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CONTENTS

POTENTIALLY PHYTOTOXIC EFFECT OF AQUEOUS EXTRACT OF *ACHILLEA SANTOLINA* INDUCED OXIDATIVE STRESS ON *VICIA FABA* AND *HORDEUM VULGARE*

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Determining the mode of action of allelochemicals is one of the challenging aspects in the allelopathic studies. Recently allelochemicals have been proposed to cause oxidative stress in target tissue and induced an antioxidant mechanism. *Achillea santolina* is widely distributed in northern western desert of Egypt, it grows well in crop fields under fig and olive trees. A field survey during spring 2008 indicated that the considered species exhibited a deleterious effect on the performance and yield of non tillage broad bean and barley field in the region. Aqueous shoot extracts of *Achillea santolina* decreased chlorophyll a and b levels, increased carotenoid content, increased levels of CAT, GPX, SOD and GR activity. (APX) increased up to 8% concentration of *Achillea* extract, a decrease at higher concentrations indicating the enhanced generation of reactive oxygen species (ROS) upon *Achillea santolina* exposure. Increased level of glutathione indicates their induction as secondary defence mechanism in response to *Achillea santolina* extract. Data revealed that broad bean was more sensitive to *Achillea* aqueous extract than barley. In conclusion, reactive oxygen species accumulation caused cellular damage despite the activation of the antioxidant system by *Achillea santolina* phytotoxins. There was an interspecies difference in the recognition of allelochemicals between *Vicia faba* and *Hordeum vulgare*. In order to determine the allelopathic potential against the two tested species, the experiment of decaying residues bioassay and test the soil under pure stand of *Achillea santolina* was conducted. Residues of *Achillea santolina* inhibited the growth of *Vicia faba* and *Hordeum vulgare* aqueous extract of residues of *Achillea santolina* soil inhibited seedling length of both plants. The phytotoxicity started after one week of decomposition and persisted for 6 weeks.

Key words: Phytotoxicity, Reactive oxygen species (ROS), *Vicia faba*, *Hordeum vulgare*, antioxidant enzymes, oxidative stress.

INTRODUCTION

Plants produce various secondary metabolites known as allelochemicals/ allelopathins, which are released in the environment by means of volatilization, leaching, decomposition of residues and root exudates and mediate plant to plant interactions (Hadacek, 2002). Allelochemicals have usually been considered to be

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secondary metabolites or waste products of the main metabolic pathways in plants, and they do not appear to play a role in the primary metabolism essential for plant survival (Rice, 1984). Although most plants produce phytotoxic allelochemicals, relatively few (*e.g. Tribulus terrestris, Achillea santolina, Fagopyrum esculentum, Oryza sativa*) have strong allelopathic properties (Bhowmik and Inderjit, 2003; Khanh *et al.*, 2005; Xuan *et al*., 2005; El-Darier and Youssef, 2007; Golisz *et al*., 2007; Salhi and El-Darier, 2008; Hatata and El-Darier, 2009).

Nishida *et al*. (2005) reported that the aromatic plant *Salvia leucophylla* contains a number of volatile monoterpenoids in their foliage, soil and airspace around. These compounds when used in their purified form inhibited cell proliferation in the meristematic cells in roots of *Brassica campestris* by interfering with organelle and nuclear DNA synthesis. In addition, it may induce oxidative stress by production of malondialdehyde (Zunino and Zygadlo, 2004).

Production of reactive oxygen species (ROS) and related oxidative stress in general has been proposed as one of the major mechanisms of action of the phytotoxins (Weir *et al*., 2004). ROS which cause Ca^2 signaling cascades triggering genome wide changes leading to death of the root system (Bais *et al*., 2003). On the one hand, ROS cause oxidative stress while on the other trigger the expression and induction of defence enzymes. Production of soluble enzymatic and non enzymatic antioxidants is one of the major protective means for plants against ROS. Superoxide dismutase (SOD) is the first line of defence which detoxifies singlet oxygen (Gomez *et al*., 2004). Catalase (CAT) and peroxidase (POX) etc. scavenge toxic H_2O_2 and provide tolerance to plants against biotic and a biotic stresses (Unyayar *et al*., 2005). However, not much is known about its exact mode of action. Specific details regarding the level and extent of oxidative stress and the induction of anti-oxidative enzyme mechanisms due to *A. santolina* exposure are lacking.

The genus *Achillea* (Family Asteraceae) comprises more than 200 species, most indigenous to Europe and the Middle East (Ahmed *et al*., 1988). Two species are widely distributed in Egypt: *A. santolina* L. and *A. fragrantissima* L. The former is considered the most important species in the northern western desert of Egypt. It grows well in crop fields, under fig and olive trees and on the edges of roads. This plant from the wild plants used in Bedouin folk medicine has the Arabic common name Al-Qisum. It is characterized by a pleasant smell due to its high content of essential oils. Several products (acetylenes, essential oils, flavonoids, sesquiterpenes) obtained from *A. santolina* have been investigated for their biological activities (Fleisher and Fleisher, 1993; Weir *et al*., 2004). It is used in folk medicine for the treatment of gastro-intestinal disturbances and various infections, among them infection of the eye (Segal *et al.*, 1987), and as hypoglycemic agent (Aboutable *et al*., 1986). The young flowering branches are used as anti-diabetic while the leaves are used for tooth pain, stomach ailments, dysentery and colic pain (Barel and Yashphe, 1989; El-Darier *et al*., 2005).

A field survey during spring 2008 indicated that the considered species exhibited deleterious effect on the performance and yield of non tillage barley and broad bean fields in the region. In addition, Hatata and El-Darier (2009) reported that the aqueous extract of *A. santolina* shoots achieved a phytotoxic effect on germination efficiency and metabolite accumulation in wheat plant.

The aim of the present work is to investigate the impact of aqueous shoot extract of *A. santolina* on different biochemical and physiological parameters in *Hordeum vulgare* L. and *Vicia faba* L. plants as demonstrated in the following: [1] some growth parameters as photosynthetic pigments; [2] induction of oxidative stress in terms of lipid peroxidation, membrane integrity and hydrogen peroxide content.; [3] level of non enzymatic antioxidant such as ascorbic acid and glutathione contents and [4] level of induction of antioxidant enzyme mechanisms in terms of activities of superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase. Also, to test the impact of initial residues bioassay and residues bioassay of *A. santolina* at different periods of decomposition on biomass and seedling length to prove the allelopathic potential against the two tested species.

MATERIALS AND METHODS

I. PHYTOCHEMICAL SCREENING OF *ACHILLEA SANTOLINA* SHOOTS

Total phenolics and flavonoids contents was determined by the method described by (Meda *et al*., 2005). Alkaloids, sterols and glycosides were determined according to (Kam *et al*., 1999; Jibbons *et al*., 1967; Lewis and Smith, 1967) respectively. In addition, tannins and coumarins were estimated by the methods described by Shellaerd (1957) and Feigl (1960) respectively.

II. SAMPLING AND PREPARATION OF *A. SANTOLINA* SHOOT EXTRACT

Fresh vegetative top growth of *Achillea santolina* L. was collected during late spring 2008 from fifteen natural agro-fields distributed in the northern western desert of Egypt. The plant material was cleaned from sand particles and allowed to air dry. Tops were cut into 1–2 cm pieces and stored in plastic bottles at room temperature. 75 g of the cut material was soaked in 500ml distilled water for 24 h at room temperature to prepare fullstrength stock solution. Plant material was filtered out of the extract with cheese-cloth followed by filtering through no.1 and then 2 Whatman filter paper. The pH of the extract was about 4.8 and adjusted to 6.8 with 10% KOH (v/v). Then subsequent treatment concentrations (1, 2, 4, 8 and 16%) will be prepared. The extracts were prepared no more than 48h in advance and were stored at –5 ^{o}C. Prior to use in bioassay, the extracts were equilibrated at room temperature for 1 h.

III. GROWTH EXPERIMENT

Seeds of barley (*Hordeum vulgare* L.) cultivar 125 and broad bean (*Vicia faba* L.) cultivar 716 were obtained from the Breeding Program of the Agricultural Research Center, Giza, Egypt. All chemicals used in the enzymatic studies were of technical grade and produced from either sigma Co., or Merck Ltd. The pot experiment was conducted under laboratory conditions with different treatments of aqueous extract concentrations of *Achillea santolina* shoots.

Weighed plastic pots measuring about 1,000 cm^3 were filled with air dried soils collected from the natural community of *Achillea santolina* and planted with 10 uniform seeds for broad bean and 15 seeds for barley each in three replicates.The pots were placed in a growth chamber maintained at 30/16 ^oC day/night temperature (±2^oC) and 16/8 light/dark with a photon flux density of approximate 150 μ mol photons $n^{-2}5^{-1}$ and relative humidity 78±2%. The pots were irrigated with distilled water (according to the pre-estimated water field capacity) and left until the emergence of seedlings. Thereafter, the pots were watered with the different treatment concentrations day for a period of 4 weeks. Finally, plants were rinsed with deionized water prior to collection. The leaf photosynthetic pigments were determined as described by Moran (1982).

IV. DAMAGE INDEX

1. Hydrogen peroxide (H_20_2) *content.* H_20_2 content was determined using the method given by (Velikova *et al*., 2000). Leaf tissue (100 mg) was extracted with 5 ml of 0.1 % Trichloroacetic Acid (TCA) and centrifuged at 12,000 g for 15 minutes. Then 0.5 ml of supernatant was mixed with 0.5 ml of 10 mM phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide and the absorbance was determined at 390 nm. The amount of H_20_2, read using the extinction coefficient 0.28 $\mu m^{-1}cm^{-1}$ and expressed as nmol g^{-1} f. wt.

2. Effect on membrane integrity. Loss of membrane integrity (an indicator of cellular damage) was studied in terms of ion (electrolyte) leakage from the leaves of broad bean and barley by measuring conductivity of the bathing medium, as the method of (Duke and Kenyon, 1993). Leaf tissue (100 mg) collected from 24-d-old seedlings was dipped in 5 ml of 1 mM MES buffer (2-[N-morpholino] ethanesulfonic acid sodium salt, pH = 6.5) containing 2% sucrose (w/v). A parallel control containing all the materials was also maintained. The conductivity of the bathing medium was measured with a conductivity meter (ECOSCAN CON5; Eutech Instruments Pte. Ltd., Singapore). Leaf samples were then boiled for 15 minutes in order to measure the maximum electrolyte leakage. For each treatment there were five replicates, and the experiment was repeated.

3. Determination of lipid peroxidation. Lipid peroxidation was measured in terms of malondialdehyde content (MDA) as described by the method of Heath and

Packer (1968). Leaves (100 mg) were extracted with TCA (0.1 %, w/v) and centrifuged at 10,000 g for 10 minutes. MDA level was used as an index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight. One ml of the supematant was added to 4 ml of 0.5 % thiobarbituric acid (TBA) made in 20 % TCA. The mixture was incubated at 95 °C for 30 minutes followed by quick cooling over ice, and then centrifuged at 10,000 g for 10 minutes. The absorbance of the supernatant was determined at 532 nm and corrected for non-specific absorbance at 600 nm. MDA amount was determined using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as nmol g^{-1} fresh weight.

V. NONENZYMATIC ANTIOXIDANTS

1. Glutathione. Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined according to the method of (Griffih, 1980). Leaf tissues from control and treated plants were ground using a pestle and mortar in liquid N2 with 1 ml of 5% sulphosalicylic acid and centrifuged at 10,000 g for 5 minutes. A 300 Ul aliquot of the supernatant was removed and neutralized to pH 7.0 by adding l8 µl of 7.5 mol l^{-1} triethanolamine. A 150 µl sample was used for the determination of total glutathione (GSH+GSSG) alone. Fifty µl aliquots of samples were mixed with 700 µl of 0.3 mol l^{-1} NADPH. 100 µl of 10 mmol l^{-1} 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) and 150 µl 125 mmol l^{-1} $NaPO_4$-6.3 mmol l^{-1} EDTA buffer, pH 6.5. Ten µl of glutathione reductase (50 µl) was added and the change in absorbance at 412 nm was monitored. A standard curve prepared by using GSH and GSSG was used in the calculation of the amounts of total glutathione.

2. Ascorbic acid. The ascorbic acid (AA) concentration was measured by using the 2,6-dichlorophenol-indophenol (DCPIP) photometric method of (Guri, 1983). Fresh leaves were quickly detached and homogenized in 10-ml of ice-cold 0.005 mol l^{-1} EDTA solution containing 3% trichloroacetic acid (TCA) for 1-2 minute. The homogenate was quickly filtered through Whatman No. 4 filter paper and brought up to 20 ml with EDTA-TA extraction solution. The reaction mixture contained 1 ml of distilled water 0.2 ml of DCPIP reagent, and 2ml of filtered leaf extract. The absorbance was measured quickly at 600 nm and it was determined from the standard curve that was made previously using various known concentrations of ascorbic acid. DCPIP reagent was prepared by dissolving 13 mg of DCPIP and 3 g of reagent grade anhydrous sodium acetate in 1 L of distilled water.

VI. PREPARATION OF ENZYME EXTRACT

Enzyme extract was prepared by homogenizing 200 mg of frozen leaf tissue (from each treatment and control) in 10 ml of sodium phosphate buffer (0.1 M,

pH 7.0). The homogenate was filtered through a triple layer of cheese-cloth and centrifuged at 15,000 g at 4 °C. The supernatant was collected, stored at 4 °C and used as the enzyme extract for analysis of superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6 ascorbate peroxidase (APX; EC 1.11.1.11), guaiacol peroxidase (GPX; EC 1.11.1.7) and glutathione reductase (GR; EC 1.6.4.2).

VII. ACTIVITIES OF ANTIOXIDANT ENZYMES

Superoxide dismutase (SOD) was assayed following the method of Beauchamp and Fridovich, 1971, by measuring its ability to inhibit photochemical reduction of nitro blue tetrazolium chloride (NBT). The reaction mixture (4 ml) contained 63 μM NBT, 13 mM methionine, 0.1 mM EDTA (ethylene diamine tetraacetic acid), 13 μM riboflavin, 0.05 M sodium carbonate and 0.5 ml enzyme extract (0.5 ml distilled water in the case of the control). Test-tubes were kept under two 15 W fluorescent lamps for 20 minutes and then transferred to the dark for 20 minutes. The absorbance was determined at 560 nm and activity was expressed as enzyme units mg^{-1}. One unit of the enzyme activity was defined as the enzyme required for 50 % inhibition of the reduction of NBT in comparison with the tubes lacking the enzyme.

Catalase (CAT) activity was measured as per the method of Cakmak and Marschner (1992). The reaction mixture (2 ml) consisted of 25 mM phosphate buffer (pH 7.0), 10 mM H_20_2 and 0.2 ml of enzyme extract. The activity was determined by measuring the rate of disappearance of H_20_2 for 1 minute at 240 nm, and calculated using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ and expressed as enzyme units g^{-1} fresh weight. One enzyme unit was defined as the amount of enzyme required to oxidize 1 UM of H_2O_2 min^{-1}.

Glutathione reductase (GR) activity was determined spectrophotometrically by monitoring GSSG (glutathione oxidized)-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm as described by Foyer and Halliwell (1976). The reaction mixture (2 ml) contained 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM GSSG, 0.12 mM NADPH and 0.2 ml enzyme extract. Absorbance at 340 nm was read immediately after addition of the enzyme extract at time zero and after 5 minutes. The enzyme activity was measured in terms of NADPH left unoxidized using an extinction coefficient 6.224 $mM^{-1}cm^{-1}$, and expressed as enzyme units g^{-1} fresh weight. One enzyme unit was defined as the amount of enzyme required to oxidize 1 UM of NADPH min^{-1}.

Ascorbate peroxidase (APX) was assayed by Nakano and Asada (1981). The reaction mixture (2 ml) consisted of 25 mM phosphate buffer (pH 7.0), 0.1 mM EDTA, O.25 mM ascorbate, 1 mM H_2O_2 and 0.2 ml enzyme extract. The enzyme activity was determined using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$ by measuring the decrease in absorbance at 290 nm for 1 minute. It was expressed as

enzyme units g^{-1} fresh weight. One enzyme unit was defined as the amount of enzyme required to oxidize 1 µM of ascorbate min^{-1}.

Guaiacol peroxidase (GPX) activity was measured using the method described by Egley *et al*. (1983). The reaction mixture (2 ml) consisted of 25 mM phosphate buffer (pH 7.0), 0.05 % guaiacol, 1.0 mM H_2O_2, 0.1 mM EDTA and 0.2 ml of the enzyme extract. Increase in absorbance was measured at 470 nm due to oxidation of guaiacol. The enzyme activity was calculated using an extinction coefficient of 26.6 $mM^{-1}Cm^{-1}$ and expressed as enzyme units g^{-1} fresh weight. One enzyme unit was the amount of enzyme that catalyses oxidation of 1 µM guaiacol min^{-1}.

VIII. INITIAL RESIDUES BIOASSAY

Residues of *Achillea santolina* were separately mixed with soil at a rate of 4,8 and 16 g per kg soil and placed in pots. For control,similar rates of peat moss were added to maintain consistency of soil organic matter (Alsaadawi *et al*., 2005). Twenty-five seeds of barley and twenty seeds of beanwere added in each pot. There were 5 pots for each treatment and all pots were placed in chamber maintained at 30/16 ^{o}C day/night temperature (±2 ^{o}C) and 16/8 light/dark with a photon flux density of approximates 150 µ mol photons $n^{-2}s^{-1}$ and relative humidity 78±2% in acomplete randomized design. Two weeks after germination, the seedlings were thinned to 5 per pots, allowed to grow for 4 weeks, then harvested and oven-dried weight of whole plants was recorded.

