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Review

Transgenic *Bacillus thuringiensis* (*Bt*) chickpea: India's most wanted genetically modified (GM) pulse crop

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Chickpea (*Cicer arietinum*) is grown widely in India because the seeds are rich source of protein for the vegetarian population of country. However, chickpea cultivation is declining over the period of time due to heavy incidences of pests and diseases. *Helicoverpa armigera* is a major pest in the field and non-availability of resistant varieties lead to heavy losses of yield per year. Crop management practices such as application of bio-pesticides, insecticides and integrated pest management are less effective to control this devastating pest. Breeding for development of resistant lines is restricted by lack of resistant sources within the gene pool. Therefore, application of gene technology for chickpea improvement appears to be appropriate approach for development of *Helicoverpa* resistant lines. Genetic transformation of chickpea using various versions of *Bacillus thuringiensis* (*Bt*) insecticidal genes have been carried out and found to confer resistance to pod borers in the laboratory bioassays. The most preferred genetically modified (GM) chickpea for field release is pyramided lines having two or more *Bt* genes with diverse mode of action for effective management of *Helicoverpa*. Here we discuss about the rationale for generation of *Bt* chickpea to enhance production.

Key words: Chickpea, *Bacillus thuringiensis*, genetically modified (GM) pulse crop.

INTRODUCTION

In India, farmers grow many species of grain legumes in an area of 36 million hectare (m ha) with an annual production of 29 million tonnes. Chickpea (*Cicer arietinum* L.) is the most important pulse crop gown in an area of 8.21 m ha and producing 7.48 millon tonnes of the grains (FAOSTAT, 2011). The crop is largely grown by small farmers in rain-fed areas (>70%) which are less fertile and poor in moisture retention capacity. Although, India produces about 75% of the chickpea (Rao, 2010), the production is inadequate to meet the demand of the domestic market. According to 2011 FAOSTAT statistics, India imports about 1, 85,000 metric tons of chickpea valued at US$ 94 millons. The demand for chickpea is projected to be double from 7 to 14 millon tonnes by 2020. In the next 10 years the net import of chickpea will be close to 1.5 millon tonnes to meet the domestic requirements.

During the past two decades, area under chickpea cultivation has declined in India. The factors that discourage farmers to undertake chickpea cultivation are lack of irrigation, high incidences of insect pest (predominantly, *Helicoverpa armigera*) and diseases, lack of supply of quality seeds, non-availability of drought tolerant and short duration varieties. The production constraints have led to increase in the price of pulses in general by two to three folds during the past 10 years in India. These protein rich pulses are now less affordable to average middle class Indian. Recently, the Government

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Abbreviations: IPM, Integrated pest management; IRM, insect resistance management; ***Bt*,** *Bacillus thuringiensis*; **Vips,** vegetative insecticidal proteins.

of India has initiated various schemes to help pulse growers to improve production by providing subsidies on irrigation, seeds, fertilizers and other farm inputs. However, success of the government schemes depends on development and deployment for varieties resistant to biotic and abiotic stresses.

Chickpea is infected by nearly 60 insect species, of which the major damage is caused by pod borer, *H. armigera* (Hubner). It is a major pest of chickpea in Asia, Africa Australia and the Mediterranean region. Pod borers alone cause 25 to 40% of the crop loss amounting $325 million annually (ICRISAT, 1992; Sharma et al., 2005). *Helicoverpa* females lay eggs on leaves, flowers, and young pods. The larvae feed on the young leaves in chickpea and young seedlings of chickpea may be destroyed completely, particularly under tropical climates in southern India. Larger larvae bore into pods and consume the developing seeds inside the pod. The losses due to *H. armigera* magnify under drought condition. In addition, climate change may aggravate chickpea-*Helicoverpa* interaction. The results of *Helicoverpa* interaction on different crops under elevated CO_2 concentrations (550 to 750 ppm) showed a complex host-pest interaction (Wu et al., 2006; Coll and Hughes, 2008). Therefore, impact of deployment of high-yielding cultivars of chickpea to production and productivity under rain-fed conditions in India would be limited unless varieties are resistant to *H. armigera*.

