African Journal of Biotechnology

Volume 12 Number 51, 18 December, 2013

ISSN 1684-5315

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African Journal of Biotechnology Vol. 12(51), pp. 7038-7042, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3290
ISSN 1684-5315 ©2013 Academic Journals

Full Length Research Paper

Nature of gene action and genetic parameters for yield and its components in chickpea

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Accepted 30 January, 2012

To determine the gene action and genetic parameters of agro-morphological traits in chickpea, five genotypes of chickpea as a half diallel crossed with each other in 2008, five parents and 10 progenies were planted as randomized complete block design with three replications and some traits including days to flowering and maturity were recorded on row basis when 50% plants flowered or matured. Plant height, biomass, harvest index, number of primary branches, number of pods per plant, number of seeds per plant, 100-seed weight and seed yield were recorded for each plant (10 observation). Based on analysis of variance, variance due to additive gene effects showed significant differences for plant height and number of primary branches per plant. Both additive and dominance genetic effects were significant for days to flowering, days to maturity, biomass, 100-seed weight, harvest index, number of pods, seeds and seed yield per plant. Higher values (>1) of the average of dominance $(H1/D)^{1/2}$, a measure of over dominance, were observed in this study except for plant height and harvest index. Higher values of narrow-sense heritability was also observed for harvest index (67%), 100-seed weight (56%) and plant height (42%) indicating that huge genetic gain could be achieved for these traits.

Key words: Additive, chickpea, diallel, dominance, heritability.

INTRODUCTION

According to Upadhyaya et al. (2001), chickpea (*Cicer arietinum L.*) is a major food legume and an important source of protein in many countries in Asia. However, chickpea production in these countries is still low (0.78 t ha^{-1}) and limited by biotic and abiotic stress factors (Saxena, 1993; Upadhyaya et al., 2001). Genetic improvement of yield in these environments has been recognized to be difficult for plant breeders as compared to favourable environments. Seed yield is a complex character controlled by several genetic and environ-mental factors and also depends on interaction of many other characters. Breeders always look for genetic variation among traits to select desirable types. Some of these characters are highly associated with seed yield. As in all cultivated plants, the main objective of chickpea breeding is to improve cultivars having high yield and quality. Since genotypic and environmental factors are the main components for determining yield and quality in plants, the primary aim should be the determination of effects of genotypic factors for selection.

Plant breeders and geneticists often use diallel mating designs to obtain genetic information for an interest trait from a fixed or randomly chosen set of parental lines. The diallel mating design and its modifications has been used by several chickpea breeders to evaluate the potential of populations for intra population improvement and the usefulness of parents in inter population breeding programs, and to select inbred lines in hybrid breeding programs. The estimation of additive and non-additive gene action through this technique could be useful in determining the possibility of commercial exploitation of heterosis and isolation of pure lines among the progenies of the good hybrids (Stuber, 1994). The diallel cross has

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Abbreviations: DF, Days to 50% flowering; **DM,** days to maturity; **100-SW,** 100-seed weight; **HI,** harvest index; **No. PB,** number of primary branches; **No. P/p,** number of pods per plant; **No. S/p,** number of seeds per plant; **PHT,** plant height; **SY/p,** seed yield per plant.

Table 1. Analysis of variance of a 5 × 5 half-diallel cross set for quantitative characters in chickpea (according to Walters and Morton, 1978).

Source	Df	DF	DM	PHT (cm)	No. PB	No. P/p	No. S/p	Biomas	100-SW (g)	SY/p (g)	HI
rep	2	4.01^{ns}	0.67^{ns}	82.7**	0.19*	812.5*	980.9*	331.6*	4.7 ns	94.3**	30.5ns
a	4	124.9**	128.9**	152.23**	0.44**	2490.3**	2876.5**	565.9**	19.8**	206.2**	408.7**
b	10	120.6**	120.8**	25.14^{ns}	0.09^{ns}	593.9**	846.11**	183.13*	17.6**	74.7**	168.6**
b1	1	39.34**	8.4 ns	8.5^{ns}	0.23*	100.02^{ns}	658.9^{ns}	82.4ns	58.4**	98.4**	531.9**
b2	4	139.8**	156.7**	37.4*	0.17*	1351.1**	1365.01**	364.6**	5.4^{ns}	68.3*	36.04**
b3	5	121.5**	114.5**	18.7ns	-0.01^{ns}	86.9^{ns}	468.5^{ns}	58.10^{ns}	19.2**	74.9**	201.9*
Error	24	4.54	3.02	11.7	0.04	168.5	188.9	64.3	2.03	14.7	12.4

*, **Significant at the 0.05 and 0.01 probability levels, respectively. a, Additive effect; b, dominance effect; b1, mean dominance deviation; b2, dominance deviation due to each parent; b3, dominance deviation due to each crossing combination. DF, Days to 50% flowering; DM, days to maturity; PHT, plant height; No. PB, number of primary branches; No. P/p, number of pods per plant; 100–SW, 100-seed weight; No. S/p, number of seeds per plant; SY/p, seed yield per plant; HI, harvest index.

been defined as the group of all possible crosses among several genotypes (Griffing, 1956). Diallel analysis has been used in chickpea to provide important information on general and specific combining ability, determining genetic variances, estimating heritability and maternal effects (Singh et al., 1999; Hovav et al., 2003; Şakar and Biçer, 2004; Anbessa et al., 2006).

Grain yield is a quantitative trait which is controlled by several genes. Knowledge of genetic components of multi genetic traits and environmental effects is important for choosing suitable breeding methods, size of populations and intensity of selection (Biçer and Şakar, 2008). With this view, the present study was undertaken to understand the genetics of agro-morphological traits in chickpea using 5 × 5 half-diallel cross in order to select suitable parent and crosses to evolve them in chickpea breeding programs.

MATERIALS AND METHODS

The experiment was conducted at the experimental farm of the Sara-Rood Dry Land Research Station in Kermanshah (west of Iran) during the spring of 2008. Five genotypes (Arman, Hashem, ILC588, $ICCV_2$ and ILC3979) of Kabuli chickpea were chosen. The choice of the genotypes was based on their differences for many agronomic characters. The experimental materials comprised of F_1 (obtained generation from two genotypes hybridization) populations from a 5 × 5 half-diallel cross and five parents.

Agronomic practices

The experimental materials were sown by hand during early spring of 2008 in a randomized complete block design with three replications. Each replication comprised of 5 parents and 10 F_1s. The number of seeds per row was 20. Weeds were removed by hand.

Studied traits

To eliminate marginal effects, observations were recorded only on 10 plants located in middle of the rows. Mature plants were individually harvested. Days to flowering and maturity were recorded on row basis when 50% plants flowered or matured. Plant height, biomass, harvest index, number of primary branches, number of pods per plant, number of seeds per plant, 100-seed weight and seed yield were recorded for each plant.

Statistical analysis

Analysis of variance (ANOVA) in the half-diallel set was performed based on the method described by Walters and Morton (1978) using the microcomputer program "DIALL win 98" developed by Ukai (1989) and estimated genetic parameters analysis using D_2 program.

RESULTS AND DISCUSSION

Gene effects

Analysis of variance (ANOVA) of half-diallel according to Walters and Morton (1978) is given in Table 1. The significant a and b effects shows genetic variations due to additive and dominance gene effects, respectively. The b_1 effect is the average indication of heterosis that was significant for days to flowering, 100-seed weight, harvest index, number of primary branches and seed yield per plant; the b_2 parameter is specific heterosis relative to each parent and indicates different dominance and recessive gene frequencies in parents for all traits except 100-seed weight. The b_3 parameter that is mainly an indication of dominant part and is similar to specific combining ability (SCA) in Griffing method, was significant for days to flowering, maturity, 100-seed weight, harvest index and seed yield per plant (Table 1).

ANOVA analysis showed that only additive gene effects were found to be significant for plant height and number of primary branches. In addition, additive gene effects were also significant for days to flowering, maturity, biomass, 100-seed weight, harvest index, number of seeds, pods and seed yield per plant. The magnitude of the additive gene effects was much higher than dominant effects. These findings show the possibility of early generation selection for some characters in this study (Table 1). According to Malhotra and Singh (1989), Singh et al. (19992, 1993), Şakar and Biçer (2004) and Biçer

Table 2. Genetic parameters of quantitative traits in chickpea.

Parameter	DF	DM	PHT(cm)	No. PB	No. P/p	No. S/p	BIOMAS	100-SW(gr)	SY/p(gr)	HI
ß-1	0.005 ± 0.14*	0.29± 0.13*	1.12 ±0.19 ns	0.76 ± 0.27 ns	0.30±.29*	0.47 ±0.29ns	0.21±0.23*	0.69 ± 0.18ns	0.47 ± 0.25 ns	0.79 ±0.1ns
D±S.E.(D)	37.9 ± 15 *	42.05±16.9*	46 ±4.3*	0.16 ±0.021*	727.02 ±145.2*	849.6 ±145*	148.5±44*	6.08 ±0.89*	58.03 ±10.5*	131.7 ±6.3*
H2±S.E. (H2	150.1* ±36.7	1604±1.5*	25.3±10.6*	0.11±0.052*	1147.6± 255.7*	1218.9 ±354.7*	325.2±108*	2.2*±7.4	78.5 ±25.6*	69.12 ±15.4*
F±S.E. (F))	54± 37.4ns	67.5±42.3ns	30.8±10.8*	0.16± 0.054*	959.6 ± 362.7*	929.03 ±261.8*	213.5±110ns	-1.3 ±2.25ns	40.7 ±26.12 ns	19.6± 15.7ns
h2 ± S.E.(h2)	12.8±24.8ns	2.3± 28ns	5.7 ±7.2ns	0.06 ±0.04ns	5.6± 240.2ns	156.2 ±239.5ns	12.1± 72.6ns	14.3 ± 1.5*	23.6± 17.3 ns	130.4 ± 10.4*
E ± S.E.(E)	4.5±6.2ns	3.02± 7ns	11.7±1.8*	0.044±0.009*	168.5 ±59.3*	188.9 ±59.13*	64.5± 18*	1.85 ±0.37*	14.7 ± 4.2*	12.4 ± 2.6*
(H1/D)1/2	2.23	2.19	0.88	1.011	1.47	1.39	1.7	1.24	1.32	0.77
H2/4H1	0.199	0.19	0.18	0.174	0.182	0.183	0.19	0.198	0.195	0.22
KD/KR	1.93	2.16	2.2	2.9	2.62	2.3	2.43	0.85	1.72	1.22
K	0.1	0.015	0.2	0.5	0.0048	0.1	0.037	1.9	0.3	1.9
R(Yr, Wr+Vr)	-0.73	-0.41	0.76	-0.25	0.00	-0.32	-0.34	-0.75	-0.75	-0.97
h2NS	0.21	0.19	0.42	0.26	0.18	0.27	0.15	0.56	0.37	0.67
h2BS	0.91	0.94	0.62	0.55	0.70	0.72	0.62	0.78	0.73	0.86

*, **Significant at the 0.05 and 0.01 probability levels, respectively. DF, Days to 50% flowering; DM, days to maturity; PHT, plant height; No. PB, number of primary branches; No. P/p, number of pods per plant; 100–SW, 100-seed weight; No. S/p, number of seeds per plant; SY/p, seed yield per plant; HI, harvest index.

and Şakar (2008), plant height, days to maturity and 100-seed weight could be selected by breeders in early generations but it is not possible for days to flowering, number of pods and seeds per plant. Days to flowering and maturity are very important traits for drought escape in terminal drought environments (Toker et al., 2007). These charac-ters cannot be used in early generation selection because they are regulated by additive and dominance gene actions. Biçer and Şakar (2008) reported similar findings for days to maturity. Breeding for plant height is very important for machinery harvesting and chickpea cultivation development in the world. Fortunately, because of the essential role of additive gene effects in plant height heritability, it seems early generation selection is effective (Table 1). Malhotra and Singh (1989), Singh et al. (1992, 1993), Şakar and Biçer (2004) and Biçer and Şakar (2008) reported similar findings for plant height but biomass was controlled by additive and dominance gene effects, however the additive component had higher value. Additive gene effects contributed to the variation in number of primary branches (Table 1); indicating that genetic gain in selection for this trait could be possible. Both additive and dominance gene effects were significant for 100-seed weight, harvest index, seeds and seed yield per plant.

However, additive gene effects were higher than dominance gene effects. According to Singh et al. (1982), Upadhyaya et al. (2006), Dhaiwal and Gill (1973), Şakar and Biçer (2004) and Biçer and Şakar (2008) both additive and dominance gene effects play role in the heritance of 100-seed weight, harvest index, number of seeds and seed yield per plant but number of pods and seeds per plant play a role only in additive gene effects.

Genetic parameters estimated

Estimates of genetic components were calculated for each trait using D_2 program. These results are based on Hayman's (1954) method of deviation of regression coefficient from one (slope) and are given in Table 2. Significant values were observed for days to flowering, maturity, biomass and number of pods per plant (Table 2). The other method was based on Hayman's (1954) method of analysis of variance for Wr–Vr values. The Wr–shown here) was significant for days to flowering and maturity, demonstrating additive-dominance model inadequacy. Hence, in addition to additive and dominance gene effects, epistasis gene effects were effective for controlling days to flowering and maturity. These epistatic effects can cause bias in the estimates of the additive and dominance components. For other characters, additive-dominance model appears to be ade-quate. This result shows that breeding for most important traits in chickpea could be simplified because of the absence of epistatic effects in the control of these traits. Similar results have been published by different researchers (Kidambi, 1988; Malhotra and Singh, 1989; Singh et al.,

1992; Anbessa et al., 2006). Analysis of variance Wr+Vr value was significant for days to flowering, maturity and harvest index (not shown here). These results show the presence of dominance gene effects for these traits. Additive variance was significant for all characters in this assay. According to Zafar and Abdullah (1971), Singh et al. (1992) and Biçer and Şakar (2008) reports, both additive and dominance variance are the main components for most agronomic characters such as days to flowering, maturity, plant height, basal pod height, number of branches, pods and seeds per plant, seed yield, 100-seed weight and seed size.

Environmental variance was found to be significant for all traits except for days to flowering and maturity that indicated high level of environmental pressure. The average degree of dominance $(H1/D)^{1/2}$ for all characters, except for plant height and harvest index was higher than unity, indicating over dominance for most traits studied in this research. However, the degree of dominance value for plant height and harvest index was less than unity, indicating the presence of partial dominance for these traits. Biçer and Şakar (2008) reported occurrence of partial dominance for most characters but Muhelbauer and Singh (1987) reported that number of branches, pods and seeds per plant show over dominance.

Dhaiwal and Gill (1973) reported that number of pods per plant and grain yield exhibited positive over dominance but 100-seed weight showed no dominance effect. The proportion of positive and negative genes (H2/4H1) was unequal, showing different distribution of genes among parents. The (H2/4H1) component ranged from 0.17 for numbers of primary branches to 0.23 for harvest index, indicating that negative genes are more frequent. Proportion of dominant and recessive genes in the parents (KD/KR) indicated that parents carry more dominant than recessive genes for most characters but 100-seed weight (KD/KR) value was lower than the one showed by parents that carry more recessive than dominant genes for this trait (Table 2). Greater ratio of dominant to recessive genes (KD/KR) with positive value (F) indicates that dominant genes except for 100-seed weight are more numerous for most studied traits (Table 2). Days to flowering and maturity were controlled by at least one group of genes (k = 0.1). Kumar and Van Rheenen (2000), Or et al. (1999) and Cho et al. (2002) reported that days to flowering was determined by one major gene, but Biçer and Şakar (2008) and Anbessa et al. (2006) reported that days to flowering was governed by three and two major genes, respectively. 100-seed weight and harvest index were controlled by at least two genes (K=1.9, 1.5, 1.9), respectively (Table 2).

These finding are in agreement with results of Biçer and Şakar (2008) and Upadhaya et al. (2006). Other characters studied in this research were controlled by at least one group of gene (Table 2). The sign of correlation coefficient (R) between averages of joint parent for each row (Yr) and (Wr+Vr) value indicated dominance direction (Table 2). The coefficient of correlation between (Yr) and (Wr+Vr) was negative and high, for days to flowering, maturity, biomass, 100-seed weight, harvest index, number of primary branches, seeds and seed yield per plant. Hence, for these traits, amplifier alleles were dominance but as for plant height and number of pods, with positive and high (R), reducer alleles were dominant. These results correspond to previous findings in this research. The highest and lowest narrow-sense heritability was obtained for harvest index (0.67) and biomass (015), respectively. The narrow-sense heritability was relatively high for plant height (42%), 100-seed weight (56%), seed yield per plant (37%) and harvest index (67%), indicating that great genetic gain could be achieved for these traits in chickpea breeding. Biçer and Şakar (2008) reported higher values of narrow-sense heritability for 100-seed weight (96%), days to flowering (84%), seeds (78%) and pods per plant (74%).

The narrow - sense heritability was low for days to flowering (20%) and maturity (19%), these results must be considered by breeders who want to create drought tolerance lines with early maturity in chickpea. The broad-sense heritability was found to be more than narrow-sense heritability for all traits, ranging from 55 to 94% (Table 2). This indicate that both additive and non additive components of genetic variances are involved in governing the inheritance of almost all the quantitative traits in chickpea, as dominance component appeared to be high in magnitude. Tambal et al. (2000) reported that broad-sense heritability ranged from 11 to 87% and seed yield and plant height had the lowest heritability values. Hence, early generation selection could not be used for most characters in chickpea. However, Joshi et al. (2004) reported that both additive (fixable) and non-additive (non-fixable) components of genetic variances are involved in governing the inheritance of all quantitative and qualitative traits in wheat

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Vol. 12(51), pp. 7112-7119, 18 December, 2013
DOI: 10.5897/AJB2013.12962
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Preventive effect of zinc on nickel-induced oxidative liver injury in rats

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Accepted 30 September, 2013

This study pertains to the potential ability of zinc, used as nutritional supplements, to alternate oxidative stress induced by nickel. Male rats were randomly divided into four groups of eight each. Group I served as the controls; group II received in their drinking water $ZnSO_4$ (227 mg/l); group III received $NiSO_4$ (2 mg/100b.w/day intraperitoneally); group IV was treated with $ZnSO_4$ and $NiSO_4$. The exposure of rats to nickel sulfate for 21 days resulted in a significant decrease in body weight gain and absolute liver weight, relative liver weight. Nickel treatment also produced oxidative liver injury characterized by increasing serum glucose concentration, glutamate-pyruvate transaminase (GPT), alanine aminotransferase (GOT) and alkaline phosphatase (ALP) activities. Meanwhile nickel supplementation decreased serum total protein and albumin in animals. In addition, liver glutathione level, catalase and glutathione peroxidase (GSH-Px) activities were diminished. The administration of zinc with nickel (Ni + Zn) corrective effects on Ni-induced oxidative stress in liver was observed. In conclusion, this study demonstrates that intraperitoneally injection with Ni caused reduction in enzymes activities in rat's liver and treatment with zinc offers a relative protection against nickel induced oxidative liver injury and lipid peroxidation probably due to its antioxidant proprieties.

Key words: Nickel, zinc, rats, oxidative stress, liver.

INTRODUCTION

The rapid development of science, industry, medicine, and agriculture has exposed man and his environment to number of exotic heavy metals. Nickel is the major components of the alloys employed in the plate and screw used for connecting bones in orthopaedic surgery and in the manufacture of artificial organs (Kocijan et al., 2004). However, excessive amounts of this transitional metal ion are toxic. Numerous authors have studied the impact of nickel on health. It can cause dermatitis to certain persons (Accominoti et al., 1998). Particle of nickel may cause some morphological transformations in numerous cellular systems and chromosomal aberrations (Coen et al., 2001). The salts of nickel as particles of nickel can be allergens and carcinogens in man while forming the oxygenated radicals (Lansdown, 1995). This cytotoxicity was investigated in numerous micro-organisms (Wu et al., 1994). Nickel was also found to be responsible on many sexual disorders (Chakroun et al., 2002). After entering the body,

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Abbreviations: GOT, Glutamate oxaloacetate transaminase; **GPT,** glutamate pyruvate transaminase; **ALP,** alkaline phosphatase; **GSH,** reduced glutathione; **GSH-Px,** glutathione peroxidase; **DTNB,** 5, 5'dithiobis-(2-nitrobenzoic acid; **ROS,** reactive oxygen species; **MT,** metalothionine.

nickel penetrates all organs and accumulates primarily in bone, liver, kidney and excreted through bile and urine (Kusal et al., 2007). Liver is the primary target for environmental and occupational toxicity and the major site for detoxification. Nickel induced severe liver and kidney damage by altering several marker enzymes and ascorbate-cholesterol metabolism. One of the harmful effects of nickel action in the body is to induce formation of reactive oxygen species (ROS) and increase lipid peroxidation in the cells (Sunderman et al., 1985). Free radicals and intermediate products of lipid peroxidation are capable of damaging the integrity and altering the function of biomembrane, which can lead to the development of many pathological processes (Kusal et al., 2007).

Zinc is ubiquitous in sub-cellular metabolism and is an essential component of catalytic site(s) of at least one enzyme in every enzyme classification (Coyle et al., 2002). Others have clearly demonstrated the hepatoprotective role of zinc under different toxic conditions (Cabre et al., 1999). Zinc is involved in stabilizing the cell membrane and prevents oxidative destruction caused by free radicals (Bettger and O' Dell, 1981; Ludwig and Chvapil, 1982) at least under certain conditions, may have antioxidant properties (Powell, 2000). It can protect against oxidative damage caused by certain xenobiotics (Fukino et al., 1986). In addition, zinc is also known for inducing methallothionein (MT) synthesis, a protein that is able to bind heavy metals and to scavenge hydroxyl radicals (Cousins and Hempe, 1990). The indications of biological antagonism between nickel and zinc have also been reported (Kasprzak et al., 1986). Nickel apparently affects zinc metabolism as evidenced by altered urinary excretion patterns (Clary, 1975) and organ distribution (Whanger, 1973). Therefore, the present study was designed to evaluate whether zinc supplementation could have a protective effect against nickel-induced oxidative liver injury in male albino rats.

MATERIALS AND METHODS

Chemicals

Zinc sulphate ($ZnSO_4$ $7H_2O$) and nickel sulphate ($NiSO_4 6H_2O$), 5, 5'dithiobis-(2-nitrobenzoic acid (DTNB) and reduced glutathione were purchased from sigma Chemical Co (St Louis, France) and all other chemicals used in the experiment were of analytical grade.

Animals

Thirty-two male albino (Wistar) rats of ten weeks of age with a body weight of 180-205 g were obtained from the Pasteur Institute (Algiers, Algeria). Animals were acclimated for two weeks for adaptation under the same laboratory conditions of photoperiod (12h light/12 h dark) with a relative humidity of 40% and room temperature of 22 ± 2°C. Food (Standard diet, supplemented by the ONAB, EL-Harouch, Algeria) and water were provided *ad-libitum*.

Experimental design

Animals were randomly divided into four different groups of eight animals each. One served as normal control. The second group (Zn) was given zinc sulphate $ZnSO_4$ $7H_2O$ in drinking water at a dose level 227 mgZn/l, while the third group (Ni) was intraperitoneally given nickel sulphate ($NiSO_4 \cdot 6H_2O$) at a dose of 2 mg/100g b. w./day . Finally, the fourth group (Ni + Zn) was treated daily with both zinc sulphate and nickel sulphate as in group two and three. The treatment of all groups lasted for three consecutive weeks.

The dose of $NiSO_4$ $6H_2O$ and the period of treatment were selected on the basis of previous studies (Kusal et al., 2001), whereas $ZnSO_4$ $7H_2O$ dose was chosen based on clinical application and on results from previous studies (Sidhu et al., 2004). The experimental procedures were carried out according to the National Institute of Health Guide-lines for Animal Care and approved by the Ethics Committee of our Institution. The treatments of rats continued for a period of three weeks. At the end of the experiment, total body weights were recorded and animals were sacrificed by decapitation without anesthesia to avoid animals stress. At the time of sacrifice, blood was transferred into ice cold centrifuged tubes. Tubes were then centrifuged for 10 min at 3000 rpm and serum was used for glucose, total protein, albumin, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) and alkaline phosphatase (ALP) assays. Livers were removed immediately and one part of the lobe was processed immediately for assaying glutathione and antioxidant enzymes activities. The other lobe was used for light microscopic studies.

Analytical methods

Determination of biochemical parameters

Serum glucose level was estimated with a commercial kit (Spinreact, Spain, ref; 41011) and determined by enzymatic colorimetric method using spectrophotometer (Jenway 6505, Jenway LTD, UK). However, GOT, GPT and ALP activities were determined with commercial kits from Spinreact, Spain, refs: GOT-1001161, GPT-1001171 and ALP-1001131, respectively. Total protein and albumin concentrations were also measured using commercial kits (Spinreact, Spain, refs: total proteins-1001291 and albumin-1001020.

Tissue preparation

About 1 g of liver was homogenized in 2 ml of buffer solution of phosphate buffer saline 1:2 (w/v; 1 g tissue with 2 ml TBS, pH 7.4). Homogenates were centrifuged at 10000 x g for 15 min at 4°C and the resultant supernatant was used for the determination of reduced glutathione and protein levels in one hand and the estimation of catalase and GSH-Px activities in the other hand.

Estimation of reduced glutathione level (GSH)

Liver GSH content was estimated using a colorimetric technique, as mentioned by Ellman (1959) modified by Jollow et al. (1974), based on the development of yellow colour when DTNB is added to compounds containing sulfhydryl groups. In brief, 0.8 ml of liver supernatant was added to 0.3 ml of 0.25% sulfosalycylic acid, and then tubes were centrifuged at 2500 x g for 15 min. Supernatant (0.5 ml) was mixed with 0.025 ml of 0.01 M DTNB and 1 ml phosphate buffer (0.1 M, pH 7.4). The absorbance at 412 nm was recorded. Finally, total GSH content was expressed as n mol GSH/mg protein.

Determination of glutathione peroxidase (GSH-Px)

GSH-Px (E.C. 1.1.1.1.9) activity was measured by the procedure of Floche and Gunzler (1984). Supernatant obtained after centrifuging 5% liver homogenate at 15000 x g for 10 min followed by 10.000 x g for 30 min at 4°C was used for GPx assay. 1 ml of reaction mixture was prepared which contained 0.3 ml of phosphate buffer (0.1 M. pH 7.4). 0.2 ml of GSH (2 mM), 0.1 ml of sodium azide (10

mM), 0.1 H_2O_2 (1 mM) and 0.3 ml of liver supernatant. After incubition at 37°C for 15 min, the reaction was terminated by addition of 0.5 ml 5% TCA. Tubes were centrifuged at 1500 x g for 5 min and the supernatant was collected. 0.2 ml of phosphate buffer (0.1 M pH 7.4) and 0.7 ml of DTNB (0.4 mg/ml) were added to 0.1 ml of reaction supernatant. After mixing, absorbance was recorded at 420 nm.

Assay of catalase activity

The activity of catalase was determined according to the method of Aebi (1984). The reaction mixture (1 ml) that contained 0.78 ml of phosphate buffer (0.1 M, pH 7.4), 0.2 ml of liver supernatant, and 0.02 ml of H_2O_2 (0.5 M) was prepared. The reaction was started by adding H_2O_2 and decomposition was monitored by following the decrease in absorbance at 240 nm for 1 min. The enzyme activity was calculated by using an extinction coefficient of 0.043 $mM^{-1}cm^{-1}$.

Protein determination

The protein content of tissues samples were measured by the method of Bradford (1976) by using bovine serum albumin as a standard.

Histological studies

For histological examination, livers was dissected and immediately fixed in bouin solution for 24 h, processed by using a graded ethanol series, and then embedded in paraffin. The paraffin sections were cut into 5 µm thick slices and stained with hematoxylin and eosin (Haoult, 1984) for light microscopic examination. The sections were then viewed and photographed.

Statistical analysis

Data are given as means ± SEM. Statistical significance of the results obtained for various comparisons was estimated by applying one way analysis of variance (ANOVA) followed by Student's t-test and the level of significance was set at $p < 0.05$.

RESULTS

Effect of treatment on body, absolute and relative liver weights

The body, absolute and relative liver weights of rats subjected to different treatments are shown in Table 1. In this experiment, it was observed that the control body weight gain and Zn-treated group have increased progressively during the study. However, in Ni-treated animals, the results showed obviously significant decrease ($p < 0.001$) in body weight gain as compared to the control group. In addition, a significant increase of Ni-treated group in absolute and relative weights was noticed at $p < 0.001$ and $p < 0.01$, respectively. However, zinc supplementation reversed these changes.

Effects of treatments on serum biochemical parameters

Compared to the controls, total protein and albumin levels in Ni-treated animals were significantly reduced ($p < 0.001$ and $p < 0.01$), but the combination of zinc with nickel produced a recovery in above mentioned biochemical variables (Table 2). In addition, the glucose concentration, GOT, GPT and ALP activities were significantly higher ($p < 0.001$) in nickel group than those of control group, indicating liver damage. However combined treatment of nickel and zinc markedly ameliorated these variations.

Effects of treatments on hepatic oxidative stress parameters

Figure 1 shows that after nickel treatment, the liver glutathione level, catalase and GSH-Px activities were significantly diminished ($p < 0.001$) in nickel experimental comparison with the control group. The simultaneous treatment with zinc partially reversed these changes to near untreated control values.

Histopathalogical results

The mentioned biochemical alteration could be referred to as the liver histological changes. In fact, liver of the control group had a regular histological structure with a characteristic pattern of hexagonal lobules separated by interlobular septa, traversed by portal veins (Figure 2A). In contrast, liver of nickel treated group had weak pathological alteration such as the presence of cellular debris within a central vein and cytological vacuolization (Figure 2C). In addition, no histological alterations were observed in the liver of Zn-treated group (Figure 2B) as compared to the control. However, the combination group of Ni-Zn showed prominent recovery in the form of the liver histoarchitecture (Figure 2D), such as the reduced cytoplasmic vacuolization and the normal sinusoidal spaces.

DISCUSSION

In this experiment, body weight gain of nickel rats group was significantly depressed. This action of nickel may be mediated by alteration in zinc metabolism such as other heavy metals (Kuhnert et al., 1987). In fact heavy metals have been recognized as antimetabolite of zinc (Brozoska and Moniuszko-Jakoniuk, 2000). Disturbances in zinc function and metabolism may have serious consequences for health. This element plays an important role in growth, development and functioning of all living cells (Nishi, 1996; Sameeh et al., 2009). As a result, zinc supply significantly prevented the nickel induced decrease in body weight gain. In this experiment, nickel sulphate group animals showed also high level of glucose. The elevation in serum glucose is a common result of nickel toxicity and is usually linked with inhibition of insulin release from Langerhans'islets (Dormer et al., 1973; Kechrid et al., 2006; Djemli et al., 2012) or with a block of glucose utilization by cells even in the presence of elevated concentrations of insulin (Sunderman et al., 1976) or the high glycogen breakdown and new supply of glucose production from other non carbohydrate sources such as proteins (Cartana and Arola, 1992). However there is an amelioration of blood glucose concentration in

Table 1. Body weight gain, absolute and relative liver weights of control male rats, treated with zinc (zinc sulphate), nickel (nickel sulphate) and zinc coadministrated with nickel, after 3 weeks of treatment.

Parameter	Experimental groups (Mean ± SEM; n = 8)			
	Control	Zn	Ni	Ni + Zn
Initial body weight (g)	188 ± 2.4	191 ±3.1	190 ±3.4	189 ±3.1
Body weight gain (g)	53.2^a ±1.2	55.7^a ± 1.7	25.6^b ±1	37.6^c ±1.1
Absolute liver weight (g)	10.2^a ±0.1	10^a ±0.4	13.5^b ±0.3	10.4^a ±0.5
Relative liver weight (g)	2.90^a ±0.14	2.71^a ±0.16	3.56^b ± 0.08	2.78^a ± 0.14

a, b, c, Values within a horizontal line with different superscript letters were significantly different ($p < 0.05$). Values are mean ± SEM, n = number of animals.

Table 2. Changes of biochemical parameters of control male rats, treated with zinc (zinc sulphate), nickel (nickel sulphate) and zinc coadministrated with nickel, after 3 weeks of treatment.

Parameter	Experimental groups (Mean ± SEM; n = 8)			
	Control	Zn	Ni	Ni + Zn
Glucose (mg/100ml)	124^a±2.35	121^a ±2.66	190^b± 5.35	157^c± 2.76
Total protein (g/100ml)	8.8^a±0.2	8.6^a±0.3	6.6^b±0.1	8.4^a± 0.2
Albumin (g/100ml)	4.5^a±0.3	4.7^a ±0.2	3.1^b±0.2	4.3^a±0.1
GOT (U/L)	85^a±1.1	83^a±0.7	113^b±1.6	91^c±2.5
GPT (U/L)	39^a±1.6	40^a±2.7	72^b±2.4	60^c±3.4
ALP (U/L)	118^a ±3.8	116^a± 1.9	198^b±5.8	165^a ±1.8

a, b, c,Vvalues within a horizontal line with different superscript letters were significantly different ($p < 0.05$). Values are given as mean ± SEM, n = number of animals.

animals treated with both metals nickel and zinc. It is probably as a result of the glycaemia lowering effect of zinc sulphate by decreasing systemic glucose accumulation, diminishing nickel binding to biomolecules, improving insulin secretion and action (Song et al., 2006) and/or protects the enzymes and ATP involved in glucose metabolism against inactivation by nickel (Nielsen, 1980).

In the present study, significantly decrease in the total protein and albumin levels was found. These findings confirm the work of Sidhu et al. (2004), when both zinc and nickel were given together in drinking water. The decrease in these two biochemical parameters concentrations of Ni-treated rats might be due to changes in protein synthesis (Kusal et al., 2000; Dostal et al, 1989). The liver is the target organ of heavy metals toxicity and its cells spell out hepatic enzymes into blood, which are commonly used as biochemical indicator index of hepatocellular damage. In the present investigation, nickel intoxication caused a significant increase in the activities of GOT, GPT and ALP, probably due to hepatocyte membrane damage resulting in increased release and leakage out of these enzymes from the liver cytosol into the blood stream which gives an indication on the hepatotoxic effect of this metal (Gama and Eatmad, 2011). These results are consistent with previous findings by some research groups who had found an association between nickel toxicity and the increased oxidative stress of rats (Novelli et al., 1998; Al Hassan et al., 2010). Consequently, biochemical perturbations seem to be correlated with the liver histological alteration such as the presence of cellular debris within a central vein and a cytoplasmic vacuolization.

Previous histological studies on liver have documented Ni-induced changes characterized by dilated sinusoids, vacuolization and the appearance of hepatic cells with distorted nuclei (Ben Amara et al., 2010; Rabbani-Chadegani et al., 2011; Djemli et al., 2012). The combination treatment of zinc improved the histological alteration induced by nickel, which could be attributed to the antiradicals/ antioxidant and metal-chelating efficacy of this element. In addition, these findings are in good agreement with those obtained by other studies which postulated the beneficial role of zinc on histological and enzymatic changes of rats (Dhawan and Goel, 1992; Djemli et al., 2012). These reports emphasized the hepatoprotective efficacy of zinc under CCl4 induced liver injury, as zinc treatment helped in the maintaining the homeostasis through regulation of protein synthesis. Thus the supplementation of zinc had protected liver function from nickel intoxication as indicated by the significant restoration of serum total protein, albumin, GOT, GPT and alkaline phosphatase.

The diminution of glutathione level in nickel rats may be as a result of oxidative stress, which has been occurred, in nickel toxicity (Djemli et al., 2012). In other words the

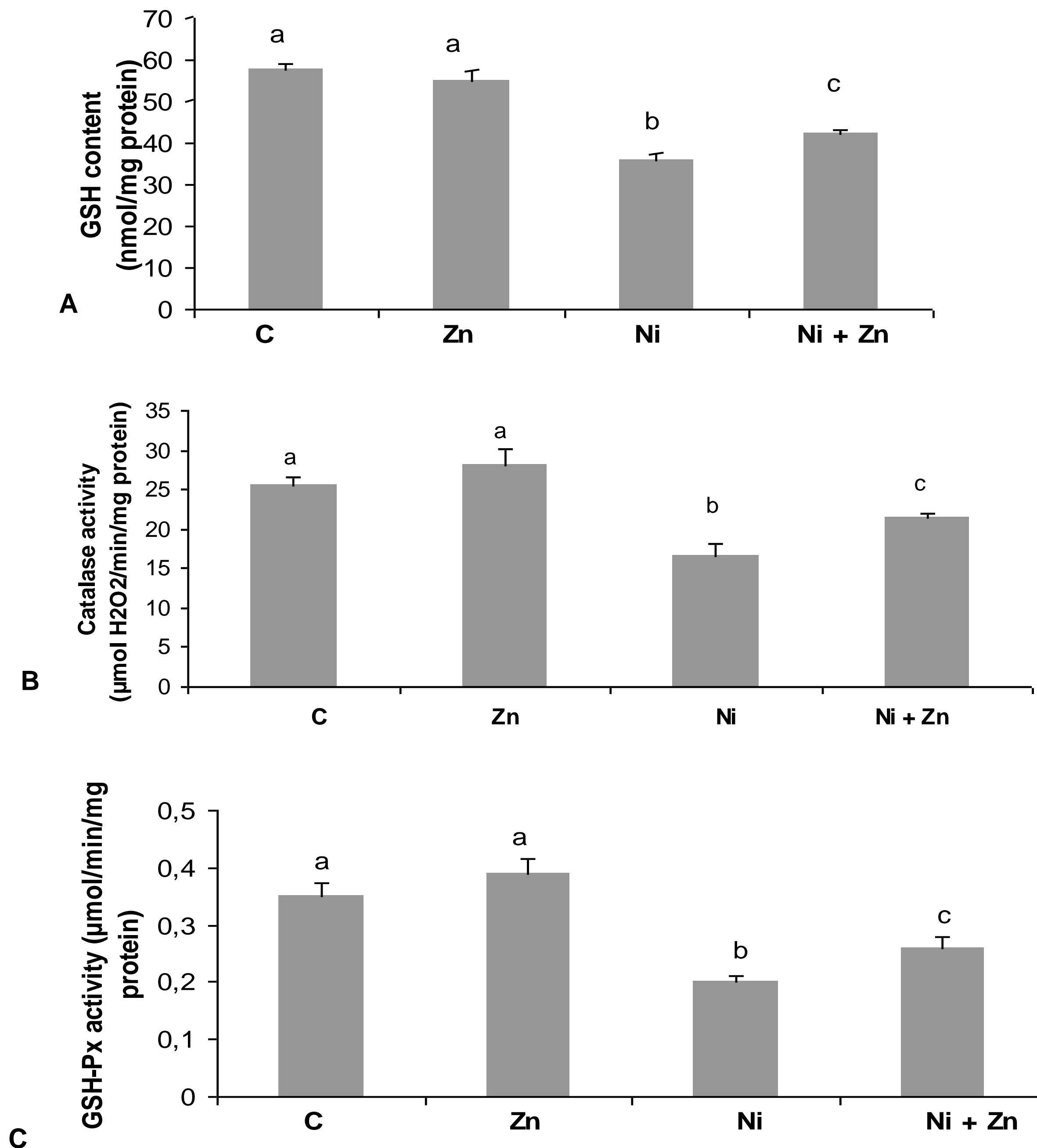

Figure 1. Values of glutathione, catalase and GSH-Px in liver of control and rats treated with zinc (zinc sulphate), nickel (nickel sulphate) and zinc coadministrated with nickel, after 3 weeks of treatment. a, b, c, Values with different superscript letters were significantly different ($p < 0.05$). Values are given as mean ± SEM for group of 8 animals each.

reduction of antioxidant production was due to the increased oxygen metabolites and the elevated free radicals, which cause a decrease in the activity of the anti-oxidant defense system (Gstraunthaler et al., 1983; Iscan et al., 2002) and several pathways have been proposed to show the depletion of GSH level in heavy metals toxicity (Mohandas, 2010). Firstly, the sulfhydryl group of cysteine moiety of glutathione has a high affinity of metals, forming thermo-dynamically stable mercaptide complexes with several metals (Aposhian, 1989). Secondly, GSH may be oxidized due to the interaction with the free radicals induced by nickel. These complexes are inert which can be excreted via the bile, and therefore GSH level could be consumed during Ni detoxification (Manna et al., 2008; Mohandas, 2010). In addition the decreased activity of hepatic CAT and GSH-Px in nickel treated animals,

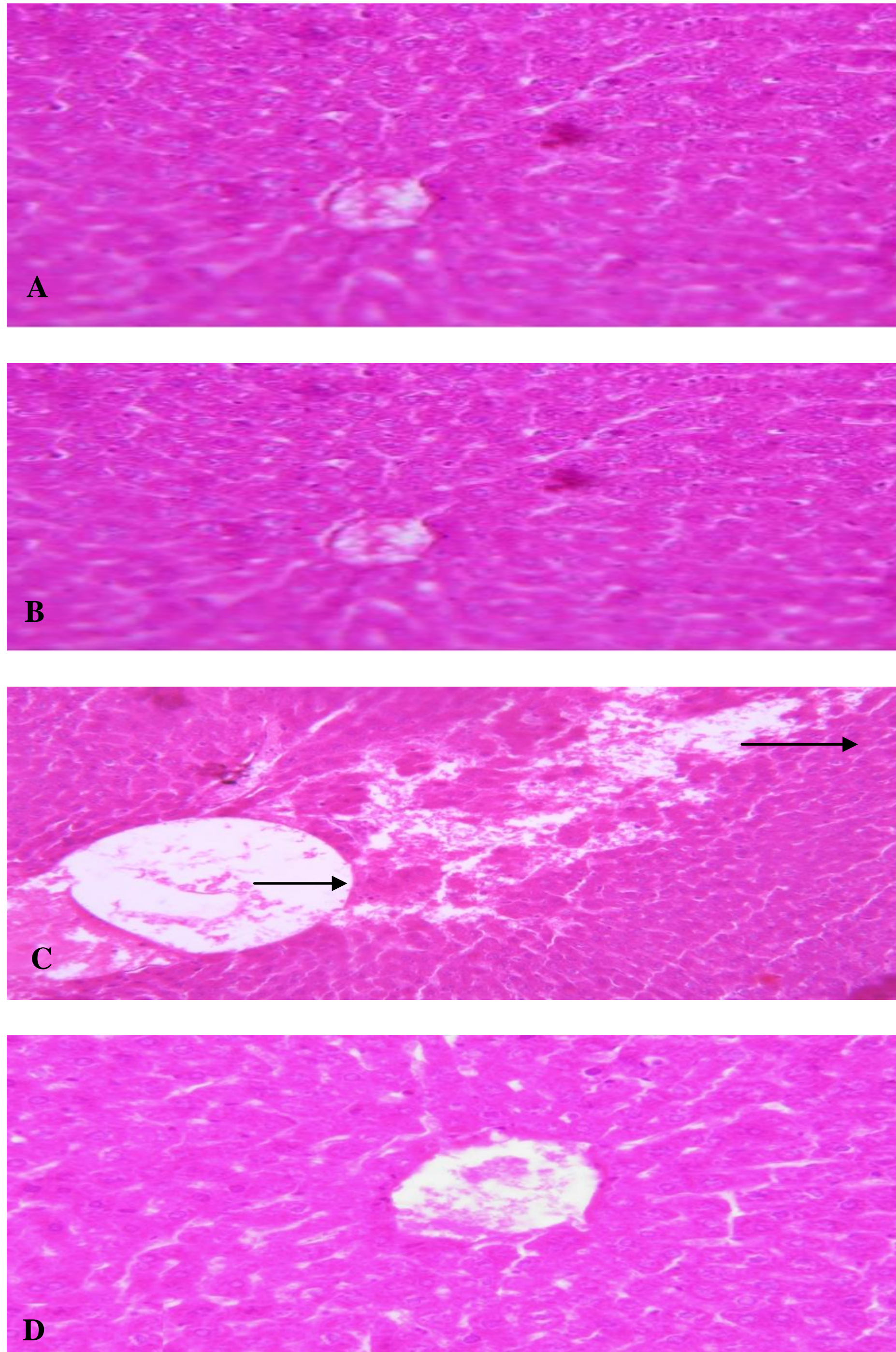

Figure 2. Effect of nickel (nickel sulphate) and zinc (zinc sulphate) coadministrated with nickel on histological damage in the liver. Control (A), treated with Zn (B), Ni (C) and Zn co-administrated with Ni (D). Optic microscopy: sections were stained using the haematoxylin-eosin method (400 x). Arrows: - indicate a presence of cellular debris within a central vein and- indicate cytoplasmic vacuolisation. Zn coadministrated with Ni maintained granular cytoplasm and normal hepatocytes.

suggests that there is an interaction between the accumulated free radicals and the active amino acids of this enzymes (Kusal et al., 2001). In Group III (nickel sulphate + zinc sulphate), the significant improvement of the glutathione level was noticed when compared with that of Group II. The observed normalization of GSH levels and GSH-Px and catalase activities following zinc treatment could be because of its property to induce metallothionein

(S-rich protein) as a free radical scavenger, or its indirect action in reducing the levels and accumulation of oxygen reactive species (Seagrave et al., 1983; Cousins and Hempe, 1990).

Conclusion

In conclusion, this study demonstrates exposure to nickel provoked oxidative liver injury by inducing lipid peroxidation, which led to depletion of liver reduced glutathione, reduction in antioxidant enzyme activities and biochemical parameters variations of rats. However, zinc treatment could protect liver against nickel toxicity by increasing GSH level and the activities of antioxidant enzymes and ameliorated some biochemical parameters and approached them closer to their normal values.

ACKNOWLEDGEMENTS

This work was supported by the research project N° F01120070054 funded by the Ministry of Higher Education, Algeria. The authors should thank members of *Algiers Pasteur Institute* for providing rats.

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Vol. 12(51), pp. 7081-7085, 18 December, 2013
DOI: 10.5897/AJB09.936
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Ground water quality evaluation in Beed city, Maharashtra, India

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Accepted 2 November, 2009

The development activities cause depletion and degradation of ground water. A survey was undertaken to assess the quality of ground water in Beed district of Maharashtra taking both physico-chemical and bacteriological parameters into consideration. The present investigation is aimed to calculate Water Quality Index (WQI) of ground water and to assess the impact of pollutants due to agriculture and human activities on its quality. The WQI varied from 329.27 (winter) to 141.56 (monsoon) indicating level of nutrient load and pollution in the handpumps and borewells. The existing results revealed that water from handpumps and borewells are not safe for human use.

Key words: Ground water, pollution, water quality index, Seasonal analysis.

INTRODUCTION

Of all natural resources, water is unarguably the most essential and precious. Life began in water, and life is nurtured with water. There are organisms, such as anaerobes witch can survive without oxygen. But no organism can survive for any length of time without water. The crucial role of water as the trigger and sustained of civilizations has been witnessed throughout the human history it is common knowledge that our planet is faced with a major problem in the available water resources (Gleick, 2008; Witkowski et al., 2007). This problem has two dimensions:

1. The first is with respect to the quantity of water available. With increasing population, the demand for water, both for human consumption and agriculture, has been steadily increasing. Also, the melting of glaciers, deforestation and general environmental degradation, in particular, of rivers, has cut the retentivity, flow and availability of water on the planet.
2. The not so obvious problem, which is perhaps more serious, has to do with the quality of water, which has deteriorated over the last 50th years, so as to render most of it is unfit for drinking. How has this happened?

Excessive urban migration has inflated cities beyond manageable limits, to produce such quantities of effluents so as to render both the local groundwater and rivers flowing by cities to be criminally polluted. This has happened mostly due to leaching of contaminants from landfills, indiscriminately disposed anthropogenic toxic waste, unplanned application of agrichemicals and surface run-off from farm lands (Datta, 1999).

At presents, it is estimated that almost half the world's population has no access to good drinking water (Soni et al., 2009). But, uptill as late as 1960s, the overriding interest in water has been *vis-a-vis* its quantity. Except in mamfestly undesirable situations, the available water was automatically deemed utilizable water. Only during the last three decades of the twentieth century the concern of water quality has been exceedingly felt so that, by now, water quality has acquired as much importance as water quality.

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Table 1. Water Quality Index (WQI) range.

WQI	Status
0 - 25	Excellent
26 - 50	Good
51 - 75	Poor
76 - 100	Very poor
100 and above	Unsuitable for drinking

Source: Mishra and Patel, 2001

A water quality index is an indicator of the quality of water. It is useful for a variety of purposes, such as:

a) Planning tool for managing water resources use,
b) Assessing changes in the quality of the water at different times, places and seasons,
c) Evaluating the performance of pollution control programmes, and- communicating water quality information to the public and to decision makers.

MATERIALS AND METHODS

The present investigation is aimed to calculate Water Quality Index (WQI) in Beed city. For this reason, ten physic-chemical parameters such as DO (Dissolved Oxygen), BOD (Biochemical Oxygen Demand), pH, Cl (Chlorides), NO_3 (Nitrate), Total Alkalinity, Total Hardness, COD (Chemical Oxygen Demand), and TC (Total Coliform) were selected and analyzed as per standard procedure of APHA (1998); Trivedy and Goel (1986); Kodarkar et al. (1998).

Water samples were collected for physico-chemical analysis from 12 sampling station of handpumps and borewells, from November, 2005 - October, 2006. Water samples were collected in one litre plastic bottles. Sample collection was usually completed during morning hours between 8 - 11:00 am every time. pH and dissolved oxygen were monitored at the sampling spots, while other parameters were analyzed in the laboratory.

Water quality index (WQI)

Water quality Index is an important parameter for the assessment and management of ground water. Water Quality Index is a single number (like a grade) that expresses the overall water quality at a certain location based on several water quality parameters. The concept of indices to represent by Horon (1965). It is defined as a rating reflecting the composite influence of different of water quality parameters on the overall quality of water. For calculaton of WQI, selection of parameters has great importance. Since selection of too many parameters might widen the quality index and importance of various parameters depends on the intended use of water (Table 1).
Weighted arithmetic index has been used for calculation of WQI, in the following steps:

Calculation of sub index or quality retting (q_n)

Let there be n water quality parameters and quality rating or sub-index (q_n) corresponding to n^{th} parameter is a number of reflecting the relative value of this parameter in the polluted water with respect to its standard permissible value. The q_n is calculated using expression:

$$q_n = 100[(V_n - V_{io})/(S_n - V_{io})]$$

Where, q_n = quality rating for the nth water quality parameter, V_n = estimated value of the nth parameter at a given sampling station, S_n= standard permissible value of nth parameter, and V_{io} = ideal value of nth parameter in pure water.
All ideal value s (Vio) are taken as zero for the drinking water except for pH = 7.0 and dissolved oxygen = 14.6 mg/L.

Calculation of quality rating for pH

For pH, ideal value is 7.0 (for neutral water) and permissible value is 8.5 (for polluted water). Therefore, quality rating for pH is calculated from the following relation:

$$q_{pH} = 100[V_{pH} - 7.0)] / [(8.5 - 7.0)]$$

Where, VpH = observe value of pH.

Calculation of quality rating for dissolved oxygen

The ideal value (V_{Do}) for dissolved oxygen in 14.6 mg/L and standard permissible value for drinking water is 5 mg/L. Therefore, quality rating is calculated from following relation:

$$q_{Do} = 100[V_{Do} - 14.6)]/[(5 - 14.6)]$$

Where V_{Do} = measured value of dissolved oxygen.

Calculation of unit weight (W_n)

The unit weights (W_n) for various water quality parameters are inversely proportional to the recommended standards for the corresponding parameters:

$$W_n = K / S_n$$

Where W_n = unit weight for n^{th} parameter, S_n = standard value for n^{th} parameter, and K = constant for proportionality.
WQI is calculated from the following equation:

$$\text{WQI} = \sum_{n=1}^{n} \text{qn wn} / \sum_{n=1}^{n} \text{wn}$$

RESULTS AND DISCUSSION

The physico-chemical and bacteriological quality of drinking water totally depends of the geological condition of the soil and ground water pollution of the area. The physico-chemical parameters value and total coliform count are presented in Table 2. The seasonal average values of various physico-chemical parameters, drinking water standards, unit weights (W_i), quality rating (q_i), Subindex value (q_iW_i) and WQI value of handpumps and borwells are calculated during different seasons are recorded in Tables 3 to 5.

The pH value of handpumps and borewell water sample was found on 7.19 (winter) to 7.32 (monsoon). The pH of all water samples were within the normal range (WHO, 1984). High TSS values in surface water might be due to mixing of sewage and industrial effluents (Chatterjee and Raziuddin, 2002).

Table 2. Seasonal values of some water quality parameters of Handpumps and borewells at twelve sampling stations (S_1 - S_{12}) during different seasons (all values are mg/L except pH).

Parameter	Season	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
DO	Win.	4.4	5.8	4.2	8.2	8.2	6.4	6.2	10.1	5.8	8.1	12.8	4.8
	Sum.	2.4	3	4.2	5.4	4.8	3	4.4	5.6	2.4	4.4	5.8	3
	Mon.	7.7	7.2	7.5	7.1	6.5	6.1	6.8	6.9	5.1	6.6	7.3	5.8
BOD	Win.	10.8	12.8	12.2	9.8	7.5	12.8	11.8	14.4	8.5	8.3	14.6	8.37
	Sum.	4.1	3.5	4.2	2.9	3.1	5.1	3.2	4.5	2.8	3.2	4.8	3.3
	Mon.	7.5	7.8	8	5.6	5.4	8.7	6.5	7.8	5.6	6.4	8.1	5.4
pH	Win.	6.97	6.9	8.08	8.79	6.9	7.08	7.24	6.84	6.94	6.72	6.94	6.88
	Sum.	7.19	7.19	7.36	7.7	7.59	7.37	7.44	7.1	7.17	7.02	7.14	7.13
	Mon.	7.1	7.11	8.1	8.5	7.62	7.1	7.2	6.9	7.11	6.92	7.1	7.11
Cl.	Win.	355	497	248.5	426	497	603.5	461.5	674.5	887.5	532.5	497	461.53
	Sum.	525.4	440.2	511.2	426	553.8	383.4	468.6	497	468.6	568	894.6	908.8
	Mon.	504	469	416	409	524	584	465	529	612	537	678	599
NO_3	Win.	0.62	0.71	0.81	0.92	0.76	0.63	0.86	0.72	0.68	0.82	0.92	0.97
	Sum.	0.72	0.63	0.73	0.86	0.91	0.74	0.81	0.65	0.93	0.95	0.88	0.97
	Mon.	0.43	0.52	0.53	0.73	0.81	0.48	0.78	0.59	0.84	0.76	0.82	0.87
T. Alk.	Win.	180	186	245	360	348	220	270	358	290	275	285	192
	Sum.	380	409	306	484	356	304	288	400	486	348	404	322
	Mon.	183	185	228	356	309	199	197	336	276	268	244	188
TH	Win.	610	744.2	554.6	488	793	610	671	866.2	744.2	427	585.6	512.4
	Sum.	418	663	609	367	360	437	379	547	534	399	573	508
	Mon.	605	752	588	492	402	572	617	718	651	414	581	509
TSS	Win.	45	37	47	62	50	51	84	48	38	41	82	88
	Sum.	48	52	42	47	40	37	46	55	62	54	48	58
	Mon.	75	62	70	64	58	72	92	82	91	66	88	77
COD	Win.	40	43.2	32	46.4	36.2	28.2	36.4	43.2	32.9	40.1	44.2	44.4
	Sum.	27.2	26.5	21.8	19.6	21.0	22.5	18.8	28.1	18.9	21.7	25.5	23.5
	Mon.	6.2	5.5	6.5	5.8	6.2	6.8	5.6	7.5	6.7	7.2	8.5	6.0
TC	Win.	143	410	625	180	216	420	325	32	82	212	440	390
	Sum.	120	402	392	140	160	220	150	40	90	490	320	260
	Mon.	160	460	670	196	230	435	340	40	102	222	460	392

Table 3. WQI of Handpumps and Borewells during in water season.

Parameter	Unit weight W_n)	ICMR (S_n)	Observed values (V_{io})	Quality rating (q_n)	Sub-index value ($q_n W_n$)
DO	0.2000	5	7.08	78.33	15.67
BOD	0.2000	5	11.02	220.4	44.08
pH	0.0040	7.0 - 8.5	7.19	12.67	0.05
Cl	0.0040	250	511.79	204.72	0.82
T.Alk.	0.0083	120	267.42	222.85	1.85
TH	0.0033	300	633.85	211.28	0.69
TSS	0.0020	500	56.08	11.22	0.02
COD	0.2000	5	38.89	777.8	155.56
TC	0.1000	1	290.08	351.31	35.13
SUM	0.7716	1206.00	1824.19	2094.53	254.07
Average	0.08	134.00	182.41	209.45	25.40
Water quality index = 329.27					

Table 4. WQI of Handpumps and Borewells during summer season.

Parameter	Unit weight (W_n)	ICMR (S_n)	Observed values (V_{io})	Quality rating (q_n)	Subindex value (q_n W_n)
DO	0.2000	5	4.03	110.10	22.02
BOD	0.2000	5	3.73	74.6	14.92
pH	0.0040	7.0 - 8.5	7.28	18.67	0.07
Cl.	0.0040	250	553.80	221.52	0.89
NO_3	0.0500	20	0.82	4.1	0.21
T.Alk.	0.0083	120	373.92	311.6	2.59
TH	0.0033	300	482.83	160.94	0.53
TSS	0.0020	500	49.80	9.82	0.01
COD	0.2000	5	22.93	458.6	91.72
TC	0.1000	1	207.00	321.36	321.13
SUM	0.7716	1206.00	1705.42	1691.31	32.13
AVERAGE	0.08	134.00	170.54	169.13	3.21
Water quality index = 213.95					

Table 5. WQI of Handpumps and Borewells during monsoon season.