IX. RESIDUES BIOASSAY AT DIFFERENT PERIODS OF DECOMPOSITION

Residues of *Achillea santolina* were separately chopped into pieces of approximately 2 cm length, mixed with the soil at a rate of 4,8 and 16 per kg soil. The pots were placed in chamber maintained at 30/16 ^{o}C day/night temperature (±2 ^{o}C) and 16/8 light/dark with a photon flux density of approximately 150 µ mol photons $n^{-2}s^{-1}$ and relative humidity 78±2% in acomplete randomized design and just enough distilled water was added to each pot to keep soil moist throughout the decomposition period. Soil samples were taken from each pot weekly and 100 ml of soil – water extract were prepared (Allen *et al*., 1974). For the bioassay twenty-five seeds of target plants were placed in a Petri dish containing 75 g of sand and 18 ml of appropriate test solution. The petri dishes were sealed with Para film stripes and placed in an incubator at 28 ^{o}C and 12 h light. Ten days after planting, seedlings length was recorded.

STATISTICAL ANALYSIS

All data were subjected to standard one-way analysis of variance (ANOVA) using SPSS (ver. 10; SPSS Inc., Chicago, IL, USA). Appropriate standard error of

means (±SE) was calculated for presentation with tables and graphs. The treatment means were separated by Duncan's multiple range test (DMRT) at $p < 0.05$.

RESULTS

Phytochemical screening of *Achillea santolina* shoots. The present data indicated that *Achillea santolina* contains about 8 mg/g, 29.70 mg/g and 0.3% of total flavonoids, total phenolics and essential oil respectively. On the other hand, coumarins, saponins and alkaloids were not detected. With respect to sterols and glycosides the samples contain a moderate content (++) while the content of tannins was low (+).

Effect of aqueous shoot extract. Aqueous shoot extract caused a decrease in pigment content. Chlorophyll a and b were found to be maximum in control. A decrease in chlorophyll was evident with the increase in extract concentration. Higher concentration of aqueous shoot extract adversely affected the amount of Chl a, Chl b. Maximum decrease of chlorophyll was recorded in seedlings treated with 16% concentration. The two species responded similarly as the reduction in chlorophyll a was the same, but in chlorohyll b there was a slight reduction in barley 13%, the corresponding value in boad bean was 26%. A different pattern was observed in the case of carotenoids in comparison to other concentrations. It is noteworthy that the carotenoid content was higher in barley than in broad bean plants at control level (about 1.25 and 11.33 mg/g fresh weight respectively) thereafter, at 16% concentration level, the two values were increased by about 1.7- and 14- fold respectively compared to the control (Table 1).

Table 1

Effect of allelochemical stress produced by aqueous shoot extract of *Achillea santolina* on pigment contents (mg g^{-1} fr.wt) of *Vicia faba and Hordeum vulgare*. Each value is a mean of three replicates with standard error. Values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test)

Concentration	Chlorophyll a		Chlorophyll b		Carotenoids	
	Vicia faba	*Hordeum vulgare*	*Vicia faba*	*Hordeum vulgare*	*Vicia faba*	*Hordeum vulgare*
0%	0.829±0.1163	0.605±0.085	0.454±0.061	0.267±0.036	1.251±0.17	11.330±1.54
1%	0.64±0.0898	0.556±0.078	0.551±0.075	0.253±0.034	2.090±0.28	9.645±1.31
2%	0.649±0.0910	0.621±0.087	0.527±0.071	0.296±0.040	1.208±0.16	13.195±1.79
4%	0.438±0.0614	0.343±0.048	0.555±0.075	0.177±0.024	4.156±0.56	16.793±2.28
8%	0.293±0.0411	0.25±0.035	0.42±0.057	0.272±0.037	12.825±1.74	14.763±2.00
16%	0.200±0.0344	0.210±0.027	0.337±0.045	0.233±0.049	16.790±2.28	19.223±2.61

Table 2

Effect of residues of *Achillea santolina* incorporated in soil on the biomass of whole plant (g plant^{-1}) of broad bean and barley. Each value is a mean of three replicates with standard error. Values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test)

Rate of residues g/kg^{-1} soil	Control		Treated samples	
	V.	H.	V.	H.
4	$0.903^{a}\pm0.0203$	$0.87^{a}\pm0.0289$	$0.480^{a}\pm0.0577$	$0.580^{a}\pm0.0577$
8	$1.507^{b}\pm0.0581$	$1.820^{b}\pm0.0608$	$0.360^{ab}\pm0.0577$	$0.413^{ab}\pm0.0521$
16	$2.600^{c}\pm0.115$	$2.800^{c}\pm0.0577$	$0.253^{b}\pm0.0291$	$0.320^{b}\pm0.0115$

Table 3

Effects of residues bioassay at different periods of decomposition of *Achillea santoliana* on the seedling length (cm) on broad bean and barley. Each value is a mean of three replicates with standard error. Values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test)

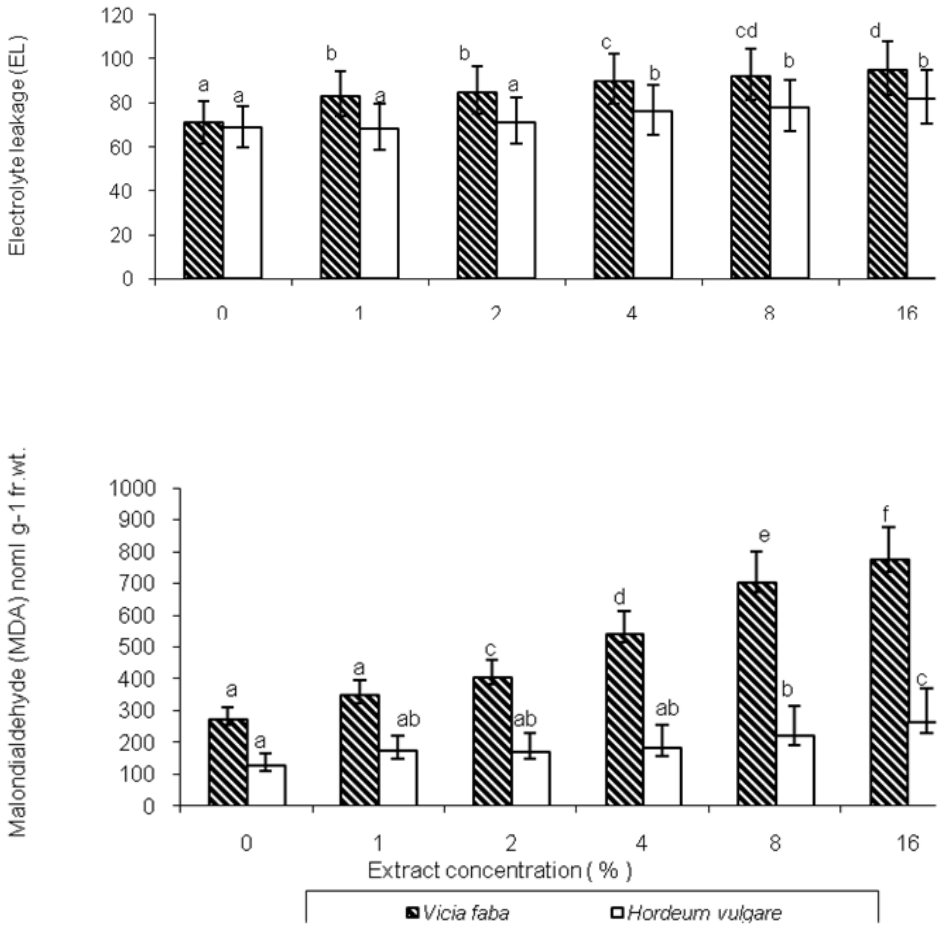
Time	4g/kg		8g/kg		16 g/kg	
	V.	H.	V.	H.	V.	H.
1 week	$30.12^{a}\pm1.96$	$28.18^{a}\pm2.01$	$27.2^{ab}\pm1.92$	$26.2^{ab}\pm2.07$	$25.1^{b}\pm1.99$	$24.2^{b}\pm2.11$
2 weeks	$26.1^{a}\pm1.77$	$27.1^{a}\pm1.82$	$22.1^{ab}\pm1.52$	$24.1^{ab}\pm1.99$	$19.2^{b}\pm1.85$	$22.4^{b}\pm1.78$
3 weeks	$24.2^{a}\pm1.62$	$25.2^{a}\pm1.66$	$20.4^{ab}\pm1.44$	$22.3^{ab}\pm1.55$	$18.3^{b}\pm1.62$	$20.2^{b}\pm1.55$
4 weeks	$20.1^{a}\pm1.558$	$22.8^{a}\pm1.41$	$18.3^{ab}\pm1.62$	$20.2^{ab}\pm1.40$	$16.2^{b}\pm1.44$	$18.4^{b}\pm1.40$
5 weeks	$18.2^{a}\pm1.03$	$19.2^{a}\pm1.33$	$16.2^{ab}\pm1.06$	$18.3^{ab}\pm1.33$	$14.2^{b}\pm1.06$	$16.3^{b}\pm1.31$
6 weeks	$15.2^{a}\pm1.00$	$17.4^{a}\pm1.01$	$13.1^{ab}\pm0.98$	$14.1^{ab}\pm1.16$	$10.2^{b}\pm0.95$	$12.2^{b}\pm1.08$
P	0.001*	0.001*	0.0001*	0.001*	0.0001*	0.001*

Measurements of damage. Treatment with *Achillea* phytotoxins endures a significant excessive ion leakage in the studied species as measured by increased conductivity of the bathing medium (MES buffer) (Fig. 1A).

We monitored changes in lipid peroxidation by measuring the thiobarbituric acid reactive substance in the leaves of both species. Increased lipid peroxidation was observed with increasing *Achillea santolina* concentration in the medium in both species.This increase was significantly higher in broad bean in all treatments of A.s. When exposed to 16% TBARS level in broad bean was three fold the control value while in barley it was doubled (Fig. 1B).

A change in H_2O_2 content is a good indicator of the ROS scavenging capacity of plants under oxidative stress. Fig. 1C shows that barley has steady state levels of H_2O_2 irrespective of the level of A.s in the medium, broad bean showed significant enhancement in the H_2O_2 content, up to 150% in the presence of 16% aqueos shoot extract.

Status of antioxidant pool. Reduced glutathione and ascorbic acid are common antioxidants used by plants to reduce ROS levels *in vivo*. We investigated the modulations of the levels of these antioxidants in response to varying degree of phytotoxin-induced oxidative stress (Fig. 3). With respect to ascorbic acid content in the two studied species, two distinct trends were noticed (Fig. 3). In broad bean, ascorbic acid steadily increased until reached its maximum at 4% extract concentration level then decreased to a minimum value of about 5.55 at 16% concentration level. On the other hand, barley exhibited a significant decrease from a maximum value of about 6.41 in control to a minimum of about 1.79 at 16% concentration level respectively.



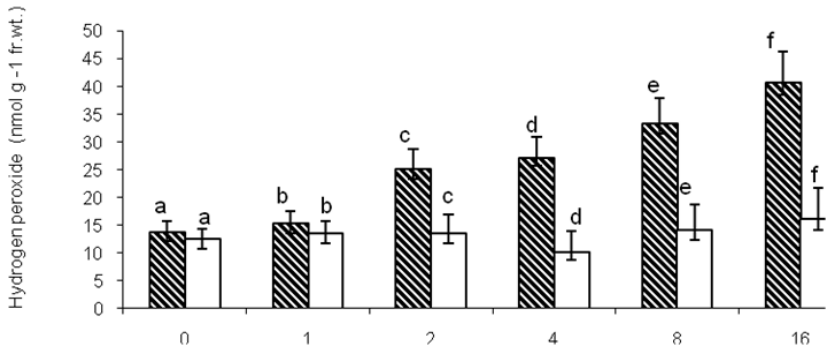


Fig. 1. Effect of allelochemical stress produced by aqueous shoot extract of *Achillea santolina* on electrolyte leakage (measured as conductivity), lipid peroxidation and H_2O_2 accumulation in broad bean and barley plants. Data are the means of three independent replicates with standard error. Values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test).

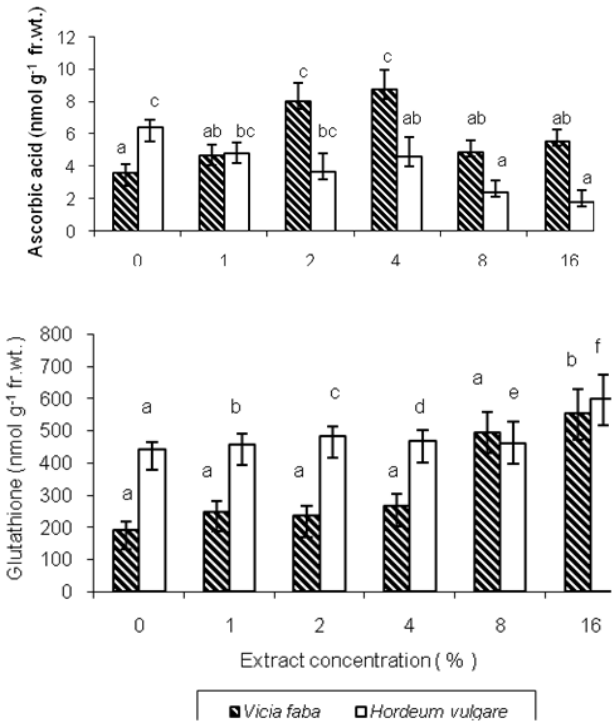


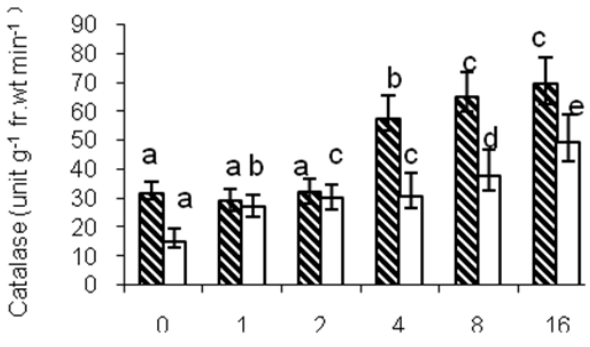
Fig. 2. Effect of allelochemical stress produced by aqueous shoot extract of *Achillea santolina* ascorbic acid content and total glutathione in broad bean and barley plants accumulation. Data are the means of three independent errors. Values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test).

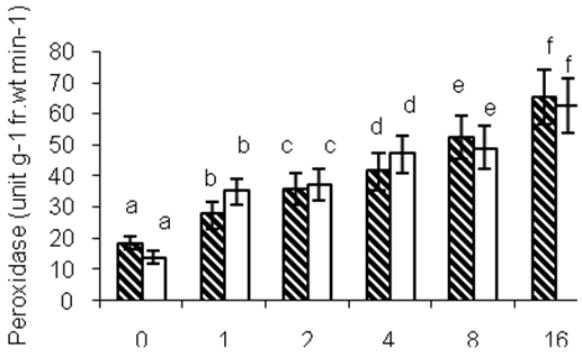
The total glutathione (including GSH and its oxidized form) content was significantly higher in barley than broad bean in control as well as stressed conditions (Fig. 3). This hints at the existence of more robust non-enzymatic ROS scavenging systems in barley. Glutathione content increased significantly by nearly 3-fold and 1.3-fold at 16% concentration of *Achillea santolina* extract in broad bean and barley respectively.

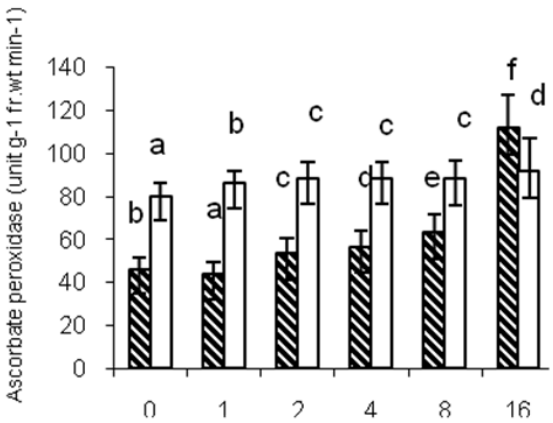
Achillea **phytotoxin-induced modulation in the activities of ROS scavenging enzymes.** Since the H_2O_2 content varied significantly between the two species, we determined the activities of major H_2O_2 scavenging enzymes, catalase and guaiacol peroxidase in both species under *Achillea* stress. CAT activity in control plants was significantly higher in broad bean than those of barley. Increase in CAT activity was recorded in all treatments of *Achillea* extract concentration with maximum activity at 16% where it reached about 2.7- and 3-fold in broad bean and barley respectively (Fig. 4).

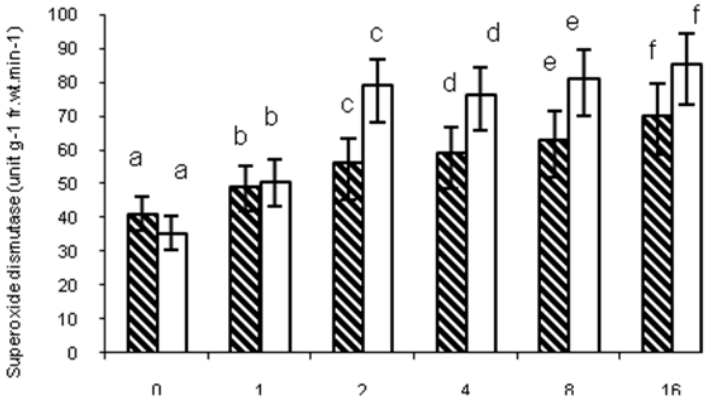
Concerning peroxidase (GPX) activity, the activity increased in both species with increasing *Achillea* phytotoxin extract. On the other hand, at 16% *Achillea* phytotoxin extract broad bean and barley achieved an increase of about 4.2- and 2.5-fold, respectively. Thus the enzymatic detoxification of H_2O_2 seemed to be variable between the two species.