MEASURES TO CONTROL *H. armigera* INFESTATION IN CHICKPEA

In order to protect the crop from *H. armigera*, various pest management practices have been adopted by Indian farmers. Efforts are being made to develop *H. armigera* resistant varieties both by conventional breeding methods as well as by using modern biotechnological tools to develop transgenic chickpeas resistant to *H armigera*.

Cultural practices, pesticides and IPM strategies

In order to reduce the survival and damage of *H. armigera*, several cultural practices are adopted such as time of sowing, spacing, fertilizer application, intercultural practices and flooding. In order to minimize extent of damage inter-cropping or strip-cropping with marigold, sunflower, linseed, mustard, or coriander is also adopted. These cultural practices are often ineffective because they are dependent on the crop husbandry practices in a particular agro-ecosystem. The chickpea trap crop is planted after the commercial crops to attract *H. armigera* emerging from winter diapause. The trap crops are destroyed before larvae commence pupation. As a result, the overall *H. armigera* pressure on summer crops is reduced, resulting in greater opportunity for adoption of soft control options, reduced insecticide use, and greater activity of the natural enemies.

The importance of biotic and abiotic factors on the seasonal abundance of *H. armigera* is poorly understood. Some parasitic wasps avoid chickpea due to dense layers of trichomes and their acidic exudates. The *Campoletis chlorideae* is an important larval parasitoid of *H. armigera* on chickpea, whereas *Trichogramma* egg parasitoids are rarely present in high numbers in India. The dipteran parasitoids *Carcelia illota*, *Goniophthalmus halli*, and *Palexorista laxa* have been reported to parasitize up to 54% of the larvae on chickpea. Predators such as *Chrysopa* spp*., Chrysoperla* spp*., Nabis* spp*., Geocoris* spp*., Orius* spp*.,* and *Polistes* spp. are common in India. Provision of bird perches or planting of tall crops that serve as resting sites for insectivorous birds such as Myna (*Acridotheris tritis)* and Drongo (*Dicrurus macrocercus)* helps to reduce the numbers of *H. armigera* larvae.

Use of chemical pesticides to control pod borers in chickpea is the most common practice, but indiscriminate use of chemicals lead to resistance development and environmental pollution (Armes et al., 1992). Integrated pest management (IPM) strategies are also being applied in order to reduce the negative effects of chemical pesticides. The IPM strategies include, timely sowing for host avoidance; intercropping with mustard, barley and linseed; use of trap crop such as *Vicia sativa* and African marigold; application of *Helicoverpa* nuclear polyhedrosis virus (HaNPV), or *Bacillus thuringiensis* (*Bt*) formulation; erection of perches; plant (Neem) bioproduct spray or limited application of chemicals like Endosulphan, Monocrotophos, Fenvelarate (Lal, 1990; Jayaraj, 1992). In India, HaNPV has been reported to be a viable option to control *H. armigera* in chickpea. However, the efficiency of the IPM strategies depends on various factors such as pest behavior, diurnal activity, weather condition, crop habitat among others. Besides, the impact of climate change could reduce the effectiveness of present IPM strategies, leading to decrease crop yield.

Wide hybridization

Breeding to transfer gene(s) conferring resistance to *H. armigera* from wild species to the cultivated species was exploited to develop resistant crop varieties. Screening of wild *Cicer* species showed resistance to *H. armigera* such as *Cicer bijugum, Cicer reticulatum, Cicer judaicum, Cicer pinnatifidium, Cicer microphyllum* and *Cicer cuneatum* (Sharma et al., 2005). It was found that the wild relatives, *C. judiacum*, *C. bijugum* and *C. pinnatifidum* have significant levels of resistance to *H. armigera* (Sharma et al., 2005, 2006), but these wild relatives were cross incompatible with the cultivated chickpea germplams. The cross incompatibility between cultivated *Cicer* and other perennial chickpeas are post-

zygotic (Mallikarjuna, 2001; Babb and Muehlbauer, 2005). Thus, cross incompatibility makes the wild relatives under-utilized in plant breeding programme.