Parameter	Unit weight (W_n)	ICMR (S_n)	Observed values (V_{io})	Quality rating (q_n)	Subindex value (q_n W_n)
DO	0.2000	5	6.72	82.08	16.42
BOD	0.2000	5	6.90	138.00	27.60
pH	0.0040	7.0 - 8.5	7.32	21.33	0.09
Cl	0.0040	250	527.17	210.87	0.84
NO_3	0.0500	20	0.68	3.4	0.17
T.Alk.	0.0083	120	247.42	206.18	1.71
TH	0.0033	300	575.08	191.69	0.63
TSS	0.0020	500	74.75	14.95	0.03
COD	0.2000	5	6.55	131.0	26.20
TC	0.1000	1	308.92	355.41	35.54
SUM	0.7716	1206.00	1761.51	1354.91	51.96
AVERAGE	0.08	134.00	176.15	135.49	5.19
Water quality index = 141.56					

The observed values of total alkalinity were found in the range of 247.42 mg/L (rainy) to 373.92 mg/L (summer). Harish et al. (2006) reported total alkalinity in the ground water between 62 and 140 mg/L. Harish et al. (1991) recorded alkalinity values in the range of 200 - 610 mg/L in city side from handpump water. The BIS (1998) accepted limit for total alkalinity is 1000 mg/L. Observed values are well within the permissible limit.

Total Hardness in handpump and borwell water samples were recorded in the rang of 482.83 mg/L (summer) to 633.85 mg/L (winter) High values of hardness can be attributed low water level and high rate of evaporation. Finding of present study is in harmony with the study of Harish et al. (1991) and Garg et al. (1990) with little variation. According to Kannan (1991), water with hardness more than 180 mg/L is very hard; in this respect, water of these sources are very hard.

Content of chlorides were noted as 511.79 mg/L (winter) to 553.80 mg/L (summer) in all above sources. Nalina and Puttaiah (2005) observed the maximum and minimum values of chloride in summer, rainy and winter season, respectively, from ground water.

Concentration of nitrate were found to vary within 0.68 mg/L (monsoon) to 0.82 mg/L (summer), and also value of nitrates in these sources are well within limits of ICMR standards (ICMR, 1975).

The level of DO varied within 4.03 mg/L (summer) to 7.08mg/L (winter); BOD ranged from 3.73 mg/L (summer) to 11.02 mg/L (winter); COD observed within 6.55 mg/L (monsoon) to 38.89 mg/L (winter); and TC count is very high in all the three seasons. Kaur et al. (1992), Rajmohan et al. (1997) and Singh et al. (2000) reported seasonal as well as yearly changes in the ground water quality. Pradhan et al. (2003) noted BOD as 1.1 mg/L (summer), 1.2 mg/L (rainy) and 1.0 (winter) in tubewell water at Rimuli, district keonjar (Orissa) India. The upper limit for BOD in drinking water is 3 mg/L, but when BOD values reach 5 mg/L, the water is doubtful in purity (Hari, 2002).

A considerable increase in COD values in some sampling stations near those locality which has poor sanitation and filthy water accumulation. Pathak (1994) reported COD values varied from 4 mg/L (rain) to 46 mg/L (winter) from handpump sample in Rewa region (M.P), India. Sharma, (2003) observed COD values 12.0 to 14.1) mg/L from tubewell water samples of Matsya Industrial area Alwar. Pathak (1994) noted t coliform high in number than the standard from handpump water samples during different seasons at Rewa region (M.P.) India. Rawat (2003) calculated MPN (coliform) per 100 ml in tubewell from 4 - 10 ml and 4 to 6/100 ml in handpumps. Fokmare (2002) also recorded increased number of coliform/100 ml in hand pumps at Akola city (M.S) India.

Application of WQI is a useful method in assessing the water quality of hand- pump and borewells. The WQI values in all three seasons are much above 100 indicating unsuitability for drinking purpose (Tables 1, 3, 4 and 5). The WQI values are maximum because for the continuous discharge of municipal sewage and industrial effluents near to the sources of water which may percolate in the ground water.

From the present observation, it can be concluded that water quality of hand pumps and borewells is under stress of severe pollution. The Beed district water is not suitable for drinking, bathing, swimming and pisciculture. In order to save these sources from further deterioration, effective pollution control measures must be taken in to consideration.

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academicJournals

Vol. 12(51), pp. 7092-7098, 18 December, 2013
DOI: 10.5897/AJB10.1751
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of accessions on the chemical quality of fresh pumpkin

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Accepted 18 June, 2013

Twenty (20) fresh pumpkin fruits were assessed for dry matter, total soluble solid, total sugar, reducing sugar, titratable acidity, pH, ascorbic acid and sugar-to-acid ratio. Statistically significant (P < 0.01) differences were found for dry matter, total soluble solid, total sugar, reducing sugar, titratable acidity, ascorbic acid, pH and sugar to acid ratio for the 20 fresh pumpkin fruit accessions. Accession 8007 had the highest dry matter, total titratable acidity, total soluble solid and total sugar content. Accession 8007 was also found to have high concentration of ascorbic acid although pumpkin fruit accession 8807 had the highest ascorbic acid. Pumpkin accession 4707 had the highest amount of reducing sugar and titratable acidity while accession 7707 had the highest pH and total soluble solid/ total titratable acid (TSS/TA) ratio. Pumpkin accession 8007 was found to be superior variety with the highest nutritional value while accession 1307 and 3907 were found to be inferior to the accessions evaluated in this study.

Key words: Pumpkin, accession, Ethiopia, nutritional value.

INTRODUCTION

Pumpkin is among the economically most important vegetable crops worldwide and is grown in both temperate and tropical regions (Pitrat et al., 1999; Paris, 1990; Sanjur et al., 2002). It is originated from Central America (Maynard et al., 2001). It was dispersed to other continents by transoceanic voyagers at the turn of the 16th century and has become a familiar and important vegetable crop in many countries (Gray and Trumbull, 1983). Depending upon the species, virtually all parts of the plant can be used for food, including leaves, shoots, roots, flowers, seeds, and immature and mature fruits (Schippers, 2000). The succulent, tasty leaves, stems, fruit and nutritious seeds make pumpkin the most popular vegetable to millions of people, ranking as one of the three most widely eaten vegetables at homes and in restaurants (Abiose, 1999). In Ethiopia, farmers produce pumpkin traditionally in their gardens together with cereals, near fences, to creep on houses, marginal or waste land, on decaying hay and heap of cow dung. The pumpkin cultivars locally produced by farmer are not identified. Suitable agro-ecologies for pumpkin cultivation for better performance of cultivars, suitable fruit and seed storage method for the extension of the shelf life and different fruit utilization method was not identified and popularized. There is wide variation in fruit size, fruit weight, shape and rind color, vine length and branching, leaf size, quality of fruit and seed size.

Despite its importance, pumpkin has not gained adequate

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Abbreviations: DM, Dry matter; **TSS,** total soluble solid; **TA,** titratable acidity; **AA,** ascorbic acid.

Table 1. List of pumpkin fruit accessions used for the study.

Number	Accession number	Origin/place of collection in Eastern Ethiopia
1	8007	Harar
2	7607	Harar
3	4707	Kulubi
4	4007	Kulubi
5	5207	Dire-Dawa
6	7807	Harar
7	3607	Hirna
8	8807	Lange
9	1907	Lange
10	8307	Lange
11	1407	Lange
12	6907	Jijiga
13	3407	Hirna
14	7207	Jijiga
15	2807	Dire-Dawa
16	7107	Jijiga
17	3907	Kulubi
18	9107	Kulubi
19	7707	Harar
20	1307	Lange

research attention in Ethiopia to harness its potential because most traditional Ethiopian vegetables do not figure very prominently in modern crop research and conservation program. The current rural development strategy of Ethiopia has given due attention to the development of vegetables production. Hence, bringing this vegetable under research stream would play a pivotal role in exploiting its potential. Since there is no research information and no work has been done on it in Ethiopia other than some characterization efforts at Haramaya University, so identifying different landraces of pumpkin that are grown in eastern Ethiopia and documenting their fresh quality is important. Accordingly, this study was initiated to generate information on fresh quality of the crop with the specific aim of determining chemical quality of pumpkin accessions.

MATERIALS AND METHODS

Experimental material

The pumpkin fruits were collected from the market based on their morphological characteristics and variation by taking the growing area into consideration. Pumpkin fruit accessions with seeds were collected from markets in eastern Ethiopia (*Jijiga, Dire-Dawa, Kulubi, Harar and Lange)* and twenty accessions (Table 1) were grown in the Haramaya University research station during 2007/2008 main growing season. All the 20 pumpkin accessions were produced in the same experimental plot in three replication. The University is located at latitude of 9° 26' N and longitude of 42° 03'east and an altitude of 1980 m.a.s.l. The rainfall of the area is bimodal (in April and August) type with an average annual rainfall of 790 mm. The mean annual temperature is 17°C with mean minimum and maximum temperatures of 3.8 and 25°C, respectively. The mean relative humidity is 50%, varying from 20 to 81% and the soil type of the area is well-drained deep clay loam type (Tekalign, 2005). After maturity of the fruits, 20 pumpkin accessions were used for fresh fruit quality assessment. Samples of three fruits from each accession (one fruit from each replicated plot) were taken for analysis.

Experimental design and procedures

The assessment of 20 fresh pumpkin fruit accessions for different quality parameters was made using completely randomized design with three replications. Three fruits were randomly selected from each of 20 pumpkin fruit accessions; one from each experimental plot. Each fruit was divided in to four parts, one fourth of which was used for fresh quality analysis.

Data collection

To determine dry matter (DM) content of the pumpkin, samples were chopped to very small size and dried in an oven at temperature of 70°C to a constant mass, and the dry matter content was calculated according to AOAC (1984). The total soluble solid (TSS) of pumpkin fruit was determined following the procedures described by Waskar et al. (1999). An aliquot of juice was extracted using a juice extractor (6001x Model No. 31JE35 6x.00777) and 50 ml of the slurry was filtered using cheesecloth. The TSS content was determined by Abbe refractrometer (B+S 60/70 Model No. A-90067, England) with a range of 0 to 32 °Brix, by placing 1 to 2 drops of clear juice on the prism. Between samples, the prism of the refractrometer was washed with distilled water and dried before use. The refractrometer was standardized against distilled water (0% TSS). The pH of fruit was measured using the method of

Table 2. The mean of DM, TSS, TS and RS content of pumpkin accessions collected from eastern Ethiopia.

Accession number	DM (%)	TSS (°Brix)	TS (g 100 g^{-1})	RS (g 100 g^{-1})
8007	11.00^{a}	10.03^{a}	9.002^{a}	4.785^{bc}
7607	10.60^{ab}	8.63^{bc}	7.696^{b}	5.235^{ab}
4707	10.30^{ab}	9.03^{b}	8.574^{a}	5.663^{a}
4007	10.00^{bc}	8.40^{bc}	7.437^{bc}	4.098^{d}
5207	9.70^{b-d}	8.00^{cd}	6.919^{c}	4.357^{cd}
7807	9.27^{c-e}	7.73^{cd}	6.818^{cd}	4.525^{cd}
3607	8.97^{d-f}	7.43^{de}	6.277^{de}	3.568^{e}
8807	8.83^{d-g}	7.06^{d-g}	5.207^{fg}	3.602^{e}
1907	8.50^{e-h}	7.23^{d-f}	5.692^{ef}	2.780^{fg}
8307	8.20^{f-i}	6.73^{e-h}	4.779^{gh}	2.976^{f}
1407	8.00^{g-j}	6.43^{f-i}	4.362^{hi}	1.958^{ij}
6907	7.80^{h-k}	6.27^{g-j}	4.147^{hij}	2.938^{f}
3407	7.60^{h-l}	6.13^{g-k}	3.935^{ijk}	2.510^{fgh}
7207	7.40^{i-m}	5.40^{j-l}	3.272^{lm}	2.352^{ghi}
2807	7.10^{j-n}	5.73^{i-k}	3.311^{klm}	1.643^{jk}
7107	6.90^{k-o}	6.07^{h-k}	3.541^{jkl}	2.093^{hij}
3907	6.70^{l-o}	4.10^{n}	2.178^{o}	1.074^{l}
9107	6.50^{m-o}	4.80^{l-n}	2.532^{no}	1.257^{kl}
7707	6.30^{no}	5.20^{k-m}	2.864^{mn}	1.778^{j}
1307	6.00^{o}	4.433^{mn}	2.262^{no}	1.109^{l}
SE (±)	0.22	0.23	0.16	0.13
CV (%)	4.65	5.79	4.22	4.17
LSD	0.85	0.86	0.60	0.48
Sign	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Means followed by common letters in a column are not significantly different from each other at 0.001 level of significance. Sign, Level of significance.

Nunes and Emond (1999). An extract of an aliquot of juice was prepared, the aliquot of juice was first filtered with cheesecloth and the pH value of the pumpkin fruit juice was measured with a Metrohn 691 pH meter. Total titratable acidity (TA) of the fruit was measured according to Maul et al. (2000). An aliquot of pumpkin fruit juice was extracted from the sampled pumpkin fruit with the juice extractor. The aliquot of pumpkin fruit juice was filtered through cheesecloth and the decanted clear juice was used for the analysis. The TA expressed as percent citric acid was obtained by titrating 10 ml of pumpkin fruit juice with 0.1N NaOH to pink end point (persisting for 15 s). Ascorbic acid (AA) content was determined by the 2, 6- dichlorophenol indophenols methods (AOAC, 1970). An aliquot of 10 ml of fruit juice was diluted to 50 ml with three percent metaphosphoric acid in a 50 ml volumetric flask. The aliquot was titrated with the standard dye to a pink end point (persisting for 15 s).

Compositional sugar analyses were conducted on fruit from each cultivar. Reducing (RS) and total sugars (TS) were estimated by using the calorimetric methods (Somogyi, 1945). Clear juice (10 ml) was added to 15 ml of 80% ethanol, mixed, and heated in a boiling water bath for 30 min. After extraction, 1 ml of saturated lead acetate ($Pb(CH_3COO)\ 2.3H_2O$) and 1.5 ml of saturated sodium hypophosphate (Na_2HPO_4) were added and the contents were mixed by gentle shaking. After filtration, the extract was made up to 50 ml with distilled water. An aliquot of 1 ml extract were taken and diluted to 10 ml with distilled water. Then, 0.5 ml aliquot of extract was taken and made up to 1 ml. To this, 1 ml of copper reagent was added in a test tube and heated for 20 min in a boiling water bath. After heating, the contents were cooled under running tap water without shaking. Arsenomolybdate color reagent were added, mixed, made up to 10 ml, and left for about 10 min to allow color development, after which the absorbance were determined by a spectrophotometer at 540 nm. For total sugar determination, sugar was first hydrolyzed with 1 N HCl by heating at 70°C for 30 min. After hydrolysis, the determination of total sugar was made by following the same procedure employed for the reducing sugar. Blanks were prepared using distilled water instead of extract. The sugar to acid ratio of the fruits was calculated by dividing TSS to TA of the fruits.

Data analysis

Analysis of variance was carried out using MSTAT-C statistical software package (MSTAT-C 1991). Means were compared using the least significance differences test at 5% probability level.

RESULTS AND DISCUSSION

Dry matter and total soluble solid

The DM contents of the pumpkin fruits differed significantly ($P < 0.001$) (Table 2). The DM content ranged between 6.0 and 11.0%. The fruit of accession 8007 had the highest DM content (11%) which was significantly

higher than the DM content of all accessions except accessions 7607 and 4707. Pumpkin accession 1307 had the lowest DM content (6%). Such high variation in DM content of pumpkin fruit has also been reported by Paulauskiene et al. (2006) who studied the quality of pumpkin cultivar in relation to their electrochemical and antioxidant properties. The difference could be due to variation in starch content of the genotype of the pumpkin fruit; with high DM content there is high content of starch (Hazzard, 2006). According to this researcher, pumpkin fruit with high DM content and starch content was characterized by high TSS and lower fiber content.

Significant ($P < 0.001$) difference in fruit TSS content was obtained among the pumpkin accessions with a range varying between 4.1 and 10.3 °Brix (Table 2). Pumpkin fruit accession number 8007 had the highest TSS (10.03 °Brix.) which was followed by accession 4707 (9.033 °Brix) while pumpkin fruit accession number 3907 had significantly lower TSS (4.1 °Brix). The result of this study agreed with the reports of Murakami et al. (1992) and Sudhakar et al. (2003) who showed large variation in the TSS content of pumpkin cultivar that also varied from year to year.

In general, 45% of the accessions recorded DM and TSS value more than average of whole accessions while the top four accessions had DM and TSS value, 26.5 and 33.9%, more than average of all accessions. More than 85% of pumpkin fruit TSS content is sugar and it is highly related to the sensory quality of pumpkin fruit and it is used to screen pumpkin fruit (Cantwell and Suslow, 1998).

DM content and TSS were shown to be among the major traits affecting the quality of pumpkin (Cordenunsi et al., 2003; Sturm et al., 2003). Several publications reported that quality of cucurbit fruit was highly related to sucrose and TSS content of fruit (Monforte et al., 2004; Sinclair et al., 2006). In addition to the naturally occurring variation in sucrose levels within any particular cultivar, there are genetic differences among cultivars affecting fruit quality in terms of their TSS contents (Stepansky et al., 1999; Burger et al., 2002, 2006, Burger and Schaffer, 2007).

In this study, DM showed strong positive correlation with TSS ($r = 0.94$), total sugar ($r = 0.953$), reducing sugar ($r = 0.919$), ascorbic acid ($r = 0.624$) and TA ($r = 0.624$) (Table 4). The positive correlation of fruit quality and DM content of pumpkin fruit in agreement with previous reports of Hazzard (2006) and Paulauskiene et al. (2006). The positive correlation could be because of starch; the major component of DM is the substrate from which TSS, total sugar, reducing sugar is synthesize during maturation and ripening (Hazzard, 2006).

Sugar contents

The total sugar content of pumpkin fruit significantly ($P < 0.001$) varied among the fruit accessions (Table 2). In this study, the total sugar content of the accessions ranged between 2.178 to 9.0 g 100g $^{-1}$ fresh weight. Pumpkin accession 8007 recorded significantly higher total sugar (9.0 g 100g $^{-1}$) than all the accessions except accession 8007 and 4707. Pumpkin fruit accession number 3907 had the lowest total sugar content. Similar to that of DM and TSS, the top four accessions (accession 8007, 7607, 4707 and 4007) had about 62% more total sugar over the average of all accessions while accession 1307 recorded the least values total sugar. The result of this study agrees with the findings of Sudhakar et al. (2003) who reported that reducing sugar content of pumpkin fruit significantly varied from cultivar to cultivar. Cantwell and Suslow (1998) also reported variations in total sugar contents among 36 varieties of pumpkin and indicated that sugar is the major component of TSS and it determines the flavor and sensory quality of pumpkin fruit.

Analysis of variance also revealed significant difference ($P < 0.001$) in the reducing sugar content of the pumpkin fruit accessions (Table 2). The reducing sugar content of the accessions ranged between 1.074 and 5.663 g 100g $^{-1}$. Pumpkin accession 4707 had the highest reducing sugar content which was followed by pumpkin accession 7607 (5.235 g 100 g $^{-1}$) while pumpkin accession 3907 had the lowest reducing sugar content. The result of reducing sugar in this study agrees with the finding of Murakami et al. (1992) and Culpepper and Moon (1945) who reported pumpkin fruit sugar content especially reducing sugar have high relation with sweetness of the fruit. Correlation analysis also showed that reducing sugar content of the pumpkin fruit accessions was strongly associated with TSS ($r = 0.903$), total sugar ($r = 0.941$) and DM ($r = 0.919$). The reason for positive correlations of reducing sugar to these parameters was that reducing sugar is a component of total sugar and TSS. Reducing sugar is also the substrate from which TA is synthesized within the fruit (Sakiyama and Stevens, 1976).

Ascorbic acid

Pumpkin fruit accession showed highly significant ($P < 0.001$) variation in ascorbic acid content at harvest (Table 3). The ascorbic acid content ranged between 4.81 mg 100 g $^{-1}$ for accession number 7207 and 9.1 mg 100 g $^{-1}$ for accession 8807. Overall, 50% of the accessions had ascorbic acid contents more than the average for all (7.01 mg 100 g $^{-1}$) while the cultivar with the highest ascorbic acid content recorded 29.8% over the average. Similar result reported by Sudhakar et al. (2003) indicated that ascorbic acid content of pumpkin vary from cultivar to cultivar. Many researchers reported that pumpkin provides a valuable source of ascorbic acid that have a major role in nutrition in the form of vitamin C as antioxidants (Duke and Ayensu, 1985; Sudhakar et al.,

Table 3. Mean value of fruit chemical composition in 20 pumpkin accessions collected from eastern Ethiopia.

Source of variation (accession)	Ascorbic acid (mg/100 g)	TA (%citric acid)	pH	TSS/TA
8007	8.700[a-c]	1.723[ab]	6.257[i]	5.818[a]
7607	7.900[c-f]	1.727[ab]	6.023[j]	5.000[b-e]
4707	8.500[a-d]	1.733[a]	5.920[j]	5.212[a-c]
4007	7.300[f-i]	1.620[bc]	6.560[fg]	5.184[a-d]
5207	7.700[d-g]	1.630[abc]	6.497[gh]	4.908[b-e]
7807	7.500[e-h]	1.653[ab]	6.340[hi]	4.677[b-e]
3607	8.900[ab]	1.490[de]	6.690[d-g]	4.990[b-e]
8807	9.100[a]	1.540[cd]	6.600[fg]	4.588[c-e]
1907	5.600[m-p]	1.350[fg]	6.750[b-f]	5.359[ab]
8307	5.400[n-p]	1.450[def]	6.643[e-g]	4.643[b-e]
1407	7.100[f-j]	1.200[hi]	6.837[a-e]	5.371[ab]
6907	6.933[g-k]	1.410[ef]	6.710[c-g]	4.444[de]
3407	8.200[b-e]	1.300[gh]	6.777[a-f]	4.711[b-e]
7207	4.800[p]	1.237[hi]	6.823[a-e]	4.368[e]
2807	5.133[op]	1.143[ij]	6.933[a-c]	5.013[b-e]
7107	6.700[h-l]	1.223[hi]	6.853[a-e]	4.961[b-e]
3907	5.833[l-o]	1.193[hi]	6.880[a-d]	3.429[f]
9107	6.500[i-l]	1.080[jk]	6.940[ab]	4.446[de]
7707	6.300[j-m]	0.9000[l]	6.993[a]	5.840[a]
1307	6.100[k-n]	1.020[k]	6.950[ab]	4.349[e]
SE (±)	0.21	0.02	0.05	0.19
CV (%)	5.15	2.88	1.33	6.91
LSD	0.796	0.099	0.198	0.742
Significance	P < 0.001	P < 0.001	P < 0.001	P < 0.001

Means followed by common letters in a column are not significantly different from each other at 0.001 level significance.

Table 4. Correlation of chemical composition parameters of fresh pumpkin fruit

	DM	TSS	TSU	RS	AA	TA	pH
DM	1						
TSS	0.940(***)	1					
TSU	0.953(***)	0.965(***)	1				
RS	0.919(***)	0.903(***)	0.941(***)	1			
AA	0.624(***)	0.609(***)	0.633 (***)	0.631(***)	1		
TA	0.624(***)	0.886(***)	0.912(***)	0.927(***)	0.61(***)7	1	
pH	-0.854(***)	-0.814(***)	-0.870(***)	-0.907(***)	-0.573(***)	-0.864	1
TSS/TA	0.376(**)	0.579(***)	0.467 (***)	0.34(**)	0.230(NS)	0.149 (NS)	-0.240(ns)

*Significant at P <0.05, *** significant at P< 0.01 and **significant at P < 0.001. DM, dry matter; TSS, total soluble solid; TSU,

2003) suggesting the potential of cultivars assessed in this study.

Ascorbic acid had significant and positive correlation with DM (R=0.624), TSS (r = 0.609), total sugar (r = 0.633), reducing sugar (r = 0.631), TA (r=0.617) and sugar to acid ratio (r = 0.941) indicating that most of the valuable quality attributes were associated and selection could be made based on one of these trait for ease of screening pumpkin genotype.

Titratable acidity

Analysis of variance revealed significant (P < 0.001) difference in TA of the pumpkin fruit accessions (Table 3). The value ranged between 0.9 and 1.73% for pump-

kin accession 7707 and accession 7607, respectively. The result of this finding agreed with the finding of Davies and Hobson (1981). In a comparative study, Hurst et al. (1995) also found TA for six pumpkin genotypes in the range of 0.9 to 1.75% which is in agreement with the present study. Many studies support the idea that organic acids are produced within the fruit from stored carbohydrate material and that large sized pumpkin fruits with high DM content and TSS also have high acidity (Sakiyama and Stevens, 1976; Tittonell et al., 2001). High DM content and TSS also have high TA (Sakiyama and Stevens, 1976; Tittonell et al., 2001) which is inline with the present result. TA of pumpkin fruits in this study was also observed to have strong positive correlation with DM (r = 0.928), TSS (r = 0.886), total sugar (r =0.912) and reducing sugar (r = 0.927). Similarly, Saliba-Colombani et al. (2001) have also shown that fruit quality like sugar (primarily reducing sugars) was positively correlated to TA since it is synthesized within the fruit from stored carbohydrate.

pH of pumpkin

Table 3 displays the pH value of the 20 fresh pumpkin fruit accessions. Significant ($P < 0.001$) difference in pH values was observed among the accessions which ranged between 5.920 and 6.993. The highest pH value was recorded in pumpkin accession 7707 which did not significantly differ from pH values of 40% of the remaining accessions. This result appeared to be close to the pH values of pumpkin reported by Paulauskiene et al. (2006) varying between 5.87 to 6.99. Atherton and Rudich (1986) noted that there is a tremendous variation among pumpkin genotypes for pH and titratable acids. Paulson and Stevens (1974) showed that pH was highly negatively correlated with TA which agrees with the present result (r =0.864). The pH value of the pumpkin fruit accessions was observed to have strong negative correlation with DM content (r =-0.854), TSS (r =-0.814), total sugar (r = -0.870) and reducing sugar (r = -0.907). The reason for the negative correlation of pH value to these parameters could be because titratable acids is synthesized from stored carbohydrate within the fruit (Sakiyama and Stevens, 1976), so as fruit DM, TSS and sugar content increases, the content of TA also increases. Since TA has inverse relationship with pH value, so the negative relationship could be due to the increment of TA.

Total soluble solid to titratable acidity ratio

TSS to TA ratio varied significantly ($P < 0.001$) among the pumpkin fruit accessions (Table 3). The TSS/TA ratio of accessions ranged between 3.429 for accession 3907 and 5.840 for accession 7707. TSS to TA ratio of fruit and vegetable shows the balance of sugar to acid of fruit and vegetable. Although, the perception of flavor is influenced by many factors, taste is one of the most important components determined by sugars and acids (Malundo et al., 1995). Hurst et al. (1995), Daniel et al. (1995) and Harvey et al. (1997) reported that the sensory quality of pumpkin fruit is influenced by sugar and acid and their ratio is used in screening of pumpkin for sensory quality. Hence, the variation in TSS/TA ratio observed in this study indicate the presence of great potential in the local pumpkin genotypes for future improvement of this crop. Many researchers report that the TSS, TA and sugar content of pumpkin have strong relation and affect the flavor of pumpkin fruit (Cantwell and Suslow, 1998).

Conclusion

There was significant ($P < 0.001$) difference among accession in DM, TSS, total sugar, reducing sugar, TA, pH, ascorbic acid and sugar to acid ratio. Same pumpkin fruit accessions had superior compositional quality; pumpkin accession 8007 had the highest DM (11%), TSS (10.03 °Brix), total sugar (9.0 g/100 g); pumpkin fruit with accession number 8807 had the highest ascorbic acid (9.1 mg/100 g); pumpkin fruit accession number 4707 had the highest reducing sugar (5.66 g/100 g) and TA (1.73%) and pumpkin fruit accession 7707 had the highest TSS/TA ratio (5.84) and pH content (6.99). Generally, pumpkin accessions 8007, 8807, 4707 and 7707 had superior nutritional value while accession 1307 seemed to have lower concentrations of total soluble solid, total sugar and reducing sugar. Overall, accessions 8007, 8807, 4707 and 4007 are recommended for their superior nutritional quality.

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Vol. 12(51), pp. 7009-7016, 18 December, 2013
DOI: 10.5897/AJBX11.062
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

Role of alternative oxidase in postharvest stress of fruit and vegetables: Chilling injury

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Accepted 3 January, 2012

Chilling injury (CI) imposes a limitation for extending storage of fruit and vegetables. Chilling injury can result from oxidative stress caused by reactive oxygen species (ROS). Alternative oxidase (AOX) as a ROS avoidance genes play pivotal role in defense mechanism against chilling injury derived oxidative stress. Postharvest treatment of fruit and vegetables through methyl salicylate, methyl jasmonate and storage in atmosphere with high O_2 could induce AOX gene expression and led to chilling tolerance. In this review, the main topic is the role of postharvest treatments in tolerance to chilling injury via induction of AOX gene expression and AOX physiological roles in fruit ripening.

Key words: Chilling injury, alternative oxidase, methyl salicylate, methyl jasmonate, reactive oxygen species, fruit ripening.

INTRODUCTION

Low temperature storage is a postharvest technology used widely to extend the postharvest life of fruit and vegetables and allows the preservation of fruit and vegetables quality after harvest, because low temperatures decrease the speed of cell metabolism and delay plant senescence and fruit ripening (Sevillano et al., 2009). Tropical and subtropical fruit and vegetables are sensitive to low temperatures and suffer chilling injury (CI). The incidence of chilling injury as a physiological disorder limits the application of low temperature storage. Therefore, the impact of chilling injury on the agro-food industry has serious economic consequences (Sevillano et al., 2009). Chilling injury of fruit and vegetables negatively affect their quality and therefore their marketing (Lafuente et al., 2005; Lafuente and Zacarias, 2006; Mulas and Schirra, 2007). To increase the tolerance of fruit and vegetables to chilling injury, postharvest treatments such as signaling molecules: Methyl jasmonate (MeJA) or methyl salicylate (MeSA) (Fung et al., 2004, 2006) or use of storage with high (Zheng et al., 2008) had been developed. Further, the induction of the AOX gene expression by these pretreatments had been shown. Also, there is a direct correlation between the induction of AOX gene expression and the increase tolerance to chilling injury. In the present review, the literature in which the role of AOX had been studied in relation to the use of treatments for harvested fruit and vegetables was analyzed. Besides, the role of AOX in the fruit ripening and their biochemical mode of action were also discussed.

CHILLING INJURY IN HORTICULTURAL CROPS

Tropical and subtropical fruit and vegetables are sensitive to low temperatures and suffer from chilling injury. The occurrence of chilling injury limits the use of cold storage. Therefore, the impact of chilling injury on the horticultural industry has serious economic consequences. Understanding the physiological mechanisms responsible

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Abbreviations: AOX, Alternative oxidase; **ROS,** reactive oxygen species; **CI**, chilling injury.

the activation and development of this physiological disorder would allow the design of strategies to avoid or delay CI appearance (Sevillano et al., 2009). Cell membranes are primary sites for development of CI (Rui et al., 2010). Phase transitions from a flexible liquid crystalline to a solid gel structure occur in membrane of chilled tissue (Lyons, 1973). The fatty acid composition of membrane lipids can determine the existence of a temperature induced phase transition (Martin et al., 1976). In order to explain the impacts of CI on cell membranes and its manifestations at the molecular level when CI occurs, Lyons (1973) proposed the so-called 'membrane theory'. The membrane theory of CI suggests that the immediate effect of low temperatures is a global increase in the microviscosity of the membrane matrix due to a diminution of random rotation or folding movements of the aliphatic chains of fatty acids and to a reduction in the degree of desaturation of these acids. Below a critical value of temperature called the transition temperature, this event leads to a reorganization of the membrane lipids in a rigid structure called solid gel. A higher proportion of unsaturated fatty acids provide higher tolerance to low temperature, as has been reported, for example, in banana, pomegranate and loquat fruit (Mirdehghan et al., 2007; Promyou et al., 2008; Cao et al., 2009). Maintenance of membrane integrity at low temperature has been reported to be important in the resistance to chilling temperature (Wonsheree et al., 2009). As indicators of membrane damage, electrolyte leakage and malondialdehyde (MDA) content are generally considered to be indirect measurements of membrane integrity and can reflect the occurrence of CI and loss of membrane integrity (Shewfelt and Purvis, 1995). Lipolytic cascade in membrane lipids deterioration during senescence and CI was achieved by the concerted activities of a variety of membranous lipolytic enzymes such as phospholipase D (PLD) and lipoxygenase (LOX) (Pinhero et al., 1998). LOX and PLD catalyze peroxidation of polyunsaturated fatty acids and are believed to be major contributors to membrane damage and thus CI in plant (Pinhero et al., 1998; Wang, 2001). Mao et al. (2007) showed that the development of CI in cucumber fruit was accompanied by increase in PLD and LOX activities when exposed to chilling stress, and that the enhanced tolerance to CI by heat treatment was related to the reduction in activities of both enzymes. Rui et al. (2010) reported that the heat treatment increased LOX and PLD activity in response to chilling stress in loquat fruit and the reduction of internal browning (IB), main symptom of chilling injury in loquat fruit by heat treatment was associated with the reduction of PLD and LOX activities. This result suggested that these two enzymes might be associated with the initiation of CI by being involved in membrane deterioration and signalling pathway in response to chilling stress. Changes in membrane structure and composition are considered as the primary event of CI and lead to a loss of permeability control and metabolic dysfunctioning (Lyons, 1973). It was reported that membrane lipids from chilling resistant plant species showed higher content of unsaturated fatty acids than did sensitive species (Wonsheree et al., 2009). Heat treatment induced acclimatization of pomegranate and banana fruit to low temperature and thus reduced CI by maintaining higher unsaturated/saturated fatty acid ratio (Mirdehghan et al., 2007; Promyou et al., 2008). Cao et al. (2009) showed that the decrease of lipid unsaturation was involved in the induction of CI in loquat fruit. The higher unsaturated/ saturated fatty acid ratio contributed to the reduced CI in methyl jasmonate treated fruit. Rui et al. (2010) suggested that the loss of membrane integrity, decrease in membrane lipid unsaturation and increases in PLD and LOX activities may be involved in the development of IB and CI in loquat fruit. The reduction of IB in chilled loquat fruit by heat treatment might be due to maintenance of membrane integrity, higher unsaturated/saturated fatty acid ratio and reduced PLD and LOX activities.

Apart from the direct effect of low temperatures on the molecular organization of membrane lipids, the loss of integrity of the membrane itself is boosted by oxidative processes, since low-temperature stress increases the levels of reactive oxygen species (ROS) (Sevillano et al., 2009). Defense against oxidative stress consists of two lines: The first line of defense is termed ROS scavenging genes which includes superoxide dismutase (SOD), catalase (CAT), the ascorbate/glutathione (AsA-GSH) cycle, the glutathione peroxidase and thioredoxin system (Møller, 2001). SOD catalyses the dismutation of $O_2^{\bullet -}$ to H_2O_2 and CAT scavenge H_2O_2 to form oxygen and water. The AsA-GSH cycle is also an important mechanism in the removal of ROS in plants. Its activation seems to produce a positive effect by inhibiting the development of CI. Sato et al. (2001) showed that the induction of APX activity by means of a heat treatment is a key element in the protection of rice against a later exposure to low temperatures. The GSH content and GR activity are also significant in plants showing tolerance to low temperatures. Cold-tolerant genotypes of tomato (Walker aand Mckersie, 1993) accumulate more GSH, and during cooling, their GR activity is higher than that of sensitive genotypes. Moreover, the rise in GSH content and GR activity in cold-sensitive genotypes of corn causes a diminution of CI (Kocsy et al., 2001).

The second line of defense is termed ROS avoidance genes which include alternative oxidase (AOX). The plant AOX pathway branches from the main respiratory electron transport chain, bypasses the final steps of the cytochrome respiratory pathway and catalyses the oxidation of ubiquinol. It was suggested that by maintaining the flow of mitochondrial electrons, AOX maintained activation of NAD(P)H dehydrogenase and proton-pumping NADH dehydrogenase (Møller, 2001) and helps in generation of sufficient ATP required for the rapid adaptation and the maintenance of plant growth rate

homeostasis (Moore et al., 2002; Hansen et al., 2002). In this process, AOX is involved in the reduction of ROS by preventing electrons from reducing O_2 to $O_2^{\bullet-}$ and thus reduces the level of O_2 in the mitochondria (Møller, 2001; Wagner and KRAB, 1995; Mittler, 2002). The concept of AOX acting as an antioxidant enzyme has been shown in isolated bell pepper mitochondria (Purvis, 1995) and in intact tobacco cells (Maxwell et al., 1999). It was proposed that the alternative respiratory pathway mediated chilling injury by keeping the production of ROS in balance with the levels of antioxidants and active oxygen scavenging enzyme systems (Purvis, 1995).

AOX FUNCTIONS IN FRUIT AND VEGETABLES

Two main hypotheses for roles of AOX activity in plants suggested that: (i) AOX activity allows continued TCA cycle carbon flow to occur under conditions when the cytochrome pathway is inhibited or restricted by the availability of ADP (Bahr and Bonner, 1973; Vanlerberghe and Mcintosh, 1994). When mitochondrial electron transport via the cytochrome pathway is inhibited or restricted, it results in the inhibition of TCA cycle and glycolysis. Reducing power (NAD (P) H) accumulates, biosynthesis stops and aerobic fermentation takes place which may lead to tissue damage by ethanol production (Vanlerberghe et al., 1995). An alternative electron transport pathway to oxygen could prevent redirection of carbon metabolism to the fermentative pathways and allow activity of TCA cycle to continue. Palmer (1976) suggested that this was probably the primary function of the AOX. Such a function was later modified by Lambers (1985), who suggested that the alternative pathway functions as an "energy overflow". It was proposed that the alternative pathway became engaged when the cytochrome pathway was saturated (Bahr and Bonner, 1973) such as the one that occur in the presence of excess sugars. (ii) The AOX has a role in the defence mechanism against oxidative stress such as chilling injury (Sevillano et al., 2009). Mittler (2002) proposed that the AOX reduced ROS production by means of two mechanisms: preventing the reduction of O_2 to superoxide radical and reducing the amount of O_2, the substrate for the formation of ROS in cell organelles.

There are two energy dissipating systems in plants: The redox energy dissipating pathway (AOX) and the proton electrochemical gradient (H^+) energy dissipating pathway or plant uncoupling mitochondrial protein (PUMP) which leads to the same final effect such as a decrease in ATP synthesis and an increase in heat production (Sluse and Jarmuszkiewicz, 2000). Linoleic acid, an abundant free fatty acid (FFA) in plants which activates PUMP, strongly inhibits cyanide resistant respiration mediated by AOX. Sluse and Jarmuszkiewicz (2000) reported that the AOX and PUMP acts sequentially during postharvest ripening of tomato fruit, AOX activity decreases in early post-growing stages and PUMP activity is decreased in late ripening stages. Thermogenesis occurs during fruit ripening (Cruz-Hernandez and Gomez-Lim, 1995; Kumar et al., 1990; Kumar and Sinha, 1992) and FFA concentration increases during the post-growing stage (Rouet-Mayer et al., 1995). Sluse and Jarmuszkiewicz (2000) indicated a clear regulation of AOX activity through a decrease in protein expression during tomato fruit ripening, and a decrease in PUMP protein expression only after the yellow stage that parallels PUMP activity. Sluse and Jarmuszkiewicz (2000) suggested that AOX and PUMP acts sequentially. AOX would be active mainly during the growing period, thereby providing a safety balance between redox potential, phosphate potential and biosynthesis demand, whereas PUMP would start working in the post-growing stage when the FFA concentration increases, thereby providing a mechanism for heat generation via a decrease in the efficiency of oxidative phosphorylation in parallel with the termination of biosynthetic processes.

Considine et al. (2001) reported that the expression of PUMP genes is strongly enhanced during fruit ripening, suggesting a developmental stage specific regulation of PUMP. The increase in mRNA levels after the climacteric ripening phase directly correlates with the increase of mango PUMP content. In tomato, the studies on fruit ripening show that PUMP expression adapts to physiological conditions. Holtzapffel et al. (2002) showed an increase in transcript and protein levels in the later stages of on-vine fruit ripening and Almeida et al. (1999) reported that the activity of PUMP decreases during the later stages of postharvest fruit ripening. Almeida et al. (1999) also reported a higher amount of AOX in tomato fruit in postharvest ripening. However, FA concentrations also increase during the post growing stage. The PUMP and AOX pathways could therefore operate with different efficiencies under distinct physiological conditions. AOX would be active mainly during high biosynthetic activities in early stages of tomato fruit ripening, whereas PUMP would be functionally silent. With increasing FFA concentration in post growth stages, as in fruit ripening but perhaps also in senescence and flowering, PUMP activity could reach maximum velocity while AOX activity is switched off (Jarmuszkiewicz et al., 2001).

POSTHARVEST TREATMENTS, AOX GENE EXPRESSION AND CHILLING TOLERANCE IN FRUIT AND VEGETABLES

Although chilling injury can be prevented by maintaining the commodity at temperatures above the critical threshold, these temperatures can significantly reduce the product’s shelf life (Troncoso-Rojas and Tiznado-Hernandez, 2006). Fruit and vegetables that are susceptible to chilling injury have a short storage life in

low temperature storage. Low temperature storage cannot be used to slow losses and pathogen growth in this commodity, which has serious economic consequence on the agro-industry.

Valero and Serrano (2010) reported that the potential symptoms of chilling injury in fruit and vegetables are surface lesions, pitting, sunken and discoloration area, water soaking of tissue, water loss, desiccation, shriveling, internal browning, mealiness and browning in the flesh, tissue breakdown, failure of fruit to ripen or uneven or slow ripening, accelerated senescence and ethylene production, shortened storage or shelf life, loss of flavor, loss of growth or sprouting capability, wilting and increased decay due to leakage of cell metabolites.

Due to consumer concerns about the presence of chemicals in fruit and vegetables, the use of environmentally friendly technologies such as salicylic and jasmonic acids and their methyl esters to avoid the development of chilling injury have been considered in many studies. The exposure of fruit and vegetables to these signaling molecules is used as a strategy to protect the commodity from the subsequent stress represented by the cold storage. From a biochemical point of view, evidence that links this protective effect with the expression of AOX has been found. The present review discusses the application of postharvest treatments and the link between treatment application and AOX gene expression.

SIGNALING MOLECULES: SALICYLATES AND JASMONATES

The increasing demand for consumption of fresh fruit and vegetables, along with more restriction on the use of synthetic chemicals to preserve produce quality and reduce CI, has encouraged scientific research to develop new technologies based on natural product such as salicylic acid and methyl salicylate (MeSA), and jasmonic acid and methyl jasmonate (MeJA).

SA and MeSA are endogenous signal molecules, playing pivotal roles in regulating stress responses and plant development. Recently, SA has received a particular attention because it is a key signal molecule for expression of multiple modes of plant stress resistance. Although, the focus has been mainly on the roles of SA on biotic stresses, several studies support major roles of salicylates in modulating plant response to several abiotic stresses, such as UV light, drought, salinity, chilling stress and heat shock. Salicylates delay the ripening of fruits, probably through inhibition of ethylene biosynthesis or action, and maintain postharvest quality (Asghari and Aghdam, 2010). Although, there are many methods to reduce CI in various fruit and vegetables, SA and MeSA treatments are inexpensive, easy to apply and can be used on various horticultural crops (Asghari and Aghdam, 2010). SA has been shown to induce expression of AOX and increase the antioxidant capacity of the cells. Increases in AOX transcript levels using SA and MeSA before cold treatment reduces the incidence of CI in freshly green bell peppers (Fung et al., 2004). Lipid peroxidation is closely tied to CI in plant cells and this leads to MDA accumulation. MDA accumulation is prevented by SA treatment (Asghari and Aghdam, 2010). SA as a signal triggers the induction of cyanide resistance respiration in plant cells by affecting the AOX enzyme activity (Raskin et al., 1989). In fruit and vegetables, SA affects AOX activity leading to decrease in the harmful effects of different post-harvest oxidative stresses such as chilling injury, prevents fermentation and maintains low respiration rates and decreases fruit ripening and senescence rates. Respiration of harvested crops is highly dependent on ethylene production and activity and any factor increasing the production and activity of ethylene leads to increases in respiration and consequently increases the senescence rate. Effect of SA in decreasing the respiration rate is mainly due to its negative effects on ACC, ACO, PG, PME, cellulase and antioxidant enzymes leading to decrease in ethylene production and action (Asghari and Aghdam, 2010).

MeJA treatment also regulates diverse processes such as skin color development by promoting β-carotene synthesis and chlorophyll degradation, CI and ion leakage. MeJA treatment reduces the development of CI symptoms in a wide range of fruit and vegetables, including mango (González-Aguilar et al., 2000), guava (González-Aguilar et al., 2004), tomato (Ding et al., 2001) and loquat (Cao et al., 2010). Fruit and vegetables treated with MeJA have higher sugar, organic acid and vitamin C levels. MeJA stimulate ethylene biosynthesis by increasing ACS and ACO activity, which in turn enhances fruit ripening (González-Aguilar et al., 2006). MeJA treatment inhibits the gray mould rot caused by *Botrytis cinerea* and reduces decay caused by green mould *Penicillium digitatum* (Gonzalez-Aguilar et al., 2006). Recently, it was reported that MeJA induced the expression of pathogen related proteins (PRs) and AOX genes, increase the transcript accumulation of heat shock proteins (HSPs), and enhanced antioxidant system activity. These findings help to explain the mode of action of MeJA in increasing chilling tolerance in fruit and vegetables (Gonzalez-Aguilar et al., 2006).

Fung et al. (2004) reported that the treatment of pepper fruit with MeSA or MeJA vapors increased preferentially the transcription levels of AOX. These authors showed that the overnight treatment with MeSA or MeJA vapors increased transcript levels of AOX (1.5 kb) even at room temperature of 25°C, whereas no change was observed with untreated control. In addition to the expected 1.5 kb AOX transcript, RNA gel blot analysis by Fung et al. (2004) revealed an extra 3.5 kb transcript which was induced only at 0°C. At 0°C, both AOX transcripts reached maximal levels firstly in MeSA treated fruit, secondly in MeJA treated fruit and lastly in controls.

Fung et al. (2004) showed that the MeSA and MeJA vapors increased resistance against chilling injury in freshly harvested green bell pepper. They also showed that the increase in AOX transcript levels by MeJA or MeSA before cold treatment was correlated with reduced incidence of chilling injury in green bell pepper. AOX transcript, protein levels and activity were shown to respond to multiple developmental and environmental triggers (Considine et al., 2002). For example, AOX transcript and protein can be increased by low temperature (Ito et al., 1997; Djajanegara et al., 1999). Mutated AOX gene was linked to quantitative trait loci (QTL) for low temperature tolerance in a rice cultivar (Ribas-Carbo et al., 2000).

Fung et al. (2004) reported that the expression of AOX family 1 gene(s) in pepper was tightly associated with low temperature stress, suggesting that family 1 gene play a role in stress-induced conditions in plants. Within 6 h of rewarming, AOX transcripts (3.5 and 1.6 kb) in pepper fruit are depleted suggesting the presence of a high turnover rate for AOX transcripts. Low levels of CaAOX1 transcripts are detected in fruit stored at 20°C and freshly harvested. This suggests that the CaAOX1 transcript is constitutively expressed throughout fruit development and corresponds to basal levels necessary for alternative respiration (Fung et al., 2004).

Fung et al. (2004) showed that the CaAOX1 transcripts (1.5 kb) reach high levels within 2 days at 5°C but fruit at 0°C takes up to 7 days to attain the same level. Purvis (2001) reported that the higher AOX protein levels were found in mitochondria from pepper fruit stored at 4°C than at 1°C. Fung et al. (2004) suggested that the delay of CaAOX1 transcript accumulation at 0°C can be overcome by application of MeJA or MeSA vapors. Both MeSA and MeJA reduce short-term external surface pitting and long-term internal seed blackening symptoms in pepper fruit. Though, MeSA induced higher and earlier expression of CaAOX1 transcripts, MeJA (10^{-4} M) was more effective than MeSA (10^{-4} M) at alleviating chilling injury. It is possible that the beneficial effects for chilling resistance from enhancement of alternative oxidase might be compromised by the death-inducing properties of MeSA (Alvarez, 2000).

Fatty acid desaturase (18:1 FAD) gene for the production of polyunsaturated membrane lipid (Miquel et al. 1993), the catalase and alternative oxidase (AOX) genes in the oxidative stress defense mechanism (Kerdnaimongkol and Woodson, 1999; Abe et al., 2002), cold regulated (COR) genes such as dehydrins (Thomashow, 1998) and CBF1 genes, a member of AP2/EREBP transcription factor family (ZHAO et al., 2009) are mechanisms correlated with the acquisition of chilling tolerance.

AOX action maintains the flow of mitochondrial electrons and avoids production of ROS (Purvis, 1997; Maxwell et al., 1999; Møller, 2001) which was thought to be the main factor which resulted in CI (Purvis et al., 1995). MeSA and MeJA induced CaAOX1 gene expression is correlated with resistance to CI in peppers (Fung et al., 2004). One unexplained phenomenon of CaAOX1 expression was the detection of two CaAOX transcripts (1.5 and 3.5 kb) at low temperature. A longer transcript was also detected in rice and suggested to be the unprocessed transcript of AOX (Ito et al., 1997). Family 1 AOX genes typically occur as multigene families in plants (Ito et al., 1997). For example, closely related LeAOX1a and 1b gene transcripts are expressed in chilled tomatoes (Holtzapffel et al., 2003). By using a yeast expression system, the 1b protein was shown to be functional and had altered regulatory properties in comparison to 1a. The 1b protein was suggested to be a less regulated form of AOX that remains active under stress conditions (Holtzapffel et al., 2003). Overnight exposure to MeSA or MeJA was effective at increasing resistance to CI in tomatoes at the mature green maturity stage (Ding et al., 2002) and in peppers (Fung et al., 2004).

Fung et al. (2006) treated pink tomato fruit overnight with MeJA or MeSA at a concentration of 10^{-4} M before cold storage at 0°C. CI index was recorded based on overall fruit appearance by taking into account the degree of shrivel, pitting, decay and fruit color. These authors reported that the significant differences in CI index were found among treated and untreated pink tomato fruit only after 3 weeks of storage and only with MeSA treatment. For MeJA treated fruit, no differences were observed in untreated fruit except for less decay. For MeSA treated fruit, CI index was significantly lower in terms of the degree of shrivel and decay. Red color development was inhibited in both untreated and MeJA treated fruit and was characterized by the development of pale orange and yellow pigmentation. In summary, MeSA (but not MeJA) efficiently delayed CI in tomatoes treated at the pink maturity stage (Fung et al., 2006).

RNA gel blot analysis carried out by Fung et al. (2006) indicated that LeAOX1a mRNA abundance increased during the 24 h exposure to exogenous MeSA and also during the first day at 0°C in both MeJA and MeSA treated fruit. In addition to the 1.6 kb LeAOX1a transcript, a 3 kb transcript was detected when fruits were transferred at 1°C. Both 1.6 and 3 kb LeAOX1a transcript levels remained high in treated and untreated fruit throughout the first 5 days of 0°C storage. The expression profile of LeAOX Family 1 transcripts in fruit appeared to be determined almost entirely by LeAOX1a expression. The transcript levels of the LeAOX Family 2 gene appeared to be increased by MeSA and MeJA during days 1 and 2, and thereafter remained unchanged during cold storage. A 3 kb transcript was also detected for the LeAOX Family 2 gene during 0°C storage.

Fung et al. (2006) concluded that the CI index and degree of severity (shrivel and decay) correlates with the expression pattern of the LeAOX1a and LeAOX2 genes in MeSA treated fruit before and during the first day of

cold storage. The three closely related LeAOX Family 1 genes are expressed at different levels in fruit and LeAOX1a is responsible for the vast majority of LeAOX Family 1 expression in fruit during cold storage and accumulation of its mRNA correlates with chilling resistance. MeJA and MeSA treatments induced chilling resistance in mature green tomatoes (Ding et al., 2002) and green bell peppers (Fung et al., 2004). However, MeJA is not as effective as MeSA in alleviating CI of pink maturity stage tomatoes. Fung et al. (2006) results confirms previous work demonstrating that tomato fruit at different maturity stages respond differently to plant growth regulator treatment (Ding and Wang, 2003). Also, chilling tolerance of maize, cucumber and rice seedling leaves and roots was shown to be differentially affected by salicylic acid (Kang and Saltveit, 2002). The delayed induction of LeAOX1a and LeAOX2 transcript levels in MeJA treated fruit may provide one explanation for the lower degree of shrivel found among chilling resistant MeSA treated tomato fruit but not in MeJA or control fruit. Among the AOX gene family members, LeAOX1 transcripts accumulate to the highest levels during cold storage and LeAOX1a mRNA abundance is higher in comparison to that of the 1b and 1c genes in fruit throughout the course of the treatment.

HIGH OXYGEN STORAGE

Storage of horticultural crops under superatmospheric O_2 concentration may stimulate, have no effect, or reduce ethylene production and rates of respiration, depending on the commodity, maturity and ripeness stage, O_2 concentration, storage time and temperature, and concentrations of CO_2 and ethylene present in the atmosphere. Ripening of mature-green climacteric fruits was slightly enhanced by exposure to 30 to 80 kPa O_2, but levels above 80 kPa retarded their ripening and caused O_2 toxicity disorders on fruits. High O_2 concentrations enhance the effects of ethylene on fresh fruits and vegetables, including ripening, senescence and ethylene-induced physiological disorders such as bitterness of carrots and russet spotting on lettuce. While superatmospheric O_2 concentrations inhibit the growth of bacteria and fungi, they are much more effective if combined with elevated (15 to 20 kPa) CO_2, which is a fungistatic gas (Kader and Ben-Yehoshua, 2000). AOX gene expression is enhanced by elevated O_2 atmospheres (Zheng et al., 2008). Zheng et al. (2008) reported that the transcript levels of antioxidative genes including SOD, APX and CAT which were relatively constant during storage at 5°C with high oxygen treatment in freshly harvested zucchini squash. However, the expressions of AOX were induced slightly in squash treated with 60 and 100% oxygen for 3 days when compared with the control squash. These increases in AOX transcript levels were correlated with the increased chilling resistance in the treated squash. The ROS scavenging enzyme activities including SOD, APX, CAT and POD in treated zucchini squash were also higher than those in the control for the first 3 days at 5°C. Transcript levels of AOX increased substantially between 3 and 6 days in all treatments, suggesting the involvement of alternative respiratory pathway during chilling stress. All the enzyme activities in 100% oxygen treated squash started to decline after 6 or 9 days of cold storage to a level comparable or lower than those of the control. These declines were correlated to the loss of chilling resistance in the 100% oxygen treated tissue as indicated in the chilling injury index. However, squash treated with 60% oxygen maintained elevated levels of all enzyme activities except POD and sustained the least chilling injury throughout the 15 days of storage at 5°C. The oxygen radical absorbance capacity (ORAC) values and total phenol content remained high in squash treated with 60 and 100% oxygen for the first 9 days, then their levels in the 100% oxygen treated samples declined sharply while those in the 60% oxygen treated samples maintained elevated, indicating that both ORAC activity and phenolic content may also contribute to the resistance of tissue against chilling injury. The 100% oxygen treated squash showed the lowest respiration rate and 60% oxygen treated samples had the lowest ethylene production. Zheng et al. (2008) results may be an indication of the low chilling injury in the high oxygen treated squash. Zheng et al. (2008) results showed that the ORAC and total phenolic levels in zucchini skin peels were both induced by cold storage and further enhanced by 60% oxygen storage. The enhanced antioxidative enzyme activities and the overall antioxidative capacity (ORAC and phenolic levels) seem to correlate with the reduced chilling injury.

In peppers, treatment with MeJA and MeSA reduced chilling injury and induced preferentially the transcript level of AOX gene, while no change in transcript levels of ROS scavenging genes were detected during the early cold storage at 0°C (Fung et al., 2004). Zheng et al. (2008) showed that the elevated level of oxygen was effective in reducing chilling injury in zucchini. Similarly, Zheng et al. (2008) observed slight induction of only AOX gene transcript during early chilling period at day 3 when no difference in chilling injury symptom was visible among treated and control squash. The detectable differences in AOX transcript levels among treated and control zucchini and peppers (Fung et al., 2004) suggested possible correlation between AOX gene expression level and chilling injury. Zheng et al. (2008) results are consistent with the idea that global reprogramming of metabolism occurs during low temperature stress (Sung et al., 2003; Graya and Heath, 2005; Hannah et al., 2005). Zheng et al. (2008) observed that cold storage resulted in inhibition of primary metabolic pathways of respiration and induction of AOX transcript of alternative respiratory chain. This is also

accompanied by the antioxidant system as demonstrated by their enzyme activities and by the ORAC assay that presumably function to provide immediate relieve to the adverse redox status. On the other hand, the plant system quickly remobilize its primary metabolites into various secondary metabolite biosynthetic pathway (Kaplan et al., 2004; Renaut et al., 2005) as also shown by Zheng et al. (2008) data from the total phenolic compound that increased by 30% during cold storage.