We have also determined the activities of various enzymes involved in ascorbte-glutathione cycle. Minium superoxide dismutase (SOD) activity was recorded in control of both target species, SOD exhibited a significant increase in the leaves of seedlings treated with different concentrations of *Achillea* extract as compared with control in two considered species. Maximum SOD activity was achieved at 16% concentration of *Achillea* shoot extract where its value was about 2- fold in broad bean and 2.4-fold in barley relative to control.




Peroxidase (unit g-1 fr.wt min-1)
0
10
20
30
40
50
60
70
80
a a b b c c d d e e f f
0 1 2 4 8 16


Ascorbate peroxidase (unit g-1 fr.wt min-1)
0
20
40
60
80
100
120
140
b a a b c c c c e f d
0 1 2 4 8 16


Superoxide dismutase (unit g-1 fr.wt.min-1)
0
10
20
30
40
50
60
70
80
90
100
a a b b c c d d e e f f
0 1 2 4 8 16

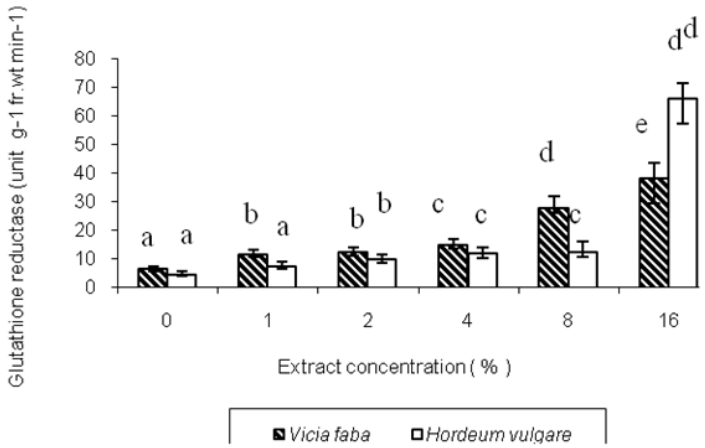


Fig. 3. Effect of allelochemical stress produced by aqueous extract of *Achillea santolina* on the activity of catalase, peroxidase, ascorbate peroxidase, superoxide dismutase and glutathione reductase in broad bean and barley plants. Data are the means of three independent errors. Values followed by the same letters within each column are not significantly different at 0.05 (ANOVA and Duncan's multiple range test).

With regard to ascorbate peroxidase (APX), a similar trend characterized by a progressive increase in the content with increasing extract concentration level was obtained for the two studied species. At 16% concentration level the increase in broad bean and barley was about 2.45 and 1.15-fold, respectively relative to control. A similar trend was observed with glutathione reductase (GR) activity. It increased in broadbean by 6- fold and in barley by about 10.5-fold at 16 % extract concentration level.

Intial residues bioassay for material selection. Residues of *Achillea santolina* inhibited the growth of broad bean and barley when compared to the control. The phytotoxicity of the residues differed among the two species (Table 2). Generally, the maximum values of dry mass of whole plant for the two studied species was attained at 4% of residue of *Achillea*. At low level, the two species responded similarly but at high level of residue concentration there was significant difference between the two species. Apparently the increase of residues in the soil increase the growth reduction significantly.

Residues bioassay at different periods of decomposition. Aqueuos extract of residues of *Achillea santolina* in soil inhibited seedling length of both plants (Table 3). The phytotoxicity started after one week of decomposition and persisted for 6 weeks. The reduction in seeding length increased with increasing rate of residues in soil during the 6 weeks of decomposition. Barley is less affected than

bean plants since at high residue concentration the reduction in seedling length was 50% whereas in bean plants it was only 40%.

DISCUSSION

The present study revealed great differences in response to the aqueous extract of *Achillea santolina* shoots between broad bean and barley in terms of photosynthetic pigments. Tammam *et al.* (2011) recorded that *Achillea santolina* extract reduced germination, radicle and plumule length and leaf area of broad bean and barley and the inhibitory effect was more noticeable in broad bean than in barley. Allelochemicals have several molecular targets and are known to affect many cellular processes in target plants, regarding the stomatal closure (Barkosky *et al*., 2000), plant-water balance (Barkosky and Einhellig, 2003), cell division (Anaya and Pelayo-Benavides, 1997), membrane permeability (Galindo *et al*., 1999), nutrient uptake (Bagavathy and Xavier, 2007), respiration (Abrahim *et al*., 2000) and other metabolic processes. The reduction in growth could be attributed to reduction in cell division and/or cell enlargement. El-Darier (2002) reported that the allelopathic effects of *Eucalyptus* aqueous extract on broad bean and maize plants were mainly on crop growth due to inhibition of some interacting physiological processes such as nutrient uptake which is highly positively correlated to the dry matter production. Such suppressive effect was more clear in dicotyledonous (broad bean) than in monocotyledonous (maize) plants. The photosynthetic pigments decreased significantly by increasing the treatment concentrations (the decrease in chlorophyll a was 86% and 64% in bean and barley respectively). The reduced Chl. under *Achillea* treatments could be attributed to the inhibition of chlorophyll biosynthesis and/or the stimulation of chlorophyll degradation (Yang *et al*., 2004). Rice (1984) has suggested that allelopathic compounds impede the synthesis of porphyrin precursors of chlorophyll biosynthesis.

Several action modes have been suggested, including direct inhibition of PSII components and ion uptake, interruption of dark respiration and ATP synthesis and ROS-mediated allelopathic mechanisms (Inderjit and Duke, 2003). Bagavathy and Xavier (2007) reported that the reduction in Chl a, Chl b and total Chl in sorghum plants when treated with Eucalyptus leaf extract. Contrarily, carotenoids increased significantly in both investigated species indicating the role of carotenoid in photoprotection of the photosynthetic pigments under stress condition (Hu *et al*., 2000). Carotenoids serve as antioxidants against free radicals and photochemical damage (Mishra *et al*., 2006).

The present study showed a significant excessive ion leakage in both studied species as measured by increased conductivity of the bathing medium (MES buffer). This may indicate that the aqueous extract of the allelopathic plant caused

stress resulting in disruption of membrane integrity. In addition, a decrease in membrane permeability could be due to peroxidation polyunsaturated fatty acids in the biomembranes resulting in the formation of several byproducts including malondialdehyde (Maness *et al.*, 1999).

MDA (as an indicator of lipid peroxidation) and electrolyte leakage resulting in loss of membrane integrity are among the key factors that determine cell injury. Generally, various types of environmental stresses (including abiotic, xenobiotic and herbicidal) mediate their impact through oxidative stress caused by generation of reactive oxygen species ROS (Smirnoff, 1995, 1998, Blokhina *et al*., 2003). ROS such as singlet oxygen (O_2), superoxide radicals (O_2^-) and hydroxyl radical (OH), hydrogen peroxide (H_2O_2) are highly reactive and toxic molecules that can cause oxidative damage to membranes, DNA, proteins, photosynthetic pigments and lipids (Apel and Hirt, 2004). Recently, ROS generation and related oxidative stress has been proposed as one of the modes of action of plant growth inhibition by allelochemicals (Weir *et al*., 2004).

To explore whether *A. santoliana* extract induces a similar response, various indicators of damages enzymatic mechanisms linked with oxidative stress were assessed in leaves of the two investigated species. The allelopathic effects on other plant species have been recognized as an important survival strategy (Canals *et al*., 2005).

In the current work MDA increased significantly in leaves of the target plant species upon treatment with *A. santolina* aqueous extract. The increase in the level of MDA is more obvious in broad bean than in barley denoting that broad bean was susceptible to the phytotoxic effect of allelochemicals. This difference could be an important factor that regulates phytotoxicity. This result was in consistence with those obtained by Scrivanti *et al*. (2003) and Zunino and Zygadlo (2004) who reported that monoterpenes enhance lipid peroxidation. Increased lipid peroxidation indicates that *A. santotiana* extract results in oxidative stress due to generation of ROS species, that causes a loss of cell integrity.

Apart from causing peroxidation of membrane lipids, high levels of H_2O_2 have been reported to cause a net reduction in photosynthesis in plants by over 50% (Kaiser, 1979). By channeling most of the photoreductants (electrons produced at photosystem II by the water oxidation complex) for the detoxification of H_2O_2 produced under stress, CO_2 fixation is compromised. This leads to poorer growth in broad bean than barley.

This large difference in ROS generation between broad bean and barley could be an important factor that regulates the occurrence of phytotoxin. Under *A. santolina* aqueous, seedlings of broad bean showed a higher level of H_2O_2 and lipid peroxidation than sensitive barley. Several studies have implied that H_2O_2 can act as mobile signal, altering plant to various biotic and abiotic stress (Neil *et al*., 2002). However, it directly damages vital processes in organelles like chloroplast and mitochondria. In chloroplast, H_2O_2 interferes with the activities of SH-group

containing enzymes such as fructose –1,6 diphosphatase and inhibits photosynthetic activity (Takeda *et al*., 1995). However, very little is known about the action of allelochemical phytotoxins in inducing ROS mediated oxidative damage.

Several reports indicated that oxidative stress may induce an increase in the glutathione (GSH) accumulation (Noctor *et al*., 1988). The present investigation revealed that glutathione concentration was higher in barley in control and stressed plants than broad bean reflecting the greater tolerance of the first than the second. Glutathione (GSH) plays an important role in the antioxidant defense system of leaves, since, it not only participates in the regeneration of ascorbate via dehydroascorbate reductase, but it can also react with singlet oxygen and OH^- radicals and protect the protein thiol groups (Asada, 1994). It is used by glutathione S-transferase to detoxify xenobiotics.

Ascorbic acid increased in low and at moderate concentration of *A. santolina* aqueous extract in broad bean leaves and then decreased at high concentration; this may be due to participation in reducing H_2O_2 to H_2O by ascorbate peroxidase. Ascorbate is an ubiquitous soluble antioxidant in photosynthetic organisms and it is also an important soluble antioxidant compound in plant cells (Nakano and Asada, 1981) and the most important reducing substrate for H_2O_2 detoxification. The low content of ascorbic acid in barley may be due to its participation in reducing H_2O_2 to H_2O catalyzed by increasing activity of ascorbate peroxidase (Apx) to dehydroascorbate and reduced back to ascorbate in the presence of glutathione (Zhang and Kirkham 1996). Hence the levels of ascorbate and glutathione play an important role in oxidative defense. Tammam (2008) reported that there is a decrease in ascorbic acid content in cotton cultivars under salt stress.

Oxidative stress could play a role in phytotoxic phenomenon (Weir *et al*., 2004). This was evident by increased activities of antioxidant enzymes under allelochemical stress. Furthermore, ROS are known to trigger activities and expression of most of the antioxidant enzymes (Apel and Hirt, 2004). Plants possess an enzymatic defence system (SOD, GR, catalases, various peroxidases) for the detoxification of various types of ROS. There are many reports indicated that oxidative stress induced an increase in the responses of enzymatic systems linked ROS scavenging process (Apel and Hirt, 2004; Jones and Smirnoff, 2005**)**. The present study showed that under control conditions broad bean leaves maintained a higher activity of GR, GPX and SOD than barley. Importantly, increases of these enzyme activities were induced by *A. santolina* aqueous shoot extract in barley more than in broad bean. This may be related to the difference in the intrinsic capacity for ROS-scavenging between two species. Barley also exhibited steady state level of H_2O_2 under *A.s* treatme. This does not mean that barley is experiencing little or no oxidative stress, since it showed elevated activities of an antioxidant enzyme like CAT, APX, GPX and GR. The insensitive

response of barley to change in *A. santoliana* suggests that *A.s.* could induce little change in ROS generation as demonstrated by measurements of damage.

SOD is the major scavenger of superoxide ion to form H_2O_2 and O_2 and plays an important role in defense activity against the cellular damage caused by environmental stress (Nakano and Asada,1981). Increased levels of SOD activity under *A. santolina* indicate an induction of oxidative stress caused by excessive generation of superoxide ion. Ye *et al*. (2006) and Yu *et al*. (2003) reported that SOD activity induced an induction of oxidative stress caused by excessive generation of superoxide ion presumably resulting from alletochemicals contained in root exudates of cucumber. Our data are in agreement with other authors for increased activities of SOD and CAT in different plants like cucumber (Macias *et al*., 2002), tomato (Romero-Romero *et al*., 2005), and mustard (Oracz *et al*., 2007) under different allelochemical stress. SOD scavenges the highly reactive free radicals by converting them into H_2O_2. Although H_2O_2 is equally toxic, H_2O_2 is further reduced to H_2O by CAT in the perioxisomes, by APX in the chloroplast and cytosol, and by GPX in the cell wall (Blokhina et al., 2003). These antioxidants cause better survival of plants under stressful condition (Mishra *et al*., 2006).

In this investigation the activities of catalase (CAT) increased steadily with increasing *Achillea* extract concentration, the values reached to about 2.7- and 3-fold in broad bean and barley respectively at 16% extract concentration level. In contrast, there was not to a large extent an increase in the activity of peroxidase (GPX) at lower extract concentrations in the two species. On the other hand, at 16% extract level broad bean and barley achieved an increase of about 4.2 and 2.5-fold respectively. However, Singh *et al.* (2009) reported that CAT and GPX activities tend to decrease in maize leaves under the influence of high concentration of aqueous leachate of *Nicotiana plumbaginifolia*.

APX increased by nearly 2.45-fold in broad bean leaves and GR increased by 13-fold in barley, GR and APX – the main component of the ascorbate glutathione cycle – that provides the main defense against oxidative damage in plants (Becana *et al*., 2000). GR is a key enzyme in providing protection against a variety of environmental and abiotic stresses (Foyer *et al*., 1991; Romero-Puertas *et al*., 2006).

Our results suggest the phytotoxic nature of *Achillea santolina* which is clearly visible by growth parameter and physiological activities. The allelopathic potential of *Achillea santolina* against the two tested species was tested by two experiments, the decaying residues bioassay and test the soil under pure stand of *Achillea santolina*. These experiments revealed the reduced dry mass and seedling length of both tested plants. The decaying residues of *Achillea* in the soil started one week after the decomposition and persisted for at least 6 weeks which is quite enough to exert its effect. The increase of residues in the soil increased the growth reduction significantly and induced dry mass accumlation of broad bean and barley.

CONCLUSION

The present study proved that in allelopathy stress induced by *A. santolina* phytotoxins suppressed the chlorophyll content except for carotenoids with a noticeable difference in tolerance between the two species. Additionally, the extract experienced an increase in the generation and accumulation of ROS in leaves, leading to increased lipid peroxidation and hydrogen peroxide contents. Increased levels of scavenging system included a non-enzymatic system as GSH and the enzymatic system of broad bean. Barley is insensitive to this chemical with little changes in ROS metabolism. Increased Levels of scavenging enzymes indicate their induction as a secondary defence mechanism in response to various allelochemicals present in aqueous shoot extract. However, further studies on allelochemicals uptake, compartmentalization and detoxification is necessary to elucidate the mechanism involved in the specific recognition ability.

Importantly, the genetic mechanisms that are involved bringing about such responses to *A. santolina* remain unknown. For this reason, we now follow-up a study concerning this molecular study.

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MERCURY INDUCED GENOMIC DISTURBANCE AND FLOWERING INHIBITION IN SESBANIA PEA (*SESBANIA CANNABINA* POIR.)

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Sesbania cannabina commonly known as Dhaincha or Sesbania pea is widely adaptable to different adverse climatic conditions. It has been found to be tolerant to heavy metal toxicity. The present investigation reports the result of the effect of mercury on seed germination, plant survival, pollen fertility and microsporogenesis of *Sesbania cannabina*. The effects of four serial doses of mercuric chloride viz. 25, 50, 100 and 200 ppm were studied. The different concentrations of mercury showed reduction in germination, survival and pollen fertility percentages along with the increase in concentrations. The microsporogenesis of plant also gets adversely affected by mercury treatment and various types of cytological aberrations were recorded and the frequency of different types of aberrations was calculated at each dose.

Key words: Metal toxicity, mercuric chloride, microsporogenesis, cytological aberrations.

INTRODUCTION

Metal mine tailing suffers from high concentrations of heavy metal toxicity, low organic matter, low water retaining capacity, poor soil structure, lack of nutrients such as nitrogen, potassium, phosphorus. Phytoremediation has emerged as an important and ecofriendly method for reclamation of metal toxic soil. Phytoremediation is the treatment of environmental problem through use of plants which are able to contain, degrade or eliminate metals, pesticides, solvents, explosives, crude oil and its derivatives and various other contaminants from the media that contain them. Phytoremediation refers to the natural ability of certain plants called hyperaccumulators.

Metals are essential for growth of plants, however excessive concentrations are toxic. Metal pollution has emerged as a big environmental problem (Schroeder and Munthe, 1998; Boening, 2002). Unlike most metals that function as nutrients, mercury has no known physiological significance and therefore is not metabolized in most of the organisms. Even a low concentration of mercury introduced into biological system may cause serious toxicity in sensitive plants and animals

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(Suszcynsky and Shann, 1995). Mercury is used in various industries such as those which manufacture paper, paint, pulp, pesticides, mining of gold, silver and mercury itself also contributes in mercury pollution to the terrestrial as well as aquatic environment (Sinha *et al*., 1996; Wang and Greger, 2004).