Germplasm screening

The crop improvement to increase production and productivity depends on identification and deployment of varieties with resistance/tolerance to pests. Therefore, screening of germplasms maintained at ICRISAT Genebank (>15,000 accessions) and identification of *Helicoverpa*-resistant chickpea lines and performing varietal trails under various agro-climatic conditions is important. Moreover, understanding the molecular basis of resistance to this pest is required to formulate appropriate strategies to manage *Helicoverpa* infestation. So far, screening of the available germplams has led to identification of only moderate levels of resistance to *H. armigera* (Lateef, 1985; Lateef and Pimbert, 1990).

GENETIC MODIFICATION STRATEGIES USING *Bt* GENES

The genome of the *B. thuringiensis* constitutes genes that encode several insecticidal proteins. The insecticidal proteins that accumulate during sporulation are known as crystalline inclusion bodies (Cry and Cty proteins) and those produced during vegetative growth are known as vegetative insecticidal proteins (Vips). Both Cry and Vips proteins are toxic to Lepidoptera, Coleoptera and Diptera insects. These toxins can be expressed in chickpea for resistance to *H. armigera.* The *Bt* proteins have been used as bio-pesticides for the past 40 years and found be species specific and non-toxic to vertebrates. One of the successful applications of recombinant DNA technology to mankind is the development and deployment of transgenic crops expressing *Bt* toxins. In India, transgenic, *Bt* cotton has revolutionized the cotton industry since 2004. The *Bt* cotton has been widely accepted by small and resource poor farmers of India, hence the area under *Bt* cotton has increased significantly from 50,000 ha in year 2004 to 8.4 millon hectare in 2009 (James, 2010). A similar strategy appears to be suitable for generation of *Bt* chickpea for resistance to pod borers.

Selection of insecticidal gene

The choice of Cry toxin for expression in the field crops is critical for pest management. Expression of *Bt* Cry1Ac is most effective against *H. armigera,* however, generation of transgenic chickpea expressing *Cry1Ac* gene alone may not be suitable in terms of insect resistance management (IRM), especially in India. The farmers in India face various challenges in terms of insect management. The marginal and small farmers cannot spare their land for refuge for proper IRM of *Bt* crop. Therefore, pyramiding two or more *Bt* genes with diverse mode of action is essential to avoid resistance buildup within insect population.

The mode of action of Cry proteins has been studied extensively (Aronson et al., 1986; Hofte and Whiteley 1989; Knowles, 1994; Schnepf et al., 1998; De Maagd et al., 2000; 2003 and Bravo et al., 2004; 2007). The *Cry* toxin interacts sequentially to receptors present on the midgut epithelium and insert into membrane forming pores that cause ionic imbalance; break the midgut cells and insect death (Schnepf et al., 1998; De Maagd et al., 2003 and Bravo et al., 2004). Recently, a signal transduction model was proposed where the toxicity is due to activation of an Mg^{2+} dependent signal cascade pathway. The Cry toxin interacts with CAD receptors which lead to activation of G protein. The G protein activates an adenyly-cyclase which results in production of intercellular cyclic adenosine monophosphate (cAMP). The cAMP activates protein kinase A that starts an intercellular pathway resulting in cell death (Zhang et al., 2006).

The first set of transgenic crop commercialized in India is cotton hybrids carrying *Cry1Ac* gene of *Bt* for resistant to cotton bollworm. The cotton industry of India received heavy benefit upon introduction of *Bt* cotton in 2002. Cotton production in India before 2002-2003 was about 2.55 to 2.75 m t, but over the past five years cotton yield has increased by 50%. In the year 2006, five new events, Bollgard II, EventI, GFM Cry1A, BNLA 601 and Event 9124, of *Bt* cotton expressing Cry1Ac, Cry1Ab, Cry2Ab, Cry1C either alone or in combination were approved for release in India (GEAC, 2009). Therefore, applying genetic engineering technologies to develop *Bt* chickpeas using bacterial '*Cry'* genes could be appropriate to protect the crop from *H*. *armigera*.