CONCLUSION

AOX is a best candid gene for manipulation of resistance to chilling injury in fruit and vegetables. Postharvest treatments increased the AOX gene expression and can be used in postharvest technology as an important method for reducing chilling injury of fruit and vegetables. The applied technologies for the reduced CI impacts via the increase of AOX gene expression are based mainly on the application of signalling molecules such as SA, MeSA and JA, and MeJA or storage with high O_2. Biotechnology has also offered way to reduce the impact of CI in sensitive fruit and vegetables with the generation of genetically modified plants by the overexpression of AOX genes that act as an antioxidant avoidance gene and controls the ROS production in fruit and vegetables under chilling temperature.

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Vol. 12(51), pp. 7017-7030, 18 December, 2013
DOI: 10.5897/AJB12.886
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Comparison of gene expression profiles in *Bacillus megaterium* treated tobacco leaves using microarray

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Accepted 19 December, 2012

The MP agent, prepared from *Bacillus* megaterium isolated from the soil near tobacco fields, can improve metabolic products, and hence the aroma, of tobacco (*Nicotiana tabacum*) leaf. To explore genes regulating metabolic responses in tobacco leaf, we used microarrays to analyze differentially expressed genes in tobacco leaves subjected to various treatments. The expressed genes were classified into six groups based on their expression profile. In total, 753 genes were significantly differentially expressed between microorganism-treated and water-treated samples. Gene ontology (GO) analyses showed that most of these genes were involved in metabolic and cellular processes. Some up-regulated genes were related to the plant defense response, such as *NtMMP1* and *NtACRE231*. Some genes were involved in metabolism responses, such as *NtDXS*. Semi-quantitative reverse transcriptase (RT)-PCR analysis of *NtMMP1* and quantitative RT-PCR analysis of *NtDXS* showed that their expression levels increased after MP agent treatment, confirming the microarray results. We evaluated *NtMMP1* and *NtDXS* in terms of their associations with Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Phylogenetic analyses of *NtMMP1*, *NtACRE231,* and *NtDXS* revealed their relationships with homologs in other species. These microarray data increase our understanding of the mechanisms by which MP agent induces a metabolic response in tobacco leaves

Key words: *Nicotiana tabacum*, Microarray, MP agent, plant defense response, aroma.

INTRODUCTION

Tobacco (*Nicotiana tabacum*) is an important economic plant that has been used as a model for plant metabolomics and green bioreactors (Zhang et al., 2011; Poethig and Sussex, 1985; Sang-Wook Park et al., 2007; Tremblay et al., 2010). In plants, secondary metabolites are important for many processes, and are closely related to plant growth and development, and adaptation to the environment. Phenolamides (PAs) are a diverse group of plant secondary metabolites that are found in many dicotyledonous plants, suggesting that they play a role in plant growth and development (Martin-Tanguy, 1985; Facchini, 2002; Edreva, 2007; Grienenberger and Legrand, 2009). Jasmonates are important plant hormones that mediate plant responses to attack from herbivores and necrotrophic pathogens (Howe and Jander, 2008; Bari and Jones, 2009). In tobacco cell cultures, methyl jasmonate (MeJA) strongly induces the expression of genes related to alkaloid and phenylpropanoid biosynthetic pathways (Goossens et al., 2003). Salicylic acid (SA) is another important signaling compound in plant defense response

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(Verberne et al., 2007). In tobacco, products of secondary metabolism not only play roles in growth, development, and defense, but also give a characteristic aroma. The main substances contributing to the aroma are phenolic compounds, terpenoids, and alkaloids, which are formed via the phenylpropanoid metabolic pathway, isoprenoid biosynthetic pathway, and alkaloid biosynthetic pathway, respectively (Wang et al., 2008).

In tobacco, treatments with microorganisms have been applied to alter metabolic products, to modify various aspects of plant development and regulation, and to improve processing and storage of the tobacco crop. Microorganism treatments were first used by Koller to increase the aroma of tobacco (Koller, 1858). Subsequently, Tamayo used microorganisms to enhance tobacco aroma and reported that *Bacillus* and *Micrococcus* improved the aroma and decreased the protein content of tobacco leaves (Tamayo and Cancho, 1953). Other studies suggested that pleasant odor components could be produced quickly when tobacco was treated with individual or mixed strains of *Bacillus subtilis* (English et al., 1967). Several bacteria, such as *Pseudomonas convexa* (syn. *Pseudomonas putida*) PC1 (Thacker et al., 1978), *Achromobacter nicotinophagum* (Hylin, 1959) and *Arthrobacter oxydans* (Gherna et al., 1965), can degrade nicotine. In the genus Arthrobacter, the mechanisms of metabolic degradation of nicotine have been characterized in detail (Gherna et al., 1965; Schenk et al., 1998; Sachelaru et al., 2005; Ganas et al., 2008). One type of bacillus, *Bacillus megaterium*, has been used to prepare an 'MP agent', which, when sprayed onto tobacco, resulted in marked increases in the concentrations of the most important aroma components and decreases in protein content (Wang et al., 2006). However, the mechanism by which these changes occur is still unknown.

Microarray technology has become one of the most important approaches to examining thousands of genes simultaneously. Microarray analyses reveal the global biological functional differences among plants subjected to various treatments, which is the key to deciphering the networks of genes and their regulators that lead to a wide variety of defense responses (Lodha and Basak, 2011). This method has been applied extensively to determine the gene expression patterns in many different organisms, including *Arabidopsis thaliana, Oryza sativa, Zea mays, Glycine max, Solanum lycopersicum* and *Vitis vinifera* (Coughlan et al., 2004; Ma et al., 2005; Lund et al., 2008; Hayes et al., 2010; Mathias et al., 2010; Rohrmann et al., 2011). *Arabidopsis thaliana* is a powerful model system in the plant kingdom, and the relationship between the plant itself and the microorganism has been particularly well studied. Recent microarray studies have showed that the plant immune response is the same among many species at the gene expression level (Wang et al., 2010; Weisman et al., 2010; Van Verk et al., 2011). For example, Arabidopsis BRCA2 and RAD51 proteins are specifically involved in transcription of defense genes in plant immune responses (Wang et al., 2010). Another two genes, WRKY28 and WRKY46, are both rapidly induced by pathogen elicitors and are related to systemic acquired resistance (Van Verk et al., 2011; Eulgem et al., 2000). In addition, enhanced disease susceptibility1 (EDS1) which interacts with two related proteins encoded by Phytoalexin Deficient4 (PAD4) and Senescence Associated Gene101 (SAG101), is an important regulator of plant basal and receptor-triggered immunity (Rietz et al., 2011). It has been reported previously that Arabidopsis CRT2 is another regulator of plant innate immunity that plays a role in regulating plant defense against pathogens (Qiu et al., 2011). Microarray technology has been used widely in studies on tobacco. For example, an Affymetrix tobacco expression microarray was generated from a set of more than 40 k unigenes and was used to measure gene expression in 19 different tobacco samples to produce the Tobacco Expression Atlas (TobEA) (Edwards et al., 2010). A cDNA microarray prepared from 2831 clones was used to compare gene expression levels in trichome and leaf tissues of tobacco (Cui et al., 2011). In addition, transcript levels in leaves and flowers of transgenic tobacco plants were analyzed using Agilent microarray techniques (Soitamo et al., 2011).

Previous research has suggested that an MP agent prepared from Bacillus megaterium might improve the metabolic products of tobacco leaves (Wang et al., 2006). Therefore, a systematic understanding of genes in *Nicotiana tabacum* and the mechanisms that underlie the improvement of its metabolic products of tobacco leaves is of great interest. In this study, we performed microarray analysis to identify differentially expressed genes between MP agent-sprayed and water-sprayed tobacco leaves.

MATERIALS AND METHODS

Microorganism cultivation

MP agent was prepared from *Bacillus megaterium* according to the method of Wang et al. (2006). The *Bacillus megaterium* strain was incubated on beef extract peptone medium at 37°C with shaking at 180 rpm until the concentration reached 108 CFU/mL. The supernatant and pellet were harvested after 24 h.

Agilent microarray design

The Agilent "4×44K" microarray chip was designed for *Nicotiana tabacum* cDNA sequences in an effort to cover most tobacco genes with at least one 60-mer oligonucleotide probe. The probes were designed by CapitalBio Corporation (Beijing, China). To investigate differences in gene expression between leaves subjected to various treatments and the control, we sprayed tobacco leaves with four different preparations: supernatant, pellet, and whole bacterial liquid of the MP agent, and water.

Experimental tobacco leaves and RNA isolation

The flue-cured tobacco variety K326 was cultivated in water at a

stable temperature of 25°C. The tobacco leaves were sprayed with supernatant, pellet, whole bacterial liquid of the MP agent, or water at 10 days before maturity. The treated leaves were snap-frozen and stored in liquid nitrogen for RNA extraction. Total RNA was isolated from each sample by TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA templates were quantified by spectrophotometry and subjected to 1.0% formaldehyde denatured agarose gel electrophoresis. The average yield of RNA in each sample was approximately 0.5 μg/mg.

Probes labels and microarray hybridization

The cDNA targets were prepared from 5 μg of total RNA and then labeled with a fluorescent dye (Cy5 and Cy3-dCTP, GE Healthcare Cat. No. PA 55021/ PA 53021). The samples were divided into four groups. In the first group, cDNA from tobacco leaves sprayed with supernatant was labeled with cy5 and cDNA from tobacco leaves sprayed with water (control) was labeled with cy3. In the second group, the cDNA from tobacco leaves sprayed with the pellet and water were labeled with cy5 and cy3, respectively. The labeling cDNA of the third group was fluorescence exchange according to the second group. In the fourth group, the cDNA from tobacco leaves sprayed with bacterial liquid of the MP agent and water were labeled with cy5 and cy3, respectively. The labeled cDNAs were dissolved in 80 μL of hybridization solution containing 3×SSC, 0.2% SDS, 5×Denhardt's solution and 25% formamide, then hybridized at 42°C overnight. After hybridization, slides were washed with washing solution (0.2% SDS, 2×SSC and 2×SSC, respectively) at 42°C for 5 min. Chips were scanned with feature extraction software and images were then analyzed by GeneSpring software (both from Agilent Technologies Co., Ltd.).

Bioinformatics

Microarray analysis

In this research, results were filtered according to the marked flag of feature extraction software. Points marked as "detected" were defined as "active genes". Using the above fluorescent labeling method, we set "sprayed with MP agent": "sprayed with water" (M: W) as the ratio. Then, we averaged the two ratios of the second and third groups, and converted the obtained ratios by a log function to generate data for analyses. When the M:W ratio showed a difference of more than 2-fold, the genes were considered to be differentially expressed (that is, M:W≥2, genes were up-regulated; M:W<0.5, genes were down-regulated).

When the analysis data was obtained, whole hierarchical clustering of the average signal intensities was performed using the program Cluster (Cluster 3.0) and the results for all ratios were visualized using the program TreeView. Then, genes were systematically annotated using the following bioinformatics tools: BLASTX was used to search for homologs and Gene Ontology was used for functional classifications (Ashburner et al., 2000). Gene sequences were BLASTXed to the non-redundant (nr) database in GenBank. GO classifications were performed for these species using the BGI-WEGO (Web Gene Ontology Annotation Plotting) web service (http://wego.genomics.org.cn).

Phylogenetic analyses

We used MEGA version 4 software for phylogenetic analyses (Tamura et al., 2007), and ClustalW for multiple alignments of protein sequences (Thompson et al., 1994). All protein sequences used for phylogenetic analyses were downloaded from NCBI.

Pathway analyses

NtMMP1 and NtDXS involved in the plant defense and MEP metabolism pathway were then clarified and graphically displayed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway method (http://www.genome.jp/kegg/).

Semi-quantitative RT-PCR and Quantitative RT-PCR

To investigate the expression level of the *NtMMP1* and *NtDXS* genes, we used RT-PCR and quantitative real-time PCR, respectively. RT-PCR was performed for 25 cycles. The primers designed for NtMMP1 (GenBank accession no. DQ508374.1) was 5'-GAACGGTCTGACGGATAA-3' (sense) and 5'-GCTAAACTCGGGAACATAA-3' (anti-sense).

Quantitative real-time PCR was used to confirm the expression level and microarray data. The primer sequences of NtDXS (GenBank accession no. FN429979.1) was 5'-TATTGGTCCTGTGGATGGT-3' (sense) and 5'-AAACTTGGCTACTCCGTGA-3' (anti-sense). The real-time PCR was carried out using the SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) and each reaction was prepared in 25 μL containing 2 μL complementary DNA, SYBR Premix Ex Taq 12.5 μL, 10 mmol/L primers 0.5 μL (sense and anti-sense). The PCR was set with the following steps: started with 10 s template denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 5 s, and then combined primer annealing/elongation at 60°C for 31 s according to the manufacturer's instructions. Then the results were read by ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, US). The real-time PCR was performed in duplicate for at least three biological replicates. The amplification of NtGAPDH cDNA sequence was taken as the inner control.

RESULTS

Differences in gene expression among leaf samples from four different treatments

Using the Agilent microarray, we analyzed the gene expression profiles of tobacco leaves subjected to four different spray treatments: supernatant, pellet, and whole bacterial liquid of the MP agent, and water (control). According to "feature extract" analysis software, the points marked as "detected" were defined as "active genes". As a result, we detected expressions of 9,565 genes. Next, we calculated gene-expression ratios (log-ratios) of "sprayed with MP agent":"sprayed with water" (M:W). The genes were clustered into six groups according to their expressions. Group 1 contained all of the up-regulated genes (2867 in total); group 2 contained all of the down-regulated genes (2770 in total); group 3 contained 996 genes that were up-regulated in leaves sprayed with supernatant.

Some of these genes were up-regulated in leaves sprayed with the pellet, and were down-regulated in leaves sprayed with the whole bacterial liquid of the MP agent. Group 4 contained 701 genes that were down-regulated in leaves sprayed with supernatant. Some of these genes were up-regulated in leaves sprayed with the pellet and with whole bacterial liquid. Group 5

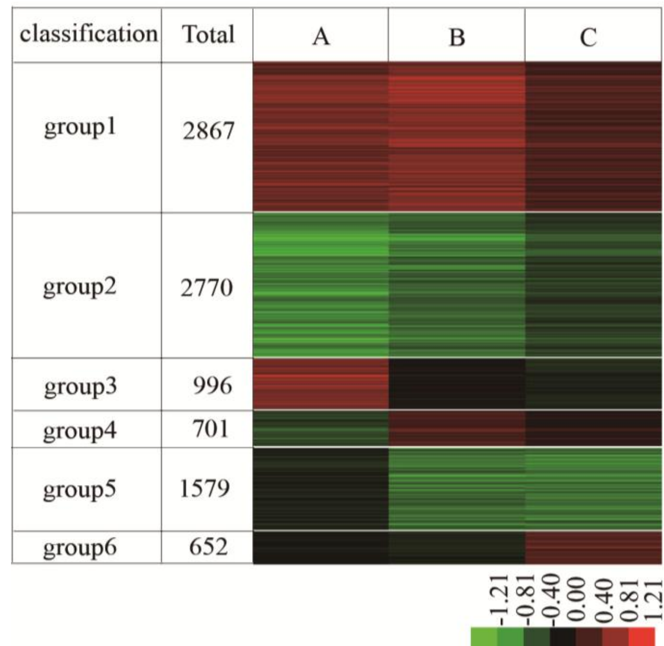
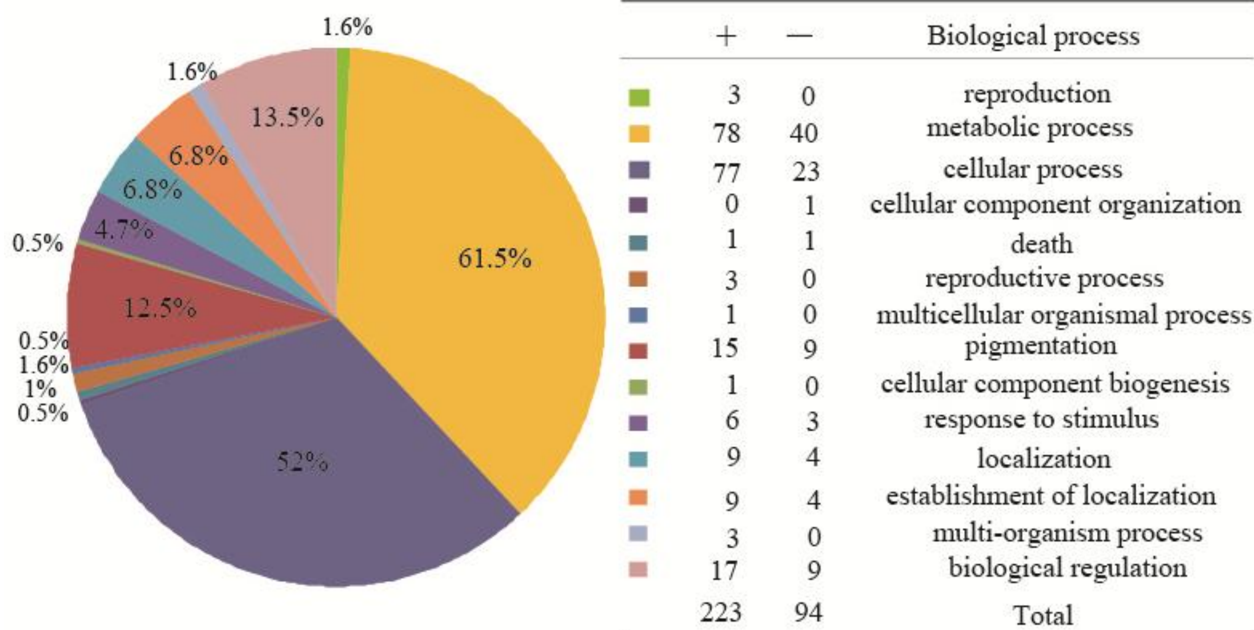


Figure 1. Hierarchical cluster analysis of genes expressed in response to MP agent- and water-treatments. Gene expression levels are represented by red and green boxes (denoting those with up-regulated and down-regulated expressions, respectively). Horizontal lines represent differential expression of genes of interest. Vertical rows represent different treatments of tobacco leaves (A, B, and C). Ratios represent "Sprayed with microorganism": "sprayed with water" (M: W). A: Sprayed with supernatant of MP agent: sprayed with water; B: sprayed with pellet of MP agent: sprayed with water; C: sprayed with whole bacterial liquid of MP agent: sprayed with water. Genes were classified into six groups based on their expression profile: Groups 1 and 2 included all up-regulated and down-regulated genes, respectively. Group 3 included genes that were up-regulated in leaves sprayed with supernatant of MP agent. Group 4 included genes that were down-regulated in leaves sprayed with supernatant and up-regulated in those sprayed with pellet and bacteria liquid of MP agent. Group 5 included genes that were significantly down-regulated after spraying with pellet and bacteria liquid of MP agent, except for some genes that were up-regulated after spraying with supernatant. Group 6 included genes that were up-regulated only in response to spraying with whole bacterial liquid of MP agent.

contained 1579 genes that were significantly down-regulated; however, some of these were up-regulated after spraying with supernatant. The 652 genes in Group 6 were up-regulated only after spraying with whole bacterial liquid of the MP agent (Figure 1). Among the six groups of genes, 753 genes were identified to differentially express. When expressions were compared between leaves sprayed with MP agent and those sprayed with water, there were 368 up-regulated genes 385 down-regulated genes.

Gene ontology annotations and analysis

The 753 differentially expressed genes were subjected to gene ontology (GO) analysis, and GO annotations were obtained for 192 genes. We also analyzed these genes in terms of their participation in biological processes. The results show that most of these genes were involved in metabolic process (118 genes) and cellular process (100 genes), accounting for 61.5 and 52% of total annotated genes, respectively. There was only one gene classified into each of the cellular component organization, multicellular organismal process, and cellular component biogenesis categories (Figure 2). Among the 192 annotated genes, there were many more up-regulated genes than down-regulated genes. The up-regulated genes were involved in processes such as reproduction, reproductive process, multicellular organism process, cellular component biogenesis, and multi-organism process. One



	+	—	Biological process
	3	0	reproduction
	78	40	metabolic process
	77	23	cellular process
	0	1	cellular component organization
	1	1	death
	3	0	reproductive process
	1	0	multicellular organismal process
	15	9	pigmentation
	1	0	cellular component biogenesis
	6	3	response to stimulus
	9	4	localization
	9	4	establishment of localization
	3	0	multi-organism process
	17	9	biological regulation
	223	94	Total

Figure 2. Gene ontology categories of the differentially expressed genes. Numbers of up (+) or down.

down-regulated gene was involved in cellular component organization. The analysis suggested that the genes with unknown or other annotated functions may be involved in biological processes.

Differentially expressed genes in response to MP agent treatment

Based on the microarray data, some differentially expressed genes were present in all six groups. According to their functional annotation, some of the up-regulated expression genes were closely related to plant defense response, hormone metabolism, cell cycle regulation and enzyme regulation. For instance, expres-sions of NtMMP1, NtACRE231, elicitor inducible LRR protein (EILP), and five WRKY transcription factors, which are involved in the plant defense response, were up-regulated after MP agent treatment (Yamamoto et al., 2004; Schiermeyer et al., 2009; Durrant et al., 2000; Takemoto et al., 2000; Rowland et al., 2005; Park et al., 2006). Some up-regulated genes were closely related to metabolic regulation (example, genes encoding P450 mono-oxygenase (Simon-Mateo et al., 2006) and pyruvate kinase (Grodzinski et al., 1999) and to cell cycle regulation (example, genes encoding cyclin-dependent kinase B1-2 (Sorrell et al., 2001), ribonucleotide reductase (Chaboute et al., 1998), and cyclin A-like protein (Reichheld et al., 1996). At the same time, 385 genes were down-regulated after MP agent treatment. Some down-regulated genes were associated with metabolic regulation, such as genes encoding an ATP-binding cassette (ABC) transporter (Yazaki, 2005, 2006) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Munoz-Bertomeu et al., 2010). Some down-regulated genes were related to cell cycle regulation, for instance, the gene encoding the ribosomal protein (Turkina et al., 2011), cytochrome C oxidase (Yamashita and Voth, 2011). Other down-regulated genes were involved in plant defense responses (for example, pectinesterase inhibitor, which plays a role in the plant defense mechanism via cell wall fortification (Hong et al., 2010; Jolie et al., 2010), and polyphenol oxidase, a multifunctional enzyme involved in the defense response
(Thipyapong et al., 2004; Poiatti et al., 2009). Further analysis of these differentially expressed genes will provide new insights into the molecular mechanisms underlying different responses among tobacco leaves subjected to various treatments.

Analyses of selected genes associated with plant defense responses

There are a number of potential genes related to growth and anti-disease responses in plants. When plants perceive some kind of pathogenic stimulation, expressions of some genes are induced leading to various reactions including resistance or growth stimulation. Previous studies indicated that after spraying with a microorganism MP agent, the levels of some proteins and amino acids decreased in tobacco leaves,

while the main aroma components significantly increased (Wang et al., 2006). According to our microarray results, spraying with a microorganism MP agent resulted in up-regulated expressions of genes involved in the plant defense response such as NtMMP1, NtACRE231, EILP, and WRKY transcription factors, but down-regulated expressions of pectinesterase inhibitor and polyphenol oxidase (Hong et al., 2010; Jolie et al., 2010; Thipyapong et al., 2004; Poiatti et al., 2009; Durrant et al., 2000; Takemoto et al., 2000; Eulgem and Somssich, 2007; Schiermeyer et al., 2009).

Matrix metalloprotease 1

Plant matrix metalloproteinases (MMPs) are conserved proteolytic enzymes that are widely distributed in the plant kingdom. MMPs play crucial roles in many aspects of pathogen defense (Liu et al., 2001), senescence (Delorme et al., 2000) and growth, and development (Golldack et al., 2002). In Nicotiana tabacum, tobacco MMP1 (NtMMP1) participate in the pathogen defense (Schiermeyer et al., 2009). In our research, we analyzed the evolution of MMPs or an unknown protein that contained a characteristic MMP domain in 15 plant species belonging to 10 families. We constructed a phylogenetic tree to examine the relationships of these proteins (Figure 3). The result indicates that NtMMP1 (red, marked with an asterisk) clustered with MMP from *Nicotiana benthamiana*. This group clustered with a zinc metalloproteinase of *A. thaliana* (Cruciferae), a matrixin family protein of *Brassica oleracea* (Cruciferae), a matrix metalloproteinase of *A. thaliana*, and a predicted GPI-anchored protein of A. thaliana. We analyzed whether NtMMP1 exhibited different transcriptional responses after treatment with the MP agent. Semi-quantitative RT-PCR showed that the highest transcript levels of NtMMP1 were observed after spraying with the pellet of the MP agent, followed by the bacterial liquid of the MP agent, then the MP agent supernatant. The lowest transcript levels were observed after spraying with the water control (Figure 4). To determine whether the function of NtMMP1 is similar to that of MMP1, we carried out a pathway analysis. The results indicate that NtMMP1 participates in the Toll-like receptor signaling pathway (Supplementary Figure S1).

Avr9/Cf-9 rapidly elicited protein 231

As mentioned above, plant cells induce an array of defense responses upon perceiving pathogens. Previously, Avr9/Cf-9 rapidly elicited (ACRE) genes from tobacco were identified to encode putative signaling components and may play important roles in the initial development of the defense response (Rowland et al., 2005; Durrant et al., 2000). In our research, we found that the expression level of *Nicotiana tabacum* ACRE231 (NtACRE231) was up-regulated in response to MP agent treatment. Subsequently, we analyzed the evolution of NtACRE231 and its homologs in 16 plant species. We found that NtACRE231 clustered with the glycosyltransferase of *Panax notoginseng* (Supplementary Figure S2).

Analysis of metabolism-related genes in tobacco

Plant secondary metabolites play important roles in plant growth and development, and in adaptation to the environment. As a model plant, tobacco is well characterized in terms of its metabolic responses (Martin-Tanguy, 1985; Facchini, 2002; Edreva, 2007; Goossens et al., 2003; Verberne et al., 2007). In our research, we found that some differentially expressed genes between MP agent-treated and water-treated tobacco were closely related to metabolic responses. These included 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Walter et al., 2000, 2002; Estevez et al., 2001), and genes encoding P450 monooxygenase (Simon-Mateo et al., 2006), pyruvate kinase (Grodzinski et al., 1999), an ABC transporter (Yazaki, 2005, 2006), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Munoz-Bertomeu et al., 2010).

1-Deoxy-D-xylulose-5-phosphate synthase

Isoprenoids are a large and highly diverse family of natural products involved in primary and secondary metabolism (Buckingham, 1998). The key pathway for production of isoprenoids is the methylerythritol phosphate (MEP) pathway (Walter et al., 2002), in which 1-deoxy-D-xylulose-5-phosphate synthase (DXS) plays a central role (Estevez et al., 2001; Walter et al., 2000, 2002). We found that the expression level of the N. tabacum DXS gene (NtDXS) increased after spraying with MP agent. Considering that DXS is an important player in the MEP pathway, we further examined NtDXS to clarify its role in the regulation of plastidic isoprenoid biosynthesis (Supplementary Figure S3). Quantitative PCR analyses showed that the highest level of NtDXS expression was after spraying with the bacterial pellet of the MP agent, followed by whole bacterial liquid, then the supernatant. The lowest expression was observed after spraying with water (Figure 5). A phylogenetic tree of the gene and its homologs showed that NtDXS clustered with the DXS of Capsicum annuum (CaDXS) (Supplementary Figure S4).

DISCUSSION

Tobacco is a dicot, and is a member of the Solanaceae. Secondary metabolic substances produced by tobacco

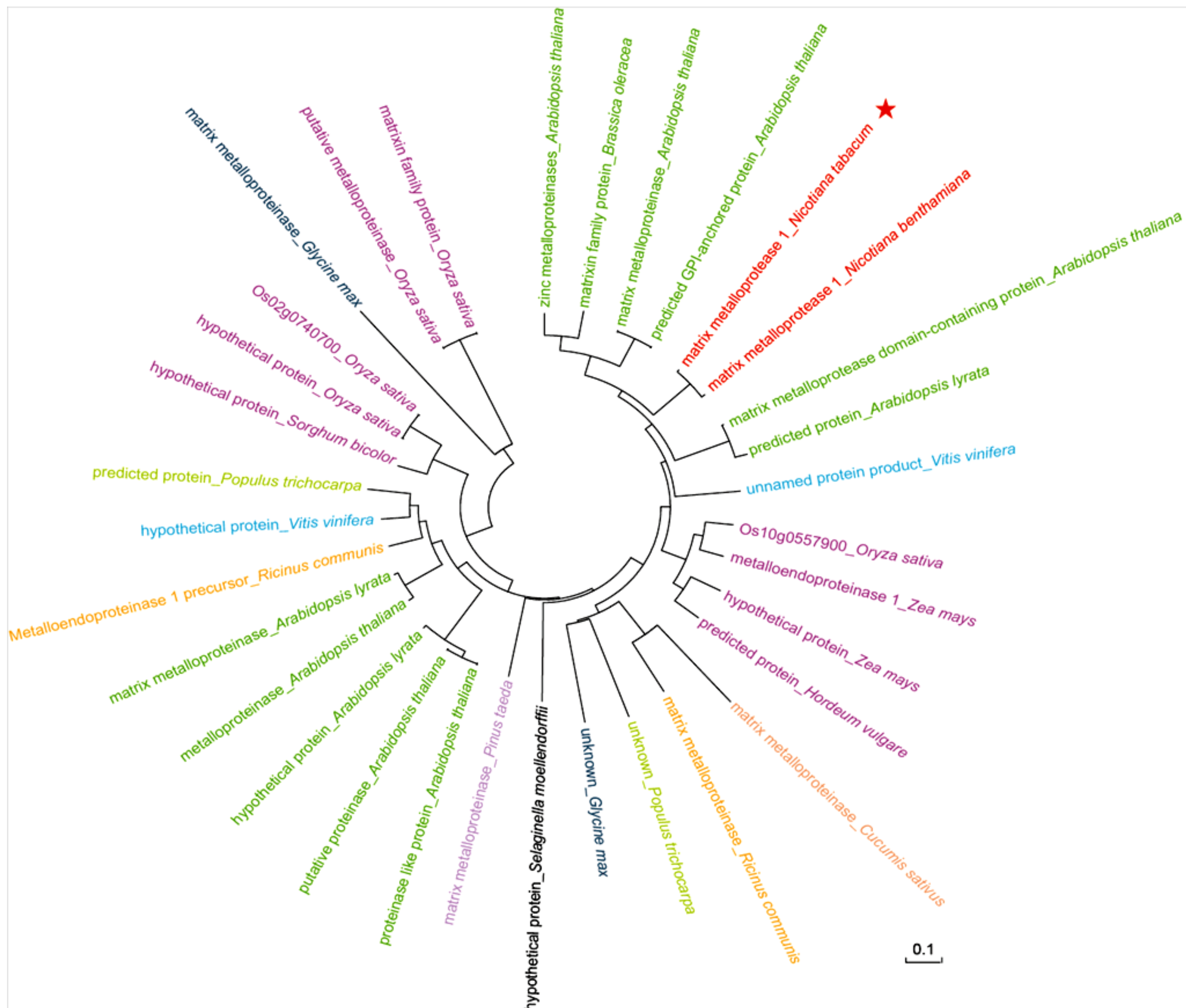


Figure 3. Molecular phylogeny of matrix metalloproteinases (*MMP*). Phylogram was constructed by the Neighbor-Joining method using MEGA4 software and reflects the relationship between *MMP* and its homologs in 15 plant species. Different colors show different plant families: Solanaeae shown in red (tobacco); Brassicaceae shown in green (*Arabidopsis thaliana*, *Brassica oleracea*, and *Arabidopsis lyrata*); Poaceae shown in purple (*Oryza sativa*, *Zea mays*, *Sorghum bicolor,* and *Hordeum vulgare*); Salicaceae shown in light green (*Populus trichocarpa*); Vitaceae shown in brilliant blue (*Vitis vinifera*); Euphorbiaceae shown in orange (*Ricinus communis*); Pinaceae shown in light purple (*Pinus taeda*); Selaginellaceae shown in black (*Selaginella moellendorffii*); Leguminaceae shown in blue (*Glycine max*); and Cucurbitaceae shown in pink (*Cucumis sativus*). Asterisk represents *matrix metalloprotease 1* of *Nicotiana tabacum*. See Materials and Methods for details of the phylogenetic analysis.

play roles in growth, development, defense responses, and in producing an aroma (Onkokesung et al., 2011; Verberne et al., 2007; Lackman et al., 2011; Liu and Thornburg, 2011; Naoumkina et al., 2008). Previously, it was reported that an MP agent produced by Bacillus megaterium, which was screened from soil in tobacco fields, was useful for increasing the aroma of tobacco. biosynthesis (Supplementary Figure S3). Quantitative PCR analyses showed that the highest level of NtDXS

The MP agent was applied to upper leaves in fields, while water was applied to controls, and then the normal processing and maturing steps were conducted. The leaves treated with MP agent showed increased contents of aroma components and decreased total amino acids and protein contents (Wang et al., 2006). In this study, we developed and validated a new 4×44k Agilent microarray, and used it to analyze gene expression after treatment with MP agent. Our results reveal that many

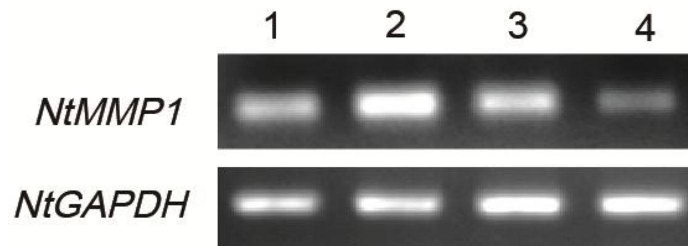


Figure 4. Expression profile of *NtMMP1* in tobacco leaves subjected to different treatments. Lane 1, tobacco leaves treated with supernatant of MP agent; lane 2, tobacco leaves treated with pellet of MP agent; lane 3, tobacco leaves treated with whole bacterial liquid of MP agent; lane 4, tobacco leaves treated with water. *NtGAPDH* mRNA was the internal control.

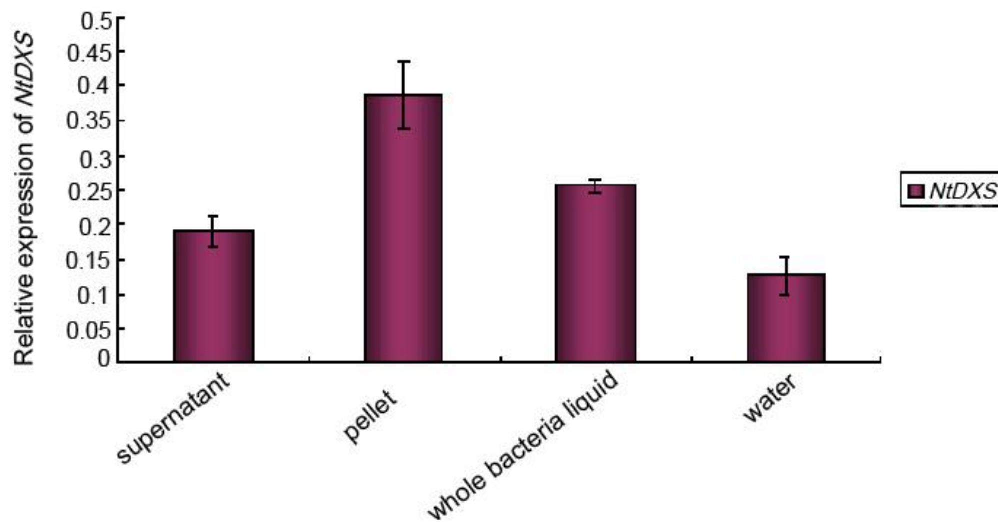


Figure 5. *NtDXS* expression was detected using real-time RT-PCR analysis after treatment with MP agent and water.

genes are differentially expressed between MP agent-treated and water-treated hydroponic seedlings of tobacco. These genes can be classed into six main groups (Figure 1). Our study reveals 753 differentially expressed genes, of which 368 were up-regulated and 385 were down-regulated. Functional analyses using Gene Ontology demonstrated that they are mainly involved in metabolic and cellular processes (Figure 2). We annotated these genes, and found that several genes have been reported previously to participate in plant defense responses. These include genes such as MMP1 (Schiermeyer, Hartenstein et al., 2009), ACRE231 (Durrant et al., 2000; Rowland et al., 2005), EILP (Takemoto et al., 2000), and those encoding WRKY transcription factors (Yamamoto et al., 2004; Park et al., 2006), a pectinesterase inhibitor (Hong et al., 2010; Jolie et al., 2010) and polyphenol oxidase (Thipyapong et al., 2004; Poiatti et al., 2009).

In the course of growth and development, plants face a variety of pathogens, including bacteria, fungi, viruses, and oomycetes. Plants have developed a variety of defense mechanisms against their attackers. However, disease resistance is often governed by a gene-for-gene interact-tions (Dangl and Jones, 2001). Gene-for-gene relation-ships, which are codetermined by a resistance gene (R) and an avirulence gene (Avr), are an important part of plant resistance. The interaction between R and Avr often leads to the hypersensitive response (HR) (Flor, 1971; Keen, 1990). Most R genes have conserved domains, such as leucine rich repeat (LRR), WRKY, and nucleotide-binding site (NBS) domains. Interestingly, we found EILP and five WRKY transcription factors containing LRR and WRKY domains, respectively, showing up-regulated expressions after leaves were sprayed with MP agent. Previous research showed that the product of EILP and WRKY transcription factors may play a central role in

plant immune responses (Bhattarai et al., 2010; Takemoto et al., 2000). Therefore, we can speculate that EILP and WRKY transcription factors play a similar role to that of the R gene after treatment with MP agent; that is, they recognize the complementary pathogen product of Avr, produce a resistance signal to activate a series of signal transduction processes, and activate expressions of defense genes leading to resistance.

The MMP1 gene is involved in a variety of physiological processes including senescence, pathogen defense, and growth and development (Delorme et al., 2000; Liu et al., 2001; Golldack et al., 2002). Our microarray data provide valuable insights into the expression level of NtMMP1 genes after treatment with MP agent and water. Subsequently, semi-quantitative RT-PCR analysis validated that transcript levels of NtMMP1 were significantly increased in response to MP agent treatment, confirming the results from the microarray analysis (Figure 4). Previous research showed that NtMMP1 plays a role in pathogen defense (Schiermeyer et al., 2009). We conducted pathway analysis for this gene and found that it is involved in the Toll-like receptor signaling pathway (Supplementary Figure S1). Plant matrix metalloproteinases (MMP) are collagenases, which are conserved proteolytic enzymes with the ability to degrade proteoglycan and accelerate degradation of the extracellular matrix (ECM) (Nagase and Woessner, 1999). NtMMP1 can degrade the pharmaceutical protein DSPAa1 (Mandal et al., 2010). In our research, we speculate that the increased expression level of NtMMP1 might result in not only enhanced resistance to pathogens, but also increased degradation of some proteins in tobacco leaves. This result reflects the underlying function of NtMMP1 in the metabolic pathway of tobacco.

Most of the aroma components of tobacco are produced via secondary metabolism. Previous studies indicated that a microorganism MP agent could significantly increase main aroma components (Wang et al., 2006). In our research, we found that some of the differentially expressed genes were closely related to metabolic responses. We focused on analyzing the expression level of the *Nicotiana tabacum* DXS gene (NtDXS). Previous research has shown that DXS is a limiting enzyme for plastidic isoprenoid biosynthesis in plants (Estevez et al., 2001; Walter et al., 2002). Isoprenoids participate in a variety of biological functions such as plant defense, photosynthesis, respiretion, growth, cell cycle control, and adaptation to environmental conditions (Estevez et al., 2001). In tobacco, isoprenoids also affect the formation of aroma (Cui et al., 2011). However, DXS, which participates in isoprenoid biosynthesis, is also a candidate gene for the trait of high levels of monoterpenols, which is associated with a distinctive aroma related to the composition of volatiles (Battilana et al., 2011). In our research, we found that the expression levels of NtDXS were increased by MP agent treatment, consistent with our microarray data (Figure 5). DXS is an important enzyme in regulation of the MEP pathway in plants (Estevez et al., 2001; Walter et al., 2000, 2002). Our pathway analysis of NtDXS yielded similar results to those for other DXSs, which are involved in the regulation of plastidic isoprenoid biosynthesis (Supplementary Figure S3). Our results suggest that NtDXS has a similar function to those of NtMMP1, NtACRE231, EILP, and WRKY transcription factors, all of which show increased expression levels after MP agent treatment, leading to increased resistance of tobacco and improved aroma characteristics of tobacco leaves. These results provide valuable information about NtDXS and allow us to clarify its possible function.

In summary, this study summarizes microarray data for tobacco leaves treated with MP agent and water. Analyses of gene expression showed that many genes were differentially expressed, and some of them were involved in plant defense responses. Further analyses of the genes identified in the microarray will increase our understanding of the role of MP agent in the growth and metabolism of *N. tabacum*.

Abbreviations

KEGG, Kyoto Encyclopedia of Genes and Genomes; **rpm,** revolutions per minute; **CFU/mL,** colony-forming units per milliliter; **Cy5 and Cy3,** reactive water-soluble fluorescent dyes of the cyanine dye family; **SSC,** standard saline citrate; **SDS,** sodium dodecyl sulfate; **BLAST,** Basic Local Alignment Search Tool; **BGI-WEGO,** Web Gene Ontology Annotation Plotting; **NCBI,** National Center for Biotechnology Information; **MEP,** methylerythritol phosphate; **RT-PCR,** reverse transcriptase polymerase chain reaction; **GO,** Gene Ontology; **ECM,** extracellular matrix.

ACKNOWLEDGEMENTS

This work was supported by Post-doctoral project, National High-Tech Research and Development Program (2010AA101506-6), Project in Sichuan Province (2009JY0156), and the Fundamental Research Funds for the Central Universities (XDJK2010C094).

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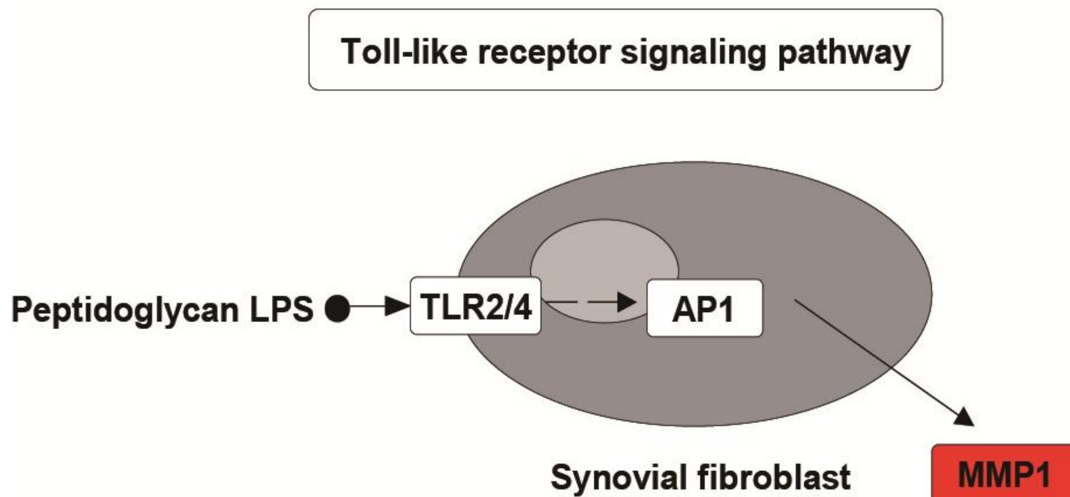
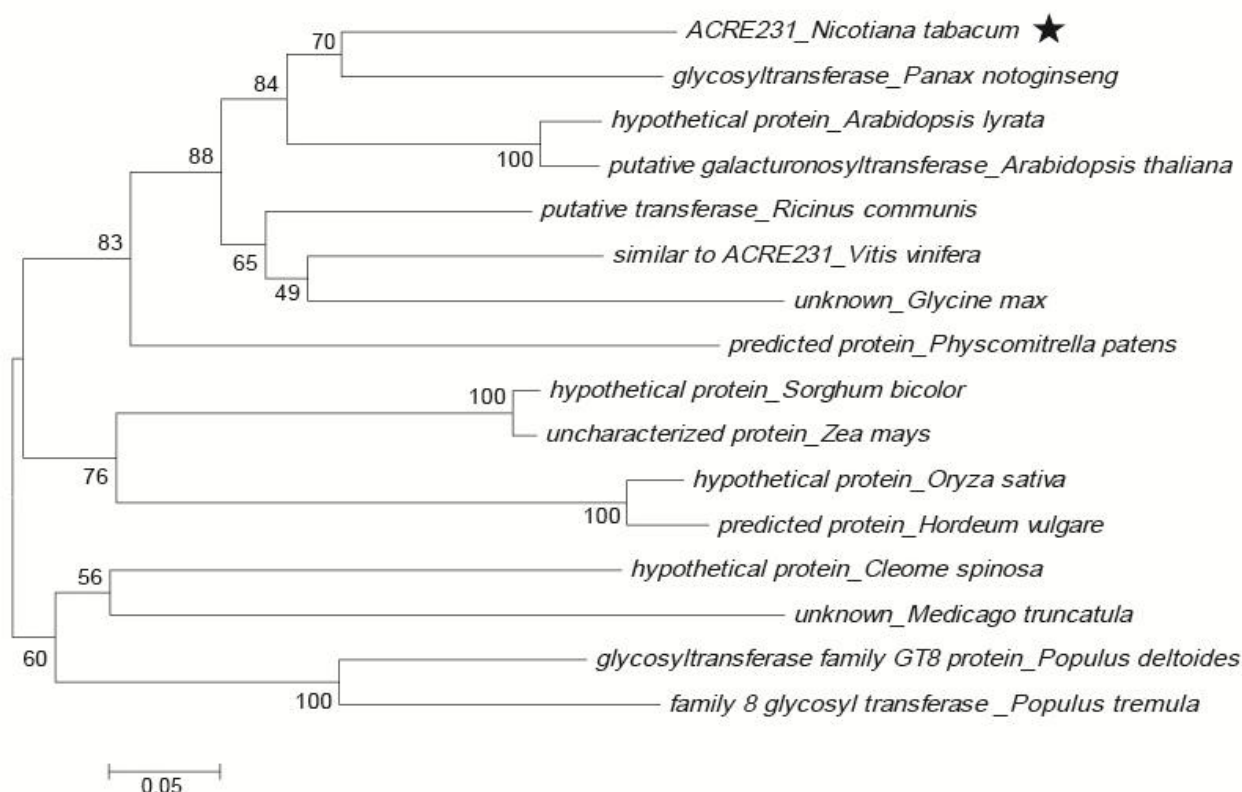
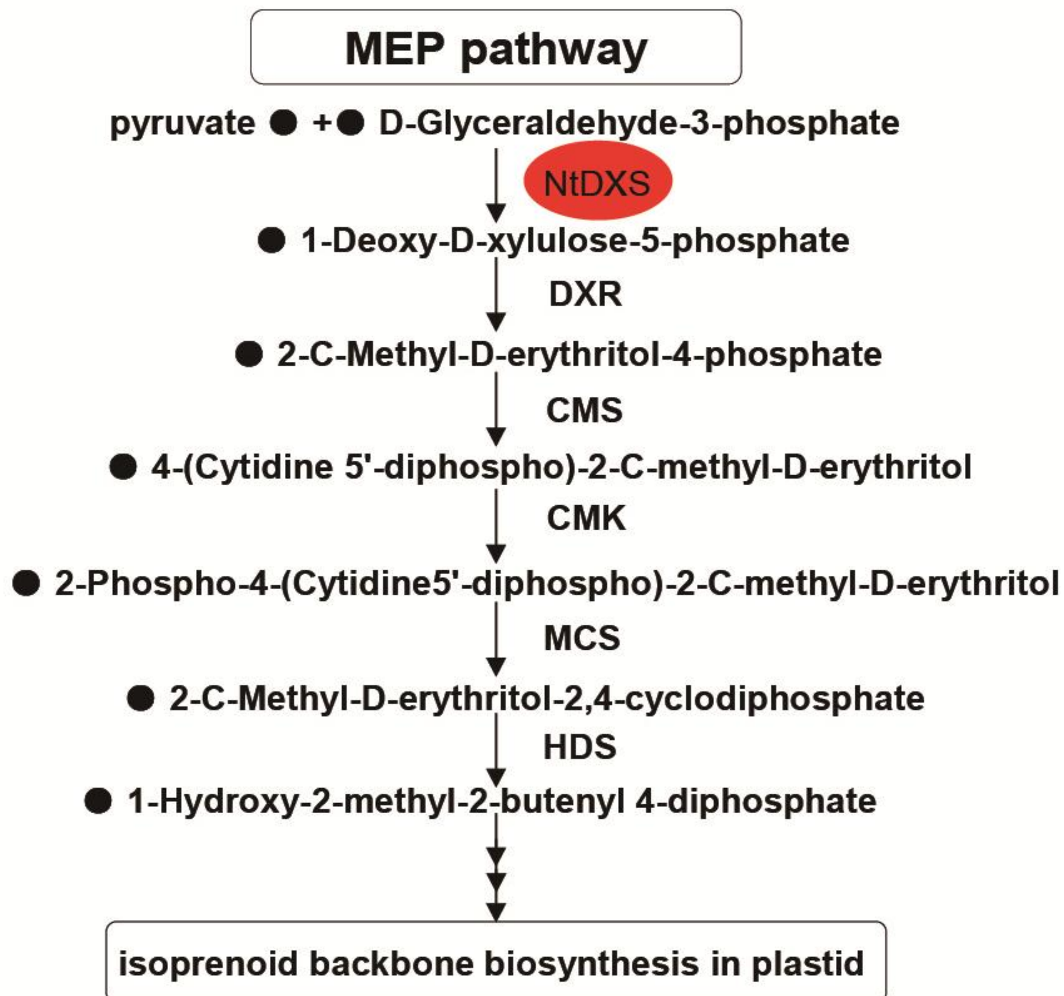


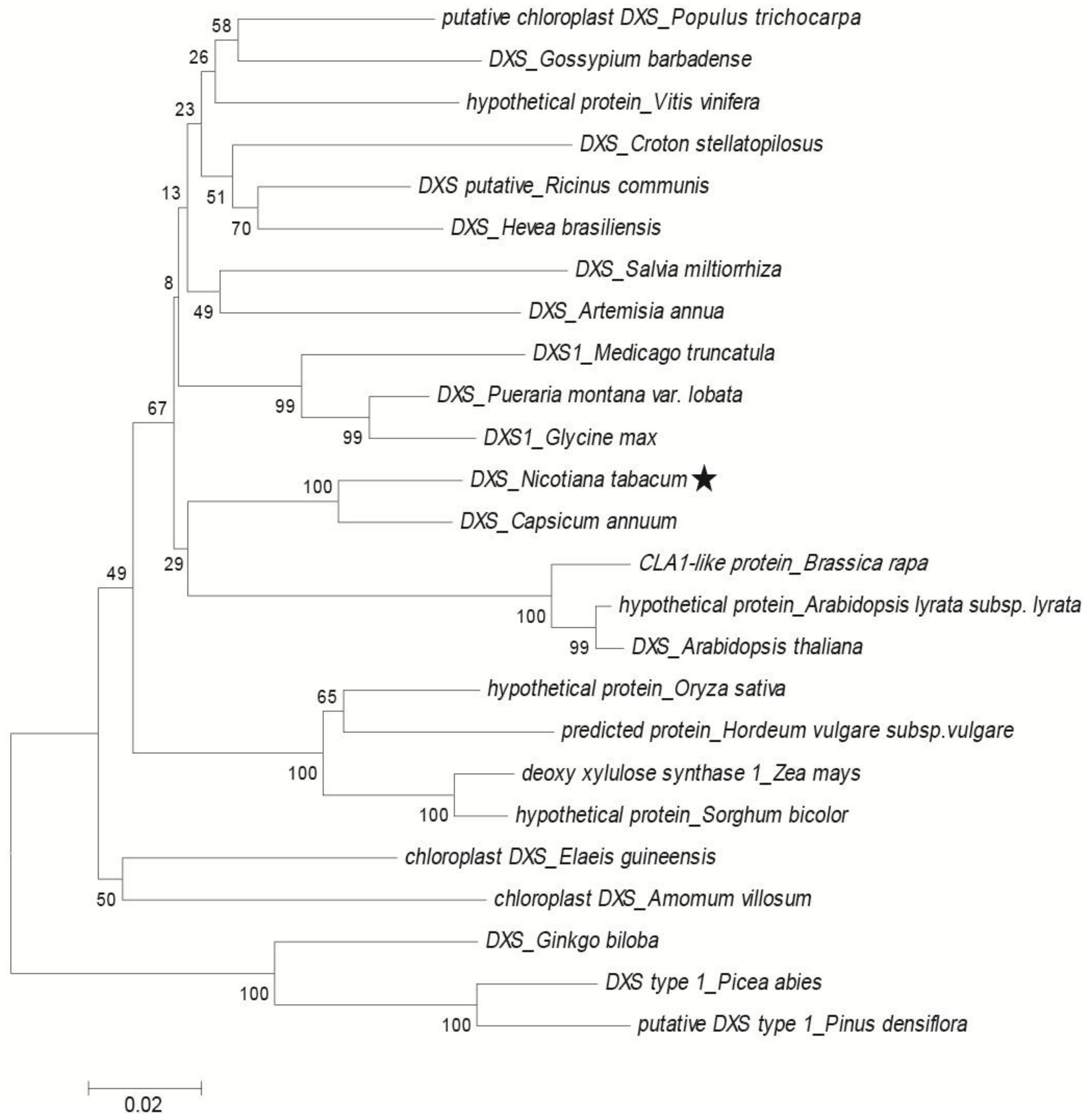
Supplementary Figure S1. Toll-like receptor signaling pathway which *MMP1* participate in. TLR2/4, Toll-like receptor 2/4; AP1, activator protein 1.



Supplementary Figure S2. Phylogenetic tree of the *Nicotiana tabacum* ACRE231 and homologous proteins. Phylogram was constructed by the Neighbor-joining method using MEGA4 software and shows relationships among 16 amino acid sequences of ACRE231-like proteins. Support for each branch, as determined from 1,000 bootstrap samples, is indicated by value at each node (in percent). Only bootstrap values above 50% are shown. Scale bar indicates evolutionary distance estimated by amino acid substitutions per position.



Supplementary Figure S3. Isoprenoid biosynthetic pathways in *Nicotiana tabacum*. Diagramatic representation of the plastidic MEP pathways is shown. NtDXS, 1-deoxy-d-xylulose 5-phosphate reductoisomerase in *Nicotiana tabacum*. DXR, 1-deoxy-d-xylulose 5-phosphate reductoisomerase; CMS, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; CMK, 4-diphosphocytidyl-2C- methyl-D-erythritol kinase; MCS, 2C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; HDS, hydroxymethylbutenyl diphosphate synthase.



Supplementary Figure S4. Phylogenetic tree of *Nicotiana tabacum DXS* and its homologs. Phylogram was constructed using the Neighbor-joining method with MEGA4 software and reflects relationships among 25 amino acid sequences of *DXS*-like proteins. Support for each branch, as determined from 1,000 bootstrap samples, is indicated by value at each node (in percent). Only bootstrap values above 50% are shown. Scale bar indicates evolutionary distance estimated by amino acid substitutions per position.

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Vol. 12(51), pp. 7031-7037, 18 December, 2013
DOI: 10.5897/AJB2013.13115
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity studies in common bean (*Phaseolus vulgaris* L.) using molecular markers

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Accepted 25 November, 2013

Molecular characterization of thirteen common bean genotypes was done with random amplified polymorphic DNA (RAPD) markers. Initially, 15 primers were screened out of which only seven were selected which generated a total of 65 amplification products out of which 63 bands (96.62%) were polymorphic indicating fair amount of polymorphism. The genotypes shared 43% genetic similarity among themselves. Cluster analysis delineated the genotypes into three groups with seven, five and one genotype in cluster-I, II and III, respectively. The maximum similarity index (82.35) based dice similarity coefficient was obtained between SKUA-R-21 and SKUA-R-19, while it was minimum (27.72) between genotypes PBG-29 and SKUA-R-01.

Key words: Genetic divergence, common bean, random amplified polymorphic DNA (RAPD).

INTRODUCTION

Common bean is regarded as “Grain of hope” as it is an important component of subsistence agriculture and feeds about 300 million people in tropics and 100 million people in Africa alone. Besides, it is emerging as an important income generation especially in Central America where beans are No. 1 income generators among field crops. Globally, with 21 million tons produced from about 26 million hectares, it accounts for about half of the total pulse production. In India, common bean is grown over an area of about 6 million hectares with a production of about 2.5 million tons (FAO, 2010). Among the pulses, *Phaseolus* genus contains approximately 70 species and within this genus, common bean (*Phaseolus vulgaris* L.) is an excellent food choice with its nutritional composition includes carbohydrates, proteins, vitamins, minerals and antioxidants (Svetleva et al., 2006). Common bean is a diploid ($2n = 2x = 22$) and predominantly self crossing species although 3% or more out crossing ratio has also been observed (Ibarra-Perez et al., 1997). Studies using molecular, physiological and morphological analyses in common bean strongly support the existence of two distinct centers of genetic diversity known as the Mesoamerican or small-seeded type and Andean or large seeded type gene pools (Blair et al., 2007; Burle et al., 2010). Today common beans are grown in many countries but widely cultivated in the tropics, subtropics, and temperate regions (Burle et al., 2010).

A complete understanding of the genetic diversity and population structure of the common bean is essential for its conservation and management, but limited germplasm characterization is a major challenge for systematic use of common bean diversity in genetic breeding programs.