Phytostabilization appears to be an environmentally friendly approach to stabilize toxic metal mined sites in long run (Baker *et al*., 1994). Traditional methods for treating this metal toxicity in environment are often expensive and may cause secondary pollutions (Sekhar *et al*., 2003). Phytoremediation, on the other hand, is usually a low cost and ecofriendly method. It does not produce secondary harmfull pollutants and there may be possibility of recovery of valuable metals and plants can also be easily monitored. Therefore, at present time it is gaining most potential method for environmental pollution, while there are certain limitations regarding the selection of hyperaccumulative plant species such as plants with low growth, possibility of transfer of these heavy toxic metals into food chain and survival of plant is also affected and to remove heavy metals from polluted soil in a timely manner (Cunningham *et al*., 1995). Therefore, it is important to select a plant species which follows a lesser number of these categories of limitations. Mercury accumulation has been studied in many plants such as *Pisum sativum* L., ryegrass (*Lolium perenne*), spearmint (*Mentha spicata* L.), norway spruce (*Picea abies* L.), spinach (*Sinacia oleracea*), rice (*Oryza sativa* L.) and willow (Beauford *et al*., 1977; Al-Attar *et al*., 1988; Godbold and Huettermann, 1988; Chunilall *et al*., 2004; Wang and Greger, 2004; Du *et al*., 2005).

Sesbania cannabina is a multipurpose leguminous crop with high growth rate and high biomass production. It is widely used as a green manure crop for wheat, rice, maize, etc. It is widely adaptable to climatic conditions such as water logging, drought, heavy metal toxicity and high alkalinity. They can also grow and adapt varied soil environments, control soil erosion and enhance soil fertility where they are grown. It is tolerant to heavy metal toxicity partly due to their root nodules and partly due to their inherent tolerance property. Because of these valuable characteristics, it is a promising multipurpose plant resource. The plant species which are tolerant to heavy metal toxicity and can grow in nutrient difficient soil, have better chance to establish and grow on mine tailings. The assessment of chromosome damage is an efficient, reliable and economical criterion to measure genetic damage. Therefore, the present study aimed to analyse the genotoxic potential of mercury in *Sesbania cannabina* and to investigate its effect on germination, growth and pollen fertility.

MATERIAL AND METHODS

Seeds of *Sesbania cannabina* variety ND-1 were offered by Sunn Hemp Research Station, Pratapgarh, U.P., India. The seeds were treated with four serial

doses of mercury (25, 50, 100 and 200 ppm) for three hours. After treatment, the seeds were thoroughly washed in running water to remove mercuric chloride solution and then they were sown in their respective pots in triplicate to raise the population. Alongwith the treated seeds, untreated seeds were also sown for control set. Germination percentage was taken after 15 days of sowing and survival percentage, after 30 days of sowing.

On the onset of budding the young floral buds of appropriate size were fixed in Carnoy's fixative (1:3, Glacial acetic acid: abs. alcohol) and after 24 hr they were changed into 90% alcohol and then used for cytogenetical analysis. The slides were prepared using other squash technique in 2 % acetocarmine solution. Pollen fertility was also calculated by using acetocarmine stainability test.

RESULTS

The data presented in Table 1 revealed that the germination and survival percentage showed a direct relationship with treatment concentrations. As the mercury concentration increases, germination and survival percentages decreased. Germination at lowest dose was not affected by mercury treatment while at highest dose it was significantly reduced as compared to control. Control and 25 ppm mercury treated seeds exhibited 100% germination whereas at 200 ppm, germination percentage was observed as 50%. Survival of plants at all the doses was affected by mercury treatment. Control exhibited 100% survival and it is followed by 90%, 87.5%, 66.66% and 25% survival value at 25, 50, 100 and 200 ppm, respectively. The plant treated with mercury showed a dose dependent reduction in pollen fertility percentages. At 25 ppm it was 88.33% and reduced to 77.39% at 100 ppm. At 200 ppm, vegetative growth of plant occurred but budding and flowering were totally absent.

Table 1

Showing germination, survival and pollen fertility percentages at different concentrations of mercuric chloride

Treatment Mercuric chloride	Germination (%)	Survival (%)	Pollen fertility (%)
Control	100	100	94.05
25 ppm	100	90	88.33
50 ppm	80	87.5	82.68
100 ppm	60	66.66	77.39
200 ppm	50	25	–

Table 2

Effect of mercuric chloride on different meiotic phases of pollen mother cells of *Sesbania cannabina*

Mutagen	Doses	Total no. of PMCs scored	Total no. of abnormal PMCs	Abnormalities at Metaphase I/II (%)				Abnormalities at Anaphase I/II (%)				*Other abnormalities (%)	Total abnorm-ality (%)
				Un	Sc	Pr	St	Un	Br	Lg	Asy		
Mercuric chloride	Control	290	-	-	-	-	-	-	-	-	-	-	-
	25 ppm	300	28	1.00	1.00	1.33	1.33	0.66	1.33	0.66	0.66	1.33	9.33
	50 ppm	205	30	1.46	0.97	2.92	0.97	1.46	1.95	2.43	0.97	1.46	14.63
	100 ppm	210	40	1.90	0.95	3.80	2.38	1.90	1.42	3.33	1.90	1.42	19.04
	200 ppm	-	-	-	-	-	-	-	-	-	-	-	-

Un – Unorientation, Sc – Scattering, Pr – Precocious movement, St – Stickiness, Br – Bridges, Lg – Laggards, Asy – Asynchronous.
*Other abnormalities: Cytomixis, Tripolarity.

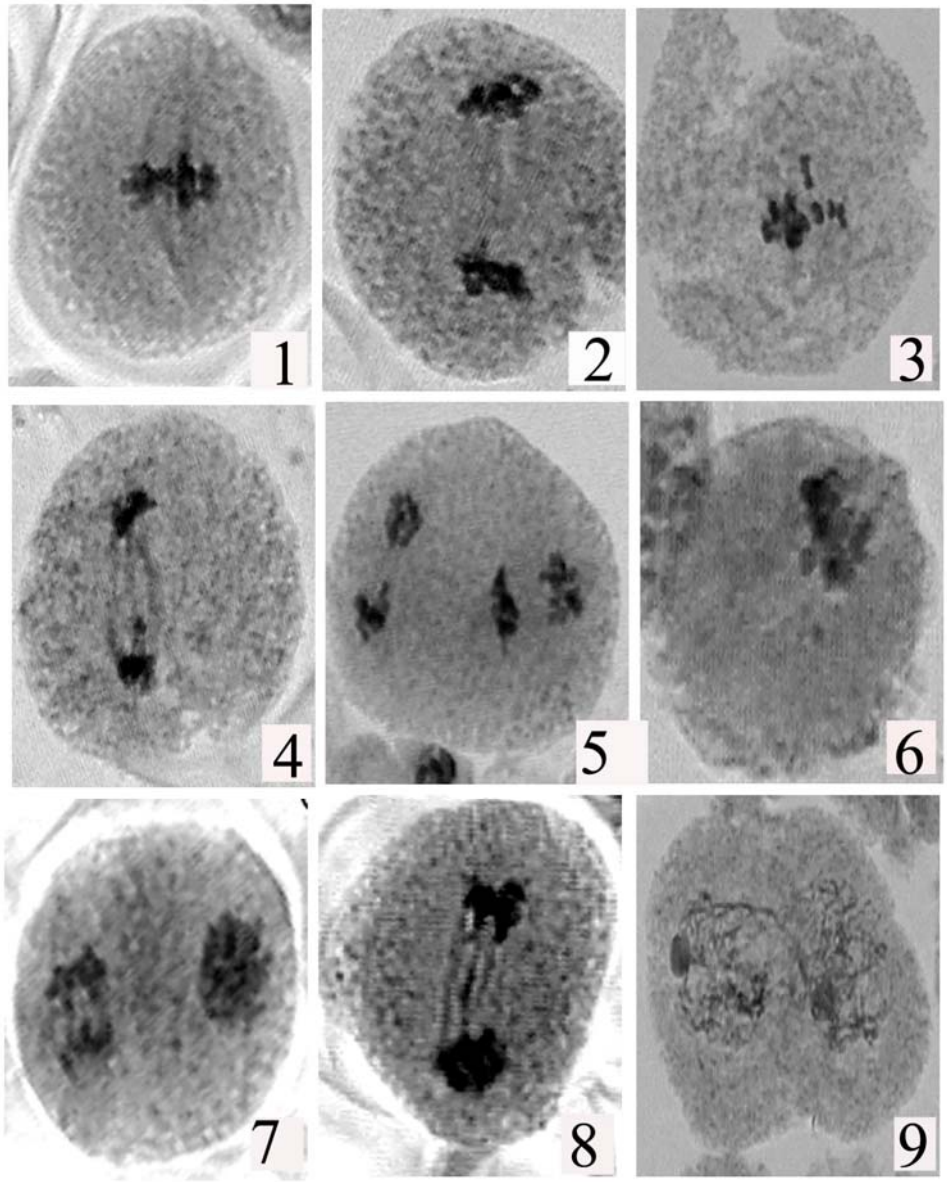

Fig. 1

1. Normal metaphase I; 2. Normal anaphase I; 3. Precocious movement at metaphase II; 4. Laggard at anaphase I; 5. Unorientation at anaphase II; 6. Unorientation at metaphase I; 7. Asynchronous division at anaphase II; 8. Bridge formation at anaphase I; 9. PMCs showing cytomixis.

Meiosis was perfectly normal in the control plants with 12 bivalents at diakinesis and at metaphase I (Fig. 1.1.) and 12:12 separation at anaphase I (Fig. 1.2). However, the plants in treatment sets displayed varying degrees of chromosomal abnormalities at each dose. A dose-based increase in meiotic abnormalities was observed. The most frequent chromosomal aberrations were precocious movement (Fig. 1.3) at metaphase I/II, laggards at anaphase (Fig. 1.4) I/II, stickiness at metaphase I/II. Although a number of abnormalities were present, *i.e.* unorientation at metaphase I/II (Fig. 1.6), unorientation at anaphase I/II (Fig. 1.5), asynchronous division at anaphase I/II (Fig. 1.7), bridge at anaphase I/II (Fig. 1.8) and scattering at metaphase I/II. The most prominent abnormality, *i.e.* precocious movement of chromosomes at metaphase I/II was 3.80% at 100 ppm of treatment while it was 1.33% at 25 ppm. Scattering at metaphase I/II was found to be highest at lowest dose, *i.e.* 25 ppm while it was lowest (0.95%) at highest dose (200 ppm). Bridges at anaphase I/II were highest at 50 ppm dose while lowest at 25 ppm, *i.e.* 1.33%.

In addition, other abnormalities like tripolarity at anaphase I/II and cytomixis (Fig. 1.9) were also encountered. Table 2 gives a detailed account of various types of aberrations and their percentages at different doses.

Pollen fertility was also reduced in treated plants as compared to control and in present investigation it has been found that, at the highest dose (200 ppm) budding and flowering were totally inhibited by mercuric chloride treatment. Pollen fertility at different doses has been shown in Table 1.

DISCUSSION

Increasing levels of heavy metals in the environment affect various physiological and biochemical processes in plants. Observations on the effect of metal genotoxicity are scarce and majority of the cellular and molecular aspects of metal toxicity in plants are unknown, even though deleterious effects of heavy metals on crop production have long been recognized. For more than 60 years or more, numerous experiments have been carried out to study the genetic effects of mercury compounds in experimental test systems (De Flora *et al*., 1994). Plants growing on metal-contaminated sites need to develop some degree of tolerance to metal toxicity in order to survive. Tolerance to metals can either be achieved by avoiding the metal stress, by tolerating it or both (Levitt, 1980).

It is obvious from the present experiment that heavy metal stress had a prominent effect on phenotypic and genetic structure of *Sesbania cannabina*. The study revealed various types of chromosomal aberrations in mercuric chloride treated plants as well as reduction in germination, survival and pollen fertility percentages as compared to control, which clearly show the mutagenic and toxic behavior of mercury. At the lowest dose, *i.e.* 25 ppm germination percentage was

not affected by mercuric chloride treatment or it may tolerate concentration up to 25 ppm as it was reduced dramatically at higher doses and similar is in the case of survival which was also reduced dramatically. In this respect, inhibition of seed germination and reduction in survival percentages were either due to the toxicity of the metal ions through disturbance of the physiological processes and induction of chromosomal aberrations (Morris and Hamilton, 1984; Datta and Biswas, 1985), or it may result from the fixation of mercuric ion by the plant tissues (Sahi *et al*., 1998). Reduction in germination may also be due to disturbance of the osmotic relationship which in turn affects some metabolic parameters during the early phase of germination (Espen *et al*., 1997).

Cytological study clearly revealed the chromotoxic behaviour of mercury and it induced various types of aberrations at each dose at different extent. Genotoxic behaviors of different toxic metals have been studied by many authors (Kiran and Sahin, 2006; Zhang and Yang, 1994; Caetano-Pereira *et al*., 2005). At metaphase I/II, the precocious movement and at anaphase I/II laggards were recorded in considerable frequencies at all the doses. The most noticeable and consistent effect of mercury was the induction of precocious movement at metaphase I/II which may be due to the effect of chemicals in breaking the protein moiety of the nucleoprotein backbone (Kumar and Rai, 2006). Laggards may be due to abnormal spindle activity and induction of lagging chromosomes leading to the separation of unequal number of chromosomes in the daughter nuclei and subsequently formation of daughter cells with unequally sized or irregularly shaped nuclei at interphase. The induction of bridges could be attributed to chromosome breaks, stickiness and breakage and reunion of the broken ends. Chromosome bridges were somewhat damaged and the damage was reversible or irreversible depending on breakage and rejoining of chromosomes (Liu *et al*., 1996). Stickiness arises due to increased chromosome contraction and condensation (Ahmed and Grant, 1972) or possibly from the deploymerization of DNA (Darlington, 1942) and partial dissolution of nucleoproteins (Kufman, 1958).Unorientation and Scattering at metaphase I may be due to disturbance in spindle apparatus or due to suppression of spindle formation (Kumar and Rai, 2007)

The present study also demonstrates the hazardous after effects of mercury exposure on *Sesbania cannabina* which ensures its mutagenic and cytotoxic behavior and it may cause genetic alterations in other organisms. Conclusively, the present study indicated that Hg was highly toxic at the level of 200 ppm as at this dose, germination and survival both were very low as well as flowering was totally inhibited. The present study revealed that the upper limit of Hg tolerance in *S. cannabina* is 100 ppm. This indicates that *Sesbania cannabina* may have a detoxification mechanism to cope with such a high concentration of mercury and this plant may be used to help reclamation of metal contaminated soil and it could serve as appropriate pioneer species to be used in short term remediation projects,

to modify the harsh environment by providing additional organic matter and nutrients.

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IN VITRO EVALUATION OF ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF *JATROPHA CURCAS* L.

J. SUNDARI [1] and R. SELVARAJ[1]

In the present study the effectiveness of *Jatropha curcas* extracts on inactivation of some microorganisms, *i.e.* gram positive bacteria *Staphylococcus aureus* and gram negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhimurium, Pseudomonas aeruginosa* and *Vibrio cholerae* and fungal pathogens, namely *Candida albicans*, *Aspergillus niger, A. flavus, A. fumigatus* and *Rhizopus* sp., were determined. The filter paper disc method was used for screening of crude extracts of leaves of *J. curcas* for antimicrobial activity. The paper discs saturated with extract were placed on the surface of the sterilized nutrient agar medium that had been inoculated with the test organisms by using a sterile swab. The diameters of inhibition zones were measured after 24 hours of incubation at 37 °C. The ethanol extract of *J. curcas* leaves was shown the largest inhibition zone (*i.e.* 18 mm) against *S. typhimurium*, when compared to other solvent extracts.

Key words: *Jatropha curcas*, leaf extract, bacteria, fungi, medicinal plant.

INTRODUCTION

The world population relies on traditional medicine for their primary healthcare. Use of herbal medicine in Asian countries represents a long history of human interaction with the environment. Plants used in traditional medicine contain a wide range of ingredients that can be used to treat for many infectious diseases. A vast knowledge of how to use the plants against different illneses may be expected to have accumulated in areas where the use of plants is still of great importance (Diallo, 1999).

Jatropha curcas L. belongs to family Euphorbiaceae and is a medicinally potent plant of great economic value. It is a drought-resistant, photo insensitive perennial plant. *J.curcas* is a multipurpose tree with a long history of cultivation in the tropical and subtropical regions of the world (Gubitz *et al.*, 1999). *J. curcas* is a potential source of non-edible biodiesel producing energy crop (Deore and Johnson, 2008).

The plants is reported to be abortifacient, anodyne, antiseptic, cicatrizing, diuretic, emetic, hemostat, lactagogue, narcotic, purgative, rubefacient, vermifuge.

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J. curcas is also used in folk remedy for alopecia, anasorca, ascites, burns, carbuncles, convulsions, cough, dermatitis, diarrhea, dropsy, dysentery, eczema, fever, gonorrhea, inflammation, jaundice, paralysis, pneumonia, rheumatism, scabies, sciatica, sores, syphilis, stomachache, tetanus, tumors, ulcers and yellow fever (List and Horhammer, 1969). Bark, fruit, leaf, root and wood are reported to contain Hydrogen-Carbon-Nitrogen, (HCN) (Watt and Breyer-Brndwijk, 1962). The sap (latex) has antimicrobial properties against *Staphylococcus* and *Streptococcus* sp. and *E. coli*.

Therefore, this research regarding antimicrobial activity is expected to enhance the use of *J. curcas* against diseases caused by the test pathogens. It is expected that screening of plant extract against wide variety of test organisms will be helpful in obtaining the new antimicrobial substances.

MATERIALS AND METHODS

Collection of plant materials. The leaves of *J. curcas* were collected from Agriculture farm, Faculty of Agriculture, Annnamalai University, Cuddalore district of Tamilnadu, India, during May 2009, and air-dried at room temperature.

Preparation of crude extracts. The dried leaves were coarsely powdered and stored in an airtight container. About 50 grams of leaf powder were taken in a clean sterile soxhlet apparatus and extracted with 250 ml of different solvents such as acetone, chloroform, ethanol and methanol. The extracts were collected and filtered using Whatman No. 1 filter paper. Then the extracts were dried in a vacuum evaporator to obtain a concentrated crude extract. This was suspended in Dimethyl Sulfoxide (DMSO) and used for antimicrobial activity.