Pyramiding two or more *Bt* genes such as *Cry1Ac* and *Cry2A* in chickpea could be a preferred option to delay evolution of resistant insects due to different mode of action for these two genes. However, reports suggest that baseline frequency of *Cry2Ab* resistance gene within populations of *H. armigera* (Mahon et al., 2007) is substantially higher than expected. Expressing *Cry1Ac* gene in combination with *Cry1F* gene may be effective to delay insect resistance because *Cry1Ac* in combination with *Cry1F* gives an additive effect against *H. armigera* (Ibargutxi et al., 2008). Moreover, use hybrid Cry proteins such as Cry1Ab- Cry1C also conferred resistance to lepidopteran pest, *Spodoptera exigua* (de Maag et al., 2000). Hybrid *Bt* protein containing domain I and II from Cry1Ba and domain II for Cry1IA was found effective against potato tuber moth and Colorado beetle (Naimov et al., 2003). Development of transgenic plants expressing Vips has been found more effective against many lepidopteran pests, including *H. armigera*. In the

case of maize it was found that Vip3A in combination with Cry1Ab provide complete resistance to *Helicoverpa zea* under field condition (Burkness et al., 2010). Transgenic chickpea stacked with *Bt* genes such as Cry1A along with Vip3A or hybrid *Bt* protein in combination with Vip3A, could be a suitable combination for Indian Agriculture.

The first successful genetic transformation of nuclear genome of chickpea was reported in 1997 using the *cry1Ac* gene (Kar et al., 1997). Subsequently, various research groups within India initiated genetic transformation of chickpea using *Cry1Ac* gene and reported generation of transgenic chickpeas (Sanyal et al., 2005, Indurker et al., 2007; Mehrotra et al., 2011). A second gene, *Cry2Aa*, was also introduced in chickpea to facilitate pyramiding with existing *Cry1Ac* lines (Acharjee et al., 2010). Mehrotra et al. (2011) generated pyramided *Cry1Ac* and *Cry1Ab* gene chickpea; however, pyramiding two or more genes with different mode of action is preferred.

Non *Bt* strategies for *H. armigera* control in chickpea

Exploitation of new genes is essential to avoid reliance on expression of only *Bt* endotoxin in the transgenic plants. A new strategy such as up-regulating secondary metabolites, which are toxic to or repel insects, to escape from insect damage (Gatehouse, 2002) or applying RNAi technology for insect control by silencing endogenous genes of insects could be new strategy to develop GM chickpea. A suitable candidate gene which was found to be effective was cytochrome *P450* gene (*CYP6AE14*) which expresses in the midgut of *H armigera.* Gene silencing in lepidoptera by RNAi technology have been found to be difficult to trigger which may be due to factors absence of RNA dependent RNA Polymerase orthologs (Gordon and Waterhouse, 2007) barrier in uptake of double stranded RNA (dsRNA), improper sorting of dsRNA during endosome trafficking to dsRNA-processing machinery among others. Mao et al. (2007) reported significant growth reduction of *Helicoverpa* larvae reared on transgenic tobacco and Arabidopsis. The efficacy of RNAi silencing can be enhanced by using a tobacco rattle virus vectors (Kumar et al., 2012).

CONCLUSION

Conventional methods of protecting chickpea for insect pest are inadequate to meet the challenges of the present agricultural scenario in India. The limitation of conventional technologies are lack of resistant germplams, enhanced susceptibility of high yielding varieties to pests, barriers to cross cultivated varieties with wild relatives to acquire resistant genes. In order to protect the chickpea yield from losses due to pest infestation resistant gene transfer across the sexual barriers through recombinant DNA technology is mostly preferred. However, selection of suitable gene or combination of genes for genetic modification of chickpea will remain critical to protect from *H. armigera* damage in chickpea.

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Genetic variability among *Andrographis paniculata* in Chhattisgarh region assessed by RAPD markers

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Random amplified polymorphic DNA (RAPD) markers were used to estimate the genetic variability and dissimilarity among the *Andrographis paniculata* (family- Acanthance), an important medicinal herb. Twenty-four (24) plants were collected from five districts of different places of Chhattisgarh region. Sixteen (16) primers generated a total of 159 polymorphic bands out of 182 total bands (79.95% polymorphism), with an average of 11.37 amplified bands per primers and 23 bands showed monomorphic banding pattern with an average of 1.43 per primers. A dendrogram was constructed based on the unweighted pair group method using arithmetic averages. Cluster analysis of data using UPGMA algorithm placed the 24 accessions of *A. paniculata* into two major clusters I and II which further sub-divided into many subclusters. Genetic dissimilarity coefficients calculated from RAPD data ranged from 0.3635 to 2.0160, with the highest value of 2.0160 between AP10 and AP16 and the lowest value of 0.3635 between accessions AP3 and AP5. The principal component analysis (PCA) clustering pattern corresponded well with the dendrogram. The results indicate that RAPD could be efficiently used for genetic diversity study in wild species of approaching value as it is quick, unswerving and superior to those on pedigree information.