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Abbreviations: RFLP, Restriction fragment length polymorphism; **RAPD,** random amplified polymorphic DNA; **CTAB,** cetyl trimethyl ammonium bromide; **PCR,** polymerase chain reaction.

Table 1. List of germplasm lines used in the study.

Genotype	Status	Place of collection
SKUA-R-01	Breeding line	PRS, Habbak
SKUA-R-11	Landrace	-do-
SKUA-R-19	Landrace	-do-
SKUA-R-21	Landrace	-do-
SKUA-R-28	Landrace	-do-
SKUA-R-34	Landrace	-do-
PBG-01	Landrace	Assar Doda
PBG-03	Traditional cultivar	Gool-Gulab Gad Doda
PBG-09	Traditional cultivar	Kishtward
PBG-16	Traditional cultivar	Beejic Poonch
PBG-29	Traditional cultivar	Khangund Pulwama
PBG-30	Traditional cultivar	Shangund Pulwama
Canadian Red	Breeding line	PRS, Habbak

Classical methods for characterizing genetic diversity in plants include the use of morpho-agronomic traits to establish genetic relationships among commercial cultivars, landraces and wild. Several types of DNA markers, developed to study genetic diversity and crop evolution, are now considered to be better for documenting the organization of diversity, when compared to former methods, such as morphologic markers (Blair et al., 2009; Kwak and Gepts, 2009; Burle et al., 2010). Human-directed selection of common-bean populations has influenced crop evolution, with cultivars originating through domestication of adjacent areas now being conceived as showing higher mutual similarity than germplasm from distant regions. Molecular characterization is required, not only to corroborate previous findings based on morpho-agronomic characterization, but also to increase the efficient use of germplasm for crop breeding. Molecular markers would also be beneficial towards improving representation in the core collection, by using a reduced number of cultivars.

Since restriction fragment length polymorphism (RFLP) were abundant and were more informative due to their co-dominant nature. Their application to breeders, however, was restricted by the costly and sophisticated techniques required. Conversely, the advantage of random amplified polymorphic DNA (RAPD) markers as a rapid, cost effective tool for the indirect selection of economic traits was immediately recognized by breeders, despite initial problems in reproducibility between laboratories. In addition to their value in genetic mapping (Grisi et al., 2007) and 'gene tagging studies', RAPD have been deployed extensively in different plant species for germplasm classification and have proved to be more useful in detecting genetic variation and classification of germplasm accessions (Ender et al., 2008; Tiwari et al., 2005). In recent years, molecular techniques including RAPD analysis have been used to characterize variability in *Phaseolous* spp. (Martins et al., 2006; Marotti et al., 2007). In view of this, the present study was undertaken to estimate the genetic diversity in common bean genotypes using molecular markers (RADP).

MATERIALS AND METHODS

Plant material

The experimental material for the present study comprised of 13 genotypes of common bean including one check viz., Canadian Red. All the genotypes were local landraces/traditional cultivars collected from different common bean growing areas of state of Jammu and Kashmir (India). The list of the genotypes is presented in Table 1. The experiment was laid out during *kharif* 2008 and 2009 in a randomized complete block design with three replications. The experimental materials were provided the cropping geometry of 30 cm between the rows and plant to plant spacing of 10 cm. The experimental fields were well prepared and standard recommended package of practices were followed to raise a good crop.

DNA extraction

Plant DNA was isolated using cetyl trimethyl ammonium bromide (CTAB) method as modified by Saghai-Maroof et al. (1984). In this method, young leaves at trifoliate stage were harvested from 5-8 plants from each row of individual genotypes (approximately 5-7 g of fresh weight). The leaves were ground to fine powder using pre-chilled pestle and mortar after adding liquid nitrogen to make leaves brittle as well as to stop DNase activity. The powder was transferred immediately to a 50 ml autoclaved polypropylene centrifuge tube containing 15 ml of pre-warmed (65°C) 2% CTAB extraction buffer. The powder was suspended in the buffer by inverting and rotating the tubes properly. The tubes were incubated at 65°C for 30-40 min in a water bath. The samples were mixed occasionally while maintaining at 65°C. After incubation, 15 ml of chloroform: isoamyl alcohol (24:1) was added and tubes were swirled, till it made a dark green emulsion. The tubes were placed on a rotary shaker for 30 min and then centrifuged at 13,000 rpm for 5 min at room temperature. The supernatant was transferred to a clean sterile 50 ml falcon tube. Four microliter (4 μl) RNase (10 mg/ml) was added to each tube and incubated at 37°C in water bath for 1 h.

Chloroform: isoamyl alcohol extraction and centrifugation step was repeated after RNase treatment if required. Following centri-

Table 2. Base sequence of primers used for DNA fingerprinting.

Primer	Sequence (5′ to 3′)
OPA-01	CAG GCC CTT C
OPA-02	TGC CGA GCT G
OPA-03	ATG CAG CCA C
OPA-04	AAT CGG GCT G
OPA-05	AGG GGT CTT G
OPA-06	GGT CCC TGA C
OPA-07	GAA ACG GGT G
OPA-08	GTG ACG TAG G
OPA-09	GGG TAA CGC C
OPA-10	GTG ATC GCG T
OPA-11	CAA TCG CCG T
OPA-12	TCG GCG ATA G
OPA-13	CAG CAC CCA C
OPA-14	TCT GTG CTG G
OPA-15	TTC CGA ACC C

Table 3. Temperature profile used in PCR.

Step	Temperature	Time (minutes)	Number of cycles
Initial denaturation	94	5	1
Denaturation	94	1	
Annealing	37	2	40
Elongation	72	2	
Final extension	94	5	
Hold	4	5	1

fugation, the upper aqueous phase was transferred to a clean sterile 50 ml falcon tube. About 0.8 volume of chilled isopropyl alcohol was added and the tubes were inverted gently several times. The DNA formed white cotton like precipitate and good quality DNA floated atop. The floating DNA was hocked out using a sterile hocked Pasteur pipette, if the DNA was not hockable, it was pelleted by centrifugation. The hocked or pelleted DNA was transferred into a clean sterile 2.0 ml microfuge tubes and was rinsed with 70% ethanol for 5 min so as to remove any residual salts followed by re-centrifugation. Pellet was collected and the left over ethanol was dried up completely by turning down microfuge tubes on a blotting paper and was allowed to air dry (at room temperature) for 1 h. Then 50-80 μl volume of 1x TE (Tris EDTA buffer 10 mM tris HCl, 1mM EDTA, pH 8.0) was added. The tubes were left for few hours at room temperature to allow DNA to dissolve. The quality and quantity of DNA was checked by agarose gel electrophoresis.

RAPD analysis

Primer selection

Fifteen 10-mer oligonucleotides primers (Operon Technologies Inc., CA. USA) were used for characterization of genotypes. The selected RAPD primers along with their base sequence are presented in Table 2.

Polymerase chain reaction (PCR) amplification

In vitro amplification using PCR was performed in a 96 well Biometra Mode-II T-gradient thermoblock using 50 ng of genomic DNA of each genotype in a final volume of 20 μl per reaction. Amplification was performed using temperature profile mentioned in Table 3.

Visualization of PCR products

To 20 μl of the amplified product, 3.33 μl of 6x loading dye was added so as to make the final concentration of the loading dye in the reaction samples to 1x. The PCR products were resolved on 1.55 super fine resolution agarose gel. The gel was prepared in 0.5x TBE buffer. Ethidium bromide was added at concentration of 0.4 mg/μl. The gel was run at 10 v/cm^2, visualized under UV light and photographed using utra cam digital imaging (A6 rc canon camera).

Scoring of RAPD fragments

The size of the DNA fragments was estimated by comparing the DNA bands with a 1 Kb DNA ladder. The amplified bands were scored as present (1) or abscent (0) and were assembled in a data matrix table.

Table 4. Primers, total number of bands, polymorphic band and percentage of polymorphism obtained by PCR amplification of DNA of *Phaseolus vulgaris* L. genotypes.

Primer	Total Number of bands	Number of polymorphic bands	Percentage of polymorphism
OPA_1	12	12	100
OPA_2	11	11	100
OPA_3	8	6	75
OPA_{10}	12	12	100
OPA_{11}	11	11	100
OPA_{13}	6	6	100
OPA_{15}	5	5	100
Total	**65**	**63**	**96.92**
Mean per primer	**9.28**	**9.00**	

Cluster analysis

Cluster analysis of the germplasm, using binary data generated by RAPD markers was conducted using computer software programme numerical taxonomic and multivariate analysis system (NTSYS-PC) version 2.02e (Rohlf, 1997). RAPD marker amplification profile for all the genotypes under study was compared to each other and DNA fragments were scored as present (scored as 1) or absent (scored as 0). Data from all the 15 primers were used to estimate the similarity based on the number of shared amplified bands. Similarity was estimated using SIMQUAL function of NTSYS, which computes a variety of similarity coefficient for quantitative data (nominal data). Similarity matrix value based on Nei and Li (1979) coefficient of similarity (D_{ij}) were calculated as

$$D_{ij} = 2a/(2a + b + c)$$

Where, ‘a’ represents matched fragments b and c are unmatched fragments. The 2a + b + c are the total number of fragments amplified in a particular set. The similarity matrix was then generated and dendrogram was constructed using unweighted pair group method using arithmetic averages (UPGMA) available in NTSYS.

RESULTS AND DISCUSSION

The present investigation revealed a high level of polymorphism in the tested genotypes. Initially 15 decamer oligonucleoticle primers were screened out of which 8 showed unclear or non-reproducible behaviour and did not agree with the criteria of Lynch and Milligan (1994) and hence were rejected. These criteria were considered because it is an objective way to limit the bias resulting from the dominant biallelic properties of RAPD’s. However, the rest of the 7 primers were selected based on robustness of amplification, reproducibility, scorability of banding patterns and were used for diversity analysis in all the 13 genotypes. The seven selected decamer oligonucleotide primers generated 65 amplification products, out of which 63 bands (96.92%) were polymorphic, which is slightly of higher percentage than the value obtained by Maciel et al. (2001) in common beans. These high values could be due to the nature of RAPD markers. Total number of bands obtained per primer in 13 genotypes ranged from 5 to 12 with an average of 9.28 bands per primers (Table 4). The maximum number of scorable bands 12 was obtained from primers (OPA_1 and OPA_{10}), followed by 11 bands from (OPA_2 and OPA_{11}). The present study is well supported by Jose et al. (2009) who investigated 20 common bean landraces using RAPD markers with respect to their genetic diversity. After initial screening of 72 primers, only thirteen primers were selected which generated a total of 102 amplicous with (63.55) polymorphism. Zhang et al. (2008) carried out genetic diversity study of 229 landraces with 30 micro-satellite markers and reported detection of 166 alleles with an average of 5.5 alleles per locus for all markers. Similarly, Sharma et al. (2006) investigated 46 common bean accessions with RAPD markers and reported a generations of 43 amplicons with (70%) polymorphism.

Banding pattern of genotypes were obtained after PCR amplification with primer (OPA_2) (Figure 1). The scorable bands were subjected to cluster analysis, which generated a dendrogram (Figure 2). The results revealed that the high level of genetic diversity was obtained among the 13 genotypes of common bean. The dendrogram was constructed using the tree plot option available an NTSYS-pc software. The genotypes were divided into three clusters in which cluster-I contained maximum of 7 genotypes (PBG-03, PBG-16, PBG-01, PBG-09, SKUA-R-01, SKUA-R-21 and SKUA-R-19), cluster-II contained 5 genotypes (SKUA-R-34, Canadian Red, SKUA-R-28, PBG-30 and SKUA-R-11) and cluster-III contained only one genotype, that is, PBG-29 (Table 5). All the three major clusters viz., cluster-I, cluster-II and cluster-III merged into a single cluster at 43 per cent similarity.

The phonetic representation of similarity coefficient among 13 genotypes of common bean is presented in Table 6. It is evident from the study that the similarity index, based on Dice similarity coefficient, ranged from 27.72 to 82.35 and the maximum similarity index was obtained among genotypes SKUA-R-2 with SKUA-R-19 and SKUA-R-11 (82.35) followed by genotypes PBG-30 and SKUA-R-28 (78.37). The minimum similarity index was obtained between genotypes PBG-29 and SKUA-R-01 (27.72). The

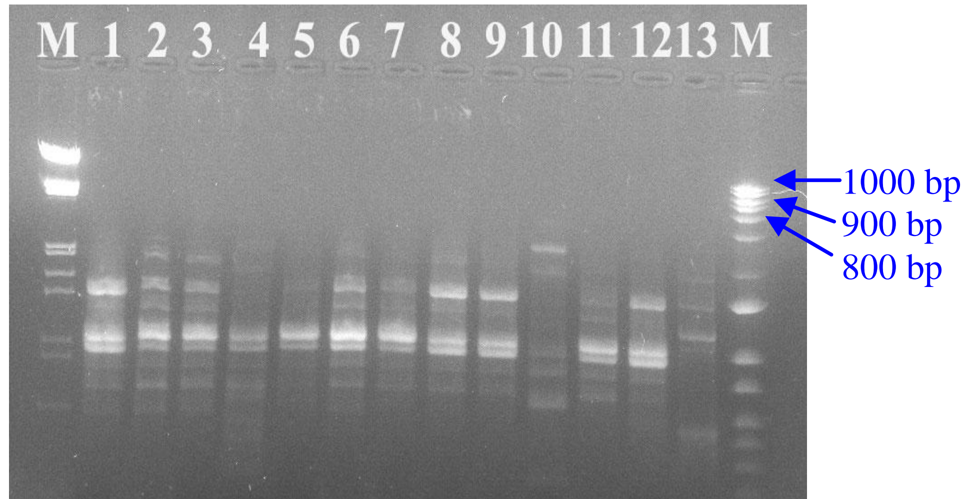

Figure 1. A typical RAPD banding pattern amplified with primer (OPA_2). Lane 1, PBG-03; lane 2, PBG-01; lane 3, PBG-09; lane 4, PBG-16; lane 5, SKUA-R-01; lane 6, SKUA-R-19; lane 7, SKUA-R-11; lane 8, SKUA-R-21; lane 9, SKUA-R-34; lane 10, Canadian Red; lane 11, SKUA-R-28; lane 12, PBG-30; lane 13, PBG-29; M, marker.

PBG003
PBG016
PBG001
PBG009
SKUAR1
SKUAR21
SKUAR19
SKUAR34
CanRed
SKUAR28
PBG030
SKUAR11
PBG029

0.40 0.51 0.63 0.74 0.85
Dice Similarity Coefficient

Figure 2. Dendrogrm depicting genetic relationship among 13 genotyeps of common bean (*Phaseolus vulgaris* L.) based on RAPD data using UPGMA (Dice coefficient).

Table 5. Classification of common bean (*Phaseolus vulgaris* L.) genotypes on the basis of RAPD data.

Cluster	Genotype	Number of genotypes in the cluster
I		
Ia	PBG-03, PBG-16, PBG-01	7
Ib	PBG-09	
Ic	SKUA-R-01, SKUA-R-21, SKUA-R-19	
II		
IIa	SKUA-R-34, Canadian Red	5
IIb	SKUA-R-28, PBG-30	
IIc	SKUA-R-11	
III	PBG-29	1

Table 6. Similarity coefficient matrix for 13 genotypes of common bean (*Phaseolus vulagaris* L.) using UPGMA (dice coefficient).

Genotype	PBG-03	PBG-09	PBG-01	PBG-16	SKUA-R-01	SKUA-R-19	SKUA-R-11	SKUA-R-21	SKUA-R-34	Canadian Red	SKUA-R-28	PBG-30	PBG-29
PBG-03	1.00												
PBG-09	60.71	1.00											
PBG-01	77.55	62.74	1.00										
PBG-16	78.04	69.38	70.00	1.00									
SKUA-R-01	40.00	53.84	31.11	38.09	1.00								
SKUA-R-19	51.42	74.41	52.94	57.89	59.45	1.00							
SKUA-R-11	71.42	58.62	62.74	65.11	50.00	75.67	1.00						
SKUA-R-21	69.38	58.82	54.54	61.11	56.52	82.35	82.34	1.00					
SKUA-R-34	61.53	51.85	55.31	63.15	43.47	58.82	69.09	72.34	1.00				
Canadian Red	57.62	49.18	48.14	66.66	42.27	60.00	65.57	72.72	67.92	1.00			
SKUA-R-28	50.00	51.42	34.92	59.25	40.62	41.66	57.14	53.96	64.40	71.23	1.00		
PBG-30	53.00	54.83	43.63	70.83	35.71	57.14	61.29	54.54	67.85	64.61	78.37	1.00	
PBG-29	45.83	40.00	32.55	61.53	27.72	48.48	44.00	46.51	50.00	56.60	58.06	6.66	1.00

difference at molecular level can be explained on their long span of cultivation in different areas which differ on the basis of soil types, climatic conditions and cultivation practices. The recombination event in support of adaptation to the environment leads to the creation of distinct genotypes. Molecular markers, unlike morpho-agronomic traits, are not influenced by environmental conditions and, therefore, are more reliable tools not only to characterize genotypes, but also to measure genetic relationship more precisely. Present study established the utility of DNA fingerprinting in genotypes using RAPD markers which revealed the presence of genetic diversity among the genotypes studied. In spite of the fact that

common bean described as an autogamous plant, recent evidences raises the possibility that some variability exists in the reproductive system of domesticated and wild varieties (Santalla et al., 2002). The study can be well supported by the study of Chiorato et al. (2007). They studied a set of 220 common bean genotypes and reported that theses genotypes made two groups with 47 and 60% genetic similarity and interpreted that both molecular and morpho-agronomical data sets are equally effective to quantify and organize the genetic diversity of common beans. Similarly, Jose et al. (2009) found that Jaccard's pair-wise similarity coefficient value of 0.5 to 0.95 indicated an intra-specific genetic variation prevalent in landraces of common bean.

Breeding strategies need to exploit the existing variation within and between wild beans and landraces. Hybridization programme can be initiated between the identified diverse genotypes in order to create variation and for incorporation of the desire trait. The molecular markers, especially SCAR can be utilized for transfer of the desired trait in short duration. These bean germplasm could broaden the genetic base of commercial beans to develop high yielding cultivars.

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Vol. 12(51), pp. 7043-7053, 18 December, 2013
DOI: 10.5897/AJB2013.12928
ISSN 1684-5315 ©2013 Academic Journals
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African Journal of Biotechnology

Full Length Research Paper

A bibliometric analysis of global research on genome sequencing from 1991 to 2010

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Accepted 12 September, 2013

This study was carried out to evaluate the global scientific production of genome sequencing research to assess the characteristics of the research performances and the research tendencies. Data were obtained from Science Citation Index Expanded database during 1991-2010. Conventional methods including document types, journals, categories, countries and institutions were used to analyze publication output to reveal the global performance. The development of genome sequencing research during last 20 years was described by synthetically analyzing the distribution of words in article title, author keywords, and *KeyWords Plus* in different periods. The results show that disease and protein related researches were the leading research focuses, and comparative genomics and evolution related research had strong potential in the near future.

Key words: Genome sequencing, research trend, scientometrics, science citation index expanded (*SCI-Expanded*), word cluster analysis, keywords.

INTRODUCTION

Genome sequencing is a laboratory process to determine the order of chemical base pairs which make up DNA or RNA at a single time. Earlier attempts for genome sequencing research mainly concentrated on small genomes such as *Bacteriophage MS2* (Fiers et al., 1976) and *Phage Φ-X174* (Sanger et al., 1977). As the sequencing methods developed, researchers considered to take on longer and more complicated genomes (Edwards and Caskey, 1991; Roach et al., 1995). The first complete genome sequences for representatives from all three domains of life were released in mid-1990s including *Haemophilus influenzae* (Fleischmann et al., 1995), budding yeast *Saccharomyces cerevisiae* (Goffeau et al., 1996), and *Methanococcus jannaschii* (Bult et al., 1996). Lately in 2001, *Nature* and *Science* published a rough draft of the human genome, marked a milestone in genome sequencing history (Lander et al., 2001; Venter et al., 2001). With success in human genome, the sequencing of model organisms, including fruit flies (Adams et al., 2000), *Arabidopsis* (Kaul et al., 2000), Algae (Douglas et al., 2001), rice (Goff et al., 2002), microbial organisms (Stover et al., 2000; Ivanova et al., 2003), and parasites (Gardner et al., 2003) have been studied in the first five years following the human genome project (HGP). Furthermore, genomes from more organisms were sequenced in a faster pace after 2005 with dramatic leaps in sequencing technology and a preci-

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Abbreviations: HGP, Human genome project; **AIDS,** acquired immunodeficiency syndrome; ***SCI-Expanded*,** Science Citation Index Expanded; **JCR,** Journal Citation Reports; **IF,** impact factor; **SPA,** single country article; **SNPs,** single nucleotide polymorphisms.

pitous drop in costs (Mardis, 2011). As of October 2011, the complete sequences were available for: 2,719 viruses, 1,115 archaea and bacteria, and 36 eukaryotes (available in www.NCBI.com).

Despite the massive success of genome sequencing achieved in 21st century, there have been few attempts at gathering systematic data on genome sequencing research. A common research instrument for this analysis is the bibliometric method which has been widely used to measure scientific progress in many disciplines of science and engineering, such as acquired immunodeficiency syndrome (AIDS) (Macias-Chapula, 2000), and cancer molecular epidemiology (Ugolini et al., 2007). Moreover, the Science Citation Index Expanded (*SCI-Expanded*) database is used to analyze research performance from a more comprehensive perspective (Li et al., 2009). Conventional methods concerning bibliometrics mainly investigated the publication characteristics, including countries (Braun et al., 1995), institutions (Rodríguez and Moreiro, 1996), journals (Colman et al.,1995), and categories (Ugolini et al., 1997) may not be adequate to indicate the future orientation of research field (Chiu and Ho, 2007). More information, closer to the research itself, such as paper titles, author keywords, *KeyWords Plus*, and abstracts have been introduced (Xie et al., 2008; Li et al., 2009; Zhang et al., 2010) for the indepth information. Furthermore, an innovative method named “word cluster analysis” was successfully applied for finding the hotspots to evaluate research emphasis and trend (Mao et al., 2010).

In this study, bibliometric methods involving both the conventional and innovative ones were used to quantitatively and qualitatively assess the global performance and trend of genome sequencing research between 1991 and 2010. The results could give insights into the characteristics of the genome sequencing literature. More importantly, it could provide not only a potential guide for novice researchers, but also a basis for better understanding the global development tendency of genome sequencing research.

MATERIALS AND METHODS

The data were based on the online version of the *SCI-Expanded* database. According to Journal Citation Reports (JCR), it indexes 8,073 journals with citation references across 174 scientific disciplines in 2010. The database was searched using the keywords including “genome sequencing”, “genome sequence”, “genome sequences”, “genome-sequenced”, “genome sequency”, and “genome sequencings” in terms of topic (title, abstract, author keywords, and *KeyWords Plus*) within the publication year limitation from 1991 to 2010. Document information including names of authors, title, abstract, author keywords, *KeyWords Plus*, address, year of publication, categories, and journals were downloaded into spreadsheet software.

Additional coding was performed manually for the number of origin country and institution of the collaborators, and impact factors of the publishing journals. Impact factors were taken from the JCR published in 2010. Articles originating from England, Scotland, Northern Ireland, and Wales were reclassified as being from the United Kingdom (UK) (Chiu and Ho, 2005). USSR and Russia were also reclassified as being from Russia. Articles from Hong Kong published before 1997 were included in the China category (Chuang et al., 2011). Collaboration type was categorized and determined by the addresses of the authors as: Single country articles with addresses from the same country; internationally collaborative articles with author addresses from more than one country or territory (Li et al., 2009); single institution articles with addresses from the same institution; and inter-institutionally collaborative articles with author addresses from more than one institution (Malarvizhi et al., 2010). All keywords, both those reported by authors and those attributed by the Web of Science, as well as words in title and abstract were identified and separated into 5-year span (1991-1995, 1996-2000, 2001-2005, and 2006-2010). Then their ranks and frequencies were calculated. A word cluster analysis was performed in the combination of the words in titles, author keywords, *KeyWords Plus*, and words in abstracts, in which different words with identical meaning and misspelled keywords were grouped and considered as a group for one research focus (Li et al., 2009; Mao et al., 2010).

RESULTS AND DISCUSSION

Altogether 20,462 publications consist of 16 document types. Articles (15,722) dominate with the highest percentage of 77%, followed by reviews (2,984; 15%), proceedings paper articles (859, 4.2%), and editorial materials (396; 1.9%). The other 12 documents types with the percentages less than one percent were meeting abstracts, news items, letters, corrections, notes, software reviews, book chapter articles, reprints, database reviews, addition corrections, and biographical-item. Only 15,722 journal articles were extracted for subsequent analyses for its dominant position and including whole research ideas and results (Ho et al., 2010).

Publication outputs

The annual number of articles is shown in Figure 1. The annual number of articles increased nearly 100 times from only 22 articles in 1991 to nearly 2,000 articles in 2010. To be specific, the annual number of articles first exceeded 100 in 1996, and rocketed over 1,000 in 2003. The development in the past two decades could be primarily attributed to the strong support of HGP in 1990. HGP, regarded as the third massive science project after Manhattan Project and Apollo Project, was invested three billion dollars by the US Department of Energy and the National Institutes of Health (Barnhart, 1989). The massive government concern and economic investment strongly prompted to the development of genome sequencing area (Lander, 1996). Another possible reason for the fast increase is that alternative sequencing methods and instruments have been produced to reduce time and cost (Margulies et al., 2005). In the 21st century, fierce commercial competition will force manufacturers to create new faster and cheaper sequencing machines, which will benefit genome

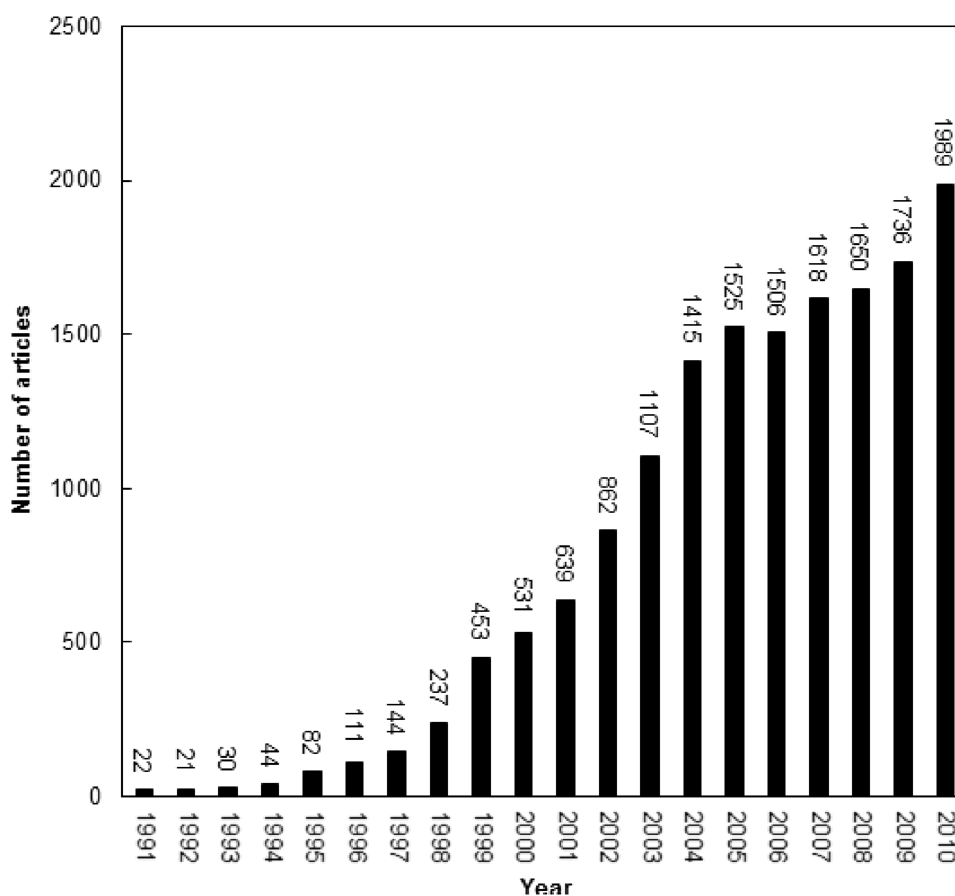

Figure 1. The growth trends of annual articles in genome sequencing research.

sequencing area deeper (Mardis, 2009).

Journals and Web of Science categories

The total articles (15,722) were published in 1,308 journals, among which, 555 (42%) journals contained only one article and 206 (16%) contained two. Table 1 shows 13 core journals that published more than 200 articles, accounting for one third of all articles. *Journal of Bacteriology* ranked first with 1,043 papers (6.6%), followed by *BMC Genomics* with 471 articles. However, the percentage of the top journal was not high, which indicated the breadth of genome sequencing research as well as the broad interest in genome sequencing from various research angles (Li et al., 2011; Wolfe and Li, 2003). In addition, as regards to the impact factor (IF) for all journals, *New England Journal of Medicine Proceedings of National Academy of Sciences of the United States of America, Nucleic Acid Research, and Genome Research* which ranked 3rd, 4th and 6th in the number of total articles, respectively, having the IFs greater than seven. Since the IF is used to evaluate a journal's relative importance of one field, these IF statistical results will help researchers select journals when publishing articles on genome sequencing related research (Ho, 2008).

Based on the classification of Web of Science categories in JCR in 2010, the publication output data was distributed in 141 Web of Science categories in science edition. Biochemistry and molecular biology, microbiology, genetics and heredity, and biotechnology and applied microbiology were the four most popular categories, which exceeded the other categories in both the cumulative number and the annual number. It is noticable that the category of biochemistry and molecular biology held primacy from 1991 to 2005, but started to decrease rapidly from 2006; while microbiology grew fast and became the first in 2010. Growth trends also appeared in the two categories of biotechnology and applied microbiology, and genetics and heredity. The annual number of articles of genetics and heredity increased continually in the study period and exceeded that of biochemistry and molecular biology after 2006. The shifting position of categories indicates that the mainstream of research is no longer restricted to the original one (Wolfe and Li, 2003), and more attention of mechanism has been transferred to the application in genome sequencing related research.

Table 1. The 13 core journals on genome sequencing, including the ranking, percentages, impact factors.

Journal	IF2010	TP (%)	Web of Science categories	Rank
Journal of Bacteriology	3.726	1,043 (6.6)	Microbiology	25/107
BMC Genomics	4.206	471 (3.0)	Biotechnology and applied microbiology Genetics and heredity	24/160 34/156
Proceedings of the National Academy of Sciences of the United States of America	9.771	458 (2.9)	Multidisciplinary sciences	3/59
Nucleic Acids Research	7.836	448 (2.8)	Biochemistry and molecular biology	30/286
Applied and Environmental Microbiology	3.778	431 (2.7)	Biotechnology and applied microbiology Microbiology	32/160 24/107
Genome Research	13.588	352 (2.2)	Biochemistry and molecular biology Biotechnology and applied microbiology Genetics and heredity	8/286 3/160 6/156
Molecular Microbiology	4.819	336 (2.1)	Biochemistry and molecular biology Microbiology	60/286 16/107
Infection and Immunity	4.098	325 (2.1)	Immunology Infectious diseases	33/134 11/58
Microbiology-SGM	2.957	310 (2.0)	Microbiology	39/107
Journal of Biological Chemistry	5.328	299 (1.9)	Biochemistry and molecular biology	50/286
PLoS One	4.411	238 (1.5)	Biology	12/86
FEMS Microbiology Letters	2.040	222 (1.4)	Microbiology	62/107
Journal of Virology	5.189	211 (1.3)	Virology	5/33

IF2010: impact factor in 2010; TP: number of total articles.

National and institutional contributors

Each author of an article has made an independent contribution to the manuscript (Coats, 2009), and therefore the institution and country the author affiliated could be consider the important contributors for the evaluation of research. Publication counts of countries is a reference for evaluating countries/territories research performance in a field, and has been used in many aspects of research such as tsunami (Chiu and Ho, 2007) and risk assessment (Mao et al., 2010). Excluding 35 articles with no author address information on the Web of Science, the 15,687 articles originated from 139 countries/territories. The distribution of the genome sequencing articles all around the world is displayed in Figure 2. America, West Europe, Japan, and China were the main production areas. Of all the 15,687 articles with author information, 4,629 (30%) were international collaborative publications and 11,058 (70%) were independent publications. The international collaborative rate of genome sequencing research is higher than that in certain studies, such as 14% biosorption technology for water treatment (Ho, 2008) and acupuncture research (Han and Ho, 2011), 16% of desalination research (Tanaka and Ho, 2011) and solid waste (Fu et al., 2010). Table 2 reveals the characteristics of the top 20 productive countries. Five indicators including the number of total articles, single country articles, internationally collaborative articles, first author articles, and corresponding author articles were displayed. The table also presented the percentage of independent articles in total articles, total articles per number of authors, and single country articles per number of authors. The USA ranked top one by all indicators. Single country articles were authored by 67 different countries, and 27 countries contributed less than ten single country articles. Furthermore, the developing countries such as India (36%), Brazil (30%), Russia (36%) had relatively low percentages of single country articles (%S); while developed countries USA and UK (%S = 61%) were more inclined or able to conduct research independently. It also appeared that the lowest average number of authors per total article (TPA) and single country article (SPA) was found to be 7.1 and 2.5 authors per article for India, while Italy, Brazil and Belgium had higher value over 14 for TPA and 4.5 for SPA.

The growth trends of the top eight productive countries are displayed in Figure 3. The USA was also dominant in the annual production, ranked first every year except 2005.

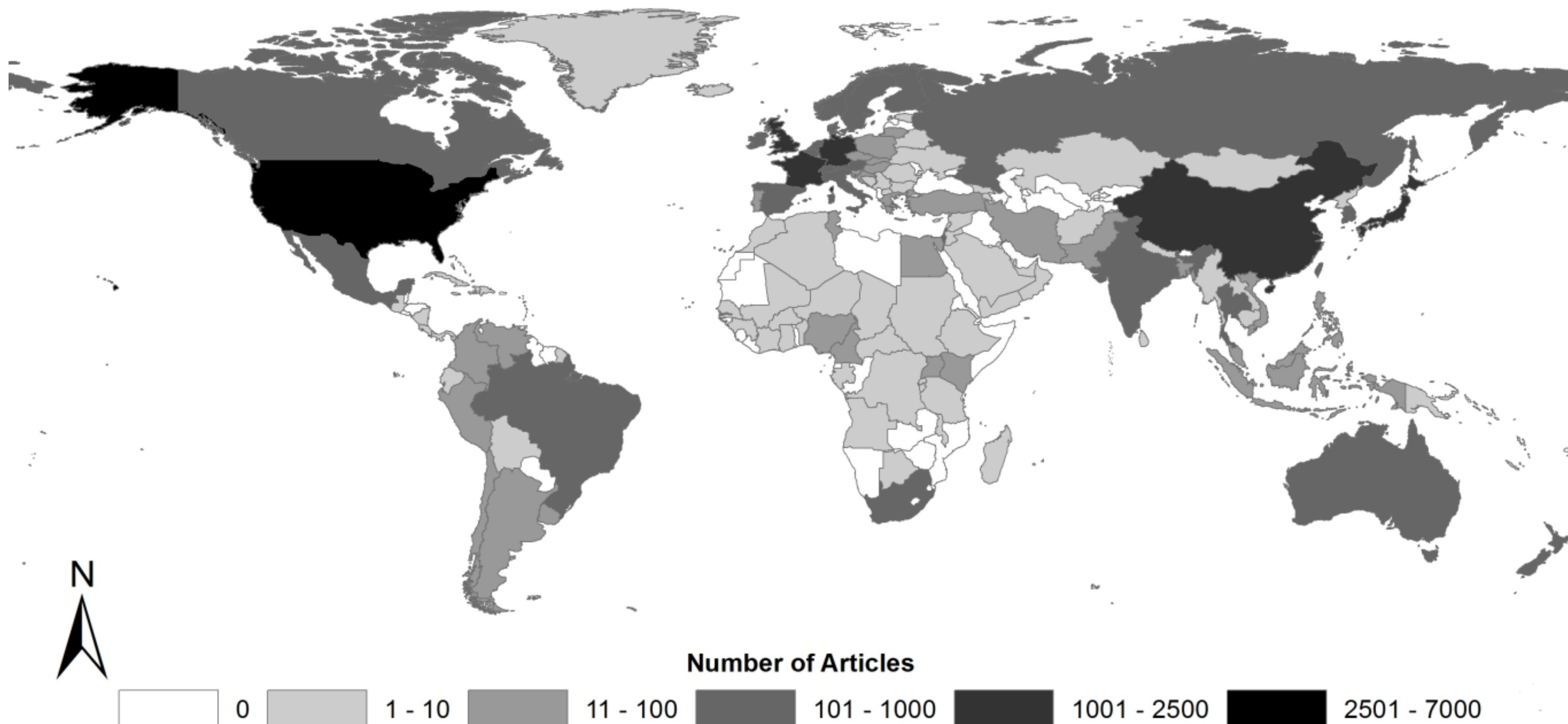

Figure 2. Distribution of genome sequencing articles in the world.

The elite performance of the USA may be due to its greater economic investment in biotech industry than other countries. For example, there were more than 1,500 biotech companies in Europe and approximately 1,300 in the United States in 2001, but revenues for European biotechs were less than one-third that of US revenues (Nagle et al., 2003). Notably, China published less than ten articles before 2001, but the annual number of articles grew sharply and ranked 4th in 2010. China is also the only one developing country which was involved in the top eight most productive countries/ territories (Figure 3). The outstanding energy of China was not surprising, because as reported, it not only experienced a sustained and remarkable increase in scientific production, as the world's second largest producer of scientific publications since 2006 (Zhou and Leydesdorff, 2008), but has also been taking a world-leading position in various fields, such as chemistry (Zhou and Leydesdorff, 2009), and nanotube technology (Kostoff, 2012). Of 15,687 articles with author addresses in Web of Science, 9,796 (62%) were inter-institutionally collaborative articles, while 5,891 (38%) were institutionally independent articles. The percentage of collaboration among institutions (62%) was twice more than that among countries (30%). The inter-institutionally collaborative rate was equal to that of global climate change with 62% (Li et al., 2011), but was larger than 53% of acupuncture research (Han and Ho, 2011), 44% of solid waste research (Fu et al., 2010), and 37% of desalination research (Tanaka and Ho, 2011). As for the top 10

Table 2. Characteristics of the top 19 productive countries/territories (TP 200).

Country	TP	TPR (%)	SPR (%)	CPR (%)	FPR (%)	RPR (%)	S%	TPA	SPA
USA	6,607	1 (42)	1 (37)	1 (55)	1 (34)	1 (34)	61	8.2	5.3
UK	2,016	2 (13)	3 (7.4)	2 (26)	3 (8.5)	3 (8.5)	61	11	5.1
Germany	1,728	3 (11)	4 (6.7)	3 (21)	4 (7.3)	4 (7.2)	47	12	4.8
Japan	1,649	4 (11)	2 (11)	7 (8.9)	2 (9.1)	2 (9.0)	45	8.3	5.7
France	1,412	5 (9.0)	6 (5.5)	4 (17)	5 (6.0)	5 (5.9)	46	11	5.8
China	1,079	6 (6.9)	5 (5.8)	6 (9.4)	6 (5.4)	6 (5.4)	56	10	7.0
Canada	888	7 (5.7)	7 (3.6)	5 (11)	7 (3.6)	7 (3.6)	44	10	4.3
Australia	600	8 (3.8)	10 (2.1)	8 (8.0)	8 (2.4)	8 (2.4)	41	11	4.5
Netherlands	509	9 (3.2)	12 (1.6)	9 (7.2)	10 (1.9)	10 (1.9)	46	12	5.1
Spain	457	10 (2.9)	11 (1.6)	10 (6.0)	11 (1.7)	11 (1.7)	50	12	4.4
Italy	411	11 (2.6)	13 (1.3)	11 (5.7)	13 (1.6)	13 (1.6)	43	14	5.9
South Korea	402	12 (2.6)	8 (2.2)	17 (3.3)	9 (2.0)	9 (2.0)	44	11	6.1
Sweden	356	13 (2.3)	15 (1.0)	13 (5.2)	15 (1.3)	15 (1.3)	41	12	3.6
Switzerland	335	14 (2.1)	18 (0.78)	12 (5.4)	16 (1.1)	16 (1.1)	40	16	4.2
India	317	15 (2.0)	9 (2.1)	24 (1.8)	12 (1.7)	12 (1.7)	36	7.1	2.5
Brazil	302	16 (1.9)	14 (1.2)	15 (3.6)	14 (1.3)	14 (1.3)	30	15	7.0
Belgium	293	17 (1.9)	17 (0.80)	14 (4.4)	17 (1.0)	17 (1.0)	30	15	4.9
Denmark	240	18 (1.5)	19 (0.65)	15 (3.6)	18 (0.76)	18 (0.74)	36	19	3.8
Russia	202	19 (1.3)	22 (0.55)	18 (3.0)	20 (0.62)	21 (0.63)	36	8.8	4.2

TP, Number of articles; TPR, the rank of total articles; SPR, the rank of single institution articles; CPR, the rank of inter-institutionally collaborative articles; FPR, the rank of first author articles; RPR, the rank of corresponding author articles; S%, the percentage of single institution articles in each institution; TPA, total articles per number of authors; SPA, single country articles per number of authors.

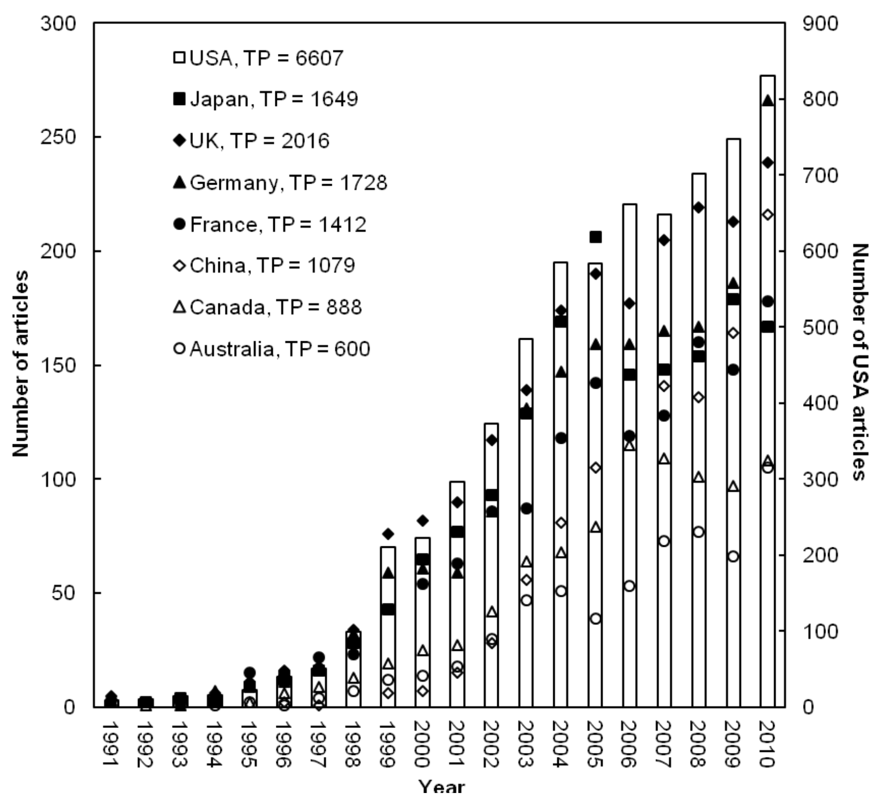

Figure 3. Comparison the growth trends of the top eight productive countries during 1991-2010.

Table 3. Characteristics of the top ten productive institutions.

Institution	TP	TPR (%)	SPR (%)	CPR (%)	FPR (%)	RPR (%)	S%
Chinese Academy of Sciences, China	289	1 (1.8)	7 (0.78)	2 (2.5)	2 (1.0)	1 (1.0)	22
University of Tokyo, Japan	288	2 (1.8)	1 (1.1)	3 (2.3)	1 (1.0)	1 (1.0)	22
University of California, Berkeley, USA	273	3 (1.7)	34 (0.42)	1 (2.5)	23 (0.49)	23 (0.47)	21
Institut Pasteur, France	264	4 (1.7)	12 (0.70)	3 (2.3)	3 (0.85)	3 (0.84)	19
INRA, France	263	5 (1.7)	6 (0.81)	6 (2.2)	4 (0.83)	4 (0.82)	19
Harvard University, USA	258	6 (1.6)	13 (0.68)	5 (2.2)	6 (0.68)	6 (0.67)	19
CNRS, France	247	7 (1.6)	19 (0.58)	7 (2.2)	16 (0.52)	16 (0.52)	19
University of Maryland, USA	243	8 (1.5)	15 (0.65)	8 (2.1)	5 (0.75)	5 (0.77)	18
University of California, Davis, USA	218	9 (1.4)	31 (0.44)	9 (2.0)	32 (0.4)	31 (0.41)	20
Washington University, USA	213	10 (1.4)	8 (0.75)	12 (1.7)	7 (0.65)	9 (0.63)	21

TP, Number of articles; TPR, the rank of total articles; SPR, the rank of single institution articles; CPR, the rank of internationally collaborative articles; FPR, the rank of first author articles; RPR, the rank of corresponding author articles; S%, the percentage of single institution articles in each institution.

institutions, a half of them were located in the USA and three were in France (Table 3). The USA, the UK, Germany, Japan, and France were the top five most productive countries. However, from Table 3, no institutions in Germany and UK could be found. Chinese Academy of Science ranked first in the total number of publications, but there is a bias because it is made up of many relatively independent institutions distributed throughout China. At present, the articles of these branches were pooled under one heading, and rankings would be different if these branches are counted as independent ones (Li et al., 2009). Thus, except Chinese Academy of Science, the leading was University of Tokyo in Japan, which also ranked first in the single insitution articles, first author articles and corresponding author articles (Table 3). University of California, Berkeley ranked first in internationally collaborative articles, but stood relatively low positions in the single institutions articles, first author articles and corresponding author articles.

Distribution of author keyword analysis

Author keywords are the words that expose the internal structure of an author's reasoning, and are used in a specific period as a bibliometric method (Chiu and Ho, 2007). Using the author keywords to analyze the trend of research is much more frequent in recent years, and proved to be important for monitoring development of science and programs (Xie et al., 2008; Li et al., 2009). Analysis of author keywords revealed that 18,030 author keywords were used from 1991 to 2010, of which 14,173 (79%) appeared only once and 1,828 (13%) appeared only twice. These once or twice only author keywords might not be standard or widely accepted by researchers (Ugolini et al., 2001). Author keywords appeared in the articles referring to genome sequencing were calculated and ranked by total 20-year period and four 5-year sub-periods (Table 4). The most frequently used keywords were identified, such as "evolution", "phylogeny", and "comparative genomics". The analysis of author keywords revealed a notable growth trend in "phylogeny", "comparative genomics", "bioinformatics", "phylogenetic analysis", "proteomics", and "functional genomics". Phylogeny is a discipline describing evolutionary relationships. It is a remarkable fact that the rank and percentage of "phylogeny" and "phylogenetic analysis" rose from 10th (1.9%), 17th (1.3%) during 1996-2000 to 1st (3.5%) and 6th (1.9%) during 2006-2010, indicating phylogeny research has been greatly prompted by the abundance of genome sequencing data (Wolfe and Li, 2003). The amount of phylogenetic information will be immense as the degree of similarities and differences between gene sequences is used as one of the most common and reliable methods to perform phylogenetic analysis, and more organisms' gene sequences information will be available from genome sequencing (Brooker, 1999; Wolfe and Li, 2003). Comparative genomics has become one of the most powerful strategies for analyzing genome sequencing data (Nelson and Cox, 2008), and its rank in author keywords increased to 1st in 2001-2005. Meanwhile, the rank and percentage of articles with "proteomics" and "functional genomics" went up respectively, which did not show up during 1991-1995, rose to 8th (1.9%) and 13th (1.4%) during 2001-2005. Proteomics is functional genomics at the protein level (Anderson and Anderson, 1998). Better understanding of proteomics would greatly aid the biological interpretation of the genome sequencing data and accelerate its medical exploitation (King et al., 2000; Weinberg, 2010).

Distribution of article titles, *KeyWords Plus*, and abstracts analysis

Article title, which always contained the information of the whole paper, is a useful tool to evaluate trend recently (Xie et al., 2008; Zhang et al., 2010). All the single words

Table 4. Top 20 most frequently used author keywords during 1991-2010 and four five-year sub-periods.

Author keyword	TP	91-10 Rank (%)	91-95 Rank (%)	96-00 Rank (%)	01-05 Rank (%)	06-10 Rank (%)
Genome sequencing	217	1 (3.2)	1 (41)	1 (14)	11 (1.5)	10 (1.4)
Evolution	211	2 (3.1)	9 (3.0)	7 (2.7)	3 (3.4)	2 (3.0)
Phylogeny	211	2 (3.1)	N/A	10 (1.9)	5 (2.9)	1 (3.5)
Comparative genomics	203	4 (3.0)	N/A	29 (0.94)	1 (4.1)	3 (2.7)
Genome	190	5 (2.8)	32 (1.0)	8 (2.4)	4 (3.1)	3 (2.7)
Bioinformatics	157	6 (2.3)	N/A	37 (0.78)	2 (4.0)	8 (1.6)
Genome sequence	152	7 (2.2)	5 (4.0)	8 (2.4)	6 (2.6)	5 (1.9)
Genomics	120	8 (1.8)	32 (1.0)	6 (2.8)	10 (1.6)	7 (1.7)
Archaea	105	9 (1.5)	N/A	4 (4.7)	9 (1.9)	19 (0.86)
Phylogenetic analysis	103	10 (1.5)	N/A	17 (1.3)	18 (1.0)	6 (1.9)
Gene expression	101	11 (1.5)	15 (2.0)	29 (0.94)	7 (2.0)	14 (1.3)
Proteomics	100	12 (1.5)	N/A	29 (0.94)	8 (1.9)	11 (1.3)
Bacillus subtilis	92	13 (1.4)	3 (17)	2 (7.5)	27 (0.91)	281 (0.16)
Microarray	78	14 (1.1)	N/A	N/A	15 (1.3)	11 (1.3)
Virulence	72	15 (1.1)	N/A	324 (0.16)	23 (1.0)	11 (1.3)
Rice	70	16 (1.0)	N/A	120 (0.31)	19 (1.0)	15 (1.2)
Saccharomyces cerevisiae	70	16 (1.0)	2 (19)	3 (6.0)	123 (0.30)	281 (0.16)
Mass spectrometry	66	18 (1.0)	32 (1.0)	29 (0.94)	17 (1.1)	18 (0.91)
Escherichia coli	62	19 (0.91)	N/A	14 (1.4)	23 (1.0)	21 (0.83)
Functional genomics	62	19 (0.91)	N/A	17 (1.3)	13 (1.4)	40 (0.59)

TP, Number of articles; N/A, not available.

in the title of genome sequencing related articles were statistically analyzed in this study. Some words which have no usefulness for the analysis of research trend were discarded such as prepositions, conjunctions. "Protein", "virus", "evolution", "comparative", and "proteins" presented in the 20 most frequently used keywords in title also appeared in the top 20 of author keywords. Mean while, the rank and percentage of "evolution" increased steeply from 106th (1%) during 1991-1995 to 14th (4.2%) during 2006-2010, similar to the results of analysis of author keywords, from 9th (3%) during 1991-1995 to 2nd (3%) during 2006-2010.

However, there is a disparity that authors might choose their title words to attract a more general or particular audience (Peters and van Raan, 1994). As a supplement, an abstract appeared as it is a brief summary of a research paper of any in-depth analysis of a particular subject or discipline, and is often used to help the reader quickly ascertain the subjective focus and emphasis specified by authors (Zhang et al., 2010). Through key words analysis in abstracts, it can be concluded that continual attention was given to "protein" and "proteins", whose rank is 6th and 8th, respectively. Proteins have been paid much attention as they perform most life functions and even make up the majority of cellular structures (Nelson and Cox, 2008). Again, "phylogenetic" showed a notable increasing trend in genome sequencing research, rising from 156th (6.4%) during 1991-1995 to 23rd (16%) during 2006-2010.

In recent years, *KeyWords Plus* were separated into different year periods to analyze the variations of trends on research topics (Xie et al., 2008). *KeyWords Plus* can provide additional search terms extracted from the titles of articles cited by authors in their bibliographies and footnotes in the ISI database, thus to augment title words and author keywords indexing (Garfield, 1990). As with the distribution of article titles, abstracts, and author keywords, "evolution", "protein", and "proteins" were emphasized in *KeyWords Plus* analysis. Moreover, "SNP" and "SNPs" exhibited growth trends in *KeyWords Plus* as well as other three kinds of keywords analysis. So far, over 1.4 million locations where single nucleotide polymorphisms (SNPs) occur in humans have been identified (Sachidanandam et al., 2001). This will allow genome-wide, high-resolution analysis of amplifications and deletions, and means significantly due to the fact that genetic variants can be examined for association with phenotypes and interpreted in clinical settings (Lander, 2011).

Research emphases and trends

The distribution of words in the article title and abstract, author keywords, and *KeyWords Plus* in different periods could provide important information for research emphases. Each research emphase related synonymic single words and congeneric phrases were summed and grouped

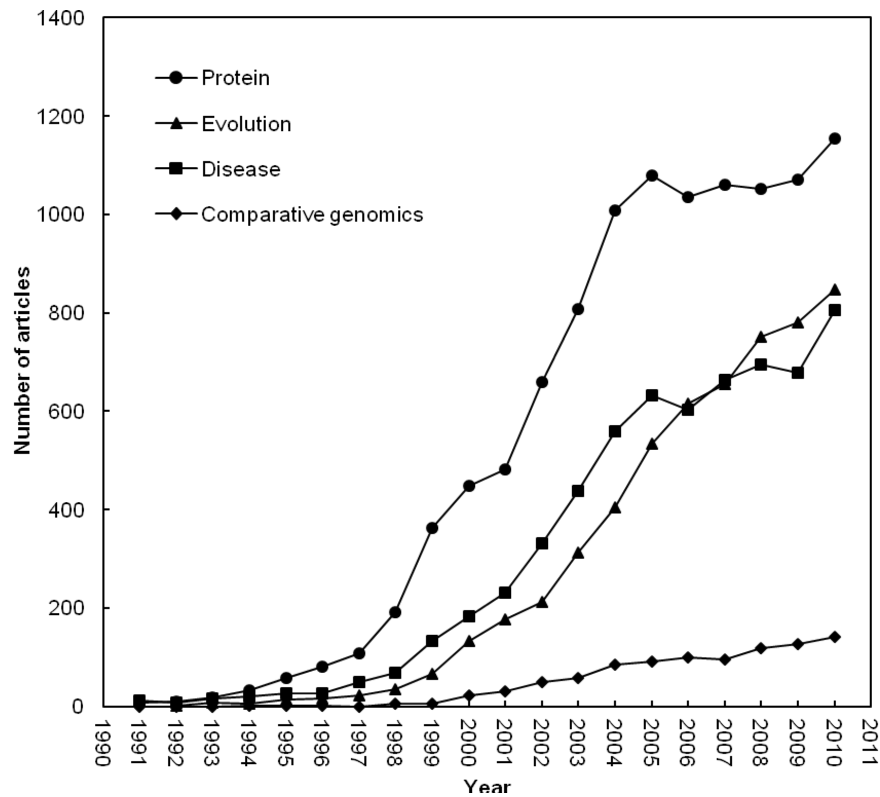

Figure 4. Growth trends of hotspot-related articles during 1991-2010.

into word clusters, so as to analyze the historical development of the science more completely and precisely, and more importantly, to discover the directions the science is taking. It was an innovative method that has been successfully used to analyze the research hotspot in several fields of science (Mao et al., 2010; Tanaka and Ho, 2011). Each hot issue in Figure 4 was supported by a word cluster, which was composed of several supporting words, including their plural forms, abbreviation, other transformations, as well as near synonyms. For example, the topic "comparative genomics" included "comparative (-) genomic (s)", "array comparative", "genome-wide association (s)", "single (-) nucleotide polymorphism (s)", "SNP (s)", and "array CGH", "blast". The growth trends of the articles concerning the supporting word clusters are displayed in Figure 4. Research emphases in genome sequencing were extracted and separated into four topics: protein, disease, evolution, and comparative genomics, among which, the topic "protein" was the most attractive. The predominant position is mainly attributed to that the success of genome sequencing speeded up protein primary structure study (Nelson and Cox, 2008). In addition, the large amount of genomic data were available for a variety of organisms facilitates proteome development, and has brought an urgent need for systematic proteomics to decipher the encoded protein networks that dictate cellular function (Ho et al., 2002). The rise in the study of disease in the field of genome sequencing started later than those of protein and is relatively slow after 2005. It indicates that although genome sequencing provides new avenues for disease genes discovery, the application is limited as gene findings from genome sequencing studies failed to explain much of the heritability of the diseases being studied (Maher et al., 2008; Ioannidis et al., 2008). Thus, it is expected that the following research in this field will turn to search evidences for the findings and elucidate the underlying mechanisms of disease in the next decade.

More attention was paid to the research on "evolution", especially after 1999. The number of articles related to "evolution" already exceeded that of "disease" in 2007. Along with gene sequences provided by whole genome sequencing, gene duplication and horizontal gene transfer (also called lateral gene transfer) are predicted to be the most familiar of the gene formation mechanisms and probably accounts for most new genes (Yang, 1998; Suzuki and Gojobori, 1999; Suzuki and Nei, 2001; McLysaght et al., 2002). Thus genome sequencing data have had an impact on the explanation for gene evolution at the scale of molecular. As the cost of genome sequencing falls and the capacity of sequencing centers grows, genome sequencing data will also allow the evolution of regulatory

elements studied in unprecedented detail (Lander, 2011; Mardis, 2011). Studies of regulatory elements will lead to answer more mysterious questions about of evolution since King and Wilson (1975) suggested that evolution of species depends more on innovation in regulatory sequences than changes in gene sequences.