Test microorganisms. The following microorganisms were used as test organisms; gram positive bacteria *Staphylococcus aureus* (MTCC 098), gram negative bacteria *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 25955), *Salmonella typhimurium* (MTCC 098), *Pseudomonas auruginosa* (ATCC 27853) and *Vibrio cholerae* and fungal pathogens *Candida albicans*, *Aspergillus niger, A. flavus, A. fumigatus* and *Rhizopus* sp. These bacterial and fungal strains were obtained from the Department of Clinical Microbiology, Rajah Muthiah Medical College (RMMC) and Hospital, Annamalai University, Tamilnadu, India.

In vitro antibacterial activity was determined by using Mueller Hinton Agar (MHA) and Mueller Hinton Broth (MHB) for bacteria. *In vitro* antifungal activity was determined by using Sabouraud Dextrose Agar (SDA) and Yeast Nitrogen Base (YNB), for yeast fungi and Sabouraud Dextrose Broth (SDB) for mould fungi and they were obtained from Himedia Pvt Ltd, Mumbai, India.

Antimicrobial assay. The disc diffusion method was used to determine the antimicrobial activity of the investigated extracts. Nutrient agar was prepared by dissolving of 25 g 1^{-1} in water. The sterile nutrient agar was inoculated with

microbial cells (200 µl of microbial cell suspension in 20 ml agar medium) and poured into sterile Petri dishes. Sterile filter paper discs of 6 mm in diameter were impregnated with 20 µl of the extract solution (equivalent to 4 mg of the dried extract). The paper discs were allowed to evaporate and thereafter they were placed on the surface of the inoculated agar plates. Then the plates were incubated at 37 °C for 24 hours for bacteria and 28 °C for 48 hours Yeast fungi, 2–4 days for mould fungi respectively, along with standard *Ciprofloxacin* (bacteria) and *Amphotroxicin* (fungi). The inhibition zone was measured from the edge of the disc to the inner margin of the surrounding pathogens. Data were analyzed using statistical tool (one way ANOVA) in SPSS.

RESULTS AND DISCUSSION

The antimicrobial activity was carried out by using leaf extracts with four different solvents, namely acetone, chloroform, ethanol and methanol. In this study six bacteria pathogens namely *S. aureus*, *E. coli*, *K. pneumoniae*, *S. typhimurium*, *P. aeruginosa* and *V. cholerae* and five fungal pathogens namely *C. albicans*, *A. niger, A. flavus, A. fumigatus* and *Rhizopus* sp., have been used. Among the six bacterial pathogens, *S. typhimurium,* showed high sensitivity to ethanol extract (18 mm), *K. pnemoniae* showed next level of ethanol extract (17 mm) and S. *aureus* showed high inhibitory zone of ethanol extract (15 mm). For all these three extracts, methanol, chloroform and acetone respectively stood next levels to sensitivity and inhibitory. *E. coli*, *V. cholera* and *P. aeruginosa* showed moderate inhibitory zone to ethanol extract and nil activity in acetone and chloroform extracts against *P. aeruginosa*. *Ciprofloxacin* was used as control for bacteria culture. The acetone, chloroform, ethanol and methanol extracts showed less inhibitory activities for all pathogens when compared to that of control.

Table 1

Screening of antibacterial activity of crude extracts of *Jatropha curcas* obtained from different solvents

Name of the pathogens	Zone of inhibition (mm) (Mean ± SD)				
	Ethanol	**Chloroform**	**Acetone**	**Methanol**	***Ciprofloxacin***
S. aureus	15±0.49	11±0.5	8±0.5	13±0.40	25±0.3
E. coli	14±0.40	8±0.35	7±0.41	12±0.36	20±0.20
K. pnemoniae	17±0.2	7±0.45	12±0.30	10±0.56	22±0.25
P. aeruginosa	12±0.36	–	–	8±0.32	21±0.4
S. typhimurium	18±0.35	11±0.30	10±0.41	11±0.2	19±0.26
V. cholerae	13±0.37	10±0.36	9±0.20	7±0.51	18±0.36

± = Standard Deviation (SD); Significant at 0.05% level

Amphotroxicin was used as control for fungal culture, all fungal pathogens showed less inhibitory zone in all solvent extracts when compared to that of control, except for *A. fumigatus*, which showed high sensitivity to ethanol extract (16 mm). All fungal pathogens showed a very less zone of inhibition in acetone extract when compared to that of other solvent extracts. Based on the results, it was observed that ethanol extract has a maximum inhibitory capacity to the fungal species studied. All data in the present study has varied significantly at 0.05% level.

Many plants or plant parts have been studied for their antimicrobial activity with different bacteria and fungi pathogens (Ahmad *et al.,* 1998). In the present investigation ethanolic extract of the leaves of *J. curcas* was screened for its antimicrobial activity. The antimicrobial activity of *J. curcas* has been studied earlier by many workers (Faria *et al.,* 2006). The ethanol extract of *J. curcas* has been reported to inhibit the growth of *Bacillus subtilis* and *Candida albicans* (Kubmarawa *et al.,* 2007) and the root extract was found to be active against *Staphylococcus aureus and Escherichia coli* (Adamu *et al.,* 2005). So, the present findings regarding the antimicrobial activity of the ethanol extract of the leaves of this plant are in agreement with the previous works.

Table 2

Screening of antifungal activity of crude extracts of *Jatropha curcas* obtained from different solvents

Name of the pathogens	**Zone of inhibition (mm) (Mean ± SD)**				
	Ethanol	**Chloroform**	**Acetone**	**Methanol**	***Amphotroxicin***
C. albicans	14±0.36	9±0.65	6±0.45	10±0.2	15±0.4
A. niger	10±0.95	10±0.25	3±0.2	6±0.4	14±0.45
A. flavus	13±0.56	8±0.36	2±0.90	9±0.5	16±0.40
A. fumigatus	16±0.80	10±0.40	6±0.49	11±0.37	17±0.30
Rhizopus sp.	11±0.65	6±0.2	3±0.95	8±0.30	15±0.20

± = Standard Deviation (SD); Significant at 0.05% level

The tested microbes were also found to be inhibited by the extracts of other angiospermic plants, *e.g. Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Bacillus subtilis* were inhibited by aqueous and organic solvents (acetone and ethanol) of *Tamarindus indica* (Doughari, 2006). *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* were inhibited by leaves extract of *Stevia rebaudiana* (Tadhani and Subhash, 2006)

and *Bacillus subtilis, Bacillus pumilus, Micrococcus luteus* and *Staphylococcus aureus* were inhibited by the methanolic extract of leaves of *Salicornia brachiata* (Manikandan *et al.*, 2009).

In the present study, the alcoholic extracts of *J. curcas* were found to be active against microbes studied. In continuous, methanolic extract of *Palustriella commutate* showed antimicrobial activity against *Micrococcus luteus, Bacillus cereus, Escherichia coli* and *Klebsiella pneumoniae* (Semra *et al.*, 2006). Whereas the extract of *Mitracarpus scaber* leaves showed antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Sarcina lutea, Candida albicans* (Abere *et al.*, 2007), *Proteus vulgaris, Serratia* marcescens (Igbinosa *et al.*, 2009), *Fusarium oxysporum, Pythium aphanidermatum, Lasiodiplodia theobromae, Curvularia lunata, Fusarium semitectum, Colletotrichum capsici, and Colletotrichum gloeosporioides* (Saetae *et al.*, 2010).

However, besides alcoholic extract, other extracts such as the organic (dichloromethane : methanol (1:1)) and aqueous extracts obtained from *Guatteria riparia* and *Gnetum leyboldii* showed antimicrobial activity against *Staphylococcus aureus* (Suffredini *et al.*, 2006); chloroform and hot water extracts obtained from *Boswellia ameero, Buxus hildebrandtii* and *Commiphora parvifolia* inhibited the growth of *Staphylococcus epidermidis,* whereas both extracts of *Withania adunensis* also inhibited the growth of *Staphylococcus aureus, Bacillus subtilis* and *Micrococcus flavus* (Mothanaa, and Lindequistb, 2005).

CONCLUSIONS

The results discussed in this article revealed that the leaves of *J. curcas* have quite number of chemical constituents, which may be responsible for many pharmacological and antimicrobial activities. Further works are required to investigate the extracts of leaves of *J. curcas* for various pharmacological activities before its commercialization for the benefit of human beings. The results lead credence to the folkloric use of this plant in treating microbial infections and show that *J. curcas* could be exploited for new potent antibiotics.

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STUDY OF CITRUS DISEASE PREVALENCE ON FOUR CITRUS VARIETIES AT THE NATIONAL INSTITUTE OF HORTICULTURAL RESEARCH (NIHORT) MBATO, OKIGWE, IMO STATE, NIGERIA

I.O. EZEIBEKWE[1]

Studies conducted to determine the prevalence of diseases on four varieties of Citrus, sweet orange (*Citrus sinensis)*, Tangelo (*Citrus paradise X Citrus reticulate*), grape (*Citrus paradise*) and lemon (*Citrus limon)* showed that tangelo and sweet oranges were more susceptible to fungal attack than grape and lemon. Disease symptoms commonly observed included dieback on the four species of citrus as follows: tangelo (65%), sweet orange (60%), grape (55%) and lemon (40%). Effective integrated management practices including use of resistance varieties are recommended.

Key words: Study, Citrus, Disease, Prevalence, NIHORT, Okigwe, Imo State, Nigeria.

INTRODUCTION

There has remained a persistent decline in *Citrus* growth and productivity associated with high level chlorosis, and shoot dieback at the National Institute of Horticultural Research, Mbato, Okigwe. There is therefore an urgent need to revamp the Citrus industry in Nigeria, in view of its numerous uses and demand for both local consumption and exportation.

Citrus, a tropical and subtropical crop, belongs to the family Rutaceae of the tribe Citrae. All the members are fruit bearing possessing juice filled vesicles known as hesparidium. They are thorny aromatic shrubs or small trees with leathery evergreen leaves. The white or purple flowers are often very fragrant (Fig. 1).

According to Okwulehie (1998), Citrus may have originated from the history of the cultivated species and cultivated from 15^{o}N–35^{o}S between sea level and 1000 m. They also require 100 cm of rainfall. Citrus plants include a large variety collection of Sweet orange (*Citrus sinensis*), Tangerine/Mandarin (*Citrus reticulate)*, Grape (*Citrus paradisi)*, Lemon (*Citrus limon)*, Lime (*Citrus auvantifolia)*, Pumelo (*Citrus grandis*).

Citrus is one of the most important fruit tree crops in Nigeria utilized for fresh consumption and for fruit industry. It surpasses all other fruits as raw materials in fruit drink industry.

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The total world population of Citrus is estimated at 36 metric tons with Nigeria producing 0.3 metric tons of the world production.

In spite of the high demand of Citrus fruits in Nigeria, its production level is low due to pests and diseases. Many microorganisms have been known to cause various diseases of Citrus trees, IITA (2003), NIHORT, (2003). These include many genera of fungi, bacteria and viruses. In the past five years or more, the decline of Citrus trees at NIHORT has drastically reduced the yield of these crops.

The symptoms of the decline include chlorosis of leaves, wilting, roots rots and diebacks. The canopy first thins out and as the diseases progress, the foliage turns yellow and permanent dieback of twigs occurs. Any new growth is weak and the affected trees may die quickly or make periodic attempts at regrowth (Bertus, 2002). Bertus (2002) showed that diseases of Citrus in New South Wales were caused by various species of the fungus *Phytophthora* that these diseases include root rot, collar rot and brown rot of fruits. He concluded that symptoms manifest first thinning out of the canopy and as the disease progresses, foliage turns yellow, permanent dieback of twigs occurs.

Most citrus trees are budded on a rootstock. Most of these rootstock are more resistant to fungal diseases than the scions (tops); so it is necessary to keep the bud union at least 4 cm to 5 cm above the soil line at planting especially in controlling *Phytophthora* disease. Budwood sources used by nursery men should be certified free from pathogen.

Some Citrus varieties are used as rootstocks in budding as they offer a good measure of resistance to pathogens when compared to other types of Citrus. Wutscher (1998) reported that with regard to variety reaction all sweet oranges and tangelo varieties, Mandarin and sweet orange hybrids were severely affected in some districts and did better in others. All varieties of Citrus stocks are not subject in the same degree of gum and foot rot disease (Brlansky, *et al*., 2003). The main objective of this study is to find out which of the varieties of Citrus under investigation is more susceptible or less resistant than the other. It is hoped that the result will enable the farmers better understand the varieties for proper handling and management.

MATERIALS AND METHODS

National Horticultural Research Institute (NIHORT) Mbato, Okigwe, Imo State is situated at longitude $7^{o}2^{ol}$E and latitude $5^{o}55^{ol}$N of Okigwe with mean annual range of rainfall 80-375mm; mean relative humidity of 79% and mean temperature of 22.7^{oC}–34^{oC} (NIHORT, 2003).

Surveys were conducted at the Citrus orchards of NIHORT on four varieties of Citrus, namely sweet orange, grape, tangelo, and lemon to assess the prevalence of diseases on the varieties.

Disease incidence was also assessed for the four varieties put together in four plots A, B, C and D according to year of planting, 1976, 1978, 1983 and 1988. Each variety occupied an area of 2,500 square meters with 100 variety crop on each plot on 7 × 7 meter gauge.

The experimental design was on Randomized Complete Block arrangement represented by the four different varieties of Citrus and four replications represented by the locations of the Citrus plantations. 30 marked crops of each variety were assessed for disease incidence and severity twice a year (during rainy and wet seasons) in three sampling years (2005, 2006 and 2007). The data recorded were the average of three determinations. Disease incidence was calculated as follows:

$$\text{Incidence} = \frac{\text{Total no. of diseased plants}}{\text{Total no. of plants observed}} \times \frac{100}{1}$$

While disease severity was calculated as an assessment using scales and ratings in percentage class infections on the citrus variety crops are as follows:

Class	**Range %**	**Scale**
0	0	Apparently healthy
1	0–25	8 – up to 25% affected
2	26–50	12 – up to 50% affected
3	51 – 75	18 – up to 75% affected
4	76 – 100	22 – up to 100 trees affected

$$\text{Severity} = \frac{\text{Sum of numerical ratings}}{\text{Total number of plants observed}} \times 100$$

Data collected in this study were subjected to analysis using percentages.

RESULTS

Dieback syndrome has largely devastated the orchards, Figs. 2, 3, 4 and 5. The symptoms of the decline include chlorosis of leaves, yellowing of leaves, wilting, root rot and dieback. Results of the field survey showed a high incidence of diseases. Symptoms are especially on sweet orange *60%) followed by tangelo (65%) and relatively lower in grape (55%) and lemon (40%), Table 1. The high percentage of disease severity followed the same trend as in disease incidence, Table 2.

The high percentage disease incidence in four plots by year of planting 1976, 1978, 1983 and 1988 of the four citrus varieties put together showed high disease occurrence in 1976 plot (77%), 1978 (67%), 1983 (63%) and 1988 (46%), Table 2.

Table 1

Incidence of diseases on varieties of *Citrus*

Variety	Incidence (%)
Sweet orange	60
Tangelo	65
Grape	55
Lemon	40

Table 2

Severity of diseases on varieties of *Citrus*

Variety	Severity (%)
Sweet orange	40
Tangelo	48
Grape	30
Lemon	22

Table 3

Disease incidence on the *Citrus* varieties in the sample plots

Year	% Incidence
1976	77%
1978	67%
1983	63%
1988	46%

Fig. 1. A Citrus tree.

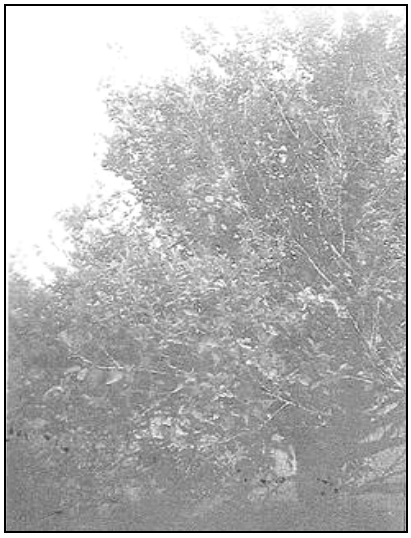

Fig. 2. Beginning of Citrus (Sweet orange).

Fig. 3. Advanced stage of Citrus.

Fig. 4. Citrus trees showing.

Fig. 5. Fully declined Grape tree.

DISCUSSION

The incidence of diseases was high in tangelo (65%), sweet orange (60%) as against 55% for grape and 44% for lemon. The percentage severity of disease symptoms showed the same trend with tangelo (48%), sweet orange (40%), grape (30%) and lemon (22%). The results showed that lemon may be more resistant to diseases followed by grape. This could be the reason why some species of lemon are used as root stocks in budding. The least resistant of the varieties observed was tangelo followed by sweet orange.

The observed differences in the incidence and severity of diseases among the varieties could have been due to different resistant capabilities in the *Citrus* species. This is in line with the work of Wutscher (1998) who worked on variety reactions of citrus showed variety resistance and also proved that fungi inoculated into seedlings were more pathogenic or caused more extensive diseases on *Citrus* previously infected or weakened by an agent than on healthy plants. Wutscher (1998) also said that reasons for differences in disease resistance between varieties of a single plant species remain largely obscure; that resistance often resides in physiological or biochemical differences between the resistant and susceptible varieties.

The percentage disease occurrence in the four sample plots 1976, 1978, 1983 and 1988 on the four *Citrus* varieties was the highest in 1976 plot (77%) and the least in 1988 plot (46%); it is likely that age could have contributed to the observable differences in percentage disease occurrences in the sample plots.

NIHORT (2003) showed that variety resistant studies at NIHORT farms indicated that disease resistance and susceptibility may be summed as follows: sweet oranges are not attacked by Citrus scab, but lemons, grape and tangelo are affected by the dieback and structural decline syndrome just like sweet oranges though grape and tangelo are not affected by scally bark, grape is easily attacked by shoot dieback.