Key words: Acanthance, dendrogram, principle component analysis (PCA), Random amplified polymorphic DNA (RAPD), variability.

INTRODUCTION

Andrographis paniculata (Burm.F) Nees, is an important medicinal herb. The whole plant is used as blood purifier and liver tonic (Chandra and Pandey, 1985; Chandra et al., 1985, 1987). Recently, it has been utilized as a treatment for HIV, hepatitis, diabetes, cancer and kidney disorders (Valdiani et al., 2012). In Chhattisgarh state, the tribal people of Raigarh districts externally applied the plant extract in snakebite (Jain and Singh, 2010), but the tribal of Balaghat district of Madhya Pradesh, used the whole plants for the treatment of chickenginia and malaria (Jain et al., 2011). It is known as a hermaphroditic, self-compatible and a habitual inbreeding plant (Latto et al., 2006) and there is an assumed rate of 28% crosses pollination for it (Sabu, 2002).

A. paniculata (Acanthance) is an annual herb commonly known as Kalmegh or Bhuineem (Sharma et al. 2009; Mishra et al. 2007). It is distributed in Southeastern Asia, including India, Sri Lanka, Pakistan, Indonesia (Mishra et al., 2007) but it is currently cultivated in Southwestern Nigeria (Fasola et al., 2010).

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In India, it is also cultivated as a Kharif season crop. Propagation is generally done through seeds; however, it can also be propagated by inducing rooting in cuttings (Zhou, 1987). The main constituents of *A. paniculata* are diterepene lactones, andrographolide, neoandrographolide, andrographiside and flavones viz., oroxylin, wogonin, andrographidines A, B, C, D, E, F (Martindale et al., 1972). Among them, andrographolide is widely used as a hepatoprotective agent. It also shows choleretic, antidiarrhoeal, immunostimulant, and anti-inflammatory activities (Kokate et al., 1999; Anonymous, 1999; Singh et al., 2003).

A. paniculata has been existing in the list of highly traded Indian medicinal plants (Sajwan, 2008) and it also has been positioned as the 17th crop among the 32 prioritized medicinal plants of India with a demand of 2197.3 tons in the year 2005-2006 and annual growth of 3.1% (Sharma et al., 2009; Anonymous, 2007; Kala et al., 2006). Priority of *A. paniculata* is regarded by herbal industries of developing countries such as Malaysia (Moideen, 2008), Thailand (Chuthaputti and Chawapradit, 2008) and Nigeria (Fasola et al., 2010). In Chhattisgarh State, Kalmegh was cultivated in various places such as Bilaspur, Rajnandgaon, Kabirdham, Bijapur, Kanker, Sukma, Khairagarh, Udanti and East Raipur. Production of Kalmegh in CG State as per market survey report 2006 by CGMFPLTD Raipur was 13950 (Qtnl) (CSMPB Raipur, 2008). Random amplified polymorphic DNA (RAPD) analysis has been applied in herbal medicine to discriminate between species in various genera (Shcher and Carles, 2008). RAPD markers were used to assess genetic variability among the various accessions of *A. paniculata.*