Like the topic "evolution", the research on comparative genomics increased remarkably in 21st century. Interspecific and intraspecific comparative genomics have widely been applied in many aspects, such as annotated the genome (Birney et al., 2007), and identified DNA variation including SNP (Kingsmore et al., 2008). The application of comparative genomics has increased the introduction of different ideas into genomics area, including concepts from systems and control, information theory, made the genome sequencing data better utilized (Via et al., 2011). Therefore, it is expected that research on comparative genomics will grow constantly since the genome sequencing data will be larger and the utilization will be more concerned in the next decades.

Conclusion

To gain a clearer insight into research focus and forecast on genome sequencing field, bibliometric analyses of annual publication outputs, journals, categories, countries, institutions, author keywords, title words, abstract words, and *KeyWords Plus* provide a synthetical overview. A total of 15,722 genome sequencing *SCI-Expanded* articles were analyzed over a period from 1991 to 2010. A fast increase was observed in the study period. 'Journal of Bacteriology' led the total 1,308 journals in the 141 Web of Science categories. Genome sequencing research tends to be utilized in a wide extent of areas. Shiftings among top categories indicated that the attention on application has been getting more popular. The national collaboration occurred more in genome sequencing in comprison with other fields. The USA held primacy using relatively less people per article, while China with a great growth rate was the most productive one among developing countries. University of Tokyo in Japan was actually the lead among the institutions. Moreover, the comprehensive analysis of author keywords, title words, abstract words, and *KeyWords Plus* provide important clues to word cluster for research emphases. The newly developed bibliometric method, "word cluster analysis", can help researchers realize the panorama of global genome sequencing research and establish future research directions. Disease and protein related research obtained stable focus on a high degree in this field. The issues "comparative genomics" and "evolution" were active during the study period and will deserve increasing concern in the future.

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African Journal of Biotechnology Vol. 12(51), pp. 7054-7056, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3749
ISSN 1684-5315 ©2013 Academic Journals

Short Communication

The isolation and characterization of twelve novel microsatellite loci from *Haliotis ovina*

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Accepted 5 July, 2012

Twelve (12) microsatellite loci were developed from *Haliotis ovina* by magnetic bead hybridization method. Genetic variability was assessed using 30 individuals from three wild populations. The number of alleles per locus was from 2 to 5 and polymorphism information content was from 0.1228 to 0.6542. The observed and expected heterozygosities ranged from 0.0000 to 0.7778 and 0.1288 to 0.6310, respectively. These loci should provide useful information for genetic studies such as genetic diversity, pedigree analysis, construction of genetic linkage maps and marker-assisted selection breeding in *H. ovina*.

Key words: Genetic markers, *Haliotis ovina*, microsatellites.

INTRODUCTION

Abalone belongs to marine gastropods and is widely distributed along the coastal waters of tropical and temperate areas (Geiger, 1999). Among about 20 commercially important abalone (Jarayabhand and Paphavasit, 1996), *Haliotis ovina*, which is also mainly distributed in tropical areas, has a high economic importance for it is both fished and farmed. Although, *H. ovina* is not a main target species for aquaculture in China, the genetic information of *H. ovina* is so essential because of the rapid decline of its natural resources due to overexploitation and the pollution of its living environment. Thus, further conservation strategies and recovery plans require the basic genetic information of *H. ovina.*

Microsatellite is an effective tool and has been applied to various genetic aspects. Up until now, microsatellite markers in *Haliotis rubra* (Huang and Hanna, 1998; Evans et al., 2000), *Haliotis asinina* (Selvamani et al., 2000), *Haliotis discus hannai* (Li et al., 2002), *Haliotis kamtschatkana* (Miller et al., 2001) and *H. discus discus* (Sekino and Hara, 2001) have been reported. Also, microsatellite markers have been applied to analyze the genetic background of *H. rubra* (Li et al., 2006; Evans et al., 2004), *Haliotis conicorpora* (Li et al., 2006), *H. discus hannai* (Li et al., 2003, 2004), *Haliotis midae* (Evans et al., 2004) and *Haliotis asinine* (Selvamani et al., 2001). To our knowledge, the genetic study of *H. ovina* is very limited (Li, 2006, 2009; Li et al., 2008; Klinbunga et al., 2003).

MATERIALS AND METHODS

One wild individual from Yingzhou population was used in SSR primer screening. Genomic DNA was extracted from foot muscle using CTAB method which was examined by agarose gel electrophoresis(1%) and ultraviolet spectrophotometer, and then was digested with *MboI* (Fermentas) for 3 h (37°C) and then, *MboI* adapter1 (5'- GATCGTCGACGGTACCGAATTCT-3') / *MboI* adapter2 (5'-GTCAAGAATTCGGTACCGTCGAC-3') were ligated to the digested products using T4 DNA ligase. The ligated product was hybridized with biotin-labeled sequence repeats (SSR) probes $(GT)_{15}$, $(CT)_{15}$, and the hybrid mixture was incubated with magnetic beads coated with streptavidin. The recovered DNA fragments were amplified using *MboI* primer (5'-GTCAAGAATTCGGTACCGTCGAC-3') and the polymerase chain reaction (PCR) products were purified by GenCleanPCR (Generay) to remove the extra dNTP and adaptors. The purified products (4.5 µl) was ligated to the pMD19-T (0.5 µl) and then transformed into *Escherichia coli*. The transformants were selected on ampicillin plates.

A total of 576 colonies were chosen to mix with 60 ul LB liquid medium, and then shake cultured for 3 h (37°C, 140 prm). DNA

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Table 1. Basic genetic information of 12 microsatellite markers in *Haliotis ovina.*

Locus ID	Primer sequence (5'-3')	Na	Repeat motif	Allele size (bp)	PIC	Ho	He	Ta (°C)	Genbank accession no.
YB4	F: ACACGAACCAAGATTAGAGG R: TGAGAGAGGAGAACAAGGAA	3	$(TC)_5(CA)_3N_2(CA)_{20}$	190-225	0.2763	0.0741	0.1426	49	JN561131
YB14	F: TGGTCGCTGGAGAATCGT R: TGCCGTGACACTGGAAAG	5	$(CTCA)_3(CA)_2(CTCA)$	180-250	0.5395	0.5517	0.5638	45	JN561132
YB15	F: GACGACACCGATAGGAGA R: AAGAGGGACAGAGGCTTG	5	$(CA)_{26}CG(CT)_{20}$	180-210	0.4013	0.1852	0.3026	40	JN561133
YB22	F: GGAACTTCAACATCCCCT R: TTCAAACTTAGAACCCGC	2	$(GA)_{18}$	275-285	0.5169	0.0769	0.5077	46	JN561134
YB23	F: ATTTCCCCGAGTACACCATACG R: TAGGACTTCAGATTGACGAGCG	3	$(GA)_9N_2(GA)_{19}$	235-285	0.3361	0.1154	0.1802	55	JN561135
YB48	F: ACTGTGTCTGAGTGGGGTATT R: AAGTTTTTTTGTGAGTGAGCA	4	$(CA)_{22}$	160-175	0.6184	0.7778	0.6101	44	JN561136
YB55	F: TTGCCTATGTCAGCACAGTTC R: AAGCAATCAACCAATCACCTG	5	$(GT)_{17}$	185-210	0.5360	0.5357	0.5143	46	JN561137
YB68	F: TGTGCTGTGCTATAAATGTCAC R: TTGTCTTTGTATCGGAGGTTG	3	$(CA)_{18}$	200-200	0.5936	0.4706	0.5419	46	JN561138
YB70	F: TCCATTTTGTGATGACTCC R: GACGACACTTTGTTGCTCT	3	$(CA)_4N_2(CA)_{21}$	160-200	0.1228	0.0667	0.1288	45	JN561139
YB77	F: GATGTAGCAAAATGTAACCCC R: ATCCCCTCGCAAACCCAG	3	$(GA)_{47}$	250-270	0.6542	0.6000	0.6310	46.5	JN561140
YB87	F: CTGATCTCTGTGCCAGGTACTA R: GACCAAACTAACATTCTCACGC	2	$(GA)_{52}$	135-150	0.5231	0.2500	0.3577	47	JN561141
YB88	F: CAAAGTTCAGTTGATTACTGGC R: TAACATTCCTGGTATTGCGAC	2	$(CA)_3N_2(CA)_8N_2(CA)_{21}$	125-140	0.1754	0.0000	0.1317	50.5	JN561142

Na, Number of alleles; Ta, annealing temperature; H_e, expected heterozygosity; H_o, observed heterozygosity; PIC, polymorphism information content.

fragments above 500 bp were selected for sequencing in Beijing Liuhehuada Company. 40 pairs of primers were designed using Primer Premier5.0 (Clarke and Gorley, 2001). 30 individuals from Yingzhou (109°51′9″ E; 18°22′37″N), Anyou (109°33′14″ E; 18°12′11″N) and Yalong Wan (109°38′36″ E; 18°11′13″N) were then used to analyze polymorphism of the loci.

The PCR amplification conditions consisted of an initial denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, annealing temperature (Table 1) for 45 s, extension at 72°C for 1 min, and final extension at 72°C for 10 min. The PCR products were electrophoresed on Sequi-Gen Sequencing Cell (BIO-RAD) and then the observed heterozygosity (H_o), expected heterozygosity (H_e), number of alleles (N_a) and the PIC (polymorphism information content) were calculated using the software GENEPOP 4.0 and CERVUS 3.0.

RESULTS AND DISCUSSION

Twelve (12) polymorphic microsatellite primers were developed. The number of alleles per locus was from two to five and PIC was from 0.1228 to

0.6542. The observed and expected heterozygosities ranged from 0.0000 to 0.7778 and 0.1288 to 0.6310, respectively. MICRO-CHECKER (Van Oosterhout et al., 2004) was applied to check microsatellite data. No genotyping error among the loci was detected.

Seven of the 12 newly developed microsatellite markers are considered to be high polymorphism (PIC>0.5) and could be useful in the further genetic studies in *H. ovina*, such as genetic diversity, pedigree analysis, construction of genetic linkage maps and marker-assisted selection breeding study. Besides, the genetic diversity index of *H. ovina* in our study was a little lower than that of *H. discus* (Zhan et al., 2008) and *Haliotis diversicolor* Reeve (Xin et al., 2008). This may be due to the overfishing of its wild resources which could result in small population size and the overexploitation of its natural environment. Also, this may relate to the samples we used to detect the polymorphism of the microsatellite loci and the microsatellite loci we chose to analyze the genetic diversity of *H. ovina*. So, in order to reveal the accurate genetic background of wild *H. ovina,* more sample areas and bigger samples size should be included in further study.

ACKNOWLEDGEMENTS

The work was supported by the National Natural Science Foundation of China (The genetic variation pattern and the conservation management unit of the endangered species, Fenneropenaeus penicillatus Alcock), Natrual Science foundation of Fujian Province (No. 2010J01213), the program of Fujian Provincial department of science and technology (No. JK2010034), the Program for New Century Excellent Talents in Fujian Province University and the Foundation for Innovative Research Team of Jimei University, China (No.2010A004).].

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Vol. 12(51), pp. 7057-7062, 18 December, 2013
DOI: 10.5897/AJB2013.13123
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Does 2,4-dichlorophenoxyacetic acid induce flowering in sweet potato?

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Accepted 27 November, 2013

Most sweet potato cultivars grown in Zimbabwe are poor in agronomic and quality traits and require improvement through breeding. However, most cultivars rarely flower yet the flowers are crucial in genetic improvements. The aim of this study was to determine the effects of different levels of 2, 4-dichlorophenoxyacetic acid (2,4-D) on sweet potato flower induction. A 3*4 factorial experiment in a randomized complete block design with three replications was used. The first factor was landrace with three different landraces and the second factor was 2,4-D with four different concentrations (0, 100, 300 and 500 ppm). The 2,4-D was applied 50 days after planting. Sweet potato landraces that were sprayed with 2,4-D showed morphological and physiological disorders that included temporal drooping, petiole epinasty, stem splitting, shoot dieback and root swelling. Extensive morphological and physiological disorders were observed on landraces that were sprayed with the high levels of 2,4-D (300 and 500 ppm). However, within 30 days, all the landraces that were sprayed with 2,4-D managed to initiate buds and set flowers while the plants that were not sprayed did not flower at all. The Friedman's tests showed no significant differences in bud and flower number among the treatment combinations used. Therefore the lowest concentration of 2,4-D (100 ppm) used in this study is probably close to the optimum concentration for flower induction in sweet potato. Although this concentration is not the actual optimum, at the moment this concentration can be used to induce flowering in sweet potato and thus allow sweet potato breeding initiatives to be launched.

Key words: Sweet potato, 2, 4-dichlorophenoxyacetic acid, flowering, seed set.

INTRODUCTION

In Zimbabwe, sweet potato consumption exceeds 3 to 5 kg per capita per annum (Mutandwa, 2008). Orange-fleshed landraces provide β-carotene, a precursor of vitamin A that is frequently lacking among children (Kapinga et al., 2010). Furthermore, sweet potato is a low input crop that performs well in marginal areas common in the drier parts of Zimbabwe (Mutandwa, 2008; Kapinga et al., 2007). Given the paramount importance of sweet potato, there is need to initiate breeding programs in the country aimed at improving sweet potato to accommodate various uses. For example, most landraces grown by farmers rarely exceed 0.5 t/ha (Mutandwa, 2008). Furthermore, most landraces are susceptible to the sweet potato virus disease (Gasura and Mukasa, 2010) and

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also lack other desirable traits that include high dry matter content and elevated levels of β-carotene and tolerance to drought (Kapinga and Carey, 2003; Mwanga et al., 2007; Gasura et al., 2008; Mutandwa, 2008; Grüneberg et al., 2009).

Sweet potato breeding in Zimbabwe is made complex by the absence of flowers or existence of scanty flowers. Under normal field conditions, some landraces do not flower at all (Huamán, 1999; Gasura et al., 2008). The production of flowers and sexual seed in sweet potato is controlled by genetic and environmental factors. Therefore, several techniques have been developed to promote sweet potato flowering and seed set. These include a short photoperiod, moderate temperature, limited water supply, trellising vines, overwintering, vine girdling, nutrition manipulation and use of growth regulators. Grafting of non-flowering sweet potato onto flowering stocks of other landraces or wild relatives of the *Ipomoea* species can induce flowering (Leopold, 1958). Although these methods of flower induction have been reported elsewhere they are yet to be tried in Zimbabwe.

The choice of a method to use in flower induction depends on its efficacy and feasibility. The advantage of a growth regulator such as 2,4- dichlorophenoxy acetic acid (2,4-D) is that it can be easily applied to a large number of landraces. At low concentration, 2,4-D stimulates flowering (Grossmann, 2007). It readily penetrate leaves, roots and stems and is rapidly transported via the symplastic and apoplastic pathways (Chinalia et al., 2007) and stimulate excessive biosynthesis of ethylene and abscisic acid (Chinalia et al., 2007; Grossmann, 2010).

Increase in endogenous ethylene and abscisic acid results in vine drooping, leaf epinasty, tissue swelling, stem cracking and leaf senescence. These physiological disorders results in the cascading of various signals that further switch on a series of genes involved in floral organs development and flowering (Tan and Swain, 2006; Ausín et al., 2005; Lohmann and Weigel, 2002). The main environmental factors influencing flowering in sweet potato are day length and temperature. Sweet potato is a short day plant and flowering is induced by using photoperiods of 8-11.5 h of intense light (Huamán, 1999). Flowering and fruit set are highest with temperatures of 20-25°C and a relative humidity of over 75% (Huamán, 1999).

Evaluation of the efficacy of 2,4-D in floral induction will assist in the initiation of sweet potato breeding program through artificial hybridization. Furthermore, this will enable genetic diversity assessment based on floral traits. Therefore floral induction will facilitate the development of landraces with superior agronomic traits thus enabling sweet potato to become an important food security crop in the country. The objectives of this study were to assess the effect of 2,4-D on sweet potato flower induction, and to determine the optimum level of 2,4-D that can effectively induce flowering in sweet potato.

MATERIALS AND METHODS

Trial site description

The experiment was carried out in the fields at the Department of Crop Science, University of Zimbabwe (17.78° S, 31.05°E). The site is in Natural Region IIa with an altitude of 1 400 m above sea level. The mean annual rainfall ranges from 800-1000 mm. The mean annual temperature ranges from 15-27°C. The soil type is predominantly red clay containing 1 and 30% organic matter and clay, respectively.

Sweet potato establishment and management

Three sweet potato landraces previously collected by the Department of Crop Science, University of Zimbabwe were planted using a 3*4 factorial experiment in a randomized complete block design with three replications. Each plot consisted of two mounds of soil with an inter-row spacing of 1.5 m and an in-row spacing of 1.0 m. Two sweet potato plants were planted at each mound. Compound D fertilizer ($7N:14P_2O_5:7K_2O$) was applied as a basal dressing at a rate of 300 kg / ha. Weeding was done by hand hoeing. Top dressing using ammonium nitrate at a rate of 60 kg / ha was applied six weeks after planting. Vines were put onto 2.0 m bamboo tripod stakes. Four levels (0, 100, 300 and 500 ppm) of 2,4-D ($C_6H_6Cl_2O_3$, molecular weight 221.04) were applied with a hand sprayer 50 days after planting. A plastic curtain was used to prevent drift of 2,4-D. The experimental site was rain-fed and irrigation water was applied to supplement the rains when necessary.

Data collection and analyses

The changes in the sweet potato plant morphology and physiology that include vine drooping, petiole epinasty, stem splitting, shoot dieback and root swelling were noted. The number of days from spraying to bud and flower formation were recorded. The numbers of buds and flowers produced per plot were counted on a daily basis and the cumulative bud and flower counts were obtained over 30 days. The bud and flower numbers were subjected to Friedman's test using Genstat software version 14 (Genstat, 2010).

RESULTS AND DISCUSSION

After spraying 2,4-D to sweet potato, various morphological and physiological changes occurred on health plants (Figure 1A) that included temporal stem drooping (Figure 1B) that recovered within 24 h, petiole epinasty (Figure 1C), root swelling (Figure 2A) and stem splitting and shoot dieback (Figure 2B). These morphological and physiological disorders were extensive in plots that received high doses of 2,4-D (300 and 500 ppm) compared to the plots that received a lower dose (100 ppm). No morphological and physiological changes were observed on non-sprayed (0 ppm) plants.

Sweet potato plants that were not sprayed with 2,4-D (0 ppm) neither initiated buds nor flowered. Buds and normal flowers were observed on all landraces that were sprayed with 100, 300 and 500 ppm of 2,4-D. However, only a few plots had plants with flowers and could not allow analyses of variance for the days to bud and flower

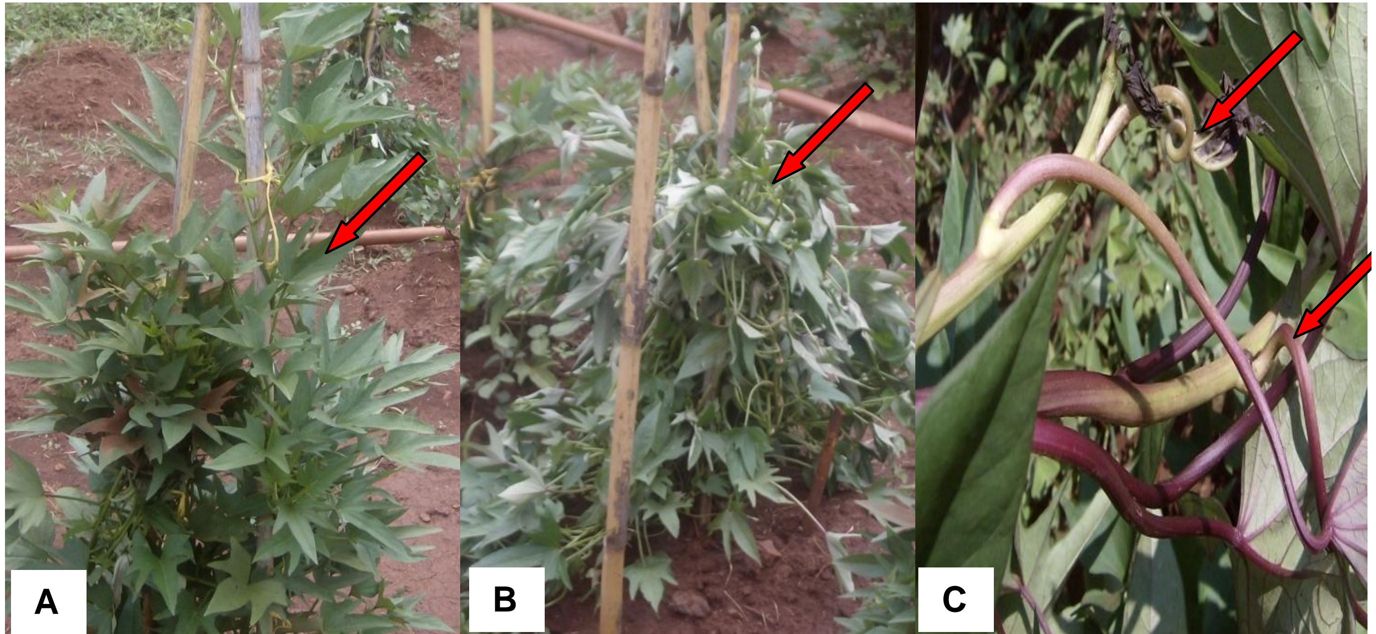

Figure 1. Healthy sweet potato (A), drooping vines following spraying with 2,4-D (B) and petiole epinasty (C).

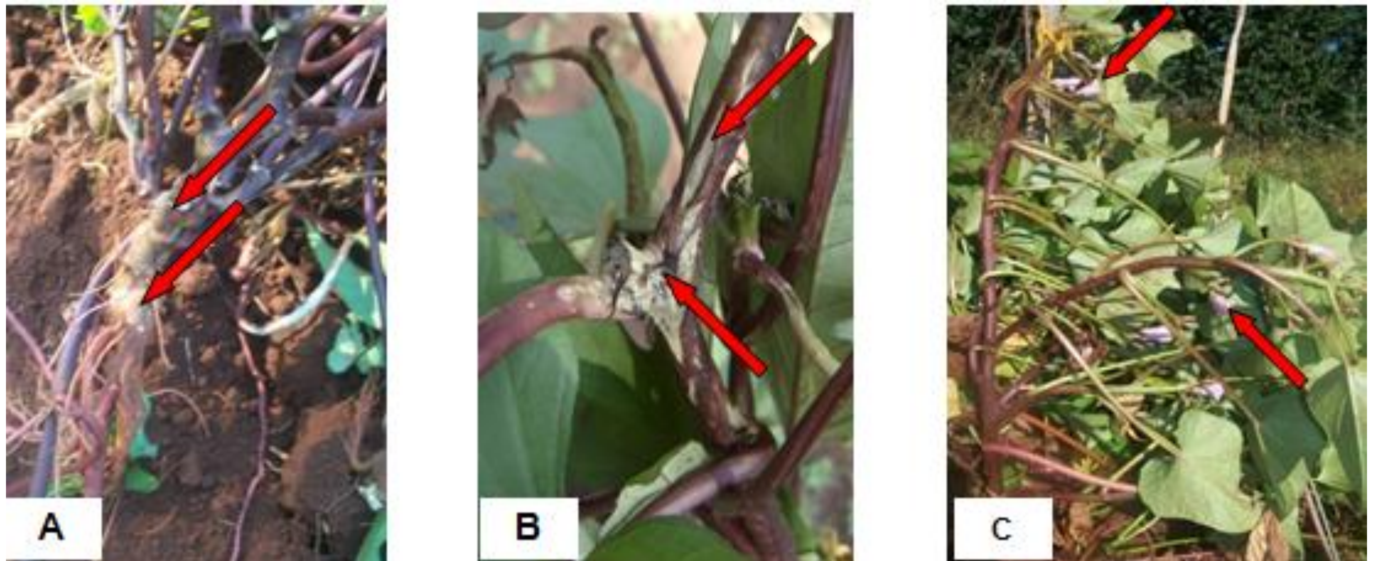

Figure 2. Sweet potato showing root swelling (A), stem splitting and shoot die back (B) and flowers (C).

initiation to be done. Nonetheless, sweet potato landrace 3 was the first to come into flower followed by landrace 1 (eight days later) and finally landrace 2 (14 days later). A total of six out of 12 treatment combinations (landrace * 2,4-D level) showed the presence of buds and flowers. These six treatment combinations included all the landraces and the three levels where 2,4-D was applied (100, 300 and 500 ppm). The six treatment combinations allowed the number of buds and flowers to be subjected to Friedman's non-parametric test. The Friedman's test of the six treatment combinations showed no significance difference in bud numbers (Figure 3) and flower numbers (Figure 4). However, landrace 3 with either 100 or 300 ppm had a relatively high number of buds and flowers although it was not significantly different from the rest.

The results demonstrate that 2,4-D is effective in inducing flowering in all three sweet potato landraces studied. This proved that flower induction is controlled by both

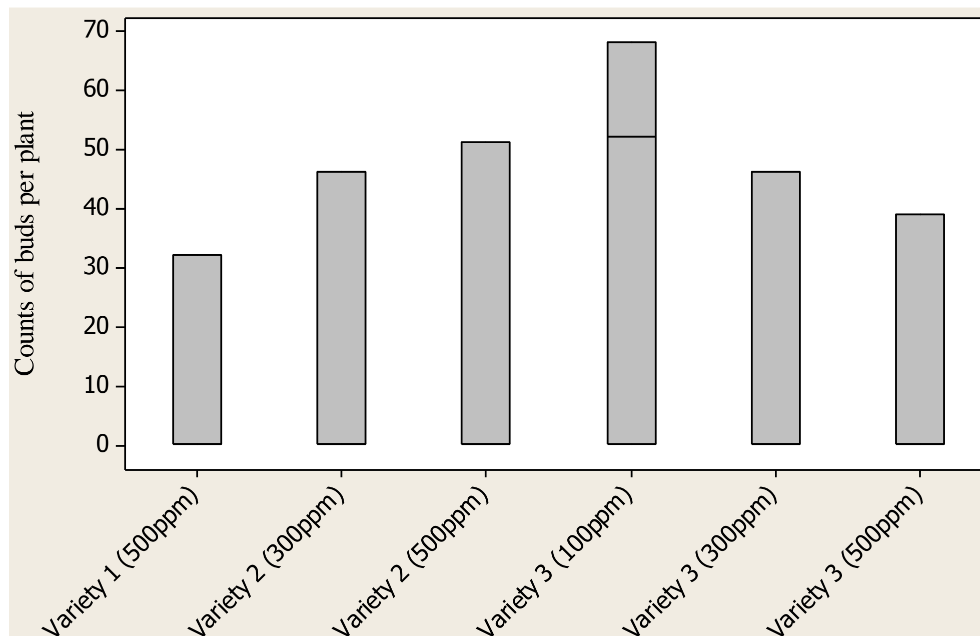

Figure 3. Box plot showing effects of variety and 2,4-D combinations on bud number of three sweet potato varieties (P=0.708).

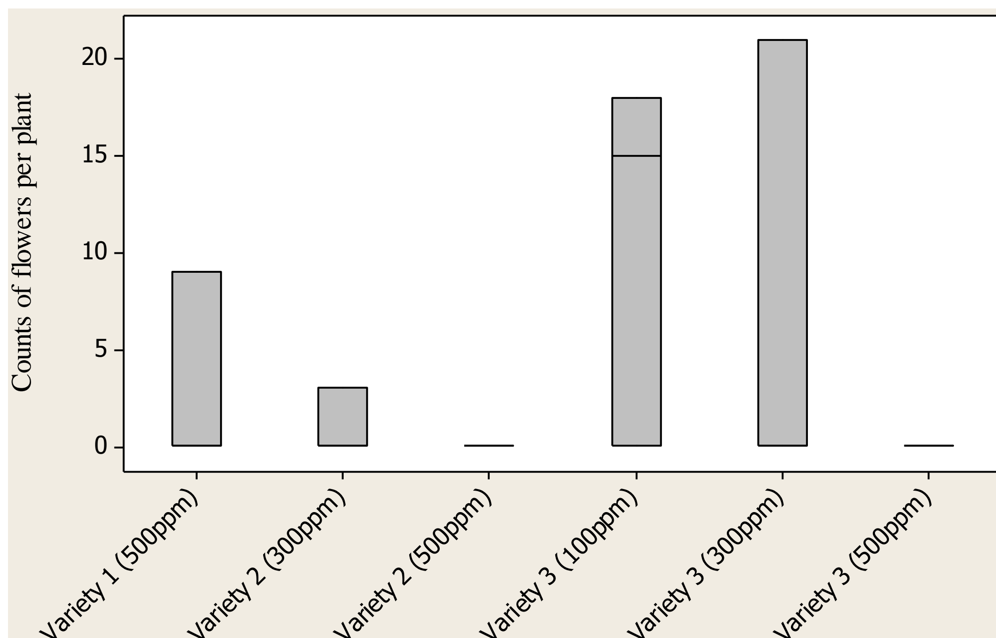

Figure 4. Box plot showing effects variety and 2,4-D combinations on flower number of three sweet potato varieties (P= 0.223).

external and internal factors (Ausín et al., 2005). The occurrence of non-significant differences among the treatments with various combinations of 2,4-D could reflect that the concentrations used went beyond the optimum, thus resulting in the absence of a clear pattern. Level two (100 ppm) is close to the optimum level of flower induction as it permits a lower 2,4-D concentration to be used while producing the number of buds and flowers that is comparable to the higher concentrations used. Thus, use of 100 ppm is not only cheap but also reduces

the extent of foliar damage as was observed at higher concentrations of 2,4-D (300 and 500 ppm). Further studies must attempt to investigate the effects of 2,4-D concentrations that are lower than 100 ppm. The morphological and physiological disorders observed after the application of 2,4-D were typical of the ethylene and abscisic acid signaling pathways as previously reported by Grossmann (2010). Ethylene is involved in plant responses to stress and the regulation of senescence. Ethylene results in re-orientation of microtubules from a transverse to a more longitudinal orientation which leads to lateral cell expansion. This expansion was observed as cracking of stems, root swelling and petiole epinasty (Grossmann, 2007). Stress induced by these abnormal hormone levels acts as a stimulant of transcriptional factors to induce flowering (Wada and Takeno, 2010). Therefore morphological and physiological disorders observed following 2,4-D sprays should be noted as a key step towards the floral induction rather than as an undesirable symptom.

The 2,4-D managed to induce flowering especially when applied at low concentration (100 ppm). At low concentration 2,4-D had a stimulatory effect on plant growth and development. Effectiveness of 2,4-D as method of flower induction in sweet potato was first reported by Howell and Wittwer (1954) and later in combination with grafting (Lardizabal and Thompson, 1990). Howell and Wittwer (1954) recorded relatively high flowering in all 2,4-D treated sweet potato plants as observed in our study. However, in their studies, Howell and Wittwer (1954) reported 500 ppm as the optimum concentration that effectively induced flower in sweet potato. However, our findings showed 100 ppm to be close to the optimum concentration. The difference in the optimum concentration observed by Howell and Wittwer (1954) and in our study could be attributed to the plot sizes and sweet potato cultivars used.

Our study used two mounds as the plot with two plants per each mound and thus the dosage applied for each plot was adequate to those few plants. Use of two plants per mound is typical of the require-ments of the nursery design to be used in breeding. Furthermore, the use of the plastic curtains also minimized drift and ensured that each application rate used was adequate for each plot. Differences in concen-trations could also depend on the foliage size of each landrace, with the landraces with huge foliage biomass requiring more dosage than those with small foliage.

The development of flower induction methods that is based on 2,4-D is a key step towards establishing a sweet potato crop improvement program. An effective method of flower induction developed in this study through use of 2,4-D sprays will enable sweet potato hybridization to take place. Controlled pollination ensures specific gene combinations to be made. Controlled sweet potato hybridization will allow the improvement of root yield, dry matter content and vitamin A content as well as tolerance to pests, diseases and drought. This will eventually lead to increased sweet potato production per unit area in the country.

Conclusions

The results show that 2,4-D can effectively induce sweet potato flowering when foliar applied at 100 ppm at 50 days after planting. Further studies must determine the actual optimum concentration of 2,4-D that can induce flowering and investigate the effects of different landraces, temperature, day length, ratoons and split application of 2,4-D on sweet potato flowering.

ACKNOWLEDGEMENTS

We are grateful to the support from the Department of Crop Science, University of Zimbabwe.

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African Journal of Biotechnology Vol. 12(51), pp. 7063-7069, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2696
ISSN 1684-5315 ©2013 Academic Journals

Full Length Research Paper

Plant regeneration through indirect organogenesis of chestnut (*Castanea sativa* Mill.)

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Accepted 15 November, 2011

To establish an effective protocol for plant regeneration through indirect organogenesis, effects of explants type, culture media and plant growth regulators on callus induction and shoot regeneration of chestnut (Castanea sativa Mill.) were investigated. Three different explants (root, nodal and internodal segment), two different media [Murashige and Skoog medium (MS) and Gamborg's B5 (B5)] and different plant growth regulators (6-benzylaminopurine (BA), thidiazuron (TDZ), indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA)) with different concentration (0.2, 0.5, 1 and 1.5 mgL^{-1}) for shoot and root induction were chosen. The results show that nodal segment was the best explant for callus induction (69.4%) when cultured on MS medium supplemented with 1 mgL^{-1} TDZ and MS was the best medium to induce callus formation (74.6%). The highest shoot multiplication (66.9%) was observed on MS medium with 0.2 mgL^{-1} TDZ. Regenerated shoots were rooted in vitro on MS containing 1.5 mgL^{-1} IBA. Also, plantlets with well developed root and shoot systems were acclimatized inside the green house and 80% of the plantlets survived on transfer to garden soil. This protocol provides a basis for future studies on genetic improvement.

Key words: Chestnut, node, internode segment, indirect organogenesis, callus formation, shoot regeneration.

INTRODUCTION

For a long time chestnut has been an important economic resource in Europe and more recently in Asia, also playing an important environmental role in many agroforestry systems (Bounous, 2005). Chestnut is a hardwood forest species of considerable agro-economic important tree species for both timber and nut production. However, this tree species is threatened by pollution, social and economic changes, and two major fungal diseases; ink disease (*Phytophthora* sp.) and chestnut blight [*Cryphonectria parasitica* (Murr.) Barr.] (Sauer and Wilhelm, 2005). Chestnut is a woody species, which is difficult to propagate either generatively by seed or vegetatively by grafting or cuttings (Osterc et al., 2005). However, as an alternative to conventional vegetative propagation methods, efforts have been made to establish reliable *in vitro* regeneration systems that allow clonal propagation (Vietez and Merkle, 2005; Troch et al., 2010). *In vitro* tissue culture techniques have been applied to chestnut regeneration since the 1980's (Rodriguez, 1982; Vieitez et al., 1983). Also *in vitro* establishment in chestnut is possible from both juvenile and mature material (Sánchez et al., 1997) and explants such as cotyledonary node (San-José et al., 2001), bud (Vieitez and Vieitez, 1980), nodal segment (Osterc et al., 2005), etc have been utilized for *in vitro* propagation of chestnut.

Production of regenerated plant through indirect organogenesis is one of the possible ways to contribute to genetic improvement because there are some advantages

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Abbreviations: BA, 6-Benzylaminopurine; **IAA,** indole-3-acetic acid; **TDZ,** thidiazuron; **IBA,** indole-3-butyric acid; **MS,** Murashige and Skoog medium; **B_5,** Gamborg's **B_5.**

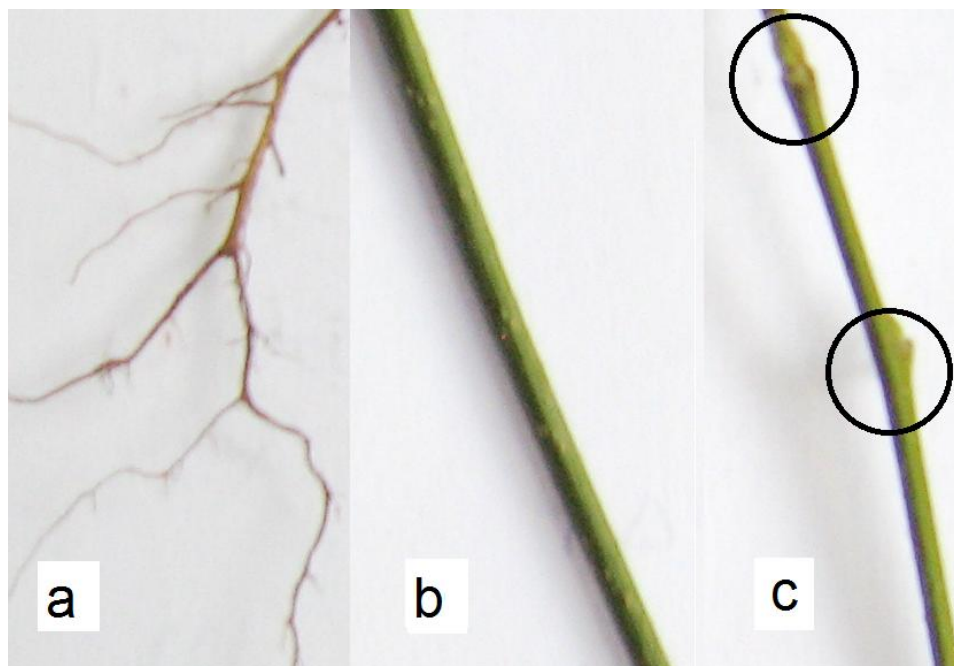

Figure 1. The explants used for experiments (a) root (b) internodal segments and (c) nodal segments.

of shoot regeneration from callus over direct shoot regeneration (Avilés et al., 2009). A callus phase is commonly included in tissue culture protocols with the objectives of generating variability to introduce new desirable traits and generating transgenic plants to introduce traits (Fereol et al., 2005; Zheng et al., 2005). Callogenic initiation implies an initial stage of differentiation from the parental tissue. Thus, to establish callus cultures, the determination of the initial tissue used is a fundamental factor in order to achieve the desired response (Bandyopadhyay et al., 1999). Earlier, zygotic embryos (Kvaalen et al., 2005), hypocotyls (Ahn et al., 2007), ovaries and ovules (Sauer and Wilhelm, 2005), internodes (Chandra and Bhanja, 2002), nodal segments (Arora et al., 2010) and cotyledonary node (Dhabhai and Batra, 2010) have been used in induction of callogenesis and indirect organogenesis.

The aim of this study was to investigate the effects of explant type, culture media and plant growth regulators on callus induction and shoot regeneration from chestnut, and also establish an efficient tissue culture protocol that would provide an efficient tool to be used in chestnut breeding programs.

MATERIALS AND METHODS

Plant materials and culture conditions

Seeds of *Castanea sativa* Mill. were collected from Visroud in Gilan, North of Iran. The area was about 350 ha with 200 to 600 m altitude. The seedlings were grown in a glasshouse under natural illumination (Hou et al., 2010). Root, nodal and internodal segments were removed from three months old seedlings as explants. The excised root, nodal and internodal segments were surface sterilized in 70% ethanol for 1 min followed by 4% sodium hypochlorite for 15 min and rinsed thoroughly three times for 3 min each in sterile distilled water. They were then placed individually (horizontal) in culture vessels (200 × 10 mm) containing 5 ml medium. The Murashige and Skoog, 1962 (MS) and Gamborg et al., 1968 (B_5) media were adjusted to pH 5.8 and 5.5 with 1 N NaOH or HCl, respectively, before autoclaving at 1.06 $kgcm^2$ pressure and 121°C temperature for 20 min. All cultures were incubated at 25 ± 2°C with 16/8 h photoperiod under light from cool white fluorescent lamps (125 µmol $m^{-2}s^{-1}$) and 60 to 70% relative humidity.

Effect of explants type, culture media and plant growth regulators on callus proliferation and shoot regeneration

Root, nodal and internodal segments excised from three-month-old seedlings (Figure 1) were placed on MS medium supplemented with 1 mgL^{-1} thidiazuron (TDZ) to evaluate the effects of different explants on callus proliferation of chestnut as the first experiment. According to the results, nodal segments were the best explants to generate callus. Therefore, the next experiments were done using nodes as initial explants.

For the second experiment, the effects of MS and B_5 basal media supplemented with 1 mgL^{-1} TDZ on callus proliferation from nodal segments were evaluated. According to the results, MS medium was better than B_5 medium to induce callus formation from nodes. Therefore, the next experiments were performed using nodal segments cultured in MS medium.

The objective of the third experiment was to investigate the effects of growth regulators on callus proliferation and shoot regeneration from chestnut. We used MS medium containing different concentration of TDZ and 6-benzylaminopurine (BA) (0.2, 0.5, 1 and 1.5 mgL^{-1}). Data collected included percentage of explants

Table 1. Effects of different explants types on callus proliferation from *Castanea sativa* Mill.

Explant	Explant forming callus (%)
Node	69.4 ± 0.40[a]
Internodal segment	39.1 ± 2.64[b]
Roots	14.2 ± 1.36[c]

Data was collected after eight weeks of culture. Explants were cultured on MS medium supplemented with 1 mgL⁻¹ TDZ. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to Student-Newman-Keuls multiple comparison test (P<0.05). TDZ, Thidiazuron; MS, Murashige and Skoog medium.

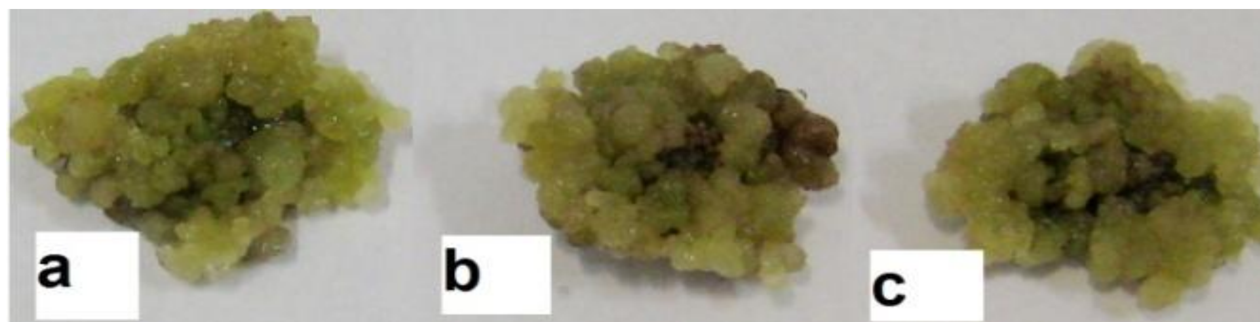

Figure 2. Plant regeneration from callus derived from *in vitro* cultured nodal and internodal segment of *Castanea sativa* Mill. (a) Callus formation from node on MS medium with 1 mgL⁻¹ TDZ after four weeks of culture. (b) Callus formation from root segments on MS medium with 1 mgL⁻¹ TDZ after four weeks of culture. (c) Callus formation from internodal stem on MS medium with 1 mgL⁻¹ TDZ after four weeks of culture. TDZ, Thidiazuron; MS, Murashige and Skoog medium.

forming callus, shoot proliferation percent, mean number and length of shoots after eight weeks of culture. Explants were subcultured onto the MS medium with same BA and TDZ concentration at two weeks for further callus and/or shoot development.

Root induction

When shoots developed from initial explants and became 20 to 30 mm in length about eight weeks after culture, they were excised and transferred to the rooting medium. The rooting medium consisted of MS medium supplemented with different concentration of IBA and indole-3-acetic acid (IAA) (0.2, 0.5, 1 and 1.5 mgL⁻¹). The percentage frequency of root formation and its length was calculated after four weeks of culture. The rooted plantlets (four to five weeks old) were then taken out from the culture vessels, washed thoroughly in running tap water to remove any remains of the nutrient-agar medium. Therefore, they were planted into pots (10 cm in diameter), containing a mixture of sterile vermiculite and sand (1:1) and maintained in the growth chamber at a temperature of 25 to 28°C, 16/8 h photoperiod and relative humidity of 80 to 90% covered with plastic bags. Once established in soil, the plants were transferred to the greenhouse at a temperature of 25 to 28°C and 16/8 h photoperiod.

Statistical analysis

The experimental unit was culture vessels. This treatment had five replications with 10 explants plated for each replication. For the comparison between MS and B_5 media, we used T-test. A completely random design was used for the data analysis. Results were analyzed statistically using the statistical analysis system program (SAS, 2001). The mean values were calculated and compared by Student-Newman-Keuls multiple comparison test (P<0.05).

RESULTS AND DISCUSSION

Effect of explants type, culture media and plant growth regulators on callus proliferation and shoot regeneration

In *C. sativa* Mill., callus formation varied significantly depending on explant type (Table 1). Nodal segments showed the earliest signs of callus formation from the cut edges on MS medium supplemented with 1 mgL⁻¹ TDZ after one week of culture, but root segments and internodal segments started to initiate callus from cut surfaces after two weeks of culture. Figure 2 indicate the callus of different explants after four weeks. The nodal segments showed higher callus formation, while roots and internodal segments exhibited a significantly lower callus induction. The highest callus induction (74.6%) was achieved when MS medium was used in comparison with 69.1% on B_5 medium (Table 2).

Table 2. Effects of different culture media on callus proliferation from node of *Castanea. sativa* Mill.

Medium	Explant forming callus (%)
MS	74.6 ± 1.67^{a}
B_5	68.1 ± 1.12^{b}

Data was collected after eight weeks of culture. Different media were supplemented with 1 mgL⁻¹ TDZ. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to T-test (P<0.05). TDZ, Thidiazuron; MS, Murashige and Skoog medium; B_5, Gamborg's B_5.

Table 3. Effects of different growth regulators on callus proliferation and shoot regeneration from nodal segments of chestnut (*Castanea sativa* Mill.)

Growth regulator (mgL^{-1})		Explant forming callus (%)	Explant regeneration shoot (%)	Mean shoot length (cm)
BA	TDZ			
0.2	0	64.3 ± 0.28^{f}	24.7 ± 2.24^{e}	0.5 ± 1.42^{e}
0.5	0	67.6 ± 0.16^{ef}	31.8 ± 2.73^{d}	0.6 ± 1.94^{e}
1	0	72.3 ± 0.43^{c}	32.6 ± 2.0^{e}	0.6 ± 2.13^{e}
1.5	0	76.9 ± 0.67^{b}	51.5 ± 2.91^{b}	1.6 ± 1.25^{c}
0	0.2	85.3 ± 0.68^{a}	66.9 ± 2.13^{a}	2.2 ± 1.44^{a}
0	0.5	71.2 ± 0.22^{d}	53.3 ± 2.66^{b}	1.9 ± 1.67^{b}
0	1	79.4 ± 0.40^{e}	41.4 ± 2.84^{c}	1.4 ± 1.25^{d}
0	1.5	60.8 ± 0.29^{e}	39.1 ± 2.0^{d}	0.9 ± 2.85^{e}

Data was collected after eight weeks of culture. Nodal segments were cultured on MS basal medium. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to Student-Newman-Keuls test (P<0.05). BA, 6-benzylaminopurine; TDZ, thidiazuron; MS, Murashige and Skoog medium.

The variations of callus induction on different media may be due to the differences of nitrate/ammonium (NO_3^-/NH_4^+) ratio, an important factor on nitrogen uptake and pH regulation during plant tissue culture (Fracago and Echeverrigaray, 2001). Vieitez et al. (1983) compared several media to obtain the cluster propagation ability in different chestnut clones from the same hybrid group *Castanea sativa* × *Castanea crenata*. The highest number of shoots per culture formed was achieved when the explants were grown on Heller's medium; explants grown on Heller's+$(NH_4)_2SO_4$ medium (Heller, 1953) and on MS medium with the addition of ammonium nitrate ($NH_4NO_3^-$) were slightly worse. However, Ballester et al. (2001) indicated that it is very difficult to recommend a mineral medium for general application; nevertheless, half strength MS media appeared to be the most suitable for multiplication through axillary shoot development. Therefore, it is concluded that the best basal medium for the callus induction was MS, although successful callus formation has been achieved on B_5 medium (Table 2). An increase in BA concentration from 0.2 to 1.5 mgL^{-1} increased callus induction from 64.3 to 76.9. The highest frequency of callus formation (85.3%) was obtained on MS medium containing 0.2 mgL^{-1} TDZ.

Shoot regeneration

Calli formed numerous shoots when they were cultured on the same medium after three to four weeks of culture (Table 3). Cytokinin type and concentration also affected the frequency of shoot induction (Maheshwari and Kovalchuk, 2011). In the present experiment, BA could induce shoot regeneration from nodal segments at the percent of 76.9% when cultured on MS medium with 1.5 mgL^{-1} BA. Increasing BA from 0.2 to 1.5 mgL^{-1} resulted in an increase in shoot regeneration ability in callus (Table 3). Vieitez and Vieitez (1980) reported that 6-benzylaminopurine (BAP) showed the most satisfactory effect on promoting the proliferation of axillary shoots, whereas zeatin slightly inhibited the development of axillary shoots but increased the induction rate and caused more vigorous shoots. Tetsumura and Yamashita (2004) achieved similar results with the addition of zeatin also causing the highest proliferation rate. In addition, Arora et al. (2010) found that it is possible to obtain shoot regeneration through indirect organogenesis from nodal segment of *Azadirachta indica* A. Juss. on MS medium supplemented with 0.2 mgL^{-1} BA. Girijashankar (2011) achieved shoot regeneration of *Acacia auriculiformis* from

Table 4. Effect of different concentrations of IBA and IAA on root induction in shoots of chestnut (*Castanea sativa* Mill.)

Growth regulators (mgL⁻¹)		Rooting (%)	Mean root length (cm)
IBA	IAA		
0.2	0	51.2 ± 1.54^{d}	0.7 ± 1.43^{e}
0.5	0	52.3 ± 1.94^{d}	1.7 ± 2.25^{c}
1	0	68.3 ± 1.11^{b}	1.6 ± 2.17^{c}
1.5	0	71.2 ± 1.88^{a}	2.1 ± 0.73^{a}
0	0.2	41.5 ± 2.27^{e}	1.0 ± 1.14^{de}
0	0.5	40.2 ± 1.01^{e}	1.4 ± 1.20^{d}
0	1	51.1 ± 1.32^{d}	1.7 ± 0.94^{c}
0	1.5	64.2 ± 1.99^{c}	2.0 ± 1.61^{b}

Data was collected after four weeks of culture. Nodal segments were cultured on MS basal medium. Values represent the mean ± S.E. Means following the same letter within columns are not significantly different according to Student-Newman-Keuls test ($P<0.05$). IAA, Indole-3-acetic acid; IBA, indole-3-butyric acid; MS, Murashige and Skoog medium.

nodal stem segments on MS containing BA (2 mgL^{-1}) + naphthaleneacetic acid (NAA) (1 mgL^{-1}). Also single nodal segments of *Castanea mollissima* cv. 'yanshanhong' induced shoot on MS medium with a half concentration of NO_3 supplemented with 0.5 mgL^{-1} BA (Hou et al*.,* 2010). The result of present study shows that TDZ induced more shoot regeneration. The highest shoot regeneration (66.9%) was obtained on MS medium supplemented with 0.2 mgL^{-1} TDZ, while further addition of TDZ concentration resulted in decreased shoot regeneration. The lowest percentage of shoot induction was observed on MS medium with 1 mgL^{-1} BA.

TDZ has been shown to be the most effective in inducing shoot regeneration in woody species and has shown to have a much stronger ability than BA on shoot induction (Huetteman and Preece, 1993). It is a synthetic polyurea which being a cytokinin-like substance, is highly effective for shoot regeneration in tissue culture of recalcitrant plant species (Liu et al., 2003). However, if concentrations of TDZ are high, hyperhydricity or morphological abnormalities could be observed among regenerated shoots (Huetteman and Preece, 1993). Lower concentrations of TDZ are thus preferable for shoot regeneration (Wang et al., 2008). In the current study, the maximum length of shoots was observed from nodal segments in MS supplemented with 0.2 mgL^{-1} TDZ (2.2 cm) (Table 3). The cytokinins, because of their role in experimentally induced cell division and differentiation, serve as a probe of hormonal involvement in differentiation (Hall, 1976). This study demonstrate the superiority of TDZ over BA as shoot-inducing cytokinin in the *in vitro* induction of adventitious shoots from nodal segments of chestnut. Ballester et al. (2001) also used 0/1 mgL^{-1} TDZ in MS medium to germinate chestnut embryonic axes.

Root induction from shoots

Rooting of the regenerated shoots did not occur on the shoot induction medium. Elongated shoots (20 to 30 mm in length) regenerated on MS medium were used for rooting experiment. Auxins had a significant influence on root formation. Depending on auxin type and concentration, roots were initiated between five to 15 days of culture. The best root formation and maximum mean length (2.1 cm) was observed during regeneration of chestnut on MS basal medium supplemented with 1.5 mgL^{-1} IBA (the root regeneration percent was 71.2%) (Table 4). IAA at 1.5 mgL^{-1} also induced 64.2% rooting in regenerated shoots, but the number of roots per shoot was considerably lower. The roots were regenerated within two weeks after transferring onto these media. The roots regenerated on the medium supplemented with 1.5 mgL^{-1} IBA were longer than others (Table 4).

Among the auxins, IAA and IBA are the most frequently applied chemicals for rooting (Harry and Thrope, 1994). Our results also show that IBA was the best one to be used for root formation. Similar results about response to IBA are observed in *Prosopis ceneria* for root induction (Kumar and Singh, 2009) and also *Azadirachta indica* A. Juss (Chaturvedi et al., 2004). Figure 3 shows the whole plantlet from nodal segment on MS with 0.2 mgL^{-1} TDZ and then 1.5 mgL^{-1} IBA. *In vitro* raised plantlets with well developed shoots and roots were transferred to pots containing sterile soilrite and acclimated, after which the successfully acclimated plants (80%) were transferred to pots under full sun where they grew well without any detectable phenotypic variation. Ahn et al. (2007) indicated that for plant regeneration from hypocotyls of *Ricinus communis* L., TDZ induced adventitious shoots at a higher rate compared to shoots induced by BA and also IBA was more efficient in root growth and shoot development than NAA.

Conclusion

The limitation of this study lies on the experimental materials, which were from two to three months old

Figure 3 Plantlet from nodal segment via callus on MS with 0.2 mgl⁻¹ TDZ and then 1.5 mgl⁻¹ IBA.

seedlings instead of mature trees with good characters. Seedling materials cannot completely represent the mature ones because of the difference of explants condition (Hou et al., 2010). However, for obtaining the physiology mechanism of adventitious root formation of *C. sativa Mill.* in a comparative short time, this experiment should be the guide of further research. We were successful in plant regeneration in *C. sativa* Mill. using MS medium supplemented with 0.2 mgL^{-1} concentration of TDZ and 1.5 mgL^{-1} IBA. This regeneration protocol will be useful not only for further research studies such as genetic cell transformation or protoplast fusion studies, but also for commercial nurseries that could use virus-free plants and agricultural practices to reduce pesticide use and increase yield production.

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African Journal of Biotechnology Vol. 12(51), pp. 7070-7073, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1199
ISSN 1684-5315 ©2013 Academic Journals

Full Length Research Paper

Protocol optimization for deoxyribonucleic acid (DNA) extraction from dried, fresh leaves, and seeds of groundnut (*Arachis hypogaea* L.)

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Accepted 3 August, 2012

Consistent isolation of best quality deoxyribonucleic acid (DNA) from peanut (*Arachis hypogaea* L.) is particularly problematic due to the presence of phenolic compounds and polysaccharides. Inconsistencies in extraction results can be attributed to the age and growth stages of the plant material analyzed. Mature leaves have higher quantities of polyphenols, tannins and polysaccharides that can contaminate DNA during isolation. In this study, we used fresh and dried leaves as well as seeds for optimization of high quality DNA isolation protocols from *A. hypogaea*. The DNA extracted with three different methods cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), and cesium chloride (CsCl) density gradient) were comparatively studied by polymerase chain reaction (PCR) analysis in terms of quantity and quality. High quality genomic DNA was obtained from fresh leaves by modified CTAB methods. The DNA obtained ranged from 1 to 2.5 ng/µl. DNA obtained by this method was strong and reliable showing its compatibility for simple sequence repeat (SSR) analyses. The SDS based methodology give large quantities of DNA contaminated with polysaccharides. Fresh leaves also gave best result in SDS method. The quantity and quality of DNA obtained was very poor in all the tested methods in case of dried leaf tissues. The current protocol will probably be useful for the extraction of high-molecular weight DNA from other plant materials containing large amounts of secondary metabolites and essential oils.

Key words: Polysaccharides, polyphenols, tannins, cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), cesium chloride (CsCl), secondary metabolites, SSR.

INTRODUCTION

Peanut is an important oil seed crops cultivated mainly in subtropical, tropical, and temperate regions globally (Proite et al., 2007). It is grown in more than 100 countries across America, Africa, and Asia on an area of about twenty five million hectares and nearly thirty five million tons were produced annually (FAO, 2007).

The development of consistent deoxyribonucleic acid (DNA) isolation protocol and polymerase chain reaction (PCR) analysis is the basic step for many biotechnological techniques, such as molecular markers and genetic engineering. Therefore, it has been used in many plant species, such as *Pinus radiate* (Claudia et al., 1998), *Arachis hypogaea* (Sharma et al., 2000), *Parkia timoriana*

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Abbreviations: DNA, Deoxyribonucleic acid; **CTAB,** cetyltrimethylammonium bromide; **SDS,** sodium dodecyl sulfate; **SSR,** simple sequence repeat.