On variety reaction, Fraser (1967) reported that all sweet orange and tangelo varieties and mandarin hybrids were severely affected by dieback. Grape fruit reaction was classed as severe. It was thought that root stocks in budding especially budding on rough lemon may have reduced damage caused by root rotting fungi rather imparting resistance to dieback.

In the light of the discussions, I recommend that apart from efficient management practices, farmers should plant more resistant varieties budded on more resistant stocks and adopt a comprehensive integrated approach to disease control involving biological, cultural and chemical agents.

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HERBICIDAL COMPARISON BETWEEN NATURALLY GROWING AND *IN VITRO* REGENERATED BISHOP'S

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Ammi majus is a medicinal plant which has allelopathic and autotoxic effect. It can affect the germination of other species and its germination. To study its herbicidal effect, it was used calluses and somatic embryos were obtained through culturing of explants on modified Murashige and Skoog (MS) solid medium supplemented with different concentrations and various combinations of N6-Benzylaminopurine (RAP) and α-Naphthaleneacetic acid (NAA). The optimum culture conditions of primary callus induction were obtained in the case of culturing hypocotyl explants on MS medium supplemented with 5.37 μM NAA and 2.22 μM RAP. The optimum culture conditions of somatic embryos induction were obtained in the case of sub-culturing the primary calluses on MS medium supplemented with 2.68 μM NAA and 1.11 μM BAP. The aqueous extract treatments showed highly significant differences among the most target species in the sense of seed germination and dry matter inhibition. The aqueous extract of primary callus was the most effective one. The aqueous extract effect of the regenerant plant was superior to the naturally growing one. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) protein profile revealed a little difference between micro-propagated plants and naturally growing ones.

Key words: *Ammi majus,* callus, embryogenesis, herbicides, medicinal plants, plant regeneration.

INTRODUCTION

Nowadays the level of sensibility to diseases is very high and people are being bombarded with thousands of unhealthy drugs. The use of medicinal plants can represent the best solution of this problem. (Barbour *et al*., 2004; Volker *et al*., 2001). *Ammi majus* L. (Apiaceae) is an important medicinal plant, commonly used as a cardiac tonic for treatment of angina, palpitations or weakness. It also used in treatment of wheezing or coughs (Ekiert and Gomółka, 2000; Pande et al., 2002). *A. majus*, as it is showing promise in cancer and AIDS therapy (El Gamal *et al*., 1993). *Ammi majus* seed set and germination is poor (Staniszewskaa *et al*., 2003). It has the potential to affect the germination of other species and itself (Pande *et al*., 2002). *Ammi majus* grows as a weed in the field crops and affects their yield (García *et al*., 2002). Its presence in the cultivated fields is not desirable (George *et al*., 2008). It competes with other species through "chemical warfare" by

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releasing chemicals inhibit the growth of its competitors (García *et al*., 2002). The secondary metabolites in cell culture are much higher than in intact plant (Ramachandra-Rao and Ravishankar, 2002). Embryos and regenerated shoots of *Ammi majus* produce an appreciable amount of these physiological active compounds (Hehmann *et al*., 2004). The possibility of use the naturally products as compounds exhibit selective allelopathic potential against weed species has received considerable attention (Abouziena *et al*., 2009). For this purpose, this study at reporting is a simple method for indirect regeneration of *Ammi majus* L. through somatic embryogenesis and shed some light on its allelopathic effect, to discuss the potential application of this approach in its economic value.

MATERIAL AND METHODS

Plant material preparation. Vegetative plants of *Ammi majus* L. were collected from the Nile River banks in Sohag Governorate, Egypt in August, 2008, while the weed species were collected from the Horticulture Research Station, Shandaweel, Sohag, Egypt. Plants were placed in paper bags and transferred to the laboratory. Plant material was air dried and kept until used. Seeds of *Ammi majus* L were soaked in water overnight, then they were surface sterilized by immersion in 70% (v/v) ethanol for 3 min, followed by soaking for 20 min in 1.5 (v/v) sodium hypochlorite and 3 rinses with sterile distilled water. Then they were placed in Petri dishes with MS (Murashige and Skoog, 1962) basal solid medium supplemented with 30 g.L-1 sucrose, without growth regulators and solidified with 1 % Difco Bacto agar. After that, they were incubated in darkness at 25±2°C for one week, and for another two weeks in a growth chamber with a 16-h photoperiod and an irradiance of 50 µE m-2 S-1.Three-weeks-old seedlings were used as source of explants. For all experiments of primary callus formation and Somatic embryos obtaining was used MS solid medium, supplemented with sucrose and growth regulators (auxins and cytokinins) and solidified with 1% Difco Bacto agar. The pH of the medium was adjusted with KOH or HCI to 5.8 before autoclaving.

Primary callus and somatic embryos obtaining. For primary callus formation, explants were inoculated into Petri plates contained with MS solid medium supplemented with different concentrations and combinations of NAA and BAP (Table 1). Then they were incubated in darkness at 25 ±2°C for 30 days. The experiment was conducted with 5 replicates per treatment. Each replicate contains 5 explants (total 25 explants). For somatic embryos induction, primary calluses were cultured on MS solid medium supplemented with different concentrations and various combinations of NAA and BAP (Table 2), then they were incubated for four weeks in a growth chamber with the same conditions of temperature (25±2°C) and photoperiod regime (16h/day) and irradiance intensity (50 µE m-2 S-1) as mentioned before. Each treatment contained 5 replicates with at least 5

explants/each. For plant regeneration Somatic embryos were transferred for germination and maturation on MS semi-solid medium supplemented with 0.02 NAA and 20 gL-1 sucrose for two weeks. Plantlets with well developed roots were transferred into plastic pots containing soil, sand and well rotted cow-dung manure in the ratio of 1:1:1 (w/v) and placed in greenhouse conditions for acclimatization for three weeks. About 82% of the plants survived in the greenhouse and grew normally. Then they were prepared for the experimental tests.

Allelopathic study. For lethal dose determination 10% tissue aqueous extracts (w/v) were used from each of primary calluses, regenerated plants and naturally growing plants, prepared according to EI-Khatib (2000). The aqueous extracts were sterilized by passing through 0.45 µMillipore filter (Wardle *et al*., 1993). The biological activity of these extracts was checked against the germination and dry matter production of weed species (*Convolvulus arvensis* L, *Imperata cylindrica* L, and *Sonchus oleraceus* L) as follows:

1. **Seed germination**. For seed germination test sterilized seeds were used as mentioned before. Ten seeds of each weed species were placed on Filter paper (Whatman No. 4) supplemented with 4 cm^3 of the aqueous extract in sterilized 9-cm Petri dishes (five replicates × plant species × extract type). Sterile distilled water was applied as control. The experiment was conducted under growth chamber conditions at temperature of 25 ± 2 °C with 170 µE m·2 s-1 photon flux density during an 11 h photoperiod. 2 cm^3 of sterile distilled water were added to the Petri dishes to keep seeds moist. Each treatment contained 5 replicates with at least 5 explants/ each. The experiment was conducted twice with the same number of replicates. The percentage of germination inhibition (PGI) of the treated seeds was calculated as follows: PGI = {100 – (% germination of treatment / % germination of control) × 100, where % of germination = (No of germinated seeds/ total number of seeds) × 100.

2. **Dry matter**. Pots of 25 cm diameter and 28 cm depth were filled with soils which have no significant differences in their physical and chemical characteristics. Soil collected shortly before the start of the experiments from the natural fields. All treatments were irrigated by the aqueous extracts except for those of control, which were irrigated by distilled water. During the course of the experiment, soil was kept under its field capacity. Seeds of *C. arvensis*, I. cylindrical and *S. oleraceus* were sown in Pots (three replicates × plant species × extract type). Then, they were kept in growth chamber conditions, at temperature of 25±2°C with 150 µE m-2 s-' photon of flux density during a 11 h photoperiod. Test was terminated when cumulative germination levelled off in all treatments. Plants were harvested after 30 days from sowing. The harvested plants were oven dried at 80 ^{0}C. Weighing was carried out for dry mass and expressed as “g/plant” Degree of inhibition was calculated as a relative percent to control, where % of DMI= Dry matter of the treated plant/Dry matter of control × 100.

Protein extraction. Leaves (1g) from both of regenerated plants and naturally growing plants was homogenized in liquid nitrogen, re-suspended in cold solution of 10% of trichloroacetic acid (TCA) in acetone with 1% ß- mercaptoethanol (ß-ME) keptat –20 °C overnight, centrifuged the next day and the pellet was re-suspended in cold acetone containing 0.1% ß-ME for 1 h. Pellet was dried lyophilizer for 30 min and stored at –20°C in aliquots.

SDS-PAGE. Were performed using 15 % acrylamide gel. Protein samples containing 40 µg of protein were mixed with an equal volume of buffer containing 0.125 M Tris-HC1, pH 6.8, 4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol and bromophenol blue as a tracking dye. The mixture was heated in a water bath (96 ^{0}C) for 90 s and loaded onto gel wells for electrophoresis (Bio Rad, Protean II XI Cell). Gels were run at 18 rnA per gel for 6 h at 4 DC in run buffer containing 0.025 M Tris.

Statistical analysis: Data were analyzed according to SAS (1996) using the L1 linear model.

RESULTS AND DISCUSSION

Primary callus formation. Production of callus was obtained successfully and rapidly in higher amount within two weeks. Most of cultures were nearly induced calluses. Calluses were appeared on the cut surface of hypocotyls explants as pustules with growth ranged from poor to good, subsequently cover the entire surface. Calluses were white to purple pigmented and friable in its appearance, later become highly pigmented within 15–20 days from culture initiation (Table 1). There was no sign of callus formation when explants were cultured on media lacked growth regulators. MS solid medium supplied with 5.37 µM NAA and 2.22 µM BAP was the most effective one, produced the best callus growth (192.2 mg/callus) and induced the highest percentage (86%) of callus induction. The results of this study are in agreement with those obtained by George and collaborators (2008) and Pande and collaborators (2002) who reported that relatively high levels of auxin and low level of cytokine stimulate the growth and development of callus in *Ammi majus* hypocotyls' explants.

Embryogenic callus obtaining. After 4 weeks from sub-culturing of friable transparent calluses on MS solid medium contained with different concentrations and various combinations of NAA and BAP, converted to embryogenic calluses. Embryogenic calluses appeared dark green and crystalline in its appearance. MS solid medium lacked growth regulators failed to induce embroids from primary calluses. The optimum culture conditions for somatic embryos induction was obtained in the case of sub-culturing primary calluses on MS medium supplemented with 2.68 µM NAA and 1.11 µM BAP. This medium was the most effective one, had the highest percentage (66.8%) of somatic embryos produced

calluses (Table 2), and associated with the highest number (11.2) of somatic embryos per callus. The results of this study are in agreement with those reported by Michael and Veit (2002) who pointed out that cytokines when used as a sole hormone for somatic embryogenesis are ineffective or only slightly promote young embryos (embryoids) growth. However, they promote growth differentiation of embryos when they combined with some auxins.

Table 1

Effect of growth regulators on primary callus induction from hypocotyls explants cultured on MS-solid medium supplemented with different concentrations and various combinations of NAA and BAP for four weeks. Two independent experiments with 5 replicates per treatment. Each replicate contains 5 explants .Data was represented as mean ±SD

Effect of growth regulators on primary callus induction								
NAA (µm)	0.0	2.69	5.37	2.69	5.37	5.37	9.89	
BAP (µm)	0.0	1.11	1.11	2.22	2.22	44.4	44.4	
% of explants developed callus	0.0	30±0.55	54±0.71	62±.0.55	86±0.84	44±0.71	38±0.84	
Callus dry weight (mg/callus)	00	22.8±5.81	54.2±5.36	77.6±5.46	192.2±7.43	76.6±3.91	104.4±5.36	

Table 2

Effect of growth regulators on somatic embryos induction from primary calluses cultured on MS-solid medium supplemented with different concentrations and various combinations of NAA and BAP for four weeks. Experiment was conducted with 5 replicates per treatment. Each replicate contains 5 explants. Data was represented as mean ±SD

Effect of growth regulators on embryogenesis of *Ammi majus* L. primary callus							
NAA (µM)	0·0	1.35	.351	2.68	2.68	2.68	
BAP (µM)	0·0	0·0	1·11	0·0	1.11	2 ·22	
% of calluses developed embryos	0·0	4±1.30	24±1.09	34±1.17	68±1.8	18±1.56	
No. of somatic embryos/callus	0·0	1.6±1.2	4.1±1.6	7.8±1.8	11.2±2.5	2.7±1.2	

Allelopathic study. The question now is what is the economic attitude of this technique? In this context, the present investigation aimed to measure the herbicidal effects of *Ammi majus* tissue culture in comparison to those of intact plant. This economical approach for using *A majus* allelochemicals as natural

herbicide emerged from the observation of García *et al*., (2002) who reported that *A. majus* is allelopathic and autotoxic plant. Nowadays the possibility of using the naturally products to control weeds has received considerable attention. These compounds from various plants exhibit selective allelopathic potenial against weed species (Abouziena *et al*., 2009). In comparison to control ones, the results (Fig. 1) clarified that, the aqueous extract of intact plant and regenerated one in addition to the aqueous extract of Primary Callus (undifferentiated tissue) clearly inhibited the seed germination of the weed plants (*C. arvensis*, *I. cylindrica* and *S. oleraceus*) which naturally invade the field and vegetables crops. Statistical treatment of the data exhibited highly significant differences among the target species in the sense of the percentage of germination inhibition (PGI) in most of treatments. The aqueous extract solution of primary callus was the most effective one. This result in agreement with those obtained by George and collaborators (2008) and Hehmann and collaborators (2004) who reported that secondary metabolites produced in cell culture in concentration much higher than those of intact plant. In some treatments particularly those of undifferentiated tissue (primary callus) delaying of the germination rate was observed. The results also clarified slightly high significant differences among the aqueous extract treatments of the regenerant plant and the naturally growing ones, that varies according to the plant treated (Fig. 1). The regenerant plant aqueous extract was superior in its effect to the intact plant aqueous extract. These results in agreement with those reported by Staniszewskaa and collaborators (2003) and Ekiert and Gomółka (2000) who pointed out that successful establishment of cell lines of *Ammi majus* capable of producing high yields of secondary metabolites depending upon culture media composition. The result of this study may be also is agreed with Wardle and collaborators (1993) and García and collaborators (2002) observation who observed that allelopathic seed germination inhibition may be the consequence of inhibition of water uptake through alters the activity of enzymes responsible for the mobilization of storage compounds during germination.

Dry matter. In this concern, I would like to refer to the role of *Ammi majus* allelochemicals on retarding growth of the plant species in the different life forms of the treated plants. Comparison was made only with control ones in the sense of dry matter inhibition. Statistical treatment of the data exhibited highly significant differences among the target species in the sense of dry matter inhibition (DMI) in most of plant species treatments. The dry matter contents of treated plant species showed lower decreasing in their dry matter contents compared with control ones. The primary callus aqueous extract was the most effective one. In most of the aqueous extract treatments the dry matter contents of roots, stems and leaves of the target species were affected compared with control ones (Fig. 2a). Many

observations were reported in the growth retardation by callus aqueous extract (Hehmann *et al*., 2004; Ramachandra-Rao and Ravishankar, 2002) who reported that secondary metabolites produced in cell culture in concentration much higher than those of intact plants. They attributed this to the somaclonal variation which takes place during the successive cell divisions of callus resulting in multiplication of chromatin groups leading to the increment of gene expression responsible for the production of allelochemicals. The regenerant aqueous extract (Fig. 2b) was superior in its effect to the naturally growing one. Since the dry matter of roots, stems and leaves showed highly decreasing in their dry matter contents compared with those of the naturally growing ones (Fig. 2c). The results of this study are in agreement with those reported by Sidwa-Gorycka and collaborators (2003) who pointed out that variety of secondary metabolites produced in embryos and regenerated shoots of *Ammi majus* in much higher concentration than those of the intact plant. Also the result of this study is agreed with those obtained by Staniszewskaa and collaborators (2003) who reported that producing of high yields of secondary products in *Ammi majus* depending upon the composition of culture media and the environmental conditions of development.

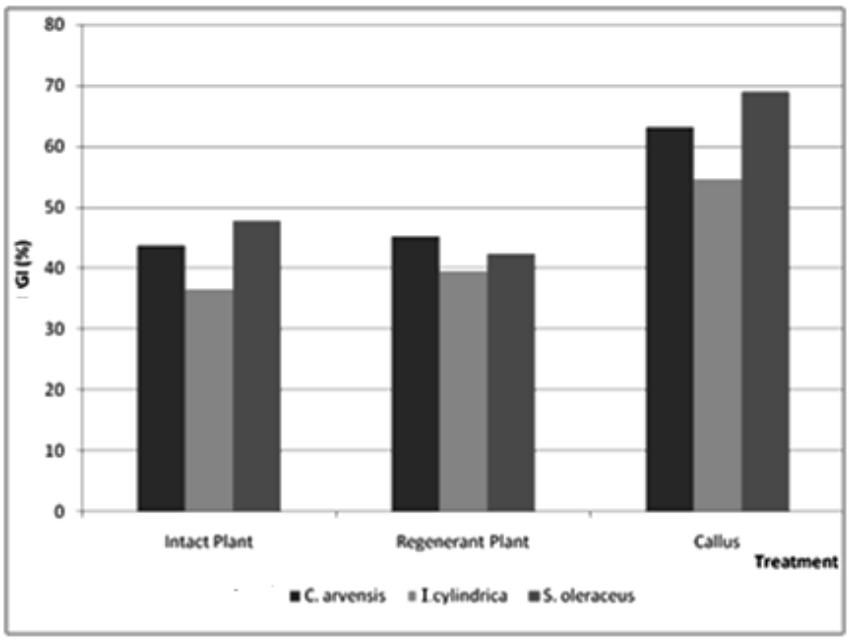

Fig. 1. Percentage of germination inhibition (PGI) of the target species treated with aqueous extract of intact plant, regenerant plant and primary callus of *Amim majus* L. The experiment was conducted twice with five replicates x plant species x extract type. Data represent means, followed by LSD test at $\alpha < 0.01$.