Genetic tools that use hybridization, polymerase chain reaction (PCR), and sequencing techniques provide more objective and reliable methods for authenticating herbal medicines (Zhang et al., 2007; Shcher and Carles, 2008). Recently, RAPD analysis has become one of the most effective methods for estimating genetic diversity in plant populations or cultivars because it can reveal high levels of polymorphism. However, it is less reproducible than other methods (Hosokawa et al., 2000; Agrawal et al., 2007; Shcher and Carles, 2008). RAPD also has many advantages, such as its high speed, low cost, and requirement of minute amounts of plant material (Williams et al., 1990; Penner et al., 1993). RAPD markers have been already successfully used on other medicinal and aromatic crops (Yang et al., 2012; Leelambika and Sathyanarayana, 2011; Kasaian et al., 2011; Khan et al., 2010; Verma et al., 2009; Bharmauria et al., 2009; Padmalatha and Prasad, 2007). The RAPD based molecular markers have been found to be useful in *A. paniculata* (Padmesh et al., 1998; Maison et al., 2005; Latto et al., 2006; Kumar and Shekhawat, 2009). Inspite of immense therapeutic value, lack of genetic variability information in *A. paniculata* occurs in Chhattisgarh State. The objective of this study was to use RAPDs to assess genetic variability and dissimilarity among *A. paniculata* of 24 accessions collected from five districts of different places in Chhattisgarh region and to estimate genetic relationships among the accessions which can be utilized for breeding program and phytochemical study.

MATERIALS AND METHODS

Plant materials

A. paniculata plants are found in pine, evergreen and deciduous forest areas, and along roads and in villages. However, it is possible to cultivate them during rainy phase of summer season. A total of 24 accessions, of *A. paniculata* collected from natural sources of five districts of different places in Chhattisgarh region were used for this study. All the accessions collected from different places were effectively maintained in the medicinal garden of the Pt. Ravishankar Shukla University Raipur (C.G) (Table 1).

DNA extraction

Genomic DNA was extracted from fresh young leaves of 24 accessions of *A. paniculata* using cetyltrimethyl-ammonium bromide (CTAB) method (Doyle and Doyle, 1990) with modifications as described by Khanuja et al. (1999). Three grams (3 g) of fresh young leaves of each 24 samples were ground to a fine powder in a mortar in liquid nitrogen and then transferred to Tarson tubes filled with 15 ml of freshly prepared and preheated 2.5% CTAB extraction buffer. Extraction buffer consisted of 100 mM Tris - HCl (pH 8.0), 25 mm EDTA (pH 8.0), 1.5 M NaCl, 1% PVP and 0.2% β - mercepto-ethanol. The suspensions of samples were incubated at 65°C for 1 h. After cooling at room temperature, an equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 10 K rpm for 10 min at room temperature. DNA from aqueous layer was precipitated by adding 5 M NaCl (30% of supernatant) and 0.6 volumes of chilled isopropanol. The mixture was centrifuged at 10 K rpm for 10 min. Pellets were washed with 80% alcohol and let the pellet dry. Pellets were dissolved in high salt TE buffer then RNase A was added and incubated at 37°C for 30 min. Again, it was extracted with equal volume of chloroform - isoamylalcohal (24:1). Two volumes of cold ethanol was added in aqueous layer and centrifuged at 10 K rpm for 10 min. Pellets were washed with 80% alcohol and dissolved in TE (Tris- Cl- EDTA) buffer after drying. DNA concentration and purity were determined by nanospectrophotometer (UK) A260/280, ratio ranged from 1.83 to 2.15 and averaged 2.04 for all the samples and electrophoresis in a 0.8% agarose gel with known standards was done. For PCR amplification, the final concentration of each DNA sample was diluted to approximately 15 to 20 ng with millique water.

PCR amplication

RAPD assays were carried out in 12.5 µl reaction mixture containing template 2 µl DNA (15-20 ng), 1.25 µl of 10X PCR Buffer (10 mM Tris HCL, 25 mM $MgCl_2$), 1 µl of 10 mM dNTPs, 0.125 µl of 20 mM primer, and 0.75 µl 1.0 of U Taq DNA polymerase, (Bangalore genei) and distilled water up to 6.25 µl. Amplification was performed in a PTC-100 programmable thermal cycler (MJ Research, USA). DNA amplification was obtained as follows: 94°C for 5 min, 32 cycles of 94°C for 30 s, 37°C for 30 s, and 72°C for 1 min, followed by one cycle of 72°C for 7 min and a hold temperature of 4°C at the end. A negative control with all PCR cocktail except the template DNA was included with each set of PCR amplification reactions. The negative control contained sterile

Table 1. Collection of *Andrographis paniculata* accessions (AP1-AP24) used for the study.

State	District	Locality	Source	Accessions number
Chhattisgarh	Korba	Kusmunda	Cultivated	AP1
		Katghora	