(Robert et al., 2003), *Mucuna pruriens* (Padmesh et al., 2006), and chickpea (Dipankar et al., 2006). In general, it is difficult to extract and purify high-quality DNA from certain plants, because of the presence of large quantities of secondary metabolites (tannins, alkaloids, and polyphenols), polysaccharides and pro-teins. These compounds interferes DNA, thus degrading its quality and reducing yields (Katterman and Shattuck, 1983; Sarwat et al., 2006).

Consistent isolation of best quality DNA from peanut (*A. hypogaea* L.) is problematic, particularly, due to the presence of phenolic compounds and polysaccharides. Inconsistencies in extraction results can be attributed to the age and growth stages of the plant material analyzed. Mature leaves have higher quantities of polyphenols, tannins, and polysaccharides that can contaminate DNA during isolation. In this study, we report the rapid and reliable procedure for extracting good quality and high quantity of genomic DNA for PCR and molecular analysis.

METHODOLOGY

A total of 70 *A. hypogaea* accessions were collected from different research institutes, such as National Agricultural Research Centre (NARC), Bangladesh Agricultural Research Institute (BARI), and Ayub Agricultural Research Institute (AARI), Pakistan. Isolation of DNA was carried out from seeds, fresh and dried leaves sources of these samples. Three different DNA isolation protocols were comparatively studied in order to optimize the best suited protocol for *A. hypogaea*.

Reagents and chemicals

Tris-HCl (1.0 M; pH 8.0, 9.5); 0.5 M ethylenediaminetetraacetic acid (EDTA; pH 8); 5.0 M NaCl; 3.0 M sodium acetate (pH 5.2); cetyltrimethylammonium bromide (CTAB; 20%); chloroform:isoamyl alcohol (24:1, v/v); and b-mercaptoethanol (AR grade). Modified CTAB extraction buffer: 0.1 M Tris-Cl (pH 9.5), 20 mM EDTA (pH 8), 1.4 M NaCl, CTAB (2%, w/v), and b-mercaptoethanol (1%, v/v) (added to the buffer just before use). Also with pure cold (-20°C) isopropanol, 70% ethanol, and absolute ethanol.

DNA extraction

We modified the pre-existing protocols described by Grattapaglia and Sederoff (1994) for the extraction of DNA from three different explants, that is, seeds, fresh and dried leaves of *A. hypogaea.*
One gram of leaves from each genotype were wiped clean with 70% ethanol. The leaves were grind in liquid nitrogen (LN) into fine powder with pestle and mortar. Fine powders were poured to 15 ml sterilized falcon tube containing extraction buffer and was properly mixed. The falcon tube was kept for 20 min in incubation bath at 65°C. After adding 15 ml sodium dodecyl sulfate (SDS) or (chloroform: isoamyl alcohol, 1:24) solution, it was shacked gently and was centrifuged at 8500 rpm for 30 min at room temperature. The supernatant was taken in a sterilized falcon tube and equivalent amount of isopropanol was added, after which it was kept at -20°C for 20 min, and was centrifuge at 8500 rpm at room temperature for 30 min. Then, the pellet was washed with 70% ethanol after removing the supernatant. DNA pellet was air-dried for about 1 h, and was dissolved in 1 ml ddH_2O and 1 µl RNase was added, and it was kept in the falcon tube at 37°C for 2 h. The amplified product on non-denaturing 1% agarose gel stained with ethidium bromide as described by Ferguson et al. (2004) for confirmation of DNA was visualized.

PCR analysis

DNA samples were diluted to 20 ng/µl for the microsatellite analysis. PCR analysis was carried out in Veriti 96-well thermal cycler (Applied Bio systems, CA) with Taq polymerase (MBI Fermentas). DNA sample were diluted to 20 ng/µl for the microsatellite analysis. PCR reaction were performed in 20 µl volumes containing 30 ng/µl genomic DNA, IX PCR buffer (MBI Fermentas) 1.6 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphates (dNTPs), 0.5 U DNA polymerase (MBI Fermentas), and 10 pmol of each primer.

DNA isolation by other methods

Isolation of DNA from the same fresh as well as dried leaf samples was carried out by two other methods, SDS and cesium chloride (CsCl) density gradient. The DNA isolated by the other methods was subjected to analysis by agarose gel electrophoresis and PCR analysis were conducted to compare the quality and quantity of isolated DNA with modified CTAB method.

RESULTS AND DISCUSSION

The DNA extracted with three different methods (CTAB, SDS and CsCl density gradient) were comparatively studied by PCR analysis in terms of quantity and quality. High quality genomic DNA was obtained from fresh leaves by CTAB methods (Figure 1). The extracted DNA showed a reading in between 1.7 to 1.8 after calculating the ratio of absorbance, 260/280 nm. The DNA obtained ranged from 1 to 2.5 ng/µl. DNA obtained by this method was strong and reliable showing its compatibility for simple sequence repeat (SSR) analyses.

The SDS based methodology give large quantities of DNA contaminated with polysaccharides. Fresh leaves also gave best result in SDS method. The quantity and quality of DNA obtained was very poor in all the tested methods in case of dried leaf tissues (Figure 2).

The results show that both CTAB and SDS methods gave intact DNA, while that of CsCl density gradient gave sheared DNA bands. PCR analysis of SSR markers in genomic DNA obtained by CTAB method resulted in good quality amplification as compared to the other (Figure 3). Similarly, PCR analysis with DNA obtained by SDS methods was not consistent and the staining with ethidium bromide gave opaque bands indicating a very low concentration of amplified products.

The employment of modified CTAB methods for isolation of DNA from high level polysaccharides containing tissues by Sharma et al. (2000) supports our results. This method was appropriate for dry as well as fresh tissues and was experienced on soybean, chickpea seeds and wheat. When high concentration of CTAB (3% w/v) and

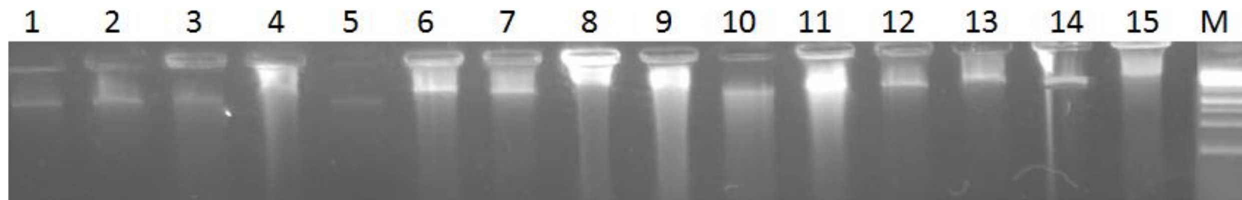

Figure 1. Ethidium bromide-stained agarose gel showing total DNA isolated CTAB method from fifteen samples of fresh leaves of (*A. hypogaea* L.). Lanes 1 to 15, Golden, Bari-2000, Banki, Chakori, Bari-89, Bard-479, 4CgOO4, 2Kcg017, 2Kcg020, Pk-90064, Icgs-09, Icgs-17, Icgs-18, Icgs-114, and Icgs-38. Lane 16, DNA ladder.

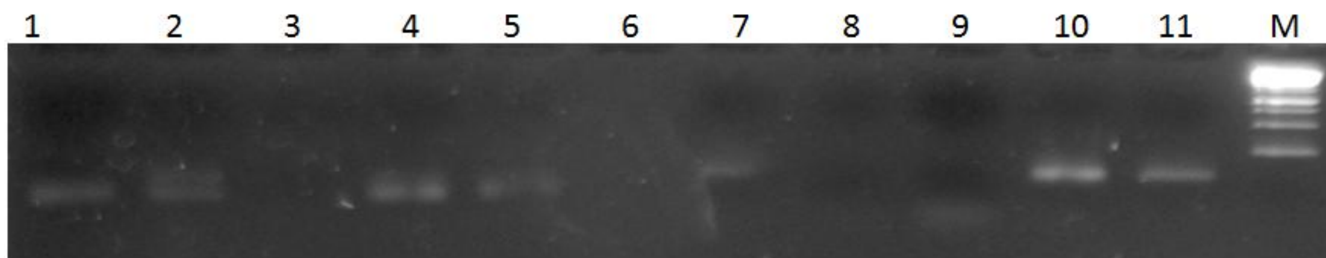

Figure 2. Ethidium bromide-stained agarose gel showing total DNA isolated by CTAB method from eleven samples of seeds powder of (*A. hypogaea* L.). Lanes 1 to 11, Golden, Bari-2000, Banki, Chakori, Bari-89, Bard-479, 4CgOO4, 2Kcg017, 2Kcg020, Pk-90064, and Icgs-09. Lane 12, DNA ladder.

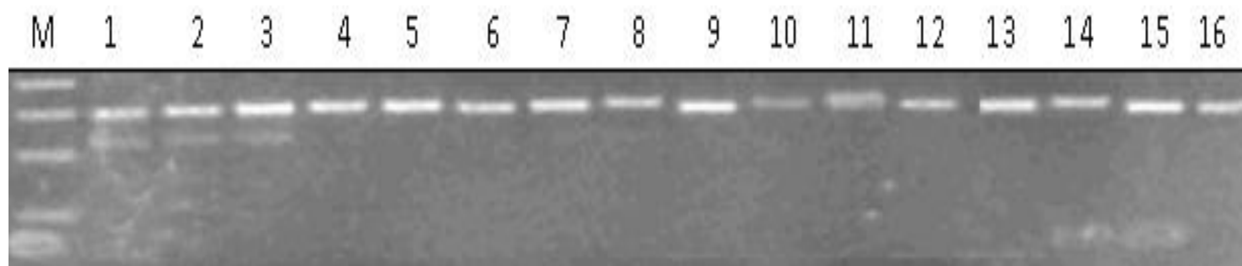

Figure 3. Ethidium bromide-stained agarose gel showing PCR-amplified products obtained from DNA isolated from fresh-leaf tissue by using ssr marker. Lane M, DNA ladder; lanes 1 to 16, Golden, Bari-2000, Banki, Chakori, Bari-89, Bard-479, 4CgOO4, 2Kcg017, 2Kcg020, Pk-90064, Icgs-09, Icgs-17, Icgs-18, Icgs-114, Icgs-38 and Icg-48

sodium chloride (3 M) were combined in extraction buffer followed by washing with phenol: chloroform:isoamyl alcohol, majority of the polysaccharides can be successfully removed (Murray and Thompson, 1980; Paterson et al., 1993; Suman et al., 1999). Protein impurities can be successfully removed with double washing with phenol: chloroform:isoamyl alcohol (Dipankar et al., 2006). The use of phenol to remove CTAB polysaccharides complex formed earlier in the reaction was also reported in literature (Dipankar et al., 2006). Suman et al. (1999) successfully used the combination of high concentration of CTAB (3% w/v) and sodium chloride (3 M) in extraction buffer, followed by washing with phenol: chloroform:isoamyl alcohol for removal of polysaccharides. Protein impurities can be efficiently removed with double washing with phenol: chloroform:isoamyl alcohol (Dipankar et al., 2006). The polyvinylpyrrolidone (PVP) containing modified CTAB buffer was also used for extraction of DNA from *Tagetes minuta*. In SDS-based extraction methodology, SDS does not bind with protein, thus degrading the purified DNA (Deshmukh et al., 2007). Maliyakal (1992) stated that the PVP and polyphenols form a complex, allowing the polyphenols to be easily removable from the samples.

ACKNOWLEDGEMENT

Te authors are thankful to National Institute for Genomics and Advanced Biotechnology, National Agricultural Research Centre, Islamabad, Pakistan for providing all kinds of technical moral and financial support.

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Vol. 12(51), pp. 7074-7080, 18 December, 2013
DOI: 10.5897/AJB12.1528
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of irrigation regimes and polymer on dry matter yield and several physiological traits of forage sorghum var '*Speedfeed*'

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Accepted 12 July, 2013

Drought is the most important limiting factor for crop production; it is becoming an increasingly severe problem in many regions of the world. Sorghum is among the most important forages used in arid and semi-arid regions of southeastern Iran, but its growth and yield is often constrained by water deficit and poor productivity of sandy soil. Irrigation water is becoming scarcer and more costly. The addition of water-saving superabsorbent polymer (SAP) in soil can improve soil physical properties, crop growth and yield and reduce the irrigation requirement of plants. This experiment was conducted on sorghum var 'Speedfeed' grass in Zahedan, Iran during 2009 and 2010 seasons. The experimental design was a split-plot with two factors including four irrigation regime (providing 40, 60, 80 and 100% from consumptive (ET crop) of sorghum) as main plots and four amounts of SAP (0, 75, 150 and 225 kg ha^{-1}) as subplots in a completely randomized block design with three replications. Irrigation level and SAP had significant effects on number of leaves per plant, number of tillers per plant, leaf area index, leaf area duration, relative water content and dry matter. The results indicate that irrigation to meet 80% of the water requirement with 75 kg ha^{-1} SAP may provide a desirable dry matter.

Key words: Dry matter yield, forage sorghum, irrigation level, superabsorbent polymer.

INTRODUCTION

Drought stress is the most important limiting factor of field crops in Iran. Most parts of Iran's cultivation land are placed in arid and semiarid regions. Drought stress limits crop growth and productivity more than any other single environmental factor (Mao et al., 2011; Todorov et al., 1998), specifically for forage production, because the cost of water and energy continues to increase (Maboko, 2006). Superabsorbent polymers are becoming more and more important in regions where water availability is insufficient (Maboko, 2006; Monnig, 2005). Applying superabsorbent polymers in agriculture have a significant role in increase of soil capacity. Polymers are safe and non-toxic and it will finally decompose without any remainder (Mikkelsen, 1994). The application of SAP for stabilizing soil structure resulted in increased infiltration and reduced water use and soil erosion in a furrow irrigated field (Lentz and Sojka, 1994; Lentz et al., 1998). Superab A200 polymer (SAP) works by absorbing and storing water and nutrients in a gel form and undergoing cycles ofhydrating and dehydrating according to for moisture's demand, increasing both water and nutrient use efficiency in crops (Islam et al., 2011; Lentz and Sojka, 1994; Nazarli et al., 2010). Superabsorbent polymer can hold 400-1500 g of water per dry gram of hydrogel (Boman and Evaans, 1991). The SAP also prolonged water available for plant use when irrigation stopped (Huttermann et al.,

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Abbreviations: DM, Dry matter yield; **LAD,** leaf area duration; **LAI,** leaf area index; **NL,** number of leaves per plant; **NT,** number of tillers per plant; **RWC,** relative water content; **SAP,** Superab A200 polymer.

1999). Thus, plant growth could be improved with limited water supply (Yazdani et al., 2007).

Sorghum is the fifth most important cereal crop grown for human consumption in the world being surpassed only by rice, wheat, barley and corn. Most of sorghum grown in Asia and the African tropics is used for human food and also fed to livestock or poultry (Gul et al., 2005). Sorghum is a drought resistant summer annual crop (Aishah et al., 2011). Forage sorghum is an important forage crop in tropical, semi-tropical and even warm-temperate regions and is cultivated over about 30,000 ha, mainly in the southern provinces of Iran such as Sistan and Baluchistan (Muldoon, 1985; Unlu and Steduto, 2000). In spite of its relatively high tolerance to drought, sorghum yields can increase by as much as four-fold if production is under full irrigation (Rai et al., 1999). Sorghum speedfeed is a crop of world-wide importance and is unique in its ability to produce under a wide array of harsh environmental condition (Moghaddam et al., 2007; Sadeghzade et al., 2012).

Relative water content (RWC) is an appropriate measure of plant water status in terms of the physiological consequences of cellular water deficit (Kramer, 1988; Shamsi, 2010). Siddique et al. (2001) reported that decreasing the soil water potential can lead to a decrease in the RWC, decreasing the plant photosynthesis and dry matter. Munamava and Riddoch (2001) reported that leaf area and dry matter yield decreased with water stress. The leaf area index (LAI) of the crop at a particular growth stage indicates its photosynthetic potential or the level of its dry matter accumulation. The higher LAI, increases dry mater accumulation in the plant (Rasheed et al.,_2003). Fischer and Wilson (1966) suggested that dry matter accumulation is closely related to the maximum LAI and sorghum yield increases up to 10 LAI. Reduction in the leaf area in response to water stress occurs either through a decline in the leaf expansion or accelerated leaf senescence (Moseki and Dintwe, 2011). The high leaf area duration (LAD) can produce higher dry matter (Sanjana Reddy, 2012) and the LAI and LAD were posi-tively correlated with dry matter production (Reddi, 2006). LAD is one of the important physiological traits that have an implication on yield potential related to increasing assimilate availability (Brevedan and Egli, 2003).

Sorghum can produce tillers, and the number of productive tillers is influenced by soil water availability (Berenguer and Faci, 2001). Drought stress reduces the number of tillers either by stopping the differentiation process or by the death of growing or grown tillers (Krieg, 1983). Tillering is controlled by hormones and factors such as temperature, photoperiod, soil moisture and plant density (Stoskopt, 1985). Water stress causes the production of abscisic acid in plant, resulting in a decrease of the tillers (Morgan and King, 1984). The tillers are more sensitive to water stress than the main stem (Krieg, 1983). The plant photosynthetic material is consumed when the tillers are generated thus tillers productions and survival depends on photosynthesis and the material stored (McCree, 1983; Krieg, 1983). The advantage of tillering in sorghum forage is the regrowth of plants after harvest. Hart et al. (2001) recognized that leaf number in sorghum was under both genetic and environmental control. Quinby and Karper (1954) pointed out that the floral differentiation of the apical meristem of sorghum terminates the differentiation of leaves and thus effectively regulates the plant size. The number of leaves determines the leaf area index and a high leaf area index with the appropriate structure could result in a high performance (Hart et al., 2001). The objectives of this investigation were to determine the effects of Superab A200 and irrigation regime on the number of leaves per plant, number of tillers per plant, leaf area index, leaf area duration, relative water content and dry matter of sorghum.

MATERIALS AND METHODS

Experimental location, irrigation treatments, SAP treatments and soil properties

The field experiment was conducted in Dashtak that is located near Zahedan, southeastern Iran (25°, 30' N and 58°, 47' E), with a mean annual rainfall of 120 mm with an arid and tropical climate. Before planting, soil samples were taken from the experimental site and were analyzed according to the procedure of Jakson (1973). Some physical and chemical properties of the soil are presented in Table 1.

The present study was conducted as a split plot randomized complete block design with three replications. The treatments included four levels of irrigation assigned to the main plots (providing 100 (I_1), 80 (I_2), 60 (I_3) and 40 (I_4) % from consumptive (ET crop) of sorghum var 'Speedfeed') and four SAP levels as a subplot [225 (S_1), 150 (S_2), 75 (S_3) and 0 (S_4), kg SAP ha^{-1}] on sorghum var 'Speedfeed' during 2009 and 2010 seasons to evaluate the effects of SAP under the irrigation regime on DM.

SAP material, SAP placement, planting seed and irrigation method

The soil amendment used was a hydrophilic polymer, SAP produced by Rahab Resin Co. Ltd., under license of "Iran Polymer and Petrochemical Institute". The chemical structure of SAP is shown in Table 2 (Abedi-Koupai and Asadkazemi, 2006; Nazarli et al., 2010; Yazdani et al., 2007).

Each plot was 15 m^2 with five planting rows, with an inter-row spacing of 50 cm, an inter-plant spacing of 6 cm and the plant average density was 34 plants per m^2. Before seed planting, SAP was placed by hand where roots were expected to have greater density (15 to 20 cm depth) in the middle of rows along the ridge (Lavy and Eastin, 1969), then the seeds were manually sown at the depths of 2 to 3 cm in the rows in early April. Soil preparation operations included plowing, disking and leveling which were carried out in early March. Thinning was done at 5 to 7 leaf stage and the seedlings distance along rows was set between 8 to 12 cm. Water requirements were determined according to FAO method using the American Class A evaporation pan data (Giovanni et al., 2009; Howell et al., 2008). The sorghum var '*Speedfeed*' evapotranspiration was calculated by Equation 1 and irrigation was assumed 80% application efficiency for the furrow irrigation distributed in the farm. The amount of irrigation in each treatment was determined using flow meters.

Table 1. Some physical and chemical properties of a representative soil samples in the experimental site before sowing (0-30 cm depth) in 2009 and 2010 seasons.

Soil property	2009*	2010*
Silt	24.9	24.8
Sand	65.3	65.9
Clay	9.80	9.30
Texture	sandy - loam	sandy - loam
Organic matter (%)	0.05	0.06
EC (1:1 extract) (ds m^{-1})	6.80	6.70
pH (1:1 suspension)	7.70	7.60
Total nitrogen (%)	0.15	0.16
Total CaCo3 (%)	0.90	1.10
NaHCO3-extractable P (mg L-1)	3.50	3.70
NaOAC-extractable K (mg L-1)	90.0	93.0

*Each value represents the mean of three replications.

$$ET_c = K_C \times ET_0 \quad (1)$$

$$K_c = \frac{ET_a}{ET_P}$$

Where, K_c, ET_a and ET_c were crop coefficients, evapotranspiration actual and evapotranspiration critical respectively. The K_c was extracted as Dorrenbos and Kassam (1979).

$$ET_0 = K_{pan} \times E_p$$

Where, ET_o, K_{pan} and E_p was evapotranspiration of the reference crop.

K_{pan} was 0.66 (Alizadeh, 2002) and E_p was evaporation of pan.

Calculating growing degree-days (GDD) and plant growth analysis

Growing degree-days were calculated using the Equation 2:

$$GDD = \frac{T\max - T\min}{2} - B \quad (2)$$

Where, Tmax and Tmin are the daily maximum and minimum temperatures respectively and B represents a base temperature value of 10°C (McMaster and Wilhelm, 1997).

LAI was measured after flowering was at a 10% level by measuring the leaf area of five plants per treatment. The LAI was calculated by Equation 3 as follows (Rasheed et al., 2003):

$$LAI = \frac{Leaf\ \ area(m^2)}{Land\ \ area(m^2)} \quad (3)$$

LAD was measured after flowering was at a 10% level by Equation 4 as follows (Rasheed et al., 2003):

$$LAD = \frac{(LAI_1 + LAI_2) \times (t_2 - t_1)}{2} \quad (4)$$

Where, LAI_1 = Leaf area Index at t_1; LAI_2 = Leaf area index at t_2; t_1 = time of first observation; t_2 = time of second observation.

To determine the DM, the harvested plants (stems and leaves) were desiccated at 75°C for two days in a ventilating oven. For calculating dry matter accumulation, five plants.

Measurement RWC, NL, NT and DM

The RWC was determined in the fully expanded topmost leaf one day before irrigation between 8 and 9 a.m. This was accomplished by excising three 1-cm disks of each sample leaf at 282, 444, 600, 766 and 907 GDD. The results were then averaged, resulting in a single value to represent that plot. The fresh weight of the sample leaves was recorded and the leaves were immersed in distilled water in a Petri dish. After 24 h, the leaves were removed, the surface water was blotted-off and the turgid weight recorded. Samples were then dried in an oven at 70°C to constant weight (Munne-Bosch et al., 2007; Schlemmer et al., 2005). The RWC was calculated by Equation 5 as follows:

$$RWC = \frac{Fresh\ Weight - Dry\ Weight}{Turgid\ W - Dry\ Weight} \times 100 \quad (5)$$

The determination of NL and NT was carried out after flowering was at a 10% level. The NL was counted randomly in one square meter area for each plot and the NT was counted in three plants in each plot, then the results were averaged, resulting in a single value to represent that plot.

Statistical analysis

The data were analyzed with SAS 9.2. The analysis of variance for each physiological variable was performed by the PROC GLM procedure. Comparison the simple effects was also conducted using Duncan's multiple range test and a comparison of the interaction effects was also conducted using the least squares means. The combined analysis of variance over years was performed on the data of two growing seasons after testing the homogeneity of the error according to Bartlett's test.

Table 2. The properties of Superab A200 material.

Appearance	White granule
Grain size (mm)	0.5-1.5
Water content (%)	3-5
Density (g cm^{-3})	1.4-1.5
pH	6-7
The actual capacity of absorbing the solution of 0.9 % NaCl	45
The actual capacity of absorbing tap water (%)	190
The actual capacity of absorbing distilled water (%)	220
Maximum durability (year)	7

Table 3. Main effects of irrigation regime on some physiological traits of sorghum var 'Speedfeed'.

Irrigation regime	Relative water content			Number of tillers per plant			Number of leaves		
	2009	2010	Average	2009	2010	Average	2009	2010	Average
I_1	81.23[a]	80.47[a]	80.85[a]	2.92[a]	2.58[a]	2.75[a]	13.17[a]	13.08[a]	13.12[a]
I_2	80.20[a]	75.04[b]	77.62[b]	2.00[b]	2.08[b]	2.04[b]	11.33[b]	11.75[b]	11.54[b]
I_3	65.44[b]	65.46[c]	65.45[c]	1.00[c]	0.92[c]	0.96[c]	8.67[c]	8.92[c]	8.79[c]
I_4	60.46[c]	59.39[d]	59.92[d]	0.83[c]	0.50[d]	0.67[d]	6.83[d]	6.42[d]	6.62[d]
Irrigation regime	**Leaf area index**			**Leaf area duration**			**Dry matter (g m^{-2})**		
I_1	8.31[a]	8.10[a]	8.20[a]	92.07[a]	87.48[a]	89.77[a]	2223[a]	2174[a]	2199[a]
I_2	6.93[b]	6.54[b]	6.73[b]	78.48[b]	73.39[b]	75.93[b]	1867[b]	1962[b]	1915[b]
I_3	4.10[c]	4.33[c]	4.21[c]	47.66[c]	50.39[c]	49.03[c]	719[c]	650[c]	684[c]
I_4	2.95[d]	3.16[d]	3.05[d]	34.84[d]	38.97[d]	36.03[d]	379[d]	359[d]	369[d]

Means in each column followed by a similar letter are not significantly different at $P<0.05$ according to Duncan's multiple range test. I_1=100, I_2=80, I_3=60 and I_4=40% of the water requirement of sorghum var 'Speedfeed' .

RESULT AND DISCUSSION

Number of leaves per plant

NL significantly decreased as irrigation water amount decreased in both seasons and averaged over the years (Table 3). Bennett (1979) reported that when leaf water potential decreased from -4 to -5, caused the number of leaves to decrease. NL increased with increasing level of polymer applied (Table 4). The interaction between the irrigation regime and SAP level were significant at the 5% level for the combined effects of 2009 and 2010 seasons and the NL in I_2S_1 was the same as I_1S_2, I_1S_3 and I_2S_4 (Table 5).

Number of tillers per plant

NT decreased as the amount of irrigation applied decreesed (Table 3). Krieg (1983) suggested that drought stress reduces the number of tillers. NT increased with increasing amount of polymer in the soil (Table 4). The interacttion between the irrigation regime and SAP level was significant at the 5% level for combined effects of 2009 and 2010 seasons (Table 5). There was a positive and significant correlation (Table 6) between NL and NT (0.86).

Relative water content

RWC decreased with decreasing irrigation water amount in both years (Table 3). Girma and Krieg (1992) reported that the RWC in sorghum var 'Speedfeed' decreased with an increase in water stress. RWC increased with increasing amount of polymer in the soil (Table 4). Mao et al. (2011) application of SAP increased RWC significantly by 15.4% when compared with the control. The interaction between the irrigation regime and SAP level was not significant at the 5% level. RWC significantly correlated (Table 6) with NL (0.92) and NT (0.88).

Leaf area index

LAI decreased with decreasing irrigation water amount in both years (Table 3). Moseki and Dintwe (2011) suggested the leaf area decreased with the increase of water stress. LAI increased with increasing amount of polymer in the soil (Table 4). Islam et al. (2011) showed that leaf area did not change under low application of superabsorbent polymer but increased remarkably following SAP application at medium and high rate by 18.9 and 32.5%, respectively. The interaction between the irrigation regime

Table 4. Main effects of Superab A200 polymer (SAP) level on some physiological traits of sorghum var 'Speed feed'.

SAP level	Relative water content			Number of tillers per plant			Number of leaves		
	2009	2010	Average	2009	2010	Average	2009	2010	Average
S_1	76.68^{a}	75.55^{a}	76.12^{a}	2.42^{a}	2.08^{a}	2.25^{a}	11.17^{a}	11.25^{a}	11.21^{a}
S_2	72.37^{b}	71.22^{b}	71.80^{b}	1.67^{b}	1.58^{b}	1.62^{b}	10.25^{b}	10.33^{b}	10.29^{b}
S_3	71.76^{b}	68.63^{c}	70.19^{c}	1.58^{b}	1.33^{bc}	1.46^{b}	9.75^{c}	9.83^{c}	9.79^{c}
S_4	66.51^{c}	64.96^{d}	65.73^{d}	1.08^{c}	1.08^{c}	1.08^{c}	8.83^{d}	8.75^{d}	8.79^{d}
SAP level	**Leaf area index**			**Leaf area duration**			**Dry matter (gm-^{2})**		
S_1	6.46^{a}	6.11^{a}	6.28^{a}	71.19^{a}	68.57^{a}	69.88^{a}	1469^{a}	1460^{a}	1464^{a}
S_2	5.54^{b}	5.93^{a}	5.70^{b}	62.57^{b}	65.37^{ab}	63.78^{b}	1348^{b}	1354^{ab}	1351^{b}
S_3	5.48^{b}	5.33^{b}	5.43^{b}	62.19^{b}	61.59^{b}	62.08^{b}	1267^{b}	1254^{b}	1260^{c}
S_4	4.80^{c}	4.76^{c}	4.78^{c}	57.09^{c}	54.70^{c}	55.89^{c}	1104^{c}	1078^{b}	1091^{d}

Means in each column followed by a similar letter are not significantly different at P<0.05 according to duncan's multiple range test. S_1=225, S_2=150, S_3=75 and S_4=0 kg SAP ha^{-1}.

Table 5. Interaction between irrigation regime and Superab A200 polymer (SAP) on some physiological traits of sorghum var 'Speedfeed' .

Irrigation regime	SAP level	Dry matter (g m^{-2})	Leaf area index	Leaf area duration	Number of leaves	Number of tillers per plant
I_1	S_1	3.33^{a}	14.17^{a}	101.5^{a}	9.50^{a}	2229.33^{ab}
	S_2	3.00^{ab}	13.17^{b}	87.20^{bc}	8.09^{b}	2256.17^{a}
	S_3	2.67^{b}	12.83^{bc}	88.73^{b}	7.93^{b}	2209.00^{b}
	S_4	2.00^{c}	12.33^{cd}	82.11^{cd}	7.29^{cd}	2100.17^{bc}
I_2	S_1	3.00^{ab}	12.67^{bc}	83.25^{bc}	7.56^{c}	2089.17^{bc}
	S_2	2.00^{c}	11.67^{e}	77.26^{d}	6.93^{d}	2011.33^{c}
	S_3	2.00^{c}	11.83^{de}	77.70^{d}	6.86^{d}	1981.60^{c}
	S_4	1.17^{de}	10.00^{f}	65.53^{e}	5.58^{e}	1576.33^{d}
	S_1	1.50^{d}	10.17^{f}	55.34^{f}	4.76^{f}	1122^{e}
I_3	S_2	1.00^{ef}	9.17^{g}	53.74^{f}	4.66^{f}	725.83^{f}
	S_3	0.67^{f}	8.33^{h}	44.56^{g}	3.89^{g}	462.33^{g}
	S_4	0.67^{f}	7.50^{i}	42.48^{gh}	3.54^{gh}	394.17^{gh}
I_4	S_1	0.67^{f}	7.80^{hi}	39.90^{ghi}	3.32^{hi}	417.17^{gh}
	S_2	0.50^{f}	7.17^{i}	36.93^{ij}	3.13^{hij}	376.83^{gh}
	S_3	0.50^{f}	6.17^{j}	37.33^{hij}	3.06^{ij}	388.17^{gh}
	S_4	0.50^{f}	5.33^{k}	33.46^{j}	2.70^{j}	292.83^{h}

I_1=100, I_2=80, I_3=60 and I_4=40% of the water requirement of sorghum var 'Speedfeed' . S_1=225, S_2=150, S_3=75 and S_4=0 kg SAP ha^{-1}. Means in each column followed by a similar letter are not significantly different at P<0.05 according to Duncan's multiple range test.

Table 6. The Pearson correlation coefficient between dry matter and some physiological traits of sorghum var 'Speedfeed' grown in 2009 and 2010 seasons.

Parameter	1	2	3	4	5	6
Dry matter (g m^{-2})						
Leaf area duration	0.93**					
Leaf area index	0.87**	0.99**				
Number of leaves per plant	0.86**	0.92**	0.92**			
Number of tillers per plant	0.84**	0.88**	0.87**	0.86**		
Relative water content	0.88**	0.86**	0.85**	0.92**	0.88**	

**Indicate significant at 0.01.

and SAP level was significant at the 5% level for the combined effects of 2009 and 2010 seasons (Table 5). LAI was significantly correlated (Table 6) with NL (0.92), NT (0.88) and RWC (0.85).

Leaf area duration

LAD decreased with decreasing irrigation water amount in both years (Table 3). Brevedan and Egli (2003) suggested that drought stress reduces the LAD. LAD increased with increasing amount of polymer in the soil (Table 4). The interaction between the irrigation regime and SAP level was significant at the 5% level for the com-bined effects of 2009 and 2010 seasons (Table 5). The LAD was significantly correlated (Table 6) with LAI (0.99), NL (0.92), NT (0.88) and RWC (0.86). In drought condi-tions, the nutrients transfers from leaves increases, accelerating the leaf senescence (Brevedan and Egli, 2003). On the other hand, Islam et al. (2011) showed that SAP could be an effective way to increase both water and nutrient use efficiency in crops and increase LAD. So LAI, LAD and DM increase.

Dry matter

Dry matter decreased with decreasing irrigation water amount in both years (Table 3). Aishah et al. (2011) reported that when the irrigation schedule changed from -1 to -1.5 Mpa, the forage yield ‘Speedfeed’ decreased by 22.2%. Dry matter increased with increasing amount of polymer in the soil (Table 4). The above-ground biomass accumulation in sorghum var ‘Speedfeed’ increased following SAP application but the effect was less for low and medium SAP rate. Islam et al. (2011) showed that the DM increased with increasing rate of superabsorbent polymer and the value increased by only 10.4% with low application of SAP, while it increased significantly by 20.5 and 32.9% with medium and high application, respectively. In sorghum var ‘Speedfeed’, the rate of dry matter production is controlled by leaf area (Peacock and Wilson, 1984). Sorghum var ‘Speedfeed’ leaf area depends on the rate and speed in which primary leaves are formed, their expansion, leaves number, and the leaf senescence rate, all of which depends on the plants water available. So, in this experiment, with an increase in the NL, LAI and LAD, the amount of DM increased (Dale, 1982; Peacock and Wilson, 1984). On the other hand, the use of polymer in soils to improve both the nutritional and water status of plants (Islam et al., 2011). The interaction between irrigation regime and SAP level were significant at a 5% level for the combined effects of 2009 and 2010 seasons and the DM content in I_1S_4 was the same as I_2S_1, I_2S_2 and I_2S_3 (Table 5). Dry matter was significantly correlated with LAD (0.93), LAI (0.87), NL (0.86), NT (0.84) and RWC (0.88).

Conclusion

Water stress decreased number of leaves per plant, number of tillers per plant, leaf area index, leaf area duration, relative water content and dry matter. Our results have shown that the applied SAP had an important effect on forage sorghum var ‘Speed feed’ and increased number of leaves per plant, number of tillers per plant, leaf area index, leaf area duration, relative water content and dry matter. Probably, the application of SAP could be an effective management practice in soils characterized by low water holding capacity where irrigation water and fertilizer often leach below the root zone within a short period of time, leading to poor water and fertilizer use efficiency by crops. Therefore, SAP increases leaf area index through increasing both water and nutrient use efficiency in crops. The higher LAI causes an increase in LAD and results in increasing dry matter accumulation in the plant. The DM yield in treatment I_2S_3 was the same as I_1S_4 that showed by using 75 kg ha^{-1} SAP as much as 20% of irrigation water was saved.

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academicJournals

Vol. 12(51), pp. 7086-7091, 18 December, 2013
DOI: 10.5897/AJB2013.13223
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro antibacterial activity of alkaloid extracts from green, red and brown macroalgae from western coast of Libya

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Accepted 28 November, 2013

Marine organisms and microorganisms are known to be a rich source of alkaloids with unique chemical feature and interesting biological activities. The current study presents the antibacterial effect of the alkaloid extracts of some green, red and brown algae were collected from western coast of Libya, against, *Escherichia coli*, *Salmonella typhi*, *Klebsiella* spp., and *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus* spp. and *Staphylococcus epidermidis* were investigated. Although alkaloid extracts of green algae inhibited all tested bacteria, maximum effect was exhibited by brown and red algae species. Thus, *Cystoseira barbata* alkaloid extract showed remarkable inhibition of human pathogen *Klebsiella* spp. *Dictyopteris membranacea* alkaloid extract also demonstrated similar considerable effect against *S. typhi* with MIC value 1.56 mg/ml. The pronounced antibacterial activity of *C. barbata* and *D. membranacea* can be attributed to their high alkaloid contents. These results suggest that red and brown algae secondary metabolites are important sources that could produce potential chemotherapeutic agents.

Key words: Macroalgae, alkaloids, antibacterial activity.

INTRODUCTION

Algae are a large and diverse group of organisms from which a wide range of secondary metabolites have been isolated. A number of these compounds possess biological activities such as toxicity, antibacterial, antifungal, antiviral, antitumour and other specific activity (Cannell, 1993). These bioactive compounds include alkaloids (Guven et al., 2010), polyphenols) Pereira et al., 2002), terpenoids (Cen-Pacheco et al., 2010), flavonoids (Stafford, 1991), tannins (Serrano et al., 2009) and acetogenins (Narkowicz and Blackman, 2006) which are applicable for antioxidant (Rocha et al., 2007), antimicrobial (Li, 2009; Saidani et al., 2011), antiviral (Romanos et al., 2002; Mayer et al., 2009), anti-inflam-matory and anticancer activities (Jaswir and Monsur, 2011; Bhakuni and Rawat, 2005; Vasanthi et al., 2004; Natarajan and Kathiresan, 2010). Nevertheless, in Libya this kind of study has not been well explored, despite the wealth of Libyan marine flora. Therefore, the present study is to investigate the alkaloid compounds extracted from macro-algae from the Libyan coast.

Alkaloids are heterocyclic nitrogen compounds, naturally occurring in plants, microbes, animals and marine

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organisms. The first medically useful example of an alkaloid was morphine, isolated in 1805 from opium *papaver somniferum* (Fessenden and Fessenden, 1982). Although, alkaloids have been extensively studied in plants and few studies in marine algae are reported due to the fact that alkaloids of marine algae are relatively rare compared with terrestrial plant alkaloids (Guven et al., 2010). The alkaloids found in marine algae can be classified into three groups: Phenylethylamine alkaloids, indole and halogenated indole alkaloids, and other alkaloids. Structurally, phenylethylamine and indole groups are the most alkaloids isolated from marine algae. Biological activities of halogenated and non-halogenated forms have been reported as bioactive compounds and as biological probes for physiological studies (Kasim et al., 2010). In addition, *Caulerpin* isolated from macroalgae, was the only indole alkaloid from marine sources which has been reported to have anti-inflammatory potentials (Carolina et al., 2011; Everton et al., 2009). In addition, there are two derivatives: lophocladine A and lophocladine B which have been isolated from a red alga *Lophocladia* spp., collected from Fijian Island, New Zealand (Gross et al., 2006) and their anticancer activity has been proved successfully in various cancer cell lines (Patricia et al., 2010).

Previous studies revealed that seaweed extracts, especially polyphenols have antioxidant activity (Chandini et al., 2008; Ganesan et al., 2008); whereas alkaloids are commonly found to have antimicrobial properties against both Gram-positive and Gram-negative bacteria (Guven et al., 2010) such as halogenated indole alkaloids which are isolated from red algae (Ayyad and Badria, 1994). These compounds have been approved for their antibacterial activity (Guella et al., 2006). The biological activity of marine indole alkaloids is clearly a product of the unique functionality and elements involved in the biosynthesis of marine natural products which increase the biological activity of seaweeds. For instance, bromination of many natural products has the potential to increase biological activity significantly (Gul and Hamann, 2005). The current study was undertaken to investigate the antibacterial effect of alkaloid extracts of 6 species of marine algae (two Chlorophyceae, three Phaeophyceae and one Rhodophyceae) collected from the Libyan western coast, against pathogenic Gram-negative bacteria: *Escherichia coli*, *Salmonella typhi*, *Klebsiella* spp., and *Pseudomonas aeruginosa* as well as Gram positive bacteria, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus* spp., and *Staphylococcus epidermidis.*

MATERIALS AND METHODS

Sample collection

Ulva lactuca, *Codium tomentosum* (Chlorophyta), *Cystoseira barbata*, *Sargassum vulgare*, *Dictyopteris membranacea* (Phaeophyta) and *Gelidium latifolium* (Rhodophyta) were collected from western coast of Libya between February and spring, 2009. The algal samples were taxonomically identified at Botany Department, Faculty of Science, Tripoli University. Algae were washed properly with distilled water, then they were shade dried at room temperature, after which they were crushed in an electric mill until a fine powder was obtained (Chiheb et al., 2009).

Bacterial strains

Eight bacterial strains (Gram positive and negative) were selected for the study. The Gram positive species were: *S. aureus* (*S. aur*) was obtained from the Clinical Microbiology Laboratory, Azzawiya Medical Center (Azzawiya, Libya). *B. subtilis* (*B. sub*) were kindly provided by Mohamed Elghazali, Department of Microbiology Biotechnology Research Center (Twaisha, Libya), while *Bacillus* spp. (*B.* spp.) and *S. epidermidis* (*S. epi*) were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Tripoli University. The Gram negative species *S. typhi* (*S. typhi*), *E. coli* (*E. coli*), *P. aeruginosa* (*P. aer*) and *klebsiella* spp. (*K.* spp.) were obtained from the Department of Microbiology, Faculty of Veterinary Medicine, Tripoli University, Libya.

Alkaloids extraction

Powdered algae materials (50 g) were extracted several times with methanol (300 ml). Methanol extraction was continued until the plant material gave a negative result for alkaloids (Mayer's test). The obtained methanolic extract was evaporated under reduced pressure at 40°C, to minimize any possible thermal degradation of the alkaloids and other thermo labile compounds. The crude alkaloid mixture was then separated from neutral and acidic materials, and water soluble ingredients by extraction with aqueous acetic acid, followed by dichloromethane extraction, then basification of the aqueous solution which was subjected to further dichloromethane extraction thereafter (Hadi and Bremner, 2001).

Thin layer chromatography (TLC)

Identification of alkaloids was further carried out by TLC using precoated silica gel 60 F264 plates (Wagner and Bladt, 2004). Different screening systems were used to obtain better resolution of the components. Dragendorff's reagent was used as a locating reagent (Harborne, 1992). R_f value of each spot was calculated as R_f = Distance travelled by the solute/Distance travelled by the solvent.

Determination of antibacterial activity

The antimicrobial activity test of algal crude extracts was performed *in vitro* using the "hole-plate diffusion method" (Sarvanakumar et al., 2009). Each test organism was maintained on nutrient agar slant and was recovered for testing by growth in nutrient broth (Biolab, Difco) for 14 h at 37°C before streaking. Cultures were routinely adjusted to a suspension of 1×10^6 to 2×10^6 CFU/ml using pre-made calibration curve representing viable cell count ($X \times 10^6$) against OD 660 nm (Y). The plates with bacteria were incubated at 37°C for 24 h. After incubation, the inhibition zones formed around the holes were measured. Methanol (100%) without seaweed extract was used as negative control and ciprofloxacin disc (30 µg) was used as the positive control.

Determination of minimum inhibitory concentration (MIC)

The MICs were determined by the agar dilution method (Daud and

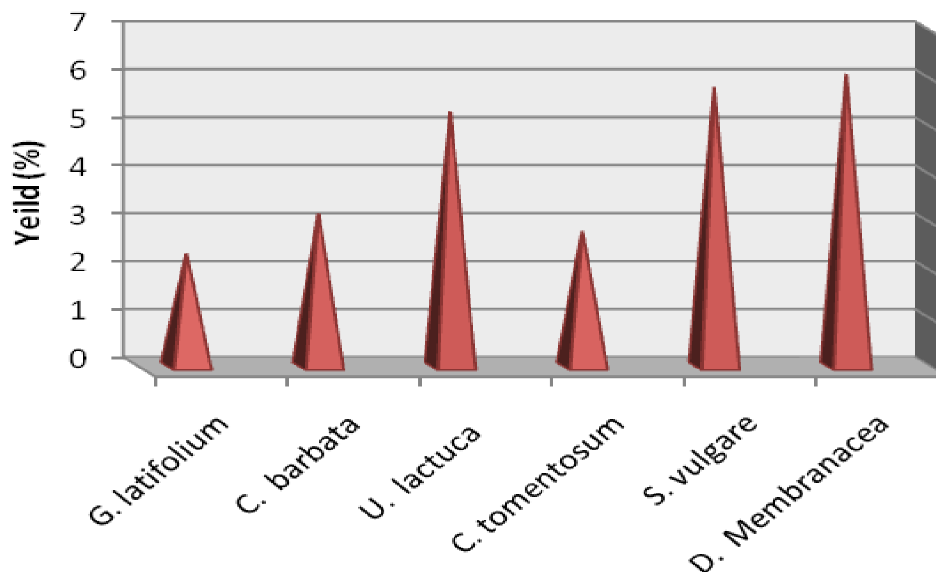

Figure 1. Percentage of alkaloid yield (mg alkaloid/g dry weigh) extracted from *U. lactuca*, *C. tomentosum*, *C. barbata*, *S. vulgare*, *D. membranacea* and *G. latifolium.*

Sanchaz, 2005). Two-fold serial dilutions of the original algae extract (100 mg/ml) were prepared in nutrient broth to obtain concentration from 100 to 1.56 mg/ml solvent. The plates incubated at 27°C for 18 h.

Bioautography method

Ten microliter (10 µl) of solutions corresponding to 1000 µg of alkaloids extract were applied to precoated Silica-gel TLC plates, developed with $CHCl_3$/ MeOH/ Na_2CO_3 (3:8:1, v/v) for each extract, and was evaporated to complete dryness. The dried plates were overlaid with nutrient agar medium seeded with *E. coli* (106 to 107 CFU/ml) and then incubated overnight at 37°C.

Statistical analyses

All assays were done in triplicate. All data are expressed as means ± S.D. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by one-way ANOVA with Turkey's b test using SPSS version 20.0.

RESULTS AND DISCUSSION

The qualitative phytochemical analysis for *U. lactuca*, *C. barbata*, *D. membranacea*, *C. tomentosum*, *S. vulgare* and *G. latifolium* showed the presence of alkaloids according to previous finding (Alghazeer et al., 2013). The present study was performed in order to extract alkaloids from the same species and then assess their antibacterial activity. Figure 1 shows alkaloid contents (% mg/g) extracted from green, red and brown algae species. The highest content was recorded for *D. Membranacea* (6.11%), *S. vulgare (*5.84%), *U. lactuca* (5.33%), (6.11%); whereas *C. barbata* and *C. tomentosum* showed moderate content of alkaloid (3.2 and 2.84%, respectively), while the lowest alkaloid content was obtained from *G. latifolium* (2.37%). Alkaloids present special interest because of their pharmacological activities. In fact, many reports revealed the presence of alkaloids in marine algae and some of them have been investigated for their biological activity (Guven et al., 2010; Kasım et al., 2010). Antimicrobial activities of alkaloid extracts from six seaweeds species represented by three Phaeophyta (*S. vulgare*, *D. membranacea* and *C. barbata*), two Chlorophyta (*U. lactuca* and *C. tomentosum*) and one Rhodophyta (*G. latifolium*) were examined against eight test bacteria (*Bacillus* spp., *B. subtilis*, *S. aureus*, *S. epidermidis*, *E. coli*, *kleb.* spp., *P. aeruginosa* and *Salmonella typhi*). The inhibition zones of brown, green and red algae extracts against Gram positive and Gram negative bacteria ranged between 13 to 35, 12 to 29 and 15 to 34 mm, respectively, all values are shown in Table 1.

The alkaloid extract of *U. lactuca* showed a relatively high mean zone of inhibition (21 ± 0.11 mm) against the Gram positive *S. aureus*, *S. epidermis* then *Bacillus* spp. (17 ± 0.3 mm), *E. coli* (16 ± 0.12 mm) and *B. subtilis* (14 ± 0.10 mm). While the alkaloid extract of *C. tomentosum* showed a remarkable high inhibition zone against *S. epidermis* (29 ± 0.35 mm) then *Bacillus* spp. (20 ± 0.3 mm), *S. aureus* (16 ± 11) and *B. subtilis* (13 ± 0.09 mm). For Gram negative bacteria, maximum zone of inhibition was recorded with alkaloid extract of *U. lactuca* against *kleb* spp. (18 ± 0.15 mm) and *S. typhi* (17 ± 0.11 mm). Also, maximum inhibition zones were recorded by *C. tomentosum* alkaloid extract against *klebsiella* spp. (27 ± 0.35 mm) then *E. coli* (23 ± 0.11 mm), *S. typhi* (21 ± 0.23 mm) and *P. aeruginosa* (12 ± 0.09 mm) (Table 1). The alkaloid extract of *S. vulgare* and *C. barbata* showed highest mean zone of inhibition against the Gram positive

Table 1. *In vitro* antimicrobial activity of the algal alkaloids extracts (100 mg/ml) against gram positive and gram negative bacteria.

Algal species	*U. lactuca*	*C. tomentosum*	*G. latifolium*	*S. vulgare*	*D. membranacea*	*C. barbata*	[a]Ciprofloxacin	[a]Chloramphenicol	[a]Neomycin
Test organism	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ (mm)	DIZ(mm)
E. coli	16±0.12	23±0.11	29±0.54*	23±0.11	30±0.31*	22±0.22	23	19	-
S. typhi	17±0.11	21±0.23	18±0.11	25±0.6	35±0.74*	26±0.45	26	21	-
Kleb. sp	18±0.15	27±0.35*	15±0.11	24±0.09	28±0.6*	35±0.54*	24	18	-
P. aer	15±0.12	12±0.09	ND	ND	ND	ND	20	-	-
B. sub	14±0.10	13±0.09	ND	ND	23±0.6	15±0.12	29	-	21
B. sp	17±0.11	20±0.35	24±0.11	19±0.21	ND	31±0.15*	24	-	26
S. aur	21±0.15	16±0.11	15±0.12	13±0.09	16±0.11	22±0.15	25	-	20
S. epi	21±0.6	29±0.35*	34±0.6*	18±0.11	23±0.15	20±0.11	23	-	23

Data are expressed as the mean ± standard deviation (SD) of three replicates. * represent the statistical comparisons between alkaloid extracts and positive control by using ANOVA followed by post hoc Tukey's b test (p<0.05). ND: not detectable.

Bacillus spp. (19 ± 0.21 mm and 31 ± 0.15 mm, respectively) then against *S. epidermis* (18 ± 0.11; 20 ± 0.11 mm, respectively), and *S. aureus* (13 ± 0.09; 22 ± 0.15). However, *D. membranacea* alkaloid extract had no effect on *Bacillus* spp., but showed high inhibition zones against *B. subtilis* and *S. epidermis* (23 ± 0.6 and 23 ± 0.15 mm). Maximum inhibition zone of Gram negative bacteria was recorded for alkaloid extract of *S. vulgare* and *D. membranacea* against *S. typhi* (25 ± 0.6 mm; 35 ± 0.74 mm) while the highest effect by the alkaloid extract of *C. barbata* was observed against *klebsiella* spp. (35 ± 0.54 mm). Whereas, *P. aeruginosa* was not susceptible to the alkaloid extracts of *S. vulgare*, *D. membranacea* and *C. barbata*.

The antibacterial activity of the alkaloid extract of *D. membranacea* and *C. barbata* were significantly high (P<0.05) compared with the positive control (Ciprofloxacin and Chloramphenicol) against Gram negative bacteria (Table 1), the antibacterial activity of green, brown and red algae is well documented (Del Val et al., 2001) as well as their isolated alkaloids (Masuda et al., 1997; Kasim et al., 2010). The alkaloid extract of *G. latifolium* showed significant high mean zone of inhibition against the Gram positive *S. epidermis* (34 ± 0.6 mm) compared with positive control (Ciprofloxacin and Neomycin) (P<0.05) which is in consistent with earlier finding where alkaloid isolated from red algae exhibited different modes of bioactivity (Sato et al., 1998; Gross et al., 2006). The recorded inhibition zone against *Bacillus* spp. and *S. aureus* were 24 ± 0.11 and 15 ± 0.12 mm respectively, while no inhibition was observed against *B. subtilis*. For Gram negative bacteria, maximum zone of inhibition was recorded with alkaloid extract of *G. latifolium* against *E. coli* (29 ± 0.54 mm), *S. typhi* (18 ± 0.11 mm) and *Kleb.* sp. (15 ± 0.11 mm), while no inhibition was observed against *P. aeruginosa* (Table 1). The activity of red, green and brown algae against both Gram positive and Gram negative bacteria may be indicative of presence of broad spectrum antibiotic compounds or simply the content of pharmacolo--gical active constituents like alkaloids (Omulokoli et al., 1997; Phang et al., 1994).

Minimum inhibitory concentrations (MICs) of the alkaloid extracted from algae (Figure 2) were found to be within the range of 100 to 1.56 mg/ml. The high levels of the MIC's of some alkaloid extracts can be attributed either to the presence of the active components in low concentrations, or to the presence of some antagonistic components that serve as growth promoters for the bacteria. The minimum inhibitory concentration (MIC) value of green algae (*U. lactuca*, *C. tomentosum*) against bacteria was ranged between 6.25 to 100 mg/ml. The lowest MIC value was recorded for *C. tomentosum* and *U. lactuca* extracts (6.25, 25 mg/ml respectively) against *S. epidermidis* while The minimum inhibitory concentration (MIC) value of brown algae (*S. vulgare*, *D. membranacea* and *C. barbata*) against bacteria was ranged between 6.25 to 100 mg/ml. The lowest MIC (1.56, 6.25 and 12.5 mg/ml) values were recorded for *C. barbata*, *D. membranacea* and *S. vulgare* extracts, respectively against *klebsiella* spp. (Figure 1). For alkaloid extracted from red algae (*G. latifolium*), the minimum inhibitory concentration (MIC) values of brown algae (*S. vulgare*, *D. membranacea* and *C. barbata*) against bacteria were

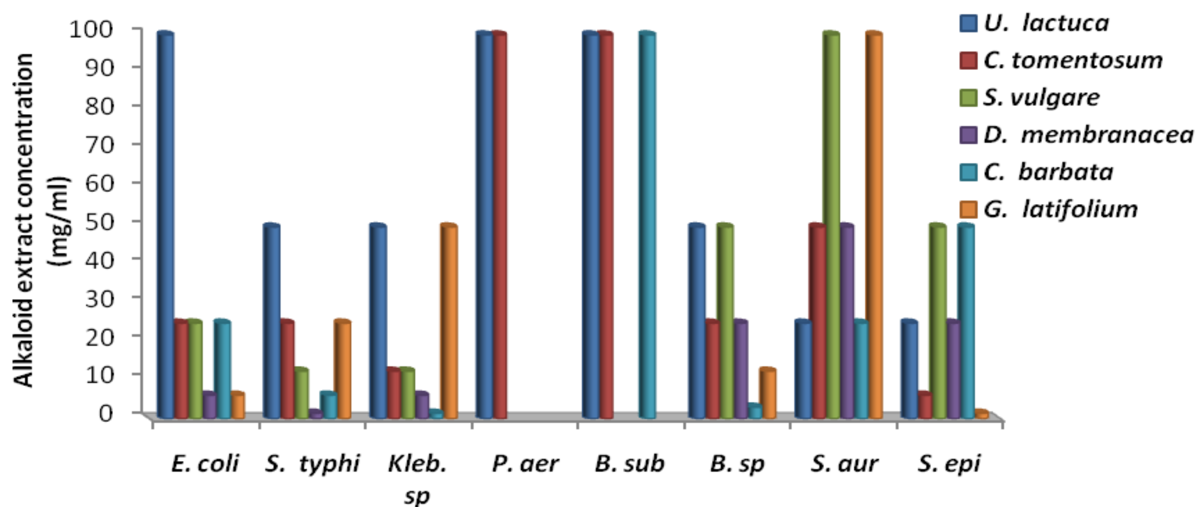

Figure 2. The *in vitro* antimicrobial activity of alkaloids extracts of tested algae expressed as minimum inhibitory concentration (MIC) (mg/ml) against some bacteria. S*. aur*: *Staphylococcus aureus*, *B. sub*: *Bacillus subtilis*, *E. coli*: *Escherichia coli*, *P. aer*: *Pseudomonas aeruginosa*, *B. sp*: *Bacillus* spp., *S. typhi*: *Salmonella typhi*, *S. epi*: *Staphylococcus epidermidis*, *Kleb*. sp: *klebsiella* spp.

Table 2. Location and prominence of zones of inhibition at different R_f values of alkaloid extracts against *E. coli*.