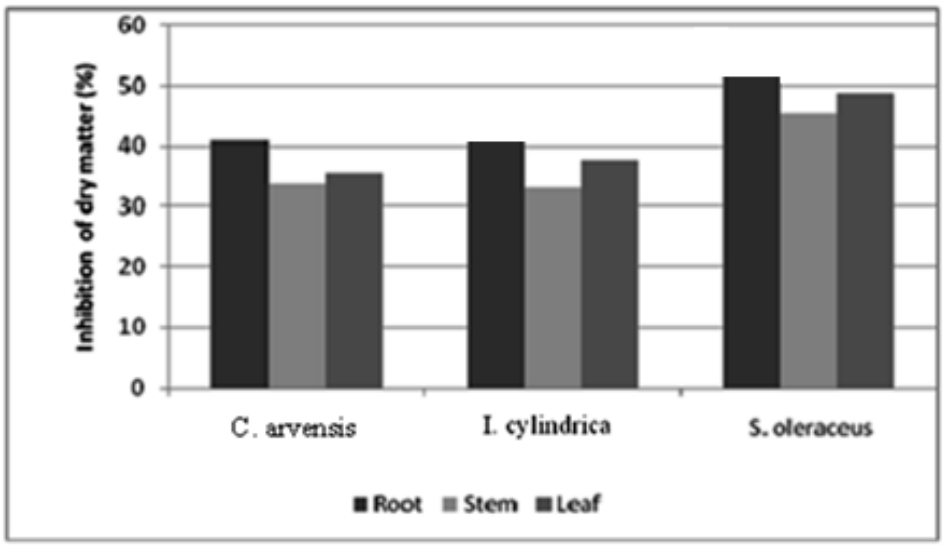

Fig. 2a. Dry matter inhibition (DMI) of the treated species with the aqueous extract of primary callus of *Ammi majus*. The experiment was conducted twice with three replicates x plant species x extract type. Data represent means, followed by LSD test at $\alpha < 0.01$.

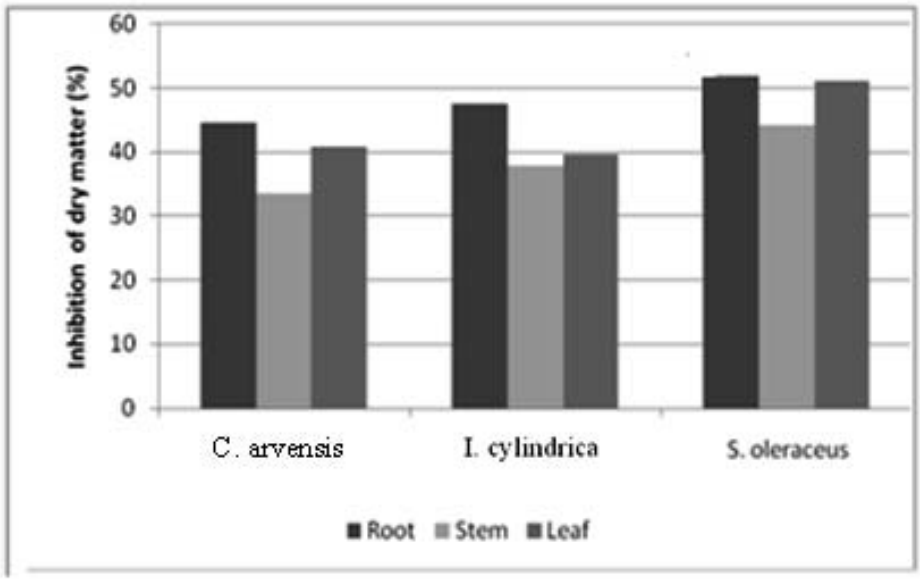

Fig. 2b. Dry matter inhibition (DMI) of the treated species with the aqueous extract of regenerant plant of *Ammi majus*. The experiment was conducted twice with three replicates x plant species x extract type. Data represent means, followed by LSD test at $\alpha < 0.01$.

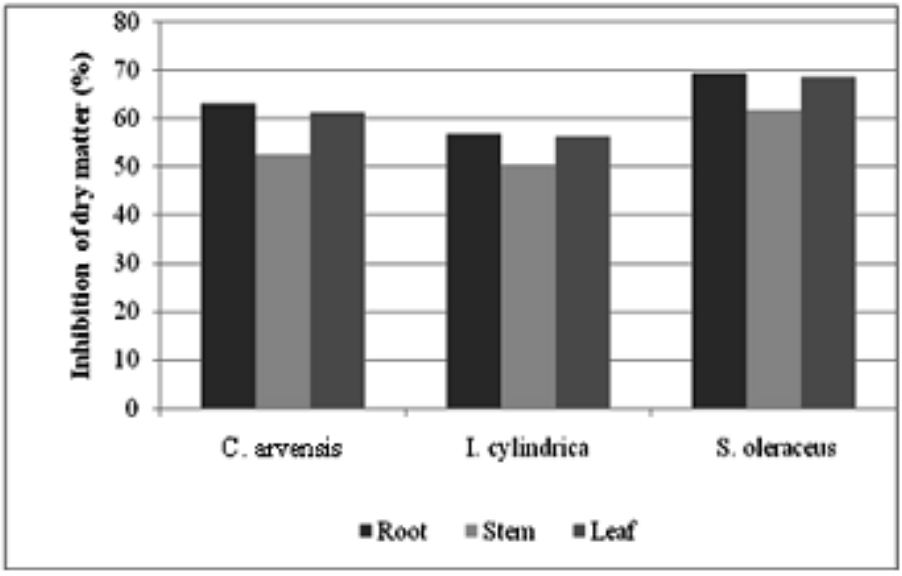

Fig. 2c. Dry matter inhibition (DMI) of the s treated pieces with the aqueous extract of naturally growing plant of *Ammi majus*. The experiment was conducted twice with three replicates x plant species x extract type. Data represent means, followed by LSD test at $\alpha < 0.01$.

***Analysis of gel electrophoresis*.** Analysis of gel electrophoresis for Soluble protein profile of naturally growing and regenerant plants, which separated by one-dimension SDS-PAGE, revealed a few major differences among the protein profile of the tested plants of *Ammi majus* genotypes (Fig. 3). The Protein profile of regenerant plant had one strong band of 32 kDa, which did not appeared in the protein profile of the naturally growing plant. It was believed that protein patterns of the regenerated plant and intact plant are stable in the course of regeneration through embryogenesis in diploid species (Vits *et al*., 1992; Chi *et al*., 1994), who reported that micropropagation through somatic embryogenesis provides an efficient means of producing a large number of elite or transgenic plants) have the same genetic makeup. The results of this study are agreed with those obtained by Staniszewskaa and collaborators (2003) who reported that protein patterns of the regenerant plant and intact plant of *Ammi maju* have few differences in their protein profile when separated by one-dimension SDS-PAGE. The result of this study is agreed with those obtained by George and collaborators (2008) who attributed the difference to the composition of culture media in the case of regenerant. This observation is agreed with those results obtained by Abouziena and collaborators (2009) who reported that high yields of secondary compounds depend upon the composition of culture media. In conclusion the results of this study proved that the regeneration of *Ammi majus* through somatic embryogenesis

provides an efficient method for its micropropagation. The production of a large number of plants which have somatic embryo's development offer an opportunity to uncover the natural variability in plants, and we can use this genetic variability for increasing its natural products.

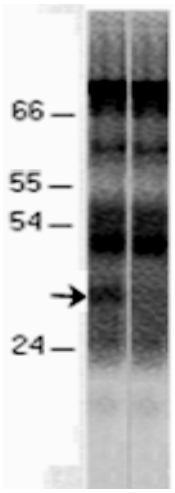

Fig. 3. Protein pattern in leaves of regenerant (lane 1), and naturally growing (lane 2) plants grown for four weeks under. Proteins were separated on 15 % polyacrylamide gel and stained by Comassie blue. Molecular mass distribution is indicated on the vertical axis. The arrow (→) indicates the protein band with a molecular weight 32 kDa.

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PHYTOCHEMICAL SCREENING OF *PLEUROTUS TUBERREGIUM* (Sing) GROWN ON DIFFERENT SUBSTRATES

C.I. ONUOHA*, G.A. OBI-ADUMANYA, I.O. EZEIBEKWE

The Sclerotia of *Pleurotus tuberregium* (Sing) were grown on four different substrates namely: humus soil, mixture of Sawdust and humus soil, sawdust and shreds of the wood of *Treculia africana*. The quantity and quality of the fruit bodies produced were measured using the following parameters: number of fruit bodies produced, height of fruit bodies, fresh weight, dry weight, diameter of pileus and length of the stipe. Sawdust which served as the control was better growth medium in terms of length of stipe, number of fruit bodies and height of mushroom. The fruit bodies from a mixture of sawdust and humus soil were better in terms of fresh and dry weight while humus soil alone produced fruit bodies with wider pileus diameter. The wood shreds of *Treculia africana* did not support the growth of the mushroom. The phytochemical composition of the sclerotium and fruit bodies from the different substrates showed that the sclerotium had more flavonoids, alkaloids, tannins, saponins and phenols than the fruit bodies from the different substrates. There was no significant difference between the alkaloid, tannin, saponin and phenolic composition of the fruit bodies from the different substrates. There was, however, a significant difference at $P \leq 0.05$ between the flavonoid content of fruit bodies from humus soil, sawdust and those from a mixture of humus soil and sawdust.

Key words: Phytochemical, screening, *Pleurotus tuberregium*, different substrates.

INTRODUCTION

Mushrooms are a group of fleshy macroscopic fungi which, recently, as other fungi were introduced into the plant kingdom of cell wall and spores. Mushroom has been valued and treated throughout the world as a special kind of food and medicine for thousands of years (Lindquist *et al*. 2005, Tribe *et al*. 1973).

There are many varieties of mushroom of which pleurotus' are characterized by a white spore print, attached to gills, often with an eccentric stipe or no stipe at all. They are commonly known as "Oyster mushroom" (Miles *et al.* 1997). *Pleurotus tuberregium* is a tropical sclerotial mushroom which has been gaining some interest in the United States. Being sclerotial, the mushroom produces sclerotium, or underground tuber, as well as a fruiting body. Both the sclerotium and the fruiting body are edible. The mushroom when matured, the cap curves upwards to form a cup-like shape. The sclerotium is spherical to ovoid and can be

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quite large-up to 30 cm (11.8 inches) or larger in diameter (Oso; 1977). It is dark on the outside and white on the inside. The sclerotium is often formed during unconducive environmental condition underground in form of a tuber.

Mushrooms are highly nutritious so they contain good quality proteins, vitamins and mineral (Khanna, *et al*. 1984, Flegg, *et al*. 1976). Mushrooms are low calorie food with little fat and are highly suitable for obese persons with no starch and very low sugars, they can serve as medicinal food for diabetic patients (Bano, 1976).

Edible mushrooms like *Pleurotus* are known to be among the largest of all fungi and so they are said to exist in a natural habitat in a mycorrhyzal relationship with a tree, and this is one of the reasons why forests are often generous to mushroom hunters. Ogundana (1978) and Stemets (2001) observed that the fungus is often found growing around the African bread fruit tree (*Treculia africana*). It attacks dead woods, on which it produces globose or ovoid sclerotia (Oso, 1977, Fasidi and Olorunmaiye, 1994).

Despite its nutritional value, mushroom cultivation is not widespread; many mushrooms are considered to be healthy food because they contain large enough protein needs of the rural poor especially during the rains. It is also rich in some essential vitamins (B_1, B_2, C) and essential minerals than most plants. They also have a low fat content and hence a high fibre content that enhances digestion of food. They have some medicinal properties as in *Pleurotus tuberregium* (Sing), are used to treat heart problem in the eastern part of Nigeria, especially among the Igbos and Edos, and in the treatment of asthma, cough and obesity (Isikhuemhen *et al*., 2000).

During the rainy seasons, different species of both edible and non-edible species usually grow on various natural substrates such as garden soil, decayed wood, termite nest, palm wastes, leaf litters, under the shade provided by cocoa, teak, coffee and rubber plantations. People in the villages (mushroom hunters) usually wake up early in the morning to look for wild edible mushroom. The collected edible species are usually sorted out, cooked or sold in the local markets. This experience of mushroom boom from the wild occurs once or twice a year and disappears within a month. During that period, the price would drop due to increase in supply only for a short period. At the end of this boom, mushroom ceases to be seen anywhere again despite the continual demand. But this important plant can be grown like any other crop, *i.e.*, like business thereby being available throughout the year.

Therefore, there is the need to ensure a balanced daily diet through the consumptions of *Pleurotus tuberregium* which is also used in hotels as flavonoids and in preparation of different kinds of delicacies and to improve businesses and income of Nigerians, there is a need to grow and cultivate this mushroom in commercial quantities outside its natural habitat using different substrates. Our forest areas are fast disappearing due to deforestation which has further reduced the quantities of mushrooms produced.

Therefore, the objectives of this study are: i) to screen some substrates such as sawdust mixture of sawdust and Humus soil, Humus soil and shredded pieces of *Treculia africana* wood on the growth of *Pleurotus tuberregium*; (ii) phytochemical screening of the grown mushroom from the above mentioned substrates and that of the sclerotium of *Pleurotus tuberregium.*

MATERIALS AND METHODS

This work has two parts. The first part has to do with the growing of the mushroom from its sclerotia on four different substrates while the second part has to do with phytochemical screening of the sclerotia and the cultivated mushrooms from the four different substrates.

The laboratory experiment was carried out at the plant science and biotechnology laboratory in Imo State University, Owerri. *Pleurotus tuberregium* was the species selected. The sclerotium of this species was sourced from Owerri old market. The materials used as substrates within were sourced within Imo State and they include:

1) Saw dust
2) Humus soil
3) Humus soil and saw dust
4) Shreds of *Treculia africana* wood.

Preparation of substrates for cultivation. The single substrates, *i.e.* saw dust, Humus soil and chopped wood of *Treculia africana*, and a mixture of Humus soil and saw dust was mixed on ratio 1:1 bases. Then the four substrates were sprinkled with distilled water moderately and the water content tested by pressing the substrates with hand to allow water drip. It was tested and it dripped, showing there was moderate water. The substrates were packed in polythene bags and then put into an autoclave for sterilization. After heating at 121 ^{o}C for 15 minutes, the substrates were allowed to cool.

1,000 g (1 kg) of each of these substrates was weighed on a weighing balance and poured in three (3) replicates into the Nursery polythene, all together there were twelve (12) replicates. The sclerotium of *Pleurotus tuberregium* was cut into miniset of 4cm^2 and then planted on each replicate, while the relative humidity was maintained at 75% to 80% required for fructification of the mushroom.

Data collection. The growth performance of *Pleurotus tuberregium* on the different substrates was determined by recording the number and size of fruiting bodies after sprouting. Measurements from these various replicates were added together and their mean recorded.

Statistical analysis. At the end of the experiment fruiting bodies of *Pleurotus tuberregium* were harvested at maturity and the following parameters were measured as follows: [1] Total number of fruiting bodies: This was done by counting the number of fruiting bodies on each substrate, [2] Height of fruiting bodies: This was measured in centimeters using a ruler from the base of the stipes to the pileus, [3] Diameter of the pileus: This was measured in centimeters with a ruler from one edge of the pileus across the stipe to the other edge, [4] Fresh weight of fruiting bodies after harvesting: This was weighed using an electronic weighing balance, [5] Dry weight of fruiting bodies: This was weighed using a weighing balance after oven drying, at 121 ^{o}C for 15 minutes and [6] Length of stipe: This was measured using a ruler in centimeters from the base of the pileus to the point where it was harvested at the base. Obtained results were recorded and subjected to statistical analysis using one-way ANOVA.

Phytochemical screening of grown mushroom. This involved phytochemical screening of both the sclerotia and the grown mushroom of *Pleurotus tuberregium* produced from the various substrates of sawdust,humus soil, humus soil and sawdust and shredded wood of *Treculia africana*. The analysis was carried out at the central laboratory unit National Crop and Root Research Institute, Umudike, Umuahia in Abia State.

Source of sample. The sample of the sclerotia of *Pleurotus tuberregium* was bought from Owerri Old Market while the sample of the mushroom of *Pleurotus tuberregium* was grown from different substrates.

Preparations of samples. The sclerotia sample bark was shredded and placed in an electric oven at 65^{o}C to dry for two hours while the fruit bodies which were already oven dried were grated into powder.

RESULTS

Ten days after planting the sclerotia, it was observed that white mycelia had colonized all the substrates. Fruit bodies were first observed on the humus soil, four days later. Two days after it was observed on the mixture of sawdust and humus soil and three days later on sawdust. The fruit bodies never grew on *Treculia africana* wood shreds. Within five days the fruit bodies were finally mature and harvested. Table 1 below shows the values of various parameters measured.

