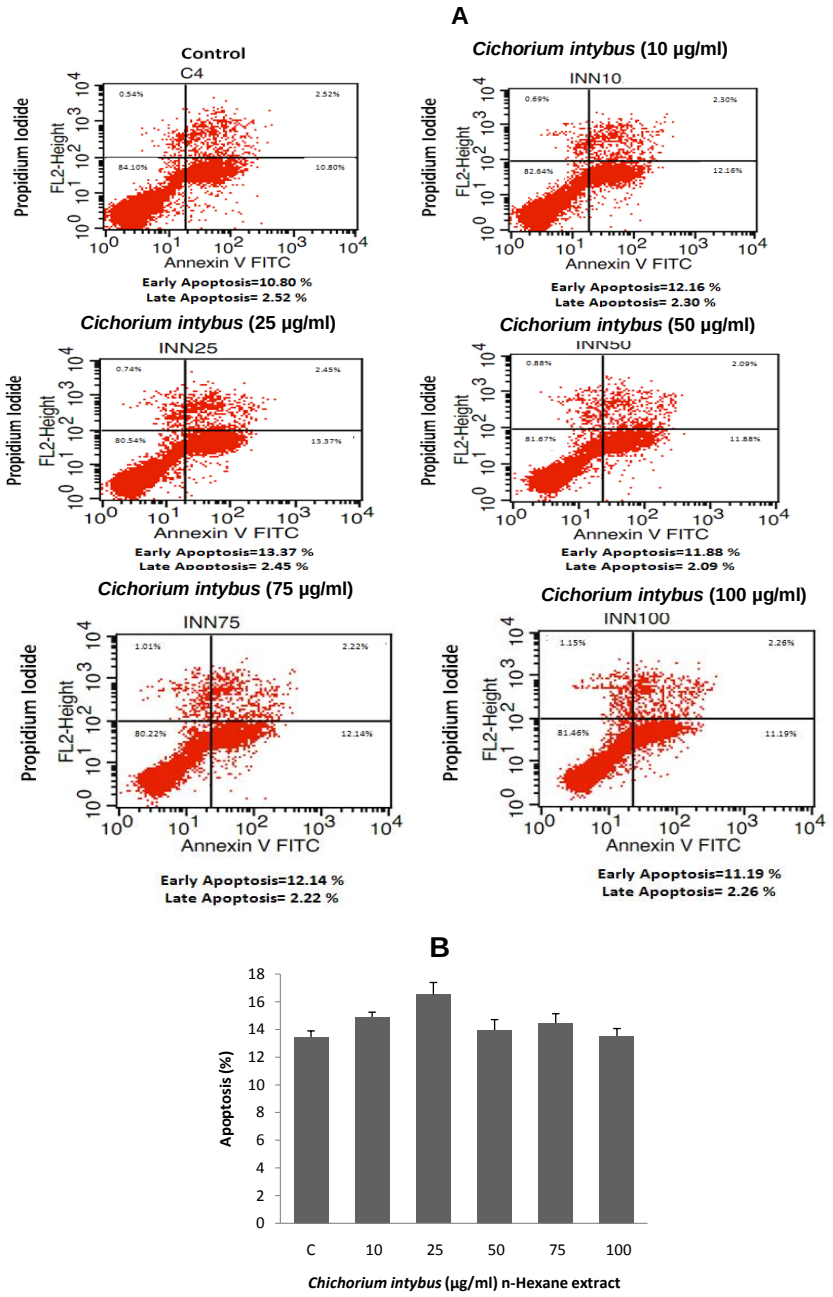

Figure 3. *C. intybus* n-hexane extract promotes apoptosis in lymphoblastic leukemia cell line, the Jurkat cells: Jurkat cells were seeded in six well plates, treated with either vehicle or different concentration of extract and incubated for 24 h. Thereafter 5 µl of Annexin-V and 10 µl of propidium iodide were added to each well, mixed well and wrapped in aluminum foil and then subjected to FACS analysis. (A) Representative flow cytometry analysis of cells. It indicated that lower left quadrant had cells which were negative for both Annexin V-FITC and propidium iodide, the lower right showed cells in early stage of apoptosis which were positive for Annexin-V, upper left showed dead cells which were positive for propidium iodide and the upper right showed cells in late stage of apoptosis and positive for Annexin V-FITC and propidium iodide-positive cells. (B) Represent corresponding cumulative data. Values are shown as means ± SEM; n = 3. P< 0.05 versus control.

cell line. Results have shown that increasing concentrations of extract were associated with increase in apoptosis up to 25 µg/ml. The number of apoptotic cells decreased at 50 µg/ml and increased at 75 µg/ml concentration. Further increase in concentration again decreased the apoptosis as shown in Figure 3A and B.

DISCUSSION

Nowadays, many drugs are showing resistance, so it is direly needed to search for and develop new drugs. Natural products may be the good source of new therapeutic agents (Saleem et al., 2014). *C. intybus* has previously been shown to be cytotoxic against breast cancer MCF-7 (Dahab and Afifi, 2007), prostate cancer LNCaP, amelanotic melanoma C32 and renal adenocarcinoma ACHN (Conforti et al., 2008). Hsieh et al. (2010) had proven its *in vitro* anti-proliferative properties and caspase 3 mediated apoptosis induction in gastric tumor cell lines.

In the present study, we have observed the cytotoxic effect of *C. intybus* n-hexane extract against Jurkat cells, a human lymphoblastic leukemia cell line, by using trypan blue assay, MTS assay and FACS analysis. It was shown from the trypan blue exclusion assay that n-hexane extract of *C. intybus* decreased cell viability by increasing concentration of the extract. Antiproliferative activity of *C. intybus* was determined by performing MTS assay. It was shown that there is decrease in cell proliferation. Apoptosis induction was evaluated by FACS analysis. The rate of apoptosis induction by *C. intybus* n-hexane extract was found maximum at the dose of 25 µg/mL. As dose of the extract was increased, apoptotic rate decreased. Thus, 25 µg/ml may be considered the most effective dose for the induction of apoptosis in Jurkat cells.

Previous studies showed that *C. intybus* n-hexane extract consists of volatile oils, fatty acids and triterpenoids (Nandagopal and Kumari, 2007). The essential oils or volatile oil have great potential of cytotoxicity, antimicrobial, analgesic, anti-inflammatory and insect repellent activities and furthermore anticancer effects are via apoptotic pathway (Sharma et al., 2013). Similarly, triterpenoids are also considered as new promising anticancer drugs (Petronelli et al., 2009). Thus, n-hexane extract of *C. intybus* may contain volatile oils and triterpenoids and cytotoxic potential of this extract may be due to the presence of these compounds.

Conflict of Interests

The authors declare no conflict of interests.

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