Algal species	Spot Number					
	S1		S2		S3	
	R_f	*E. coli*	R_f	*E. coli*	R_f	*E. coli*
U. lactuca	0.57	+	0.68	+	-	-
C. tomentosum	0.59	-	0.72	+	-	-
G. latifolium	0.57	++	0.61	-	0.68	+
S. vulgare	0.55	+	0.76	+	-	-
D. membranacea	0.54	++	0.64	-	0.75	++
C. barbata	0.52	+	0.74	++	-	-

Degree of inhibition: ++ = Prominent; + = moderate; - = Nil, *E. coli*: *Escherichia coli*; Mobile phase system is: chloroform: methanol: sodium carbonate (3:8:1, v/v).

ranged between 1.56 to 100 mg/ml. The lowest MIC (1.56 mg/ml) value was recorded against *kleb.* sp (Figure 1).

Preliminary TLC and the bioautographic assay tests were carried out on extracts to separate the compounds that were responsible for the inhibition tested the bacteria hence one spot may contain more than one compound. The results showed that the antibacterial assay can be attributed to the compounds observed at the various R_f values on the TLC separation. Although, sometimes the activity of compounds is not easily detected by this assay, if the compound does not diffuse through the agar, then the activity could be masked. The bioautoghraphy method was applied to the extracts using *E. coli* isolate to which all extracts exhibited antimicrobial activity (Table 1). The results showed good activity against *E. coli*, with prominent inhibition zones for the alkaloid of *D. membranacea*, *C. barbata*, *S. vulgare*, *G. latifolium* and *U. lactuca* extracts had two zones of inhibition, whereas, the alkaloid of *C. tomentosum* extract exhibited one zone of inhibition (Table 2).

Conclusions

The results of this work indicate the presence of alkaloids in tested algae that play an indispensable role in anti-bacterial activity, however further studies to identify and characterize the specific active compounds, as well as the evaluation of the toxic aspects are recommended.

ACKNOWLEDGMENTS

The authors wish to thank Dr Hussein and Dr Asma AlNajar for their constant encouragement and consultation.

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African Journal of Biotechnology Vol. 12(51), pp. 7099-7105, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1864
ISSN 1684-5315 ©2013 Academic Journals

Full Length Research Paper

Cactus (*Opuntia ficus indica f. inermis*) fruit juice protects against ethanol-induced hematological and biochemical damages in rats

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Accepted 23 July, 2012

A putative beneficial effect of *Opuntia ficus indica f. inermis* prickly pear juice (OFIj) was tested on ethanol-induced hematological and biochemical damages in rats. Our results show that chronic ethanol treatment (300 mg/100 g body weight for 90 days) of Wistar rats (group 2) significantly reduced red blood cells (RBC) and platelet (Plt) counts, hemoglobin (Hb) content, mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) whereas white blood cells (WBC) counts and the mean corpuscular volume (MCV) significantly increased as compared to the controls rats treated with same distilled water (group 1). In addition, serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and γ-glutamyl transpeptidase (γ-GT) as well as urea, creatinine, cholesterol and triglycerides concentrations significantly increased in ethanol-fed rats. Furthermore, serum, hepatic and renal lipid peroxidation levels were also increased in animals given ethanol compared to the controls. In alcoholic rats co-treated with 4 ml OFIj / 100 g b.w. (group 3), all the above cited parameters were maintained to near-normal values. In group 4 only 4 ml OFIj / 100 g b.w. was given, no changed parameters was shown. Therefore, OFIj appeared to be a promising agent for protection against ethanol toxicity.

Key words: *Opuntia*, alcohol, blood, liver, kidney, toxicity.

INTRODUCTION

Epidemiological, experimental and clinical investigations have shown a strong consistent relationship between alcohol abuse and liver diseases, hypertension, blood anomalies and other disorders (Husain et al., 2001; Russo et al., 2004). The ethanol-related diseases are instigated by excess production of acetaldehyde, hydrogen peroxide (H_2O_2) and reactive oxygen species (ROS), resulting from ethanol metabolism via the alcohol dehydrogenase (ADH) and the cytochrome P450-2E1 (CYP2E1) enzymes (Nordmann et al., 1992; Cederbaum et al., 2009). In clinical investigations, hematological and biochemical parameters are used to follow the evolution of the diseases resulting from ethanol abuse. Numerous studies demonstrate that fruits of some berry plants biosynthesize phytochemicals possessing antioxidant activity which could be used as a natural source of free radical scavengers (Yurt and Celik, 2011; Erukainure et al., 2011). In this respect, we were interested in the protective potential of cactus plants.

Native to Mexico, cactus plant is widespread through

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Abbreviations: OFIj, *Opuntia ficus indica f. inermis* prickly pear juice; **RBC,** red blood cells; **Plt,** platelet; **Hb**, hemoglobin; **MCH,** mean corpuscular hemoglobin; **MCHC,** mean corpuscular hemoglobin concentration; **WBC,** white blood cells; **MCV,** mean corpuscular volume; **ALT,** alanine aminotransferase; **AST,** aspartate aminotransferase; **ALP,** alkaline phosphatase; **LDH,** lactate dehydrogenase; **γ-GT,** γ-glutamyl transpeptidase.

Table 1. Scheme of drugs treatments.

Group	Treatment	
	At 9 h:00 in the morning	At 13 h:00 after noon
Control	Water (10 ml/kg b.w)	Water (10 ml/kg b.w)
EtOH	Water (10 ml/kg b.w)	Ethanol (10 ml/kg b.w)
OFIj+EtOH	OFIj (4 ml/100 g b.w	Ethanol (10 ml/kg b.w)
OFIj	OFIj (4 ml/100 g b.w)	Water (10 ml/kg b.w)

Water: distilled water, ethanol (10 ml/kg b.w.) = Ethanol (3 g/kg b.w).

out South America, Australia, South Africa, and the whole Mediterranean area (Galati et al., 2003; Tesoriere et al., 2004). *Opuntia ficus indica f. inermis* species grows throughout Tunisia and is mainly cultivated for its sweet and juicy fruit (prickly pear), which was shown to be rich in antioxidant compounds such as polyphenols, flavor-noids, betalains, and ascorbic acid. *Opuntia* fruits were found to display interesting properties such as antiulcer-rogenic (Galati et al., 2003), antioxidant (Kuti, 2004), and neuroprotective (Dok-Go et al., 2003). Moreover, prickly pear is used for the treatment of gastritis, hyperglycemia, arteriosclerosis, diabetes, and prostate hypertrophy (Agozzino et al., 2005). In Chinese medicine, cactus pear is used for inflammation and pain treatment (Zou et al., 2005). To our knowledge, there is no information hitherto about the effect of *O. ficus indica f. inermis* prickly pears or their juice on alcohol toxicity.

Therefore, the present study was undertaken to investigate the effects of *O. ficus indica f. inermis* prickly pear juice on hematological and biochemical disorders induced by ethanol treatment in rats.

MATERIALS AND METHODS

Chemicals

Ethanol 96.2% was purchased from Carlo Erba Reagents. Trichloroacetic acid (TCA), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and all other chemical products used in this study were purchased from Sigma Chemicals (Aldrich Chemical Company).

Preparation of *O. ficus indica f. inermis* fruit juice (OFIj)

Mature prickly pears of *O. ficus indica f. inermis* species (purple-skinned) were collected from local area. The whole unpeeled fruit (30 kg) was washed, ground by a Musermax double bladed mill and filtered through a colander (0.5 mm mesh size) to discard seeds. The resulting juice was centrifuged at 3000 × g for 10 min to remove hard fibers. The clarified juice (16.6 L) was then collected and stored at −21°C until use.

Animals

Adult male albino Wistar rats (n = 32) weighing 160 to 180 g were obtained from Pasteur Institute of Tunisia. Animals were quarantined and allowed to acclimatize for a week prior to experimentation. The animals were handled under standard laboratory conditions of temperature (22 ±2°C), relative humidity (70 ± 4%), and a 12 h light/dark cycle. Animals were fed with commercial pellets and given tap water *ad libitum*. Experiments were carried out according to the Tunisian code of practice for the care and use of animals for scientific purposes.

Experimental design

After acclimation period, rats were randomly divided into four groups of eight animals each, initially weighted then treated for 90 days with two consecutive intra-gastric intubations per day of (1) pure water (control group), (2) ethanol (prepared at 30% in distilled water), (3) both OFIj and ethanol or (4) only OFIj extract as described in Table 1. Food intake was daily monitored in each group during the entire treatment period. At the end of the experimental period, the final body weight of the animals, absolute liver and kidney weights were determined.

Hematological parameters

At the end of the experimental period, all animals were sacrificed by cervical dislocation. Blood samples were immediately collected in two tubes; the first was dry and the second was heparinized. The last tubes served to determine hematological parameters (red blood cell number (RBC), hemoglobin concentration (Hb), hematocrit value (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (Plt) and white blood cell number (WBC)) using a hematology analyzer Coulter MAXM (Beckman Coulter, Inc., Fullerton, USA).

Evaluation of biochemical parameters

Serum samples were obtained by the centrifugation of the blood collected in the dry tubes at 1000 x *g* for 10 min at 4°C, and were then stored at -20°C until analyses. The levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), gamma-glutamyl transferase (γ-GT), cholesterol, triglyceride, total protein, urea and creatinin were measured using provided BioMaghreb commercial kits.

Lipid peroxidation estimation

The liver and the kidney were quickly excised, rinsed with ice cold saline solution, weighted and homogenized (1:2, w/v) in 50 mmol/L phosphate buffer (pH 7.4) using an Ultra Turrax homogenizer and centrifuged at 4°C. The supernatant were frozen at -20°C in aliquots until analysis. Lipid peroxidation in the serum and tissue

Table 2. Effects of ethanol, OFIj or their combination (OFIj+EtOH) on weight gain, food intake and absolute liver and kidney weights.

Parameter	Experimental group			
	Control	**EtOH**	**OFIj+EtOH**	**OFIj**
Gain weights (%)	38.2±1.08	22.5±2.31**##	35.61±1.41	36.4±1.06
Food intake (g/rat/day)	13.6±0.97	9.2±0.31**#	11.32±0.68	11.9±0.47
A. liver weight (g)	8.67±0.25	10.86±0.14*#	8.37±0.32	8.48±0.41
A. kidney weight (g)	0.99±0.09	0.65±0.06*#	0.87±0.04	0.96±0.03

Values are expressed as means ± SD, for eight rats in each group. Significant differences were calculated at *p < 0.05, **p < 0.01 vs, control group and at #p <0.05, ##p < 0.01 vs, OFIj + ethanol group. A: Absolute.

Table 3. Hematological parameters of control and rats treated with ethanol, OFIj or their combination (EtOH + OFIj).

Parameter	Experimental group			
	Control	**EtOH**	**OFIj+EtOH**	**OFIj**
RBC (106/µl)	7.28±0.42	6.73±0.25*#	7.16±0.36	7.46±0.32
Hb (g/dl)	14.25±038	10.31±0.54*#	13.83±0.49	14.38±051
Ht (%)	44.81±1.23	37.65±1.41**##	42.37±1.32	43.92±1.21
MCV (mm^3/RBC)	57.43±2.34	65.72±3.41*#	54.68±2.21	56.83±2.89
MCH (pg/RBC)	20.16±0.67	17.83±0.38*#	19.81±0.49	20.34±0.58
MCHC (g/dl)	33.73±1.53	28.26±1.28**#	32.65±1.42	33.84±1.61
Plt (103/µl)	734.34±41.1	283.91±25.7**#	723.49±36.4	738.21± 49.3
WBC (103/µl)	10.67±0.23	15.43±0.91**#	11.23±0.43	10.53±0.16

Values are expressed as means ± SD, for eight rats in each group. Significant differences were calculated at *p < 0.05, **p < 0.01 vs, control group; and at #p <0.05, ##p <0.01vs, OFIj + ethanol group. RBC: Red blood cells, Hb: Hemoglobin, Ht: Haematocrit, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, Plt: Platelet and WBC: white blood cells.

homogenate was estimated by measuring thiobarbituric acid reactive substances (TBARS) and was expressed in terms of malondialdehyde (MDA) content which is the end product of lipid peroxidation, according to the method of Ohkawa et al. (1979). In brief, 125 µl of samples were homogenized by sonication with 50 µl of TBS, 125 µl of TCA-BHT in order to precipitate proteins and centrifuged (1000 × g, for 10 min at 4°C). The obtained supernatant (200 µl) were mixed with 40 µl of HCl (0.6 M) and 160 µl of TBA dissolved in Tris and the mixture was heated at 80°C for 10 min. The absorbance of the resultant supernatant was read at 530 nm. The amount of MDA was calculated using an extinction coefficient of 156×10^5 mM^{-1} cm^{-1}.

Statistical analysis

All data were expressed as mean ± SD. Differences among the experimental groups were assessed by one-way ANOVA followed by Duncan's test. Values were considered statistically significant when $p < 0.05$.

RESULTS

Effect of OFIj and/or ethanol on food intake, body and organ weights

Food intake, body growth and kidney weight were significantly lower in ethanol-treated rats, as compared to the controls (Table 2). By contrast, chronic ethanol administration significantly increased ($p < 0.05$) liver weight as compared to the controls. When OFIj was administered together with ethanol, the adverse effects of ethanol upon these parameters were alleviated. Administration of OFIj alone did not cause any significant alteration of the studied parameters.

Hematological parameters

Values of hematological parameters are reported in Table 3. In rats receiving ethanol, erythrocytes number (RBC), hemoglobin concentration (Hb), hematocrit value (Ht), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelets (Plt) number where significantly reduced as compared to the controls. In contrast, mean corpuscular volume (MCV) level and leucocytes number (WBC) were higher than that in the controls. No significant changes were observed in hematological parameters when animals were given both OFIj and ethanol, demonstrating a protective effect of the cactus fruit juice. Administration of OFIj alone to healthy rats did not cause any significant

Table 4. Enzyme activities in the serum of control and rats treated with ethanol, OFIj or their combination (EtOH + OFIj).

Enzyme (U/L)	Experimental group			
	Control	**EtOH**	**OFIj+EtOH**	**OFIj**
AST	134.8±2.1	214.7±5.8***##	153.6±4.1*	129.3±1.7
ALT	51.3±2.8	87.3±6.5**#	58.9±4.6*	50.8±4.8
ALP	58.5±4.2	94.6±5.7**#	64.1±4.7	59.6±5.1
LDH	928.4±21.3	1488.6±57.8***#	1106.2±41.7	935.7±52.9
γGT	2.34±0.12	5.32±0.36**#	3.14±0.28	2.31±0.16

Values are expressed as means ± SD, for eight rats in each group. AST: Aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; LDH: lacatate dehydrogenase and γGT: gamma glutamyl transferase. Significant differences were calculated at *p < 0.05, **p < 0.01, *p < 0.001vs, control group; and at #p <0.05, ##p <0.01vs, OFIj + ethanol group.

Table 5. Changes of serum biochemical parameters of the control and rats treated with ethanol, OFIj or their combination (EtOH + OFIj).

Parameter	Experimental group			
	Control	**EtOH**	**OFIj+EtOH**	**OFIj**
Cholesterol (mg/dl)	76.28±5.31	116.28±4.25**#	81.62±6.76	73.4±4.08
Triglyceride (mg/dl)	84.72±6.24	106.11±5.31**#	92.41±4.13	85.65±6.73
Total protein (g/dl)	6.98±0.48	5.15±0.41*#	6.37±0.32	6.95±0.81
Urea (mg/dl)	32.21±1.51	59.81±2.46*#	37.62±2.31	31.87±2.46
Creatinin (mg/dl)	0.42±0.07	0.86±0.05*#	0.51±0.09	0.38±0.04

Values are expressed as means ± SD, for eight rats in each group. Significant differences were calculated at *p < 0.05, **p < 0.01 vs, control group and at #p <0.05 vs, OFIj + ethanol group.

alteration of the hematological parameters either.

Biochemical parameters

The biochemical parameters of the control and experimental groups are shown in Tables 4 and 5. It appears that chronic ethanol administration significantly increases activities of transaminases (AST, ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transferase (γGT) as compared to the control group (Table 4). Data also shows that chronic ethanol administration significantly increased serum concentrations of cholesterol, triglycerides, urea and creatinine, while proteins concentration decreased (Table 5). When OFIj was administered together with ethanol, values of all theses parameters were kept close to the control values. OFIj, when given alone, did not change these values either.

Lipids peroxidation

MDA levels in serum, hepatic and renal tissues were significantly higher (p<0.01) in ethanol-treated rats than in the controls (Table 6). When rats were given OFIj together with ethanol, MDA levels in serum, liver and kidney significantly reduced as compared to the ethanol group. No significant difference in MDA levels was observed when comparing rats receiving OFIj alone and controls.

DISCUSSION

The various biochemical and hematological parameters investigated in this study are useful indices for evaluating the putative protective effect of the cactus prickly pear juice on disturbances induced by ethanol in rats hematological and biochemical parameters.

The present study showed a reduction of body weight, food intake and kidney weight in ethanol-treated rats. By contrast, ethanol intake significantly increased liver weight. Our results are in agreement with those reported by Dinu et al. (2005) and Kasdallah-Grissa et al. (2007). Obvious decrease of body and kidney weights may not result from a mere decrease in food intake, but rather could be due to the toxicity of ethanol on the gastro-intestinal tract leading to a relative impairment of nutrients digestion or to inhibition of protein synthesis (Saravanan et al., 2006). The observed increase of liver weight in ethanol-fed rats was in agreement with the observations of Navder et al. (1997) who attribute this increase to lipids accumulation. In our study, OFIj significantly mitigates the effects of ethanol on food intake and on body, liver and kidney weights. OFIj

Table 6. Hepatic, renal and serum malondialdehyde (MDA) contents of control and rats treated with ethanol, OFIj or their combination (EtOH + OFIj).

Parameter	Experimental group			
	Control	EtOH	OFIj+EtOH	OFIj
Liver MDA	0.78±0.04	1.36±0.08***##	0.81±0.02	0.76±0.01
Kidney MDA	0.76±0.02	1.09±0.05**##	0.79±0.06	0.74±0.03
Serum MDA	0.82±0.05	1.47±0.04***##	0.85±0.04	0.79±0.02

Values are expressed as means ± SD, for eight rats in each group. MDA was expressed as nmol/mg protein. Significant differences were calculated at **p < 0.01, ***p < 0.001vs. Control group; and at #p <0.05, ##p <0.01vs. OFIj + ethanol group.

provides a wide range of natural antioxidants to ethanol-fed rats including polyphenols, flavonoids, ascorbic acid, carotenoids, and betalains compounds (Alimi et al., 2012a). This kind of antioxidants could protect body from ethanol-inducing oxidative damage.

It has been reported that ethanol causes hematological disturbances in various clinical and experimental studies (Kanbak et al., 2007; Padmini and Sundari, 2008). The present study demonstrates that chronic ethanol administration induced a decrease in the levels of rats Hb, Ht, MCH, MCHC and RBC and Plt numbers. The reduction in RBCs, Hb and Ht might be due to an inhibition of erythropoiesis and hemoglobin synthesis and to an increase in the rate of erythrocytes destruction (Maruyama et al., 2001). The observed decrease of RBCs, Hb, Ht values and the increase of MCV value in rats exposed to ethanol could be the manifestation of swollen erythrocytes and macrocytic anemia (Harold and Ballard, 1997). In agreement with the above observations our previous study (Alimi et al., 2012a) showed that chronic ethanol ingestion leads to a marked anemia in rats, evidenced by a large production of deformed erythrocytes, associated to an increase in erythrocyte hemolytic percentage. This last finding could explain the reduction of Hb, MCH and MCHC values observed in ethanol-fed rats. In this study the ethanol-treated rats also exhibited significantly higher WBC number than the control animals. The increase in WBC might be the activation marker of defense and immune system and showed that there were inflammations in the tissues (Maturu et al., 2011). The decrease in Plt count in ethanol-fed rats suggested a possible effect on blood coagulation and haemostasis blood system damage. In the present study, pretreatment of alcoholic rats with OFIj significantly mitigate the obvious hematological disturbances. In our previous study, we showed that OFIj was rich in polyphenols, flavonoids, ascorbic acid, carotenoids, and betalains compounds. Our previous study demonstrated that OFIj supplement, increased plasma scavenging activity of control and ethanol-fed rats and prevented the impairment of erythrocyte osmotic stability and morphologic aspect (Alimi et al., 2012a). Such abundance in antioxidant compounds might confer to OFIj an antioxidant activity and prevent blood cells from ethanol metabolites.

The results of the present study also showed that chronic ethyl alcohol ingestion caused a significant increase in serum levels of liver biochemical markers (AST, ALT, ALP, LDH and γGT). It has been shown that the disturbances in the transport function of the hepatocytes as a result of hepatic injury causes the leakage of enzymes from cells to the blood stream (Fan et al., 2009). The serum increase of these enzymes activities in ethanol-fed rats may be due to the increase of hepatocytes permeability damaged by ethanol metabolites, this obvious results was in agreement with the findings reported by Yurt and Celik (2011). More so, ethanol-inducing hepatocytes damage was confirmed in this study by increases of cholesterol, triglyceride and total protein contents in serum. Similar changes have been reported by Pari and Suresh (2008).

In addition, kidney is vulnerable to damage because of larger perfusion and the increased concentration of excreted compounds that occur in renal tubular cells (Dinu et al., 2005). Serum levels of creatinine and urea were used as indicators of renal function. Elevated blood urea is known to be linked with an increased protein catabolism to urea as a result of increased synthesis of arginase enzyme involved in urea production (Yanardag and Sacan, 2007). Generally, the renal damage and glomerular filtration impairment were noticed in kidney as a result of ethanol toxicity (Pari and Suresh, 2008). This may account for the increased level of serum urea, creatinine as well as lowered creatinine clearance seen in alcohol-treated rats. The excess release of liver and kidney biochemical markers could be a result of ethanol-induced membrane lipid peroxidation.

Lipid peroxidation has been implicated in a number of deleterious effects such as decreased membrane integrity, increased hemolytic and erythrocyte deformation (Meagher et al., 1999; Kasdallah-Grissa et al., 2006). In correlation with previous studies ethanol-fed rats exhibited a large amount of MDA in serum, hepatic and renal tissues. Pre-treatment of ethanol-fed rats with OFIj significantly reversed serum, hepatic and renal lipid peroxidation as evidenced by the decrease of MDA content to near control levels.

Consistent with the attenuation of lipid peroxidation, treatment of ethanol-fed rats with OFIJ was associated with a corresponding reduction in levels of serum bioche-

mical markers related to the hepatic and renal damage, indicating a protective role of OFIj against ethanol toxicity in liver. The chemical analysis of OFIj demonstrate the presence of phenolics acids such as gallic, protocatechic, 4-hydroxybenzoic, vanillic and syrengic acids and flavonoids like quercetin, luteolin, kaempferol and isorhamnetin (Alimi et al., 2012b). The OFIj active principles may acts as scavengers of free radicals and which causes the oxidative process in liver and kidney cells.

It has been shown that the main nutraceutical benefit of prickly pears and their juice has been attributed to the flavonoids contents (Kuti, 2004). It was also demonstrated that flavonoids can be incorporated in cells plasma membranes, which becomes more ordered and therefore enhances their stability (Chaudhuri et al., 2007). The localization of flavonoids in the plasma membranes could strictly hinder the diffusion of free radicals, and thereby decreases resulting damage (Dobrzynska et al., 2005). Such flavonoids proprieties could explain the increase of hepatic and renal cells membrane integrity evidenced by the decrease of MDA contents, and the reduction of serum pathology markers in alcoholic rats pre-treated with OFIj. Rather than scavenging and stabilizing capacities, it has been demonstrated that flavonoids may also inhibit the CYP 2E1 activity and/or decrease its content, thereby contributing to inhibit and/or to decrease ethanol metabolism, hence the occurrence of oxidative stress (Orellana et al., 2002).

Conclusion

The present study reported for the first time that a dietary regimen enriched with *O. ficus indica f. inermis* prickly pear juice could prevent ethanol-induced toxicity on the blood, liver and kidney in rats by avoiding anemia, the decrease of hemoglobin content, the normalization of the biochemical markers related to liver and kidney integrity and the inhibition of lipid peroxidation process. Multiple mechanisms are suggested in this study to explain the beneficial effect of OFIj, from being able to scavenge ROS and stabilizing liver and kidney membrane integrity until the possible inhibitor effect on ethanol metabolism. For this reason OFIj consumption may provide a useful approach for decreasing alcoholic damages to blood, liver and kidney functions.

ACKNOWLEDGEMENT

This research was funded by the Tunisian Ministry of Higher Education and Scientific Research through the Research Unit of Macromolecular Biochemistry and Genetics, Faculty of Sciences of Gafsa and the Integrated Physiology Laboratory, Faculty of Science of Bizerte.

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Vol. 12(51), pp. 7106-7111, 18 December, 2013
DOI: 10.5897/AJB2013.12098
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Could grape seed extract modulate nephritic damage induced by methomex in male rats?

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Accepted 30 August, 2013

Methomex (Metho) is classified as a carbamate insecticide. The present study was designed to examine the influence of grape seed oil (GSO) on the histopathological changes in methomex-induced kidney damage in male rats. Rats were divided into 6 groups, the first of which was considered as the control. The 2nd group was treated with 4 ml/kg GSO. Rats from 3rd and 4th groups were treated with Metho at dose level of 2.4 and 4.8 mg/kg, respectively. Rats from 5th and 6th groups pre-administered with GSO were treated with 2.4 and 4.8 mg/kg Metho, respectively. Metho administration caused destruction of the normal pattern of the renal tissue. These damages were encountered by the presence of some glomeruli appeared atrophy with distension of Bowman's space and degeneration of their parietal epithelial cells. The lumina of distal and proximal convoluted tubules contain hyaline casts of dead cells. The renal medulla showed dilated collecting tubules stuffed with red blood cells (RBCs). Pre-administration of GSO to Metho-induced rats revealed apparent normal renal parenchyma. The proximal convoluted tubules and collecting tubules appeared near to normal with their narrow lumen. Pre-administration with GSO exhibited that it had a protective effect against methomex-induced toxic effects in the kidney. The present study advocated using GSO in the daily diets.

Key words: Grape seed oil, methomex, kidney, histopathology.

INTRODUCTION

Carbamate insecticides are widely used in industry, agriculture and for public health purposes. Numerous incidents of acute carbamate poisoning have resulted from inhalation of sprays or contamination of crops or food (Mahgoub and El-Medany, 2001). This is due to the misuse of pesticides by concerned individuals and the absent or weak national controlling methos regarding the safe use of these chemicals (Ibitayo, 2006). Methomex (Metho) is classified by the Environmental Protection Agency (EPA) as a restricted-use pesticide (RUP) or class IB (highly hazardous) (Farré et al., 2002). Methomex, a derivative of carbamic acid, has been widely marketed since 1967 as a broad-spectrum insecticide to control ticks and spiders (WHO, 1996). Its application as an insecticide is highly effective against a wide variety of pests, particularly those that are resistant to organophosphorus. It induced significant toxicity against the treated rats (El-Fakharany et al., 2011) by exerting its toxic effect via peroxidative damage to the hepatic, renal and splenic cell membranes and induces DNA damage in these organs (El-Khawaga, 2005).

Grape seed (*Vitis vinifera*) extracts are known to have high antioxidant activity and contain numerous polyphenols. The polyphenols have been shown to have positive

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Abbreviations: RUP, Restricted-use pesticide; **GSO,** grape seed oil; **SOD,** superoxide dismutase; **GST,** glutathione S-transferase; **LPO,** lipid peroxidation; **HSCs,** haematopoietic stem cells.

effects on vascular injury; it is also known to have free radicals scavenging and antimutagenic activity (Çetin et al., 2008). Grape seeds are rich sources of monomeric phenolic compounds such as catechin, epicatechin and dimeric, trimeric and tetrameric proanthocyanidins (Shin et al*.,* 2010). These molecules possess a structure that confers on them an antioxidant property, which has been demonstrated to exert a novel spectrum of biological, pharmacological, therapeutic, and chemoprotective effects against oxygen free radicals and oxidative stress, which can be used as herbal remedies especially for controlling oxidative damages (El-Ashmawy et al*.,* 2007; Dulundu et al*.,* 2007). Several studies have indicated that extracts obtained from grape seed inhibit enzyme systems that are responsible for the production of free radicals, and that they are antimutagenic and anticarcinogenic (Pinheiro et al., 2010). For this reason, grape seed extract is widely consumed as a dietary supplement in addition to the chemotherapeutic agents in cancer treatment (Çetin et al., 2008). Grape seed extracts have been reported to possess a broad spectrum of pharmacological and therapeutic effects including anti-inflammatory activity and reduced apoptotic cell death (Ashtiyani et al., 2013). The present study, therefore, investigated the protective effect of grape seed extract against nephritic damage induced by methomex in male rats.

MATERIALS AND METHODS

Reagents and doses

Methomex was obtained from Agriculture Pesticides Laboratory, Agriculture, Research Center, Giza, Egypt and used in the present study (methomex LD_{50} = 48 mg/kg. body weight orally) according to the study of Thomson (1992). Grape seed oil (GSO) was obtained from the Unit of Squeeze and extraction of National Oils in National Research Center, Dokki, Cairo, Egypt. Grape seed oil was administered to rats at a dose level (4 ml/kg b.w.) according to the study of Maheswari and Rao (2005).

Experimental animals

Adult male albino rats of the *Rattus rattus* strain weighing 100±10 g were obtained from the Egyptian Organization for Vaccine and Biological Preparations at Helwan, Egypt. They were housed in a controlled environmental room, with a 12 h light/dark cycle. The animals were classified into six groups (7 rats each).

Experimental design

The duration of the present study was eight weeks. Rats were divided into 6 groups. The 1st group served as the control; these received a daily oral administration of saline by gastric tube. The 2nd group was orally administered GSO (4 ml/kg). The 3rd and 4th groups were given daily oral dose of 1/10 and 1/20 of LD_{50}/day of methomex, respectively. Rats belonging to the 5th and 6th groups were pre-administered with GSO, then after 2 h, rats received oral dose of 1/10 and 1/20 of LD_{50}/day of methomex, respectively. At the end of treatment period, rats were anaesthetized by ether and renal tissue samples were collected for histological and histochemical investigations.

Histopathological studies

For light microscopic investigations, renal tissue specimens were fixed in 10% neutral buffered formalin, dehydrated in alcohol series, clearing in xylol and embedding in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin according to the method of Humason (1979) and examined under a photomicroscope.

RESULTS

Sections of kidney from control rats illustrated that preserved architecture of the renal tissue appeared in the preserved renal parenchyma, rounded glomeruli, distal convoluted tubules with wide lumen and proximal convoluted tubules with narrow lumen lined by cuboidal epithelium in the renal cortex (Figure 1) and the renal medulla showed normal collecting tubules (Figure 2). Sections from kidney tissue of rats treated with 4 ml/kg GSO showed no histopathological changes when compared with control animals.

The effects of methomex (Metho) with both doses on nephritis damage were evaluated by histopathologic examination of the kidney sections by H&E staining. In contrast to the normal group of rats, the administration of Metho caused extensive disruption of tissue architecture. These disturbances were more obvious in the higher dose. Oral administration of Metho (1/20 LD_{50}) caused destruction of the normal pattern of the renal tissue. These damages were encountered by the presence of hypertrophy of glomerular tuft, thickening of parietal layer of Bowman's capsule as well as focal interstitial nephritis, focal area of mononuclear cellular infiltration is clearly seen, the lumina of renal tubules containing hyaline cast and cellular debris (Figure 3). Lymphocytic infiltrations, tubulointerstitial nephritis which means necrosis of tubular cells and necrosis in the interstitial cells, also the renal tubules appeared with pyknotic nuclei (Figure 4). Kidney sections from rats administered with Metho (1/10 LD_{50}) showed necrosis of renal tubules, atrophy of glomerular tuft, distension of Bowman's space and destruction of the renal tubules (Figure 5). Congestion of blood vessel, necrobiosis of renal tubular epithelium with pyknosis of their nuclei and swelling with multivacuolations of the cytoplasm with obliteration of their lumina and focal area of severe haemorrhage was observed (Figure 6). Shrunken renal corpuscle and widened Bowman's space, degeneration of the parietal epithelial cells of Bowman's capsule was also observed. The lumina of distal and proximal convoluted tubules contain hyaline casts of dead cells and congested blood vessel can be also detected (Figure 7). The renal medulla showed dilated collecting tubules stuffed with R.B.Cs. (Figure 8).

The effects of grape seed oil (GSO) on methomex-induced kidney damage were evaluated by histopathologic examination of the kidney sections by H&E staining. The treatment of Metho caused extensive necrosis, vacuolar degeneration and disruption of renal tissue architecture.

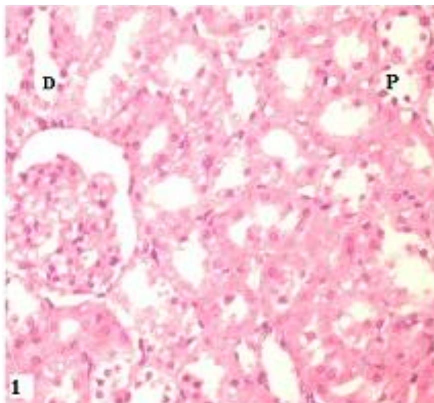

Figure 1. Photomicrograph of the renal cortex from control rat, showing the normal histological structure of renal parenchyma, rounded glomeruli, distal convoluted tubules with wide lumen (D) and proximal (P) convoluted tubules with narrow lumen lined by cuboidal epithelium. (H-E, X 400).

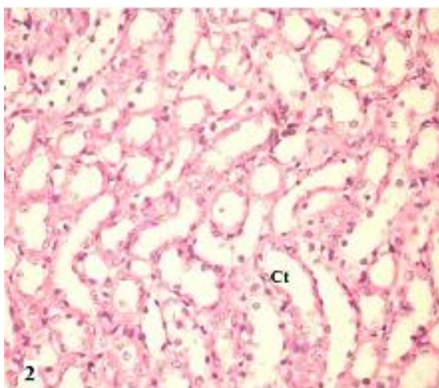

Figure 2. Photomicrograph of the renal medulla from control rat showing normal collecting (Ct) tubules. (H-E, X 400).

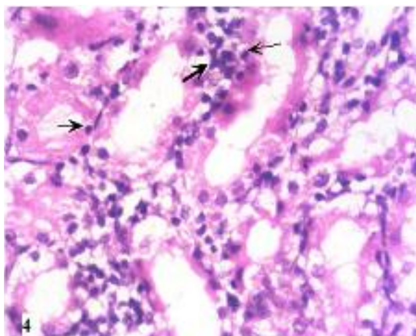

Figure 4. Photomicrograph of the kidney section from rat administered with 1/20 **LD_{50}** Metho, showing tubulointerstitial nephritis and the renal tubules appeared with pyknotic nuclei (thin arrows), beside the presence of lymphocytic infiltrations (thick arrow). (H-E, X 400).

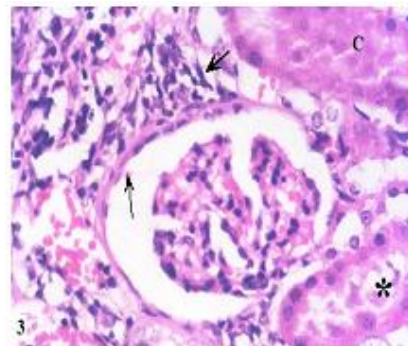

Figure 3. Photomicrograph of the renal cortex, from rat treated with 1/20**LD_{50}** Metho showing hypertrophy of glomerular tuft, thickening of parietal layer of Bowman's capsule (thin arrow) as well as focal inter-stitial nephritis, focal area of mononuclear cellular infiltration is clearly seen (thick arrow) notice: the lumina of renal tubules containing hyaline cast (C) and cellular debris (*). (H-E, X400).

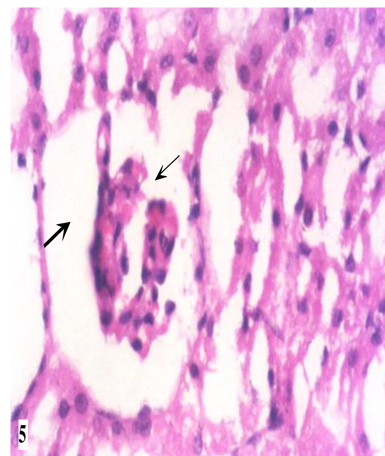

Figure 5. Photomicrograph of the renal cortex, from rat administered with 1/10 **LD_{50}** Metho, showing necrosis of renal tubules, atrophy of glomerular tuft (thin arrow), distension of Bowman's space (thick arrow) and destruction of the renal tubules. (H-E, X 400).

rats. In this study, pre-administration of GSO protected the Renal tissue from injuries and improved the renal lesions where encountered in the renal tissue as a result of Metho administration. Pre-administration of GSO to Metho-induced rats 1/20 LD_{50} (mg/kg), kidney sections revealed slight hypertrophy and vacuolation of glomerular tuft with slight distension of Bowman's capsule, also the lumen of the proximal convoluted tubules appeared filled with debris (Figure 9). The renal medulla, showed the normal structure of the collecting tubules (Figure10). Kidney sections from rats administered with GSO + 1/10

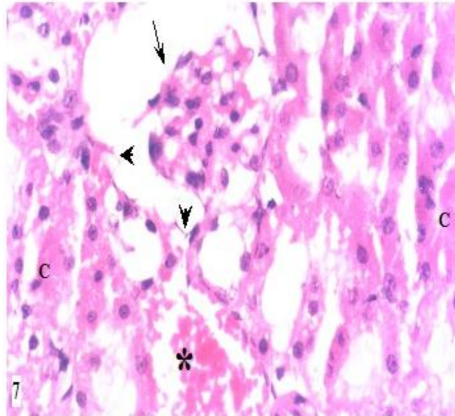

Figure 7. Photomicrograph of the renal cortex from rat treated with 1/10 **LD_{50}** Metho showing shrunken renal corpuscle (arrow) with widening of the urinary space, degeneration of the parietal epithelial cells (head arrow) of Bowman's capsule. The lumina of distal and proxy-mal convoluted tubules contain hyaline casts (C) of dead cells. Congested blood vessel can be also detecting (*). (H-E, X 400)

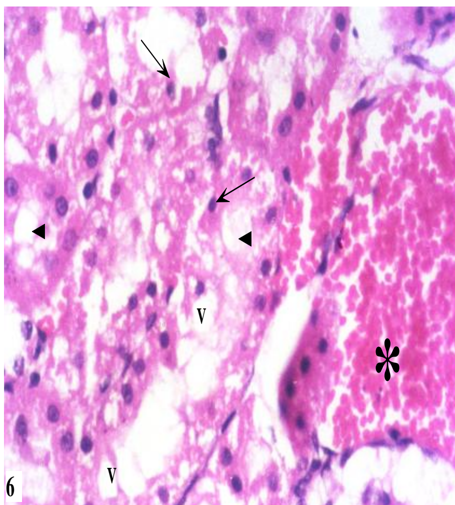

Figure 6. Photomicrograph of the renal cortex, from rat treated with 1/10 **LD_{50}** Metho showing congestion of blood vessel, necrobiosis of renal tubular epithelium with pyknosis (thin arrow) of their nuclei and swelling with multi vacuolations of the cytoplasm (V) with obliteration of their lumina (head arrows) and focal area of severe haemorrhage is observed (*). (H-E, X 400).

LD_{50} mg/kg Metho revealed apparent normal renal parenchyma, but still glomeruli appeared with slight hypertrophy. The proximal convoluted tubules appeared near to normal with their narrow lumen (Figure 11). The renal medulla delineated near to normal appearance of the collecting tubules as appeared in Figure 12.

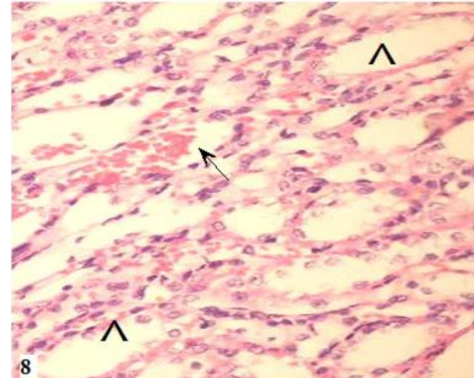

Figure 8. Photomicrograph of the renal medulla from rat treated with 1/10 **LD_{50}** Metho, showing dilated (^) collecting tubules stuffed with R.B.Cs. (arrow) (H-E, X400).

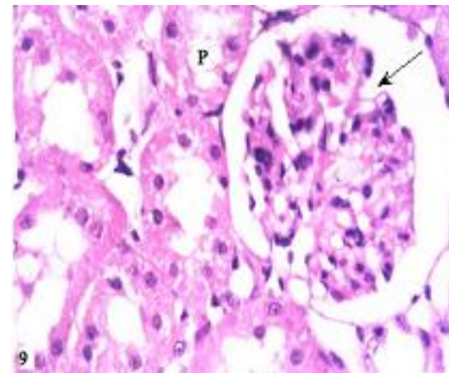

Figure 9. Photomicrograph of the renal cortex, from rat treated with GSO+1/10 **LD_{50}** Metho showing slight hypertrophy and vacuolation of glomerular tuft with slight distension of Bowman's capsule, note the lumen of the proximal convoluted tubules appeared filled with debris (P). (H-E, X400).

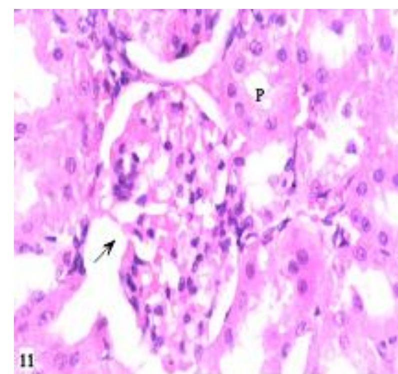

Figure 11. Photomicrograph of the renal cortex, from rat treated with GSO+1/20 **LD_{50}** Metho showing apparent normal renal parenchyma, but still glomeruli appeared with slight hypertrophy. The proximal convoluted tubules appeared near to normal with their narrow lumen (P) (H-E, X 400).

DISCUSSION

Methomex (Metho) application as an insecticide is highly effective against a wide variety of pests, particularly those that are resistant to organophosphorus (Farré et al., 2002). Metho exerts its toxic effect via peroxidative damage to the hepatic, renal and splenic cell membranes and induces DNA damage in these organs (El-Khawaga, 2005). Kidneys are responsible for the elimination of metabolic waste and the control of the amount and composition of the body fluids. Nephrotoxicity can result in systemic toxicity causing decreased ability to excrete

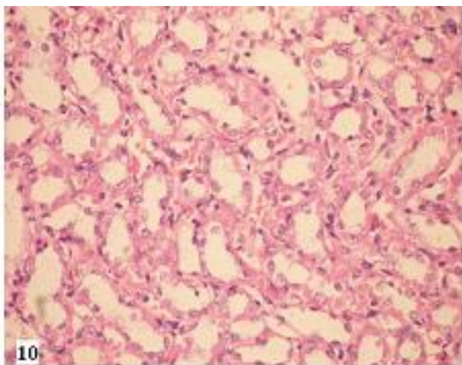

Figure 10. Photomicrograph of the renal medulla, from kidney rats administered with GSO+1/10 **LD_{50}** Metho showing the normal structure of the collecting tubules (H-E, X 400).

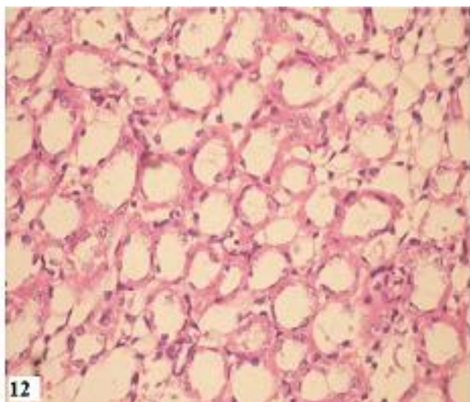

Figure 12. Photomicrograph of the renal medulla, of kidney section from rat administered with GSO+1/10 **LD_{50}** Metho showing near to normal appearance of the collecting tubules. (H-E, X 400).

body wastes, inability to maintain body fluid and electrolyte balance and decreased synthesis of essential hormones (For example, erythropoietin) (Finn 1977; Laurent et al., 1988).

The histological disturbances in the renal tissue in this study come in accordance with the results obtained by Radad et al. (2009). The authors reported that exposure of rats to Metho (2 mg/kg) markedly affected glomeruli, tubules and interstitium. Glomeruli appeared swollen Bowman'sspaces.Glomerularswellingwasprimarilycaused by congestion of glomerular capillaries and thickening of glomerular basement membranes. There were also mild proliferation of the glomerular epithelial cells and thickening of the Bowman's capsule and periglomerular fibrosis. Renal tubular epithelium appeared swollen and sometimes showed hyaline droplet degeneration and certain degree of necrobiotic changes. In some cases, renal tubules showed dysplastic changes characterized by abnormal mitotic figures and nuclear pleomorphism. There was also fibroblastic proliferation in the interstitium in some cases. Metho treated rats showed histopathologic changes in the kidney, and spleen of male and female rats. Similarly, enzymatic alterations of acetyl cholinesterase and liver glucose-6 phosphate dehydrogenase were also observed (Fayez and Bahig, 1992).

In the kidney, Metho treatment damaged the glomeruli, the tubules and the interstitium. Similarly, Nariman et al. (1995) and Selmanoglu et al. (2001) observed proliferation and swelling of glomerular endothelial cells and tubular degeneration, mononuclear cell infiltration and fibrosis in thiodicarb and carbendazim treated rats, respectively. Dysplastic changes seen in the tubules of some methomex-treated rats are of great concern. Together with increased frequency of normal mitosis in the liver, they might suggest that Metho is a potentially carcinogenic substance. However, there was no evidence of carcinogenicity in both rats and mice fed Metho for 2 years (EPA, 1987). Methomex was found to be potentially toxic to liver, kidney, lungs, spleen and testicles when applied repeatedly at a dose of 2 mg/kg. The observed renal damages could predispose to hepatic insufficiency and renal failure in exposed individuals (Radad et al., 2009).

The nephritic damage may appear due to the oxidative damages as a result of Metho administration. El- Khawaga (2005) and Mansour et al. (2009) showed that Metho decreased superoxide dismutase (SOD) and glutathione S-transferase (GST) activities and increased the level of lipid peroxidation (LPO) as well as the percentage of haemolysis. The response occurred in a concentration-dependent manner. The study suggested that methomyl has the capability to induce oxidative damage as evidenced by increasing LPO and perturbations in various antioxidant enzymes.

Pre-administration of GSO with Metho exhibited that GSO had a protective effect in preserving the architecture of the renal tissue. GSO is an extract by-product obtained from the grape seed and it contains a variety of biologically active species used for protection against oxidative stress induced by free radicals and ROS (Baiges et al., 2010; Ashtiyani et al., 2013). In relation to their polyphe-

nol compounds, GSO contains mainly flavonoids, all involved in ameliorating the oxidative stress *in vitro* and *in vivo* through their ability to balance the oxidant-antioxidant status (Sehirli et al., 2008). The damaged renal are potent sources of reactive oxygen intermediates and these compounds exert paracrine stimulation of stellate cells. Therefore, the hepatoprotective effects of GSO may decrease paracrine stimuli, which lead to hepatic fibrosis via activated haematopoietic stem cells (HSCs) (Shin et al., 2010).

Grape seed extract has a protective effect on oxidant-induced production and deposition of extracellular matrix components, which results in hepatic fibrosis (Dulundu et al., 2007). Furthermore, GSO treatment reversed all the injury parameters and the levels of inflammatory mediators while protecting the liver tissue against reperfusion-induced oxidative injury (Sehirli et al., 2008). In the same line, El-Ashmawy et al. (2007) concluded that grape seed procyanidin extract are useful herbal remedies, especially for controlling oxidative damages, by enhancing the expression profile of copper/zinc-superoxide dismutase (Cu/Zn-SOD), an enzyme that defends against oxidative stress (Puiggrãs et al., 2009). The GSO along with MTX-administration (used to treat cancer and some inflamematory diseases) significantly reversed these parameters toward to near normal. These results indicated that GSO could reduce hepatic and nephritic damage induced by MTX-treatment in young rats therefore having free radical scavenging (Pinheiro et al., 2010).

Conclusion

Methomex administration caused extensive destruction to the renal tissue. This damage was more pronounced in the higher dose which may cause renal failure. However, co-administration of the extracts of protective plants resulted in minimizing the deleterious effects of methomex toxicity on male renal tissues. It may be concluded that grape seed oil is useful as a herbal remedy, especially for controlling oxidative damages.

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African Journal of Biotechnology Vol. 12(51), pp. 7124-7132, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB09.1489
ISSN 1684-5315 ©2013 Academic Journals

Full Length Research Paper

Isolation, characterization and antimicrobial activity of *Streptomyces* strains from hot spring areas in the northern part of Jordan

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Accepted 20 April, 2010

A total of 30 *Streptomyces* isolates (28 from soil and 2 from water) were isolated and purified from hot-springs areas in the northern part of Jordan. Four strains were thermopile. They grew at 45 and 55°C but not at 28°C. Strains were described morphologically on four different media: on glycerol yeast extract, oatmeal, yeast malt-extract and starch casein agar. White and grey color series were the most frequent series on all media. The results showed that glycerol yeast extract and starch casein were the best media for sporulation. And yeast malt-extract was the best medium for the production of soluble pigment. Physiological and biochemical tests showed that the highest number of *Streptomyces* isolates were able to hydrolyze tyrosine was 26 (87%). This was followed by 25 (83%) for starch, 24 (80%) for urea, 21 (70%) for casein and 10 (33%) for gelatin. Twenty two (73%) strains showed the ability to reduce nitrate and 8 (27%) strains produced melanin. Carbon source utilization showed that 26 (87%) strains were able to utilize L- arabinose, 25 (83%) strains were able to utilize meso-inositol, 8 (27%) strains were able to utilize D-sorbitol, 18 (60%) strains were able to utilize D-mannitol, 28 (93%) strains were able to utilize L-rhamnose and all isolates exhibited the ability to utilize D-fructose and D- glucose. The ability to exhibit antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* was detected among 20 and 26% of the isolates, respectively, while the ability to exhibit antifungal activity against *Candida albicans* was detected among 23% of the isolates. Molecular identification of the 8 antibiotics producers was carried out by PCR technique using two sets of primers specific to *Streptomyces* 16S rDNA gene sequences; strepB/strepE and strepB/strepF which amplified 520 and 1070 bp, respectively. All these antibiotic producer isolates showed positive results for the genus *Streptomyces* specific primers.

Key words: Characterization, streptomyces, antimicrobial activity, hot springs, thermophile, PCR.

INTRODUCTION

Since the discovery of penicillin from the filamentous fungus, *Penicillium notatum,* by Fleming in 1929 and the observation of the broad therapeutic use of this agent in the 1940s, the so-called "Golden Age of Antibiotics", many countries around the world have developed intensive programs to increase the number of described antibiotics or to find new one's (Abussaud, 2000; Cragg and Newman, 2005).

In spite of the large number of antibiotics that have been discovered since that time, a large number of pathogenic bacteria have became resistant to antibiotics in common use (Mellouli et al., 2003; Cirz et al., 2005). As a result of the increasing prevalence of these antibiotic-resistant pathogens and the pharmacological limitations of the present antibiotics, searching for new antibiotics or modification of the present types has become an urgent focus for many researches (Rintala, 2001; Sahin and Ugur, 2003).

Filamentous bacteria belonging to the genus *Streptomyces* are well-known as the largest antibiotic-producing genus in the microbial world discovered so far (Taddei et al., 2006; Jayapal et al., 2007). Most *Streptomyces* and other Actinomycetes produce a diverse array of antibiotics including aminoglycosides, anthracyclins, glycolpeptides, β-lactams, macrolides, nucleosides, peptides,

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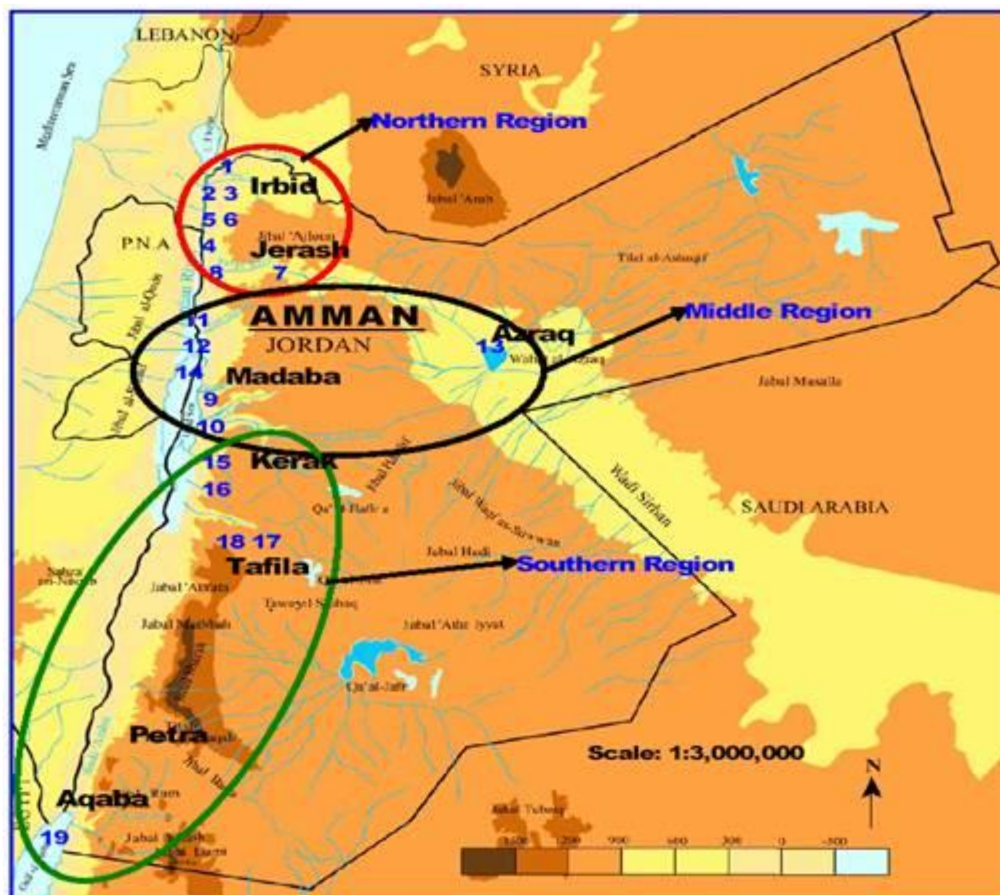

Figure 1. Distribution of hot-springs in in northern region of Jordan. 1: Al Hammah; 2: Ashouneh; 3: Abu Dablah; 4: Waggas; 5: Al Mansheyyah; 6: Abu Ziad; 7: Jerash; 8: Deir Alla.

polyenes, polyethers and tetra-cyclines. They produce about 75% of commercially and medically useful antibiotics (Mellouli et al., 2003; Sahin and Ugur, 2003).

The genus *Streptomyces* proposed by Waksman and Henrici in 1943, are a Gram-positive, aerobic, filamentous soil bacteria, produce an extensive branching substrate and aerial mycelium bearing chains of arthrospores. The substrate mycelium and spores could be pigmented, but also diffusible pigments could be produced. *Streptomyces* have high G+C (69 - 78%) content in their DNA and their cell wall is characterized as Type I (Lechevalier and Lechevalier, 1970; Williams et al., 1989; Rintala, 2001).

In the course of screening for new antibiotics, attention has primarily been concentrated to isolate *Streptomyces* from soil. Most recently, attention has been focused on greater diversity of organisms, those which are considered "rare", those which are difficult to isolate and/or culture and those which grow under extreme conditions such as thermophiles, acidophiles, halophiles etc (Yallop et al., 1997; Thakur et al., 2007).

Mesophilic *Streptomyces* are usually cultivated at temperature from 22-37°C while thermophilic *Streptomyces* grow between 25 and 55°C, they grow quite well at 50°C (Kim et al., 1999; Rintala, 2001). These organisms are useful as producer of antibiotics, enzymes and other bioactive metabolites because of their rapid autolysis of mycelium (Xu et al., 1998).

In continuing our screening program for *Streptomyces* flora in Jordan (Abussaud, 1996; Abussaud, 2000), we tend our attention to isolate *Streptomyces* strains from new locations and conditions such as hot-springs areas and test their capability to produce antimicrobial substances in order to look for the possibility of finding novel antibiotics. We started with 4 locations in the northern part of Jordan: Alshouneh, Waggas, Almansheyyah and Deir Alla springs (Figure 1).

MATERIALS AND METHODS

Collection of sample

A total of 12 soil samples and 12 water samples were collected from four different hot spring areas (Figure 1) Alshouneh, Waggas, Al-Mansheyah and Deir Alla. Six samples (3 soil samples and 3

water samples) from each location were collected as follows.

Water samples from the spring water column

About 2 l of water have been collected in sterile container, closed immediately and stored in ice box, in the presence of ice pads until shipped to the laboratory for analysis.

Soil samples

Soil samples were taken from sites along water streams at a depth of 10 cm, after removing approximately 3 cm of the soil surface. Samples were placed in polyethylene bags, closed tightly and stored in ice box as previously described. Physical factors such as temperature of water were directly measured at the sampling site by using a thermometer (Brannan co. England). The pH was also measured by using pH indicator paper (Whattman co. England).