Table 1

The means and standard deviation values of the diameter of the pileus, length of stripe, number of fruit bodies, height of the mushroom, fresh weight and dry weight of the mushrooms from the various substrates used

Substrates	Diameter of the pileus (cm)	Length of the stipe (cm)	Number of fruit bodies	Height of the mushroom (cm)	Fresh weight (g)	Dry weight (g)
1. Saw Dust	7.74 ±2.18	6.72 ±1.44	2	9.94 ±3.09	7.57 ± 3.79	2.12 ± 1.10
2. Humus Soil	8.65 ± 1.75	5.83 ± 0.47	1	6.92 ±1.13	5.95 ± 3.28	2.12 ± 0.93
3. Mixture of Humus Soil and Saw Dust	5.23 ± 1.53	4.83 ± 0.77	1	8.12 ± 0.14	8.36 ±0.93	2.38 ±0.18
4. *Treluilia africana* wood shreds	–	–	–	–	–	–

Phytochemical analysis. The results of the phytochemical analysis of various mushroom grow from the various substrate and that of the sclerotium. Where: **Specimen A:** represents the mushroom grown from humus soil; **Specimen B:** represents the mushroom grown from a mixture of humus soil and saw dust.

Specimen C: represents the mushroom grown from sawdust and **Specimen D:** represents the sclerotium of *Pluerotus tuberregium*.

Table 2 shows the mean values and their standard deviation of the percentage presence of alkaloid, flavonoid, tannin, saponin and phenols of the various specimens. The (sclerotium) contained the highest percentage of alkaloid with 501% while specimen A (mushroom got from the humus soil) contained the lowest yield with 0.55% for the flavonoid, specimen D also had the highest percentage with 2.11% while specimens A and C (mushroom got from humus soil and respectively sawdust) had the lower percentage with 0.08%. In Tannin, specimen D had the highest percentage with 1.84% while specimen C had the lowest percentage of 0.12%. For saponin, specimen D still had the highest percentage with 4.98, while specimen A had the lowest percentage with 0.12%. Phenols had specimen D also with the highest percentage of 0.42%, while specimen A had the lowest percentage of 0.06%.

Table 2

Percentage of phytochemicals in the mushrooms investigated

Specimen		Alkaloid (%)	Flavonoid (%)	Tannin (%)	Saponin (%)	Phenols (%)
A	Fruit body from Humus soil	0.55 ±0.01	0.08±0.02	0.14±0.00	0.12±0.02	0.06±0.00

Table 2

(continued)

Specimen		Alkaloid (%)	Flavonoid (%)	Tannin (%)	Saponin (%)	Phenols (%)
B	Fruit body from mixture of Humus soil and saw dust	0.60 ±0.02	0.12±0.01	0.16±0.00	0.14±0.02	0.08±0.00
C	Fruit body from saw dust	0.66 ±0.02	0.08±0.03	0.12±0.00	0.18±0.02	0.08±0.00
D	The Sclerotium	5.01±0.14	2.11±0.03	1.84±0.00	4.98±0.03	0.42±0.00

DISCUSSION

In the work, firstly, the sclerotia of *Pleurotus tuberregium* were grown on four different substrates, namely Humus soil, a mixture of sawdust and humus soil, sawdust and *Treculia africana* wood shreds. Only three of the substrates: humus soil, mixture of humus soil and sawdust and then sawdust, were observed to be good substrates for the growth of mushrooms.

Fruiting bodies got from the substrates that supported the growth of the mushroom were measured qualitatively using some parameters such as diameter of pileus, length of stipe, number of fruiting body, height of the mushroom, fresh weight of mushroom and its dry weight. All these parameters were subjected to statistical analysis using ANOVA.

Actually out of the six parameters measured in this work for the quality of the fruit bodies produced from various substrates, those produced from sawdust had more significance than others in three of the parameters (length of stipe, no. of fruit bodies and height of the mushroom), which shows that fruitbodies from this substrate could attract high market value as the fruit bodies are usually sizeable and this also agrees with (Candy, 1990), Kadiri and Fasidi (1990) and Okwujiako (1992) that sawdust is a good growth medium for *Pleurotus tuberregium*. Fruit bodies from humus soil produced larger pileus while fruitbodies from the mixture of humus soil and sawdust had higher fresh weight and dry weight. Therefore, fruit bodies from humus soil could also attract a high market value because of the size of its pileus.

In the order of fructification, mushrooms from the humus soil fruited first, followed by a mixture of humus soil and sawdust and lastly by mushrooms from sawdust. Fruit bodies from humus soil were able to fruit first perhaps because of

the richness of the soil in nutrients and organic matter which are necessary for the formation of fruit bodies as conduced by Chang and Buswell (1996).

Treculia africana wood shreds did not encourage the growth of the mushroom. This could be as a result of low absorption of water by the wood shreds since a humid environment favoured the fructification of the fungus. The mushroom is said to traditionally grow near and on the wood of *Treculia africana* and so it could be that the fungus derives some other nourishment from the soil around the *Treculia* wood which only *Treculia* wood shreds alone could not provide.

Furthermore, the mushrooms produced from various substrates were compared along side with the sclerotium of *Pleurotus tuberregium* as they were phytochemically analysed and their results subjected to statistical analysis using one-way ANOVA at $P < 0.05$.

In the phytochemical analysis the sclerotium showed high significance in the presence of all the chemicals (flavonoid, alkaloid, tannins, saponins and phenol) when compared with the mushrooms produced from the three other substrates. These show that the sclerotium of *Pleurotus tuberregium* being very rich in all the phytochemicals mentioned above could serve as a source from which industries ask for precursors for the synthesis of complex chemical substances (Moore 2003). This finding also agrees with Moore, (2003) who stated that the phytochemical found in the sclerotium of *Pleurotus tuberregium* promotes the function of the immune system, acts directly against bacteria and viruses, reduces inflammation and is associated with the treatment and prevention of cancer, cardiovascular disease and any other malady affecting the health or well being of an individual.

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ESTIMATION OF GENETIC VARIABILITY, HERITABILITY AND GENETIC ADVANCE IN AROMATIC FINE GRAIN RICE

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A study on genotypes was conducted in Randomized Block Design in three replications under rainy condition during 2010. The data were recorded for 15 qualitative characters viz., days to 50% flowering, number of tillers per hill, number of panicles per m^2, number of spikelets per panicles, panicles length, flag leaf length, flag leaf width, plant height, days to maturity, grain yield per plant, grain yield per plot, biological yield, harvest index and test weight to study genetics variability, heritability and genetic advance. Analysis of variance among 41 genotypes showed highly significant differences for yield contributing traits viz., number of panicles per m^2, plant height, days to 50% flowering, days to maturity, biological yield harvest index, number of spikelets per panicles. Grain yield per plant, panicles length, flag leaf length, number of panicles per hill, number of tillers per hill, test weight grain yield per plot and flag leaf width indicated the presence as substantial amount of genetic variability in study material and there is scope for selection. On the basis of mean performance yield traits it was conducted that "NDR-9542" was best performer for yield. The results showed higher GCV and PCV in parameters like grain yield per plot, flag leaf width, biological yield, days to 50% flowering and plant height. Maximum heritability was encountered for characters viz. plant height, days to 50% flowering, days to maturity, panicles length and number of panicles per hill. High genetic advance observed in number of panicles per m^2, plant height and days to 50% flowering. Thus these characters could be improved by selection in breeding programme for crop improvement.

Key words: Genetic variability, heritability, genetic advance, coefficient of variation, aromatic rice (*Oryza sativa* L).

INTRODUCTION

Rice (*Oryza sativa* L.) is principal food crop of South-Eastern countries and supports nearly one half of the world population. It holds the key for the food security and prosperity. India is a natural repository for long and short-grained aromatic rices, which are conserved by the formers over centuries. It is the primary staple food crop throughout Asia and other parts of the world. Today the demand

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for increase in productivity and quality of rice in available marginal land is very high. It is leading food source in terms of colonies being consumed for mankind and feed about 60% of the world's population (FAO, 2007). India share in the world 21.6% in rice production, China 1st and India 2nd position in rice production in the world. India has the area under rice 43.77 m.ha. and production 96.43 m.ha tonnes and in the world rice production 63.5 m.tonnes. (Agricultural statistics at a Glance 2008). Thus, there is a challenging need to improve rice yield to meet the growing demand. During the past two decades, significant progress has been made in increasing the yield and other qualities.

A well planned plant-breeding programme for developing high yielding genotypes requires complete knowledge on the genetic variation available in the population. In all these stages, estimation of genetic variability, heritability and genetic advance is necessary. Yield is a complex character being governed by a large number of cumulative and dominant genes and highly influenced by environment. This necessitates thorough knowledge variability during the genetic factors, actual genetic variation heritable in the progeny and the genetic advance that can be achieved through selection. Moreover, heritability estimate along with genetic advance was more useful than heritability estimate alone in predicting resultant effect for the selection of the best individual form of segregating population.

MATERIALS AND METHODS

Seeds of rice were obtained from Directorate of Rice Research, Hyderabad, A.P., India. Aromatic fine grain observational nursery consisting of 41 entries aromatic rice was grown in randomized block design with three replications each with plot size of 7.5 m^2 at Field Experimentation Center of the Department of Genetics and Plant Breeding, Sam Higginbottom Institute of Agriculture, Technology and Sciences, Deemed to-be University, Allahabad during Kharif (June–November 2009). The recommended standard culture practices were followed to raise the crop. Data on days to 50% flowering, number of tillers per hill, number of panicles per hill, number of panicles per m^2, number of spikelets per panicles, panicles length, flag leaf length, flag leaf width, plant height, days to maturity, grain yield per plant, grain yield per plot, biological yield, harvest index, and test weight were recorded following standard evaluation system or method. The mean value was subjected to analysis of variance to test the significance for each character as per methodology advocated by Panse and Sukhatem (1967). The estimates of phenotypic and genotypic coefficient of variation were classified as

low, medium and high (Sivasubramanian and Madhavamenon, 1973) (less than 10% = low, 10–20% = moderate, greater than 20% = high). Heritability (broad sense) estimates for yield component of rice were worked by Burton and De Vane 1952. The estimation method of genetic advance was by given by Johnson *et al*. (1955).

Table 1

ANOVA for different characters in 41 aromatic rice short grain genotypes

S. No.	Characters	Mean sum of squares		
		Replication (d. f. = 2)	Genotypes (d. f. = 40)	Error (d. f. = 80)
1	Days to 50% flowering	36.32	394.00*	8.03
2	No. of tillers / hill	1.03	2.34*	0.31
3	No. of panicles / hill	0.67	3.57*	0.19
4	No. of panicles / m^2	12.13	1076.76*	75.36
5	No. of spikelet / panicles	18.06	42.05*	11.60
6	Panicles length (cm)	0.48	8.39*	0.40
7	Flag leaf length (cm)	0.24	7.56*	1.66
8	Flag leaf width (cm)	0.16	0.06*	0.01
9	Plant height (cm)	4.39	462.71*	2.29
10	Days to maturity	34.76	266.97*	5.96
11	Grain yield / plant (g)	65.34	10.08*	3.700
12	Grain yield / plot (kg)	0.02	0.46*	0.05
13	Biological yield (g)	117.95	195.3*	31.15
14	Harvest index (%)	16.73	51.17*	16.74
15	Test weight (g)	1.86	1.18*	0.75

* As significant at 5 % level of significance

Abbreviations: No. – Number, g – gram, kg – kilogram, cm – centimeter, % – Percentage, d.f. – Degree of freedom.

Table 2

Estimates of Variability, Heritability and Genetic advance in Aromatic Rice

S. No.	Characters	VG	VP	GCV	PCV	h^2 (%)	GA
1	Days to 50% flowering	128.66	136.68	11.68	12.04	94.1	22.67
2	No. of tillers/hill	0.68	0.98	4.29	5.16	69.0	1.41
3	No. of panicles/hill	1.13	1.32	6.49	7.02	85.3	2.02
4	No. of panicles/m^2	333.80	409.16	6.68	7.40	81.6	33.91
5	No. of spikelets/panicles	10.15	21.75	2.19	3.21	46.7	4.48
6	Panicles length (cm)	2.66	3.07	6.13	6.58	86.8	3.13
7	Flag leaf length (cm)	1.97	3.63	4.54	6.16	54.3	2.13
8	Flag leaf width (cm)	0.02	0.02	10.62	12.89	67.9	0.21
9	Plant height (cm)	153.44	155.83	11.18	11.27	98.5	25.32
10	Days to maturity	87.00	92.96	7.89	8.15	93.6	18.59
11	Grain yield/plant (g)	2.13	5.83	2.57	4.26	36.4	1.81
12	Grain yield/plot (kg)	0.14	0.19	14.65	17.33	71.5	0.64
13	Biological yield (g)	54.73	85.87	9.86	12.35	63.7	12.16
14	Harvest index (%)	0.17	1.55	1.33	4.04	10.8	0.28
15	Test weight (g)	0.14	0.89	1.87	4.66	16.1	0.31

Abbreviations: g – gram, kg – kilogram, cm – centimeter, VG – Genotypic Variance, VP – Phenotypic Variance, PCV– Phenotypic Coefficient of Variation, GCV – Genotypic Coefficient of Variation, GA – Genetic Advance, h^2 – Heritability.

RESULTS AND DISCUSSION

The analysis of variance revealed highly significant differences among the genotypes for the entire test characters, indicating the high variability among the varieties. Thus this ample scope for selection of different quantitative characters for

rice improvement. The estimates of phenotypic and genotypic variance were obtained for different characters and they are presented Table 1. Estimates of phenotypic and genotypic variance revealed that the number of panicles per m^2 exhibited the highest phenotypic and genotypic variance followed by plant height, days to 50% flowering, days to maturity, biological yield and harvest index. Phenotypic variance was higher than genotypic variance for all characters of yield and yield contributing characters indicated the influence of environmental factors of these traits on the basis of mean performance of yield and yield contributing traits, NDR 9542 was the highest yielder followed by IR77512-2-1-2-2, WAS 197-B-4-1-25 and they are presented in Table 2.

The phenotypic coefficient of variation (PCV) and the genotypic coefficient of variation (GCV) were of the same magnitudes for all the characters, indicating the least effects of environmental factors.

High genotypic coefficient of variation and the the phenotypic coefficient of variation were observed for grain yield / plot, days to 50% flowering and plant height. These are in confirmation with the earlier finding of Chaubay and Singh (1994). Genetic coefficient of variability along with heritability gave an idea of selection (Burton, 1952). The character, which exhibited high heritability and genetic advance, indicated the broad sense of additive gene effects in its inheritance and such characters could be improved by selection (Panse and Sukhatme, 1967). In the present study, almost all the characters though had high heritability values but plant height, days to 50% flowering, days to maturity, panicles length and number of panicles per hill, suggesting preponderance of non-additive gene action in the inheritance of these traits. Similar results were also reported by Mehetre *et al.* (1996).

High heritability coupled with high genetic advance number of panicles/m2, plant height, days to 50% flowering, days to maturity and biological yield. The high heritability with high genetic advance observed for these characters might be due to additive gene effects. Hence, selection in the segregating generation would be very effective for them. Similar results were also reported by (Kavith and Reddy, 2002) and Kumar et al. (2007).

Therefore, selection based on the characters number of panicles per m^2, plant height, days to 50% flowering, days to maturity, panicles length, biological yield, number of panicles per hill, grain yield / plot, number of tillers per hill and number of spikelets per panicles having high heritability coupled with high genetic advance may bring about the desired improvement in yield.

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CORRIGENDUM

The reader is kindly asked to consider the following tables and figure of the paper "Response of temperature and pH on the growth and biochemical changes in *Spirulina platensis*" [ROM. J. BIOL. – PLANT BIOL., VOLUME 56, No. 1, P. 37-42].

Table 1

Biochemical composition of *Spirulina platensis* grown at different temperature

Temp (^{o}C)	Protein (% DW)	Lipid (% DW)	Carbohydrate (%DW)
25	57.0±0.83	8.0±0.25	23.8±0.23
28	58.3±0.30	7.1±0.10	22.6±0.2
32	61.1±0.12	8.4±0.05	23.0±0.1
35	55.2±0.27	9.2±0.11	25.3±0.1
37	48.6±0.31	10.4±0.10	28.1±0.1

Mean of five determinations +SD Temp, Temperature

Table 2

Photosynthetic pigments of *Spirulina platensis* grown at different temperature

Temp (^{o}C)	Chlorophyll-a (Mg g^{-1})	Carotenoids (Mg g^{-1})	Phycocyanin (Mg g^{-1})	Allophycocyanin (Mg g^{-1})	Phycoerythrin (Mg g^{-1})
25	12.6±0.1	1.25±0.005	134.1±0.2	53±0.2	9.2±0.15
28	13.3±0.05	2.31±0.01	132.0±0.34	48±0.1	7.4±0.2
32	16.1±0.15	1.05±0.005	128.2±0.2	39±0.15	9.7±0.05
35	15.0±0.11	1.0±0.0	123.1±0.1	32±0.3	4.4±0.1
37	10.6±0.1	1.0±0.0	95.6±0.5	23±0.36	3.5±0.11

Mean of five determinations +SD, Temp, Temperature

Table 3

Biomass production in *S. platensis* under different conditions

	pH	Temperature (^{o}C)			
	25	28	32	35	37
	Biomass g/L				
8	2.31	2.60	3.20	3.31	1.83
9	2.50	2.82	4.10	4.13	3.20
10	3.00	3.02	4.60	3.20	2.03
11	2.42	2.70	3.96	2.43	2.00
Total	10.23	11.14	15.86	13.07	9.06

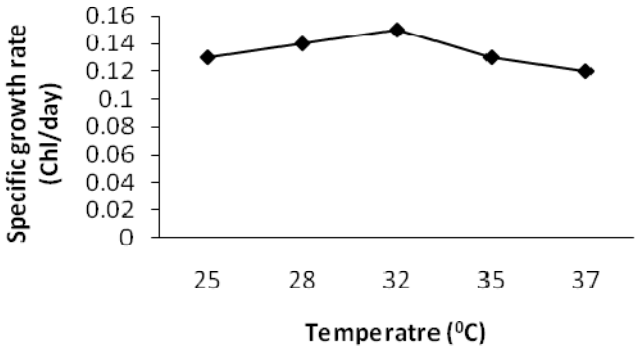

Fig. 1. Growth rates of *S. platensis* at different temperatures.