Isolation of *Streptomyces* strains

Soil samples

Soil samples were analyzed following a modification of the procedure of Abussaud and Saadoun (1991): one gram of soil was suspended in 100 ml sterile distilled water, shaked in a reciprocal shaker at 190 rpm for 30 min, and then allowed settling. Serial dilutions (10^{-1} to 10^{-6}) were made. A 0.1 ml of each dilution was pipetted and spread evenly over the surface of Starch Casein (SCM) agar plates (g/l): (starch 10, casein 0.3, $NaNO_3$ 2, K_2HPO_4 2, NaCl 2, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01 and agar powder 20, pH = 7.2) supplemented with cyclohexamide (50 ug/ml) and filter-sterilized rifampicin (0.5 ug/ml) using a sterile L-shaped glass rod. These plates were incubated at 28 and 55°C until good growth occurred. Dilutions that gave about 100 colonies per plate were chosen for the isolation of *Streptomyces* isolates.

Water samples

(a) 100 ml of each water sample was filtered through a Millipore membrane (0.22 - 0.45 µm pores, Sartorious. Germany), after that, the membranes were transferred to the surface of Starch Casein (SCM) plates and incubated at 55 and 28°C for 7 - 14 days.
(b) 10 ml of each water sample was inoculated into 90 ml starch casein (SCM) broth and Tryptone-yeast extracts broth (TYE): (g/l) Tryptone 5, yeast extract 3, pH = 7.2) in 250 ml flask, then incubated for 24 h with shaking; (225 rpm, HT shaker. Germany), at 55 and 28°C, after that about 200 µl were transferred to starch casein (SCM) plates. These plates were incubated following the previous procedure of incubation.
(c) A combination between the previous two steps was done to ensure our results, in details: 100 ml of each water sample was filtered through a Millipore membrane; the membranes were transferred to 100 ml starch casein (SCM) broth in 250 ml flask, incubated at 55 and 28°C for 24 h with shaking at 225 rpm. Then aliquots (0.2 ml) of 10^{-2} to 10^{-5} ten-fold serial dilutions were spread over the surface of dried SCM agar plates.

The plates were incubated as described previously at 55 and 28°C for 7 - 14 days.

Streptomyces colonies were then picked up and transferred to yeast malt-extract agar (g/l) (yeast-extract): (3, malt-extract 3, peptone 5, glycerol 10 ml/l, agar 20, pH = 7.0 ± 0.2), starch casein agar plates, glycerol yeast-extract agar plates (g/l): yeast-extract 2, K_2HPO_4 1, glycerol 5 ml/l, agar 20, pH = 7.2 and oatmeal agar for further purification.

Maintenance media

After purification, *Streptomyces* isolates were maintained as suspend-sions of spores and mycelia fragments in 20% glycerol (v/v) at -20°C.

Morphological characterization

Morphological characterization of *Streptomyces* isolates were done according to the ISP recommendations (Shirling and Gottlieb, 1966). A pure culture of each isolate was picked up and transferred to grow on four different media: Yeast Malt-Extract (YME) agar (ISP2), Starch Casein (SCM) agar, Glycerol Yeast-Extract (GYE) agar and Oatmeal agar (ISP3) for 5 - 10 days at 55°C for themophilic *Streptomyces* and at 28°C for mesophilic *Streptomyces*. Then, colors of the aerial and substrate mycelium and those of the soluble pigments were examined.

Cultural, physiological and biochemical tests

Determination of the cultural, biochemical and physiological characteristics was carried out according to Williams et al. (1983), Brown et al. (1999) and Babcock (1979).

Growth temperature

Also the ability of the isolates to grow at different temperatures (28, 37, 45 and 55°C) was studied.

Carbon source

The ability of the strains to use different carbon sources was determined according to the ISP recommendation (Shirling and Gottlieb, 1966); seven sugars were used as a carbon sources: L-arabinose, Meso-inositol, D-sorbitol, D-mannitol, L-rhamnose, D-fructose and D- glucose, the results were determined after 14 days incubation at optimum temperatures.

Antimicrobial activity

Antimicrobial activity on agar media

Antimicrobial activity was tested by growing *Streptomyces* strains on agar plates until good and thick growth occurred. Then agar block from these plates were transferred to plates previously seeded with the indicator organisms (*Escherichia coli, Staphylococcus aureus* and *Candida albicans)*. The plates were incubated at appropriate temperature for each indicator. Activity was measured as inhibition zone in millimeters around the agar block (Abussaud and Saadoun, 1991).

Antimicrobial activity in broth media (antibiotics fermentation)

Spores from each 10 days-old culture of isolates grown on glycerol yeast-extract agar plates were used to inoculate 100 ml of glycerol yeast-extract broth into 250-ml Erlenmeyer flasks. These cultures were grown in a rotary shaker at 150 rpm, 28°C, for eight days. Glycerol Yeast-Extract medium was preliminarily tested and was found to be suitable for antibiotic production by our isolates.

During fermentation, 2 ml sample of each culture were collected in eppendorf tube every 2 days and tested for antibiotic activity against *E. coli, S. aureus* and C. *albicans*. Activity was tested by

Table 1. Primers used for detection of *Streptomyces* in PCR reactions.

Primer	Sequence (5′ 3′)	Primer target sequence	Amplicon length (bp)	Reference
strepB(F) /strepE(R)	ACAAGCCCTGGAAACGGGGT CACCAGGAATTCCGATCT	16S rDNA	520	Rintala et al., 2002
strepB(F) /strepF(R)	ACAAGCCCTGGAAACGGGGT ACGTGTGCAGCCCAAGACA	16S rDNA	1070	Rintala et al., 2002

Table 2. Physical properties of hot springs.

Location	Temperature (°C)	pH
Alshouneh (Sh)	54	6.5
Waggas (Wg)	47	8
Almansheyyah (Mn)	48	8
Deir Alla (DA)	36	7

preparing wells into agar plates previously seeded with the indicator organism and transferring 100 microliters from the centrifuged fermentation broth into these wells.

Molecular identification of antibiotics producer isolates

Extraction of genomic DNA from pure culture

Genomic DNA was isolated from the isolates using a bacterial genomic DNA isolation kit (Biobasic Inc. Canada). One separate colony from each bacterial isolate was inoculated into 10 ml nutrient broth and incubated overnight at 28°C. Then 1 ml was taken and centrifuged at 14000 rpm for 15 min at room temperature, the pellets were suspended in 200 µl cold TE (10 mM tris base, 1 mM EDTA, pH 8.0) buffer and 400 µl digestion solution and mixed well, then a 3 µl of Proteinase K were added and incubated at 55°C for 2 h.

After incubation, 260 µl of 100% ethanol were added to the solution, and then the whole mixture was applied into 2 ml EZ-10 column provided with the Kit and centrifuged at 8,000 rpm for 1 min. The pellets were resuspended again with 500 µl of wash solution and centrifuged at 8,000 rpm for 1 min, this step was repeated again.

After that, the column was placed in a clean microfuge tube and a 50 µl of elution buffer were added to the center of the column, incubated at 37°C for 2 min and finally centrifuged at 10,000 rpm for 1 min to elute the DNA.

Polymerase chain reaction (PCR)

PCR amplification of 16S rDNA was carried out in 50 µl volumes containing: 25 µl of Econo Taq PLUS GREEN 2X Master Mix, 0.25 µl (100 pmol) of each primer, 2 µl (10 ng) of DNA template and 22.5 µl DNAse free water, each primer pair has its program that will be mentioned later.

For each PCR reaction a negative PCR reaction tube was performed where no DNA template was added (not shown in figures) and all PCR reactions were performed in a Perkin Elmer DNA thermal cycler (Perkin Elmer 480).

Identification of bacterial isolates using StrepB/StrepE primer pair specific to genus Streptomyces 16S rDNA gene sequences

The primer pairs StrepB/StrepE (sequences listed in Table 1) amplified 520 bp fragments, nucleotides 139 - 657. The PCR was programmed as follows: after the initial denaturation for 5 min at 98°C, 30 cycles of denaturation (1 min at 95°C), primer annealing 40 s at 54°C and primer extension (2 min at 72°C) were performed. A final extension at 72°C for 10 min had followed.

Identification of bacteria isolates using StrepB/StrepF primer pair specific to genus Streptomyces 16S rDNA gene sequences

The primer pairs StrepB/StrepF and StrepB/StrepE amplified 1070 and 520 bp fragments, nucleotides 139 -1212. The PCR were programmed as follows: after the initial denaturation for 5 min at 98°C, 30 cycles of denaturation (1 min at 95°C), primer annealing 40 s at 58°C and primer extension (2 min at 72°C) were performed. A final extension at 72°C for 10 min followed.

Gel electrophoresis and photography

The PCR amplified products were separated on 1% w/v ultra-pure agarose powder in 1X TBE buffer (pH 8.3) at 100 V for 60 - 70 min using mini-gel set (Bio Rad,). Gels were stained with ethidium bromide (0.5 µg/ml) and analyzed using BioDocAnalyze (Biometra, Germany). A 250 base pair (bp) molecular weight marker was included on every gel.

RESULTS

Physical properties of hot springs

Physical properties such as temperature and acidity degree (pH) for each sampling location (water sample) are indicated in Table 2.

Isolation of *Streptomyces*

Thirty different bacterial isolates were isolated during this study. Six isolates were isolated from Deir Alla, only one from water (DA1-DA6); 8 isolates were isolated from Almansheyyah (Mn1-Mn8); 9 isolates were isolated from Alshouneh, only one from water (Sh1-Sh9) (two of these isolates were thermophilic they grow at_55°C) and 7 isolates were isolated from Waggas (Wg1 - Wg7); also two of these isolates were thermophilic; (Wg6 and Wg7).

Morphological characterization

The aerial mycelium color of the isolates ranged from red,

Table 3. Physiological and biochemical characteristics of *Streptomyces* isolate (number of positive/total in each site).

Location	Starch hydrolysis	Urea hydrolysis	Casein hydrolysis	L-tyrosine hydrolysis	Gelatin lequification	Nitrate reduction	Melanin formation
DA	4/6	6/6	6/6	6/6	1/6	4/6	1/6
Mn	7/8	6/8	7/8	7/8	3/8	6/8	3/8
Sh	9/9	7/9	6/9	7/9	4/9	6/0	3/9
Wg	5/7	5/7	2/7	6/7	2/7	6/7	1/7

Table 4. Ability of *Streptomyces* isolates to grow at different temperatures.

Location	28°C	37°C	45°C	55°C
DA	6/6	6/6	3/6	0
Mn	8/8	8/8	6/8	0
Sh	7/9	7/9	5/9	2/9
Wg	5/7	6/7	4/7	2/7

white, blue and green, grey to purple. While the substrate mycelium color shows narrower diversity. On the other hand, the isolates produced different pigments: yellow, pink and grey. These differences might reflect the diversity among the isolates.

On GYE medium, out of 30 isolates, 14 were white, 10 grey, 2 red, 2 green, 1 blue and 1 purple. On the other hand, 9 isolates were found to be producers of diffusible pigment, 7 of them produced brown color while the other produced a yellow pigment.

On Oatmeal medium, out of 30 isolates, 12 isolates were white, 9 grey, 2 pink, 1 brown, 1 purple and 1 red and 4 isolates failed to sporulate on this medium. 10 isolates produced diffusible pigment, 5 of them brown, 4 yellow and one isolates produced pink pigment.

On YME media, out of 30 isolates, 10 isolates were white, 7 grey, 1 green, 1 brown, 1 yellow and 10 of them were not able to sporulate. 12 isolates were able to produce diffusible pigment, 7 produced brown pigment and the rest produced yellow pigment.

On STC media, out of 30 isolates, 13 isolates were white, 10 grey, 3 pink, 2 purple, 1 green and 1 brown, only 3 isolates were able to produce diffusible pigment, 2 produced brown color and 1 produced red color.

Physiological and biochemical characteristics of *Streptomyces* isolates

As indicated in Table 3, 25 (83%), 24 (80%), 21 (70%), 26 (87%), 10 (33%), 22 (73%) and 8 strains (27%) showed the ability to degrade starch, hydrolyze urea, degrade casein, hydrolyze tyrosine, liquefy gelatin, reduce nitrate and produce melanin, respectively.

Also the ability of the isolates to grow at different temperatures (27, 37, 45 and 55°C) was examined. The results depicted in Table 4 showed that, out of 30 isolates only 4 (Thermophilic) isolates were able to grow at 55°C, 18 isolates included thermophilic isolates were able to grow at 45°C, 27 at 37°C and 26 at 27°C.

Carbon source utilization

Table 5 represents the ability of *Streptomyces* to utilize different carbon sources (7 different sugars were used). Out of 30 isolates, 26 (87%) isolates were able to utilize L- arabinose, 25 (83.%) isolates were able to utilize Meso-inositol, 8 (27%) isolates were able to utilize D-sorbitol, 18 (60%) isolates were able to utilize D-mannitol, 28 (93%) isolates were able to utilize L-rhamnose and all isolates exhibited the ability to utilize D-fructose and D-glucose.

Antimicrobial activity on agar media

Antimicrobial activity was tested against *E. coli, S. aureus* and *C. albicans.* Out of 30 isolates 8 were antibiotics producers. These isolates were assigned: Mn1, Mn3, Mn8, Sh1, Sh3, Sh6, Wg4 and Wg5. Six of them showed antibacterial activity against G-ve (represented by *E. coli*), all of them were active against G+ve bacteria (represented by *S. aureus*) and 7 of them were able to produce antifungal activity against *C. albicans*.

Table 6 represents the antimicrobial activity during a period of 8 incubation days. After 2 days, only 1 (12.5%) isolate was active against all tested microorganisms. After 4 days, 3 (37.5%) of the isolates were active against all tested microorganisms. After 6 days, 5 (62.5%) isolates showed activity, 40% of them were active against *E. coli*, all of them exhibited activity against *S. aureus* and 80% of them exhibited activity against *C. albicans.* After 8 days, 4 (50%) of the isolates exhibited the activity, 75% of them were active against *E. coli* and all of the rest were active against *S. aureus* and *C. albicans*.

Molecular identification of the bacterial isolates

Genomic DNA was isolated from the 8 antibiotic producers (Mn1, Mn3, Mn8, Sh1, Sh3, Sh6, Wg4 and Wg5) and from the positive control (*Streptomyces halstedii* ATCC

Table 5. Utilization of carbon sources (positive/total).

Location	Arabinose	Inositol	Sorbitol	Mannitol	Rhamnose	Fructose	Glucose
DA	5/6	5/6	3/6	4/6	5/6	6/6	6/6
Mn	8/8	5/8	1/8	5/8	8/8	8/8	8/8
Sh	7/9	8/9	2/9	6/9	9/9	9/9	9/9
Wg	6/7	7/7	2/7	3/7	6/7	6/7	7/7

Table 6. Antimicrobial activity *of *Streptomyces* isolates (on agar media).

Location	*E. coli*	*S. aureus*	*C. albicans*
DA	0	0	0
Mn	3/8	3/8	2/8
Sh	3/9	3/9	3/9
Wg	0	2/7	2/7

*Number of active isolates/total number.

10897). A large amount and good quality of genomic DNA was obtained from each bacterial isolates.

Two primer pairs were used in the PCR reactions to identify the bacterial isolates as a *Streptomyces* isolates (StrepB/StrepE and StrepB/StrepF). Using 16S rDNA StrepB/StrepE primer pair, all antibiotic producers showed positive results with 520 bp PCR amplification products (Figure 2)

Using 16S rDNA StrepB/StrepF primer pair all antibiotic producers and the control *S. halstedii* showed positive results with 1070 bp PCR amplification product (Figure 3).

DISCUSSION

Streptomyces represent an important source of biologically active compounds. They are used extensively in industry as producers of antibiotics, enzymes, enzyme inhibitors and antitumour agents. However, it is important to continue the screening for novel bioactive compounds as the number of microorganisms resistant to the existing antibiotics is growing every year. However, it is becoming increasingly difficult to discover new commercially useful secondary metabolites from common streptomycetes, thereby emphasizing the need to isolate, characterize and screen novel members of the genus *Streptomyces*. *Streptomyces* from under explored habitats are proving to be a rich source of new bioactive compounds, including antibiotics (Berdy, 2005; Okoro et al., 2009). Therefore, we decided to isolate S*treptomyces* strains from Jordanian hot springs and study their capability to produce antibiotic activity.

A total of 30 different bacterial isolates were recovered during this study from non-cultivated hot spring areas in Jordan. All bacterial isolates were typically *Streptomyces*; they grew on a range of agar media showing morphology typical of *Streptomyces*.

The majority of the isolates showed good growth on SCM agar medium. This medium seems to be specific and sensitive for *Streptomyces*, since it contains starch that most *Streptomyces* use as a carbon source and the basic minerals that are needed for good growth. In addition, its transparency facilitates colony observation. Earlier studies have shown the importance of the constituents of the screening media under which the producing microorganisms were cultivated (Williams et al., 1989; Rintala, 2001).

The number of *Streptomyces* isolates that were isolated from soil samples (28 isolates) was highly greater than that isolated from water samples (2 isolates), this may by due to the presence of organic matter that make *Streptomyces* abundant in soil, it is the dominant genus in the soil that gives it it's odor (Kutzner, 1986 ; Rintala, 2001).

Streptomyces have been isolated from fresh water as well as marine environments (Delabre et al., 1998), although, it has been a subject of debate. Many scientists considered *Streptomyces* to be part of the marine ecosystem, while many others failed to isolate *Streptomyces* from water samples and did not consider *Streptomyces* to be indigenous to the marine environments (Okazaki, 2006) and the debate point was whether they are indigenous, or have been washed off from the surrounding soils, so such studies could explain the presence of few *Streptomyces* strains in hot-springs water samples, since its water coming from under ground, surrounded by rocks not soils (Goodfellow and Simpson, 1987; Rintala, 2001).

All of these isolates fitted the genus description as reported by several studies (Shirling and Gottlieb, 1966; Kutzner, 1986; Williams et al., 1989). The color of the substrate mycelium and aerial spore mass was varied which reflect the diversity of *Streptomyces* isolates.

Msameh (1992) in his study on distribution and antibiotic activity of *Streptomyces* flora in Jordan reported that the white and grey color series showed the highest percentage of occurrence (43.6 and 28.3%, respectively). In the present study, 50% of the isolated *Streptomyces* isolates were from Alshoneh and Deir Alla (30 and 20%, respectively). White and grey color series had also the percentage (46 and 33.3%, respectively).

The comparison of the physiological and biochemical characteristics of the presented isolates with the actionmycetes as described in Bergey's Manual of Determinative

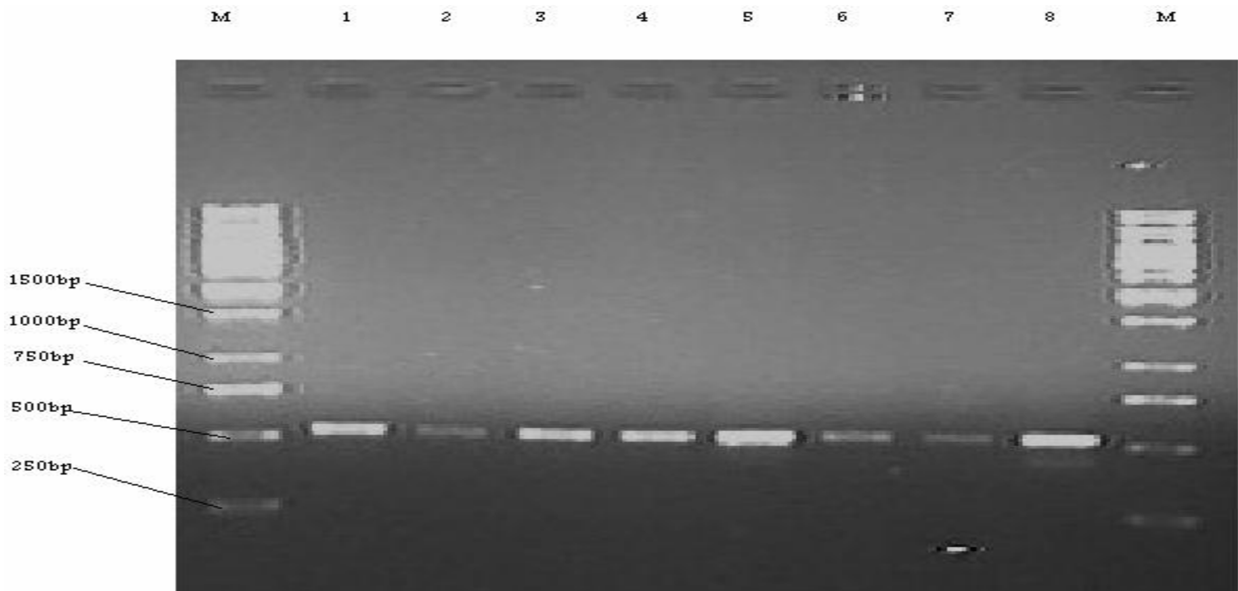

Figure 2. Agarose gel electrophoresis of PCR amplification products of genomic DNA isolated from *Streptomyces* pure culture using strepB(F)/strepE(R) of 16S rDNA gene. Lane M, 1 kb DNA ladder; lane 1, Mn8; lane 2, Sh3; lane 3, Mn1; lane 4, Sh6; lane 5, Wg5; lane 6, Wg4; lane 7, Sh1; lane 8, Mn3.

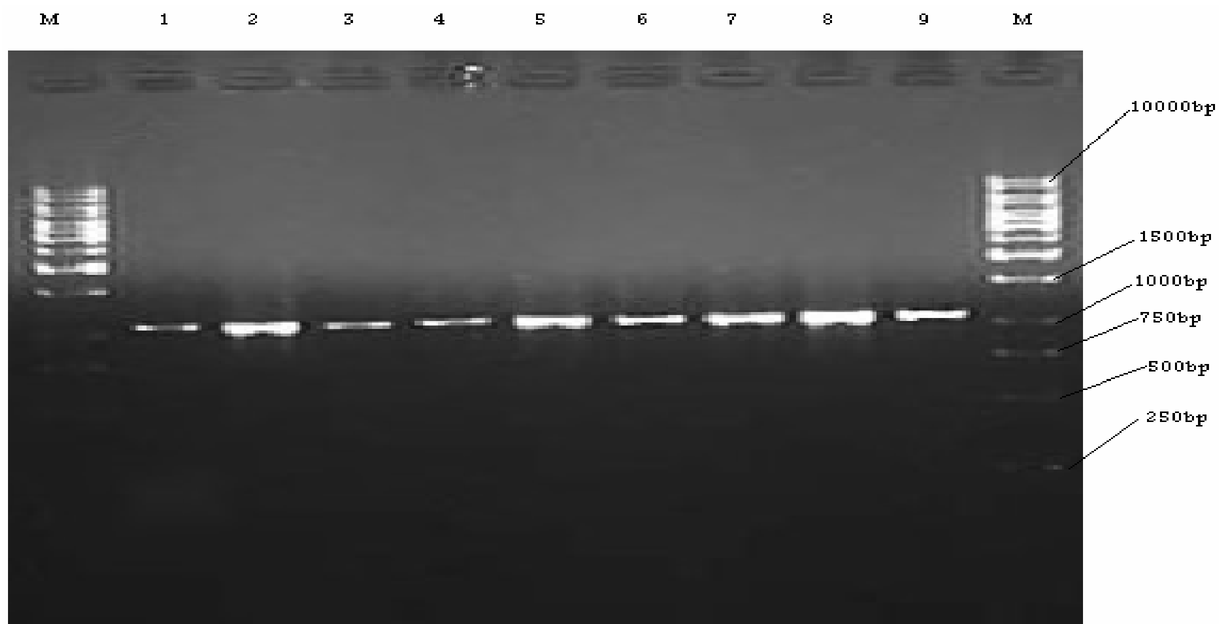

Figure 3. Agarose gel electrophoresis of PCR amplification products of genomic DNA isolated from *Streptomyces* pure culture using strepB(F)/strepF(R) of 16S rDNA gene. Lane M, 1 kb DNA ladder; lane 1, Mn8; lane 2, Sh3; lane 3, Mn1; lane 4, Sh6; lane 5, Wg5; lane 6, Wg4; lane 7, Sh1; lane 8, Mn3) and lane 9, *S. halstedii*.

Bacteriology determined that these isolates belongs to the genus *Streptomyces.*

Antibacterial activity and antifungal activity was observed in 8 (27%) and 7 isolates (23%), respectively. In former studies, it was shown that the isolation rate of *Streptomyces* with antimicrobial activity was higher than 40% (Lemriss et al., 2003) and in others less than 10% (Jiang and Xu 1996). This variation may be due to many factors example, soil type, climate, strain type and isolation methods. We found the best percentage (37.5%) of antibacterial and antifungal activity among Almansheya strains, followed by those from Alshouneh and Waggas

Table 7. Antimicrobial activity of antibiotics producers (in broth media).

Strain	2 days against			4 days Against			6 days against			7 days against		
	E. coli	*S. a*	*C. alb*	*E. coli*	*S. a*	*C. alb*	*E. coli*	*S. a*	*C. alb*	*E. coli*	*S. a*	*C. alb*
Mn1	-	-	-	-	-	-	-	-	-	-	-	-
Mn3	+	+	+	+	+	+	+	+	+	+	+	+
Mn8	-	-	-	-	-	-	-	+	-	-	-	-
Sh1	-	-	-	-	-	-	ND	+	+	+	+	+
Sh3	-	-	-	+	+	+	+	+	+	-	-	ND
Sh6	-	-	-	+	+	+	ND	+	+	+	+	+
Wg4	-	-	-	-	-	-	-	-	-	-	+	+
Wg5	-	-	-	-	-	-	-	-	-	-	-	-

E. coli: *Escherichia coli, S. a: Staphylococcus aureus*, *C. alb*: *Candida albicans.* + = Active, - = not active, ND = not determined.

areas 33.3 and 28.5%, respectively. No antibiotics producers were isolated from Deir Alla area.

The highest percentage of activity was recorded against Gram-positive bacteria followed by Yeast and Gram-negative bacteria. Some isolates did not show activity in liquid media. Out of the 8 active isolates on agar medium, only 5 (62.5%) isolates were found to exhibit antibacterial activity in liquid media (Table 7).

During the screening of the secondary metabolite, *Streptomyces* isolates were often encountered which show antimicrobial activity on agar but not in liquid culture (Thakur et al., 2007).

Molecular identification

Molecular identification was performed using polymerase chain reaction (PCR) which is currently used as a sensitive and specific detection method for micro-organisms (Rintala et al., 2002). The 16S rDNA gene was chosen as the target gene for the PCR primers in the PCR assay, aiming at the detection of the 8 antibiotic producers of the *Streptomyces* isolates. In this study two sets of primers strepB/strepE and strepB/strepF specific to 16S rDNA gene fragment were used to identify bacterial isolates; positive results were recorded for all bacterial isolates with amplification and corresponding to 520 and 1070 bp and thus, confirm that all antibiotics produce bacterial isolates belong to *Streptomyces* species.

In order to detect the presence of *Streptomyces* isolates in the water samples, the same two sets of primers were used to amplify the 16S rDNA gene collected from water samples; all samples exhibited negative results which indicated the inexistence of *Streptomyces* in these water samples.

In comparison between cultural and molecular methods for identification of *Streptomyces* isolates, we could say that molecular methods are more sensitive, rapid and not laborious in opposite to cultural methods that are laborious and time consuming.

ACKNOWLEDGMENT

This work was funded by the Deanship for Academic Research and Higher Studies at Yarmouk University in Irbid-Jordan.

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African Journal of Biotechnology Vol. 12(51), pp. 7133-7137, 18 December, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2679
ISSN 1684-5315 ©2013 Academic Journals

Full Length Research Paper

Effects of dietary conjugated linoleic acid, fish oil and soybean oil on body-fat deposition and serum lipid fractions in broiler chickens

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Accepted 27 October, 2011

An experiment was conducted on broiler chickens to study the effects of dietary fats rich in conjugated linoleic acid (CLA), fish oil (n-3 rich oil), soybean oil (n-6 rich oil), polyunsaturated fatty acids (PUFAs) alone or in dual mixtures, as well as palm oil as a more saturated fat on tissue fat deposition and serum lipid concentrations of broiler chickens. The fat included in the experiment's diets is a dose of 7% for single fats and 3.5 + 3.5% for the dual mixtures. The conjugate linoleic acid (CLA) supplement used in this study was LUTA-CLA 60, containing 60% CLA, so that dietary inclusion of 7 and 3.5% LUTA-CLA 60 were supplied with 4.2 and 2.1% CLA, respectively. The chickens fed diets containing palm oil, soybean oil or fish oil as the only dietary fat deposited more fat in breast tissue compared with other groups ($P < 0.05$). The highest fat in thigh tissue was observed in birds fed 7% fish oil ($P < 0.05$). The CLA containing diets resulted in fatter liver tissue ($P < 0.05$). The diets containing 7% fish oil effectively decreased the lipid content of chicken's liver ($P < 0.05$). The fish oil and soybean oil as n-3 and n-6 rich sources, respectively demonstrated a comparable reduction in the serum cholesterol and low density lipoprotein (LDL) concentrations ($P < 0.05$), while the 7% CLA diet increased serum high density lipoprotein (HDL) level ($P < 0.05$). The results of this study showed that dietary fish oil, and CLA effectively increased fat content of meat and liver tissues, respectively; and the dietary soybean oil and fish oil were more effective on reducing serum undesired lipoproteins, as compared with CLA.

Key words: CLA, PUFA, body fat deposition, serum lipids and broiler chickens.

INTRODUCTION

Chicken has been used as a suitable model for lipid metabolism studies, because dietary modifications, especially dietary fat type can change the chicken body composition. Broiler chickens are at risk of fatness and their liver synthesis and secrete large amount of triglyceride and lipoproteins (Griffin et al., 1991). CLA is a natural constituent of meat and dairy products from ruminants, originated from bacterial bio-hydrogenation in the rumen and its anti-lipogenic effects have been reported in different species (Ha et al., 1989). Fish oil is a rich source of n-3 PUFAs, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid which are well-known for their favorite effects on human health (Knapp, 1991). Soybean oil and palm oil are good causes of oils

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Abbreviations: CLA, Conjugated linoleic acid; **PUFAs,** polyunsaturated fatty acids; **LDL,** low density lipoprotein; **HDL,** high density lipoprotein.

rich in n-6 PUFAs and SFAs. The objective of the present study was to assess the effects of altering the dietary fat type with inclusion of CLA, fish oil, soybean oil or their dual mixtures as well as palm oil on breast, thigh and liver tissues fat deposition and serum lipid concentrations in broiler chickens.

MATERIALS AND METHODS

Animal management

A total of 560 Ross 308 make broiler chickens were used in this study. All chicks were fed on the same corn-soybean meal based starter diet for day 1 to 10 and then allocated to the experimental grower (day 11 to 28) and finisher (day 29 to 42) diets. Seven isocaloric and isonitrogenous diets were formulated and they contain: 7% soybean oil (7% SO), 7% LUTA-CLA 60 (CLA), 7% fish oil (FO), 3.5% LUTA-CLA 60+ 3.5% soybean oil (CLA + SO), 3.5% fish oil + 3.5% Soybean oil (FO + SO), 3.5% LUTA-CLA 60 + 3.5% fish oil (CLA + FO) or up to 12% palm oil (PO) (Table 1). The CLA supplement of LUTA-CLA 60 was prepared and supplied by BASF Company (Germany) and it contained 30% isomer 9c, 11t and 30% isomer 10t, and 12c of conjugated linoleic acid plus a large amount of oleic acid. However, the dietary inclusion of 7 and 3.5% LUTA-CLA 60 were supplied by 4.2 and 2.1% CLA isomers, respectively. The higher palm oil content in the latest diet was due to the lower metabolizable energy content of palm oil.

At 42 days of age, following feed withdrawal overnight to permit gut clearance and before slaughtering, 5 ml of blood samples were harvested from the wing vein of two male chicks selected randomly per pen. Serum was harvested with centrifugation at 3000 rpm for 10 min and then the serum was frozen for future analysis of serum lipid and lipoprotein concentrations, and was stored at -20°C. On day 42, two male birds per cage were weighed alive, and then slaughtered after an overnight withdrawal period. Samples of Liver, breast and thigh muscles were collected and stored at -20°C until lipid analysis. Lipids content of liver, breast and thigh tissues were measured by the method of Folch et al. (1957). The serum lipoprotein fractions were measured using an autoanalyzer (HITACHI 902 automatic autoanalyzer).

Individual chicks were the experimental units for tissue and serum analysis data. Data sets of completely randomized design with seven treatments, and eight replicate (four replicate pens and two samples per pen), were compared across the treatments using the one-way analysis of variance (ANOVA) procedure. Significant means were then elucidated using the Duncan multiple range tests. All statistical tests were conducted at 95% confidence level using the SAS program (SAS, 9.1, 2002).

RESULTS

Table 2 shows the lipid content of breast, thigh and liver tissues of broiler chickens fed different dietary fats. For lipid content of breast tissue, the treatments formed two distinct groups so that the chickens fed PO, SO or FO diets had significantly higher breast tissue lipid than those fed on the other diets ($P < 0.05$). The highest lipid deposition in thigh tissue was observed in birds fed FO diet, such that the difference was significant when compared with the birds fed CLA, CLA + SO or FO + SO diets ($P < 0.05$).

The lipid content of breast tissue in chickens fed the CLA + FO diet was closer to those fed on CLA than FO diet ($P < 0.05$), which suggest a dominant effect for dietary CLA in this respect. Combination of soybean oil with CLA or fish oil in diets resulted in less fat deposition in both breast and thigh tissues compared with the SO diet ($P < 0.05$).

The CLA containing diets resulted in more fat deposition in the liver of broiler chickens ($P < 0.05$), except for CLA + FO diet, which resulted in a comparable lipid deposition in liver to the PO and FO + SO diets. The FO and then the SO diets effectively decreased chickens liver lipid content ($P < 0.05$).

The serum lipid fractions of experimental birds is shown in Table 3. The serum triglyceride and very low density lipoprotein (VLDL) levels of birds fed FO diet were lower than those of birds fed one of the CLA containing diets (CLA, CLA + FO and CLA + SO) as well as the FO + SO diet ($P < 0.05$). The serum total cholesterol concentration of birds fed FO or SO diets were lower than other treatments ($P < 0.05$), except for the birds fed the CLA diet. The FO diet reduced the serum LDL concentration as compared with the other diets ($P < 0.05$) with the exception of SO diet.

There was no difference in serum composition between treatments containing soybean oil in combination with CLA or fish oil (CLA + SO vs. FO + SO). The serum glucose level of birds fed PO diet was higher than that of birds fed other experimental diets ($P < 0.05$) except for the 3.5% FO + 3.5% SO diet.

DISCUSSION

Breast and thigh tissues fat content

In this study, the CLA containing diets resulted in different changes in lipid contents of thigh and breast tissues. The dietary CLA effectively decreased fat deposition in broiler chickens meat, and especially breast tissue was more affected. This observation is in agreement with the report of Kawahara et al. (2009) who found that contents of total lipid and triglyceride in breast meat tended to decrease in the broilers fed 1 to 2% dietary CLA. On the contrary, Buccioni et al. (2009) couldn’t show any changes in lipid content of muscles in broilers fed different dietary CLA levels. Javadi et al. (2007) even reported a higher lipid content in meat of broiler chickens fed 1% dietary CLA as compared with the control group.

It seems that in the present study, CLA had a dominant effect over soybean oil so that the 2.1% CLA + 3.5% SO diet resulted in less fat deposition in both breast and thigh tissues, than 7% SO diet (and comparable to the 4.2% CLA diet). This dominant effect of CLA is absorbable over fish oil in breast tissue too. Previous studies have shown that CLA increases energy expenditure through increased oxygen consumption (Choi et al., 2004) and increased gene expression of uncoupling proteins, which both are indices for energy expenditure and less fat deposition (Choi et al., 2004).

Table 1. Ingredients and compositions of the experimental diets.

Ingredient	Starter	Grower (11 to 28 days of age)							Finisher (29 to 42 days of age)						
	(1-10days)	PO[2]	SO	FO	CLA	FO+SO	CLA+SO	CLA+FO	PO	SO	FO	CLA	FO+SO	CLA+SO	CLA+FO
Corn (%)	60.23	48.46	53.99	53.99	55.8	53.99	54.13	54	52.62	57.98	57.98	59.5	57.98	59.06	58.92
Soy meal (%)	30.81	30.56	32.27	32.27	28.6	32.27	31.96	32.26	27.76	30.27	30.27	26.38	30.27	28.3	28.6
Fish Meal (%)	5.37	5	3	3	5	3	3.2	3.01	3	1	1	2.99	1	1.7	1.51
Soybean oil (%)	-	-	7	-	-	3.5	3.5	-	-	7	-	-	3.5	3.5	-
Fish oil	-	-	-	7	-	3.5	-	3.5	-	-	7	-	3.5	-	3.5
Palm oil (%)	-	12.6	-	-	-	-	-	-	12.92	-	-	-	-	-	-
CLA(%)[1]	-	-	-	-	7	-	3.5	3.5	-	-	-	7.4	-	3.5	3.5
Oyster shell (%)	1.41	1.34	1.42	1.42	1.33	1.42	1.41	1.42	1.3	1.39	1.39	1.3	1.39	1.35	1.36
DCP (%)	0.51	0.48	0.66	0.66	0.52	0.66	0.64	0.66	0.69	0.84	0.84	0.71	0.84	0.81	0.82
Salt (%)	0.25	0.29	0.32	0.32	0.28	0.32	0.31	0.32	0.32	0.35	0.35	0.32	0.35	0.34	0.34
Vit-Min P(%)[3]	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
DL-Met (%)	0.26	0.23	0.25	0.25	0.23	0.25	0.25	0.25	0.17	0.18	0.18	0.16	0.18	0.18	0.18
L-Lys (%)	0.15	0.04	0.09	0.09	0.24	0.09	0.09	0.09	0.22	-	-	0.25	-	0.27	0.27
Analysis															
ME (Kcal/Kg)	2860	3175	3205	3211	3175	3208	3175	3175	3225	3235	3241	3225	3238	3225	3225
CP (%)	22.5	21	21	21	21	21	21	21	19	19	19	19	19	19	19
Crude Fat (%)	2.86	15	9.52	9.52	9.65	9.52	9.54	9.52	15.34	9.55	9.55	10.07	9.5	9.61	9.6
Linoleic a (%)	1.46	2.63	5.37	1.39	5.27	3.38	5.3	3.31	2.74	5.45	1.46	5.56	3.46	5.4	3.4
Ca (%)	0.95	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.85	0.85	0.85	0.85	0.85	0.85	0.85
Ava P (%)	0.475	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.425	0.425	0.425	0.425	0.425	0.425	0.425
Na (%)	0.152	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Lys (%)	1.368	1.23	1.23	1.23	1.34	1.23	1.23	1.23	1.2	1.02	1.02	1.2	1.01	1.2	1.2
Met (%)	0.662	0.6	0.6	0.6	0.6	0.6	0.6	0.6	0.48	0.48	0.48	0.48	0.48	0.49	0.49
Met+Cys (%)	1.0355	0.95	0.95	0.95	0.95	0.95	0.95	0.95	0.8	0.8	0.8	0.8	0.8	0.8	0.8

[1]CLA used in this experiment was CLA LUTA60 which contains 60% CLA, then 7% and 3.5% dietary inclusion of CLA will be equal to 4.2 and 2.1%, respectively. [2]PO= Diet containing palm oil, SO=diet containing 7% soybean oil, FO= diet containing 7% fish oil, CLA = diet containing 4.2% CLA, CLA+SO = diet containing 2.1% CLA+3.5% soybean oil, CLA+FO = diet containing 2.1% CLA+3.5% fish oil, FO+SO = diet containing 3.5% fish oil+3.5% soybean oil. [3]Mineral premix provided per kg of ration with 50 mg Fe, 70 mg Mn, 50 mg Zn, 7mg Cu, 0.4 mg Co, 0.17mg Se, and 0.75 mg I. Vitamin premix provided per kg of ration with 6,000,000 IU vitamin A, 1.500,000 IU vitamin D3, 15,000 IU vitamin E, 2.5 mg vitamin K3, 0.02 mg vitamin B12, 3,000 mg riboflavin, 7000 mg pantothenic.

Liver fat content

In the present study, the CLA containing diets increased liver fat accumulation, and the diet with 4.2% CLA was more effective than diets containing 2.1% CLA. The birds fed 7% fish oil (n-3 rich) as the only dietary fat source had a less fat accumulation in liver tissue as compared with the birds fed diet with 7% soybean oil (n-6 rich). Mikkelsen et al. (1993) reported that more unsaturated fatty acids are more effective on the fatty acid synthesis inhibition; DHA (22:6n-3)

Table 2. The liver, breast and thigh tissues Fat content in broiler chickens fed different dietary fats.

Parameter	Tissue fat content (% of dry matter)		
	Liver	Breast	Thigh
PO[1]	3.34[b]	2.46[a]	3.2[ab]
SO	2.85[c]	2.02[a]	3.38[a]
FO	2.42[d]	2.02[a]	3.94[a]
CLA	4.22[a]	1.11[b]	2.55[bc]
CLA+SO	4.1[a]	1.55[b]	2.16[c]
CLA+FO	3.86[ab]	1.23[b]	3.01[ab]
FO+SO	3.16[bc]	1.31[b]	2.39[bc]
SEM	0.16	0.06	0.13

[a-d]Means with different superscripts within column differ significantly at P<0.05. [1]CLA used in this experiment was CLA LUTA60 which contains 60% CLA, then 7% and 3.5% dietary inclusion of CLA will be equal to 4.2% and 2.1% respectively. 2PO= diet containing Palm oil, SO = diet containing 7% Soybean oil, FO= diet containing 7% Fish oil, CLA = diet containing 7% CLA, CLA+SO = diet containing 3.5% CLA+3.5% Soybean oil, CLA+FO = diet containing 3.5% CLA+3.5% Fish oil, FO+SO = diet containing 3.5% Fish oil+3.5% Soybean oil.

being more effective than EPA (20:5:n-3) or arachidonic (20:4n-6).

Serum parameters

The effect of n-3 rich fats, especially fish oil on serum triglyceride reduction has been previously reported in chicks (Akiba et al., 1995). In the study of Phetteplace and Watkins (1989), the chickens fed menhaden oil had lower plasma triacylglycerol levels compared with the values for those fed chicken fat; they concluded that a decrease in TG synthesis by the liver could result in lower amounts of TG in the VLDL + LDL fraction. We observed the same relationship between dietary fish oil and palm oil as a more saturated fat.

There are some reports which show that n-3 PUFAs may act in a similar way to fibrate drugs, which enhance the conversion rate of VLDL to LDL (Despres et al., 2004). These opinions are not in agreement with our observation for serum thyroglobulin (TG) and LDL levels, which can be attributed to the species differences because all the reports given in the foregoing are noted in humans.

The report which shows that the effect of 7% CLA diet on increasing serum TG concentration is higher than birds fed 7% FO, 7% SO or PO diets is in agreement with the observations of Du and Ahn (2003) in chicken and Du and Ahn (2002) in rat; but the majority of the previous reports in different species have shown a decreased serum or plasma TG following CLA administration (Bhattacharya et al., 2006; Baddini et al., 2009).

The reason for the higher serum TG concentration in birds fed dietary CLA is not clear, but it could be attributed to the alterations in activities of enzymes involved in hepatic lipid metabolism. In the study of Du and Ahn (2003), dietary CLA caused a significant increase in liver fatty acid synthase (FA synthase) activity and an increase (even though not significant) in acetyl-CoA carboxylase activity. FA synthase and acetyl-CoA carboxylase are the main enzymes regulating fatty acid synthesis. The higher FA synthase activity could be explained in part by the increased plasma TG levels. In the cultured adipose cells, FA synthase gene expression was not decreased by dietary CLA (Choi et al., 2000). These results show that dietary CLA decrease lipogenesis in mammary glands and adipose tissues but not in liver. This could be the explanation for ineffectiveness of CLA in decreasing fat deposition in birds (Du and Ahn 2002), because the liver is the main site of lipogenesis.

In the present study, the higher TG levels in the serum of birds fed on CLA containing diets indicates that the increase in hepatic steatosis by dietary CLA could be due to the action of CLA to change lipid metabolism in the liver. However, the ineffectiveness of CLA on serum total cholesterol in the present research was not surprising, because there are various previous reports on the decreasing (Zanini et al. 2006) or increasing (Szymczyk et al., 2001; Du and Ahn, 2003) effects of CLA on serum total cholesterol in broiler chickens.

Except for the one report on pigs (Stangl et al., 1999), almost the majority of the previous studies indicated a reducing effect of CLA on serum or plasma total cholesterol in different species (Bhattacharya et al., 2006; Baddini et al., 2009).

The increased serum HDL level in broiler chickens fed on CLA in this study is in agreement with previous reports in broilers (Du and Ahn., 2003), mice (Bhattacharya et al., 2006) and human (Smedman and Vessby., 2001). Although, in the research of Szymczyk et al. (2001) on broiler chickens, the level of 1% dietary CLA increased serum HDL concentrations, but the 1.5% CLA level decreased it. It seems that there is also an interaction between CLA and soybean oil effects on the serum LDL concentration; so that the combination of these two fat sources resulted in a significantly higher LDL level as

Table 3. Serum composition of birds fed on experimental diets containing different fat types.

Parameter	Triglyceride (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	Glucose (mg/dl)
PO[1]	40.2^{bc}	99.5^{a}	77.2^{a}	38.3^{ab}	8.0^{bc}	124.7^{a}
SO	40.2^{bc}	62.8^{b}	34.2^{c}	24.7^{bc}	8.0^{bc}	80.2^{bc}
FO	37.0^{c}	60.7^{b}	48.5^{bc}	21.8^{c}	7.1^{c}	82.3^{bc}
CLA	53.3^{a}	76.7^{ab}	75.2^{a}	41.7^{a}	10.7^{a}	72.2^{c}
CLA+SO	48.5^{ab}	90.8^{a}	51.5^{bc}	40.0^{a}	9.7^{ab}	85.7^{bc}
CLA+FO	44.5^{a}	92.2^{a}	63.5^{ab}	41.3^{a}	8.9^{a}	85.2^{bc}
FO+SO	44.0^{a}	94.3^{a}	65.0^{ab}	39.7^{a}	87^{a}	117.5^{ab}
SEM	1.8	3.5	2.2	1.8	0.4	4.6

$^{a\text{-}c}$Means with different superscripts within column differ significantly at $P < 0.05$. [1]CLA used in this experiment was CLA LUTA60 which contains 60% CLA, then 7% and 3.5% dietary inclusion of CLA will be equal to 4.2% and 2.1%, respectively. 2PO = diet containing Palm oil, SO=diet containing 7% Soybean oil, FO= diet containing 7% Fish oil, CLA = diet containing 7% CLA, CLA + SO = diet containing 3.5% CLA + 3.5% Soybean oil, CLA + FO = diet containing 3.5% CLA + 3.5% Fish oil, FO + SO = diet containing 3.5% Fish oil + 3.5% Soybean oil.

compared with the birds fed each fat separately. It seems that the effects of dietary CLA on glucose metabolism in human are inconsistent and probable effects of CLA on metabolic syndromes are still controversial (Bhattacharya et al., 2006).

The results of the present study showed that the dietary soybean oil and fish oil were more effective on serum lipoproteins reduction, than CLA; however, CLA more effectively increased serum HDL concentrations.

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Vol. 12(51), pp. 7120-7123, 18 December, 2013
DOI: 10.5897/AJB09.1389
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Helminth parasites of *Synodontis nigrita* at lower Niger (IDAH), Nigeria

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Accepted 31 March, 2010

The isolation and identification of helminth parasites of *Synodontis nigrita* and length–weight relationship of the fish in the lower Niger (Idah), Kogi State, Nigeria were carried out in order to describe the pattern of occurrence of the helminth and to establish the well-being of the host fish. A total of 102 randomly sampled fish were studied and three genera of helminths were recovered; 39.1, 48.7 and 12.2% respectively, and were harboured in the fish' intestine. The three genera of helminths isolates identified include two nematodes (*Capillaria* and *Contracaecum* species), Acanthocephala (*Acanthocephalus* species) and Trematode (*Posthodiplostomum* spp.). Of the 102 fish studied, 16 were infected with 80 helminth parasite giving a prevalence rate of 15.7%. The overall mean intensity and mean abundance of helminth parasite occurrence for the sampled fish were 13.6 and 1.5, respectively. The mean standard length of the fish was 7.45 ± 2.59 cm. The need for fish seeds from the wild to be examined for the helminth parasites during culture practice and the socio-economic and human health implications of eating infected fish is also recommended.

Key words: Acanthocephala, nematode, isolation, trematode, intestine.

INTRODUCTION

Catfish is a common name for about 2,200 species of fishes that make up the order Siluriformes and class Actinopterygii (ray-finned fishes). These two families of the order Ariidae and Plotosidae are primarily marine, while all other families are freshwater dwellers. Catfishes are a collection of scaleless, tenacious fish mostly nocturnal scavengers that have adapted to life in a variety of environments with some living near the bottom in shallow waters (Gunder and Fink, 2004).

Fish, like all living organisms, are susceptible to infections with various parasites (Hilderbrand et al., 2003). Chiefly among the parasites afflicting fish are the helminths. Helminths comprising nematodes, trematodes, cestodes and Acanthocephala commonly parasitize both wild and cultured fish with the former constituting heavier parasitic burden (Merck, 2006). Direct association of wild species with cultured fish farms has been established as a way of contaminating cultured fish by parasites (Okaeme and Olufemi, 1997).

The wellbeing, robustness and degree of fatness of fish is a measure of its condition factor with respect to the same specie taken from other water bodies or to other species of fish taken from the same water body (Pauly, 1983). It is expressed by relating length of fish to its weight. A plump or fat fish will give a higher condition factor than a lean and thin fish. Lower value means that the fish are in poor condition which may be a reflection of either over population or outbreak of diseases (Gupta and Gupta, 2006).

MATERIALS AND METHODS

Study area

The study area is the lower Niger (Idah), Kogi State, Nigeria. It is located on latitude 7° 06'N and longitude 6° 43'E of the Greenwich Meridian in the Guinea Savannah vegetation zones of Nigeria (Areola et al., 1992). A total of 102 fish samples of *Synodontis*

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Table 1. Pattern of helminth parasites occurrence in *S. nigrita* in relation to standard length of fish in the lower Niger (Idah), Nigeria.

Standard Length group (cm)	Total number examined	Total number infected	Total parasites recovered	Prevalence rate (%)	95% C I	Mean intensity (No)	Mean abundance (No)
0.0 - 5.4	1	1	18	100.0	-	18.0	18.0
5.4 - 19.0	20	3	12	19.0	18.96 - 19.04	2.6	5.0
5.8 - 19.0	36	2	26	33.0	32.99 - 33.00	3.1	1.0
5.4 - 26.1	22	2	14	28.9	28.78 - 29.02	4.1	1.2
5.8 - 25.1	13	4	8	6.7	6.42 - 6.98	2.0	1.3
19.0 - 26.1	10	4	2	3.7	3.31 - 4.09	53.0	26.5
Total	102	16	80	15.7	26.37 – 26.43	5.0	0.8

CI = Confidence interval.

Table 2. Identity and pattern of helminth parasites recovered from *S. nigrita* in the lower Niger (Idah), Nigeria in relation to predilection sites.

Helminth parasites		Predilection sites					Sub total	
Taxonomic group	Species	Skin	Gill	Fin	Anus	Small intestine	Number	%
Nematoda	*Capillaria* sp.	-	-	-	-	4	4	16.5
	Contraceacum sp.	-	-	-	-	11	11	22.6
Acanthocephala	*Acanthocephalus* sp.	-	-	-	-	62	62	48.7
Trematoda	*Posthodiplostomum* sp.	-	-	-	-	3	3	12.2
Grand total		-	-	-	-	80	80	100.0
Percentage (%)		-	-	-	-	100	100.0	

nigrita of different sizes caught by fishermen using gillnets, cast nest and hook and line at Idah area of the lower Niger were identified, bought and transported alive to the Biological Science laboratory, Kogi State University, Anyigba between January and August 2008 for the study.

The skin, fins, eyes, anus, intestinal organs, buccal and opercula cavities of fish were cut open and placed in 0.9% physiological saline and examined under a dissecting microscope. Helminths recovered were counted and placed in saline solution (0.9%) which was kept overnight in refrigerator to enable them stretch and relax. They were later fixed and preserved in 70% alcohol. The helminths were stained over night with weak Erlich's haematoxylin; and dehydrated in graduated alcohol (30, 50, 70, 90% and absolute) for 45 min, cleared in methyl-salicylate and mounted on a slide in Canada balsam. The occurrence (prevalence, mean intensity and abundance) of the helminths on the *Synodontis* fish hosts was determined by standard procedures described by Khalil and Polling (1997).

With the aid of a measuring board and sensitive Mettler weighing balance, the total length (cm), standard length (cm) and weight (g) of each fish sample was measured fresh to the nearest 0.1 cm and 0.1 g, respectively. The length weight relationships were estimated from the allometric formula, $W = aL^b$, where W is total body weight (g), L the total length (cm), a and b are the coefficients of the functional regression between W and L (Ricker, 1973).

RESULTS

From Table 1, grouping of the fish into 6 categories according to standard length (SL) showed that every group had helminth infection but the group with highest parasitism is the 0.0-5.4 cm group (n = 1) as it had prevalence rate of 100%; this group had mean intensity and mean abundance of 18.0. Mean intensity (number of parasite per fish) was highest for the SL group 5.8- 26. 1 cm.

A total of 80 helminth parasites comprising nematode, Acanthocephalan and trematode were isolated from one predilection site of the fish sampled, the small intestine. The types and pattern of helminth parasites isolated from *S. nigrita* species were nematodes, 15 (30.3%); Acanthocephala, 62 (55.4%) and trematode, 3 (14.3%) (Table 2).

The pattern of helminth parasites occurrence in relation to season of the year is as shown in Table 4. Sixty one (61) fish were sampled for wet season, out of which 5 (21.1%) were infected, mean intensity 11.6 and mean abundance 1.0 of helminth parasites occurrence were recorded. In dry season, forty one (41) fishes were sampled, 11 (78.9%) had infection with mean intensity of 2.0 and mean abundance of 0.5, respectively (Table 3).

The standard lengths (SL) of the sampled fish ranged from 5.4 to 26.1 cm, 5.8 to 19.0 cm and 5.4 to 26.1 cm. The mean SL for the fish were 7.29 ± 2.82, 7.66 ± 2.25 and 7.45 ± 2.59. The weights of the fish ranged from 2.6 - 379.9, 3.4-206.6 and 2.6-379.9 g with the overall mean

Table 3. Pattern of helminth parasites occurrence recovered in *S. nigrita* in relation to season in the lower Niger (Idah) Nigeria.

Season	Fish species	Total fish examined	Total fish infected	Total parasites recovered	Prevalence rate (%)	95% CI	Mean intensity (No)	Mean abundance (No)
Wet season	*S .nigrita*	61	5	58	7.5	7.11 - 7.14	11.6	1.0
Dry season	*S .nigrita*	41	11	22	8.2	8.52 - 8.63	2.0	0.5
Total		102	16	80	15.7	15.63 -15.77	13.6	1.5

CI = Confidence interval

Table 4. Length - weight relationship of *S. nigrita* in the lower Niger (Idah) Nigeria.

Fish species	Sex	Standard length (cm)			Weight (g)			n	a	b	r
		Min.	Max.	Mean ± SD	Min.	Max.	Mean ± SD				
S. nigrita	Males	5.4	26.1	7.29 ± 2.82	2.6	379.9	13.90 ± 49.41	58	0.0197	2.9948	0.9697
	Females	5.8	19.0	7.66 ± 2.25	3.4	206.6	13.31 ± 30.53	44	0.0164	3.0887	0.9694
	Combined sex	5.4	26.1	7.45 ± 2.59	2.6	379.9	13.65 ± 42.13	102	0.0182	3.0364	0.9701

n = Number of fish examined; a = intercept; b = slope; r = correlation coefficient of determination.

weight of 13.65 ± 42.13 g (Table 4).

DISCUSSION

The result of this study reveals the occurrence of four helminth parasites in *S. nigrita* in the lower Niger (Idah), Kogi State, Nigeria. The four parasites belonged to nematode, Acanthocephala and trematoda. The large number of helminths infection recorded indicated that helminths were considerable parasites of the *Synodontis* species studied. This agrees with the study of Boomker (1994), Akinsanya et al. (2008) and Owolabi (2008). The parasites recovered from the studied fish were *Capillaria* spp., *Contraceacum* spp., *Acanthocephalus* spp. and *Posthodiplostomum* species. This conforms with the studies of Boomker (1994), Khalil (1969) and Boomker (1994).

According to the host parasite checklist on African freshwater fishes of Khalil and Polling (1997) and other relevant studies, the present work is the first scientific record of *Contraceacum* spp. and *Posthodiplostomum* spp. in the *Synodontis* species examined in lower Niger (Idah) Nigeria. The high proportion of Acanthocephala (48.7%) than nematode (39.1%) and trematodes (12.2%) showed that *Acanthocephalus* spp were the commonest infection of this genus in lower Niger (Idah).

The standard lengths (SL) of the sampled fish ranged from 5.4 to 26.1 cm, 5.8 to 19.0 cm and 5.4 to 26.1cm. The mean SL for the fish were (7.29 ± 2.82), (7.66 ± 2.25) and (7.45 ± 2.59). The weights of the fish ranged from 2.6 - 379.9 g, 3.4 - 206.6 g and 2.6 - 379.9 g, while the mean was 13.65 ± 42.13 g. The result of the length-weight relationship of the fish showed that the fish exhibits isometric growth in the water body.

It is therefore recommended that fish seeds from the wild should be examined for the presence of helminth parasites prior to use and periodically during culture practice. Awareness should also be created on the socio-economic and human health implications of eating infected fish among the fisher folks and the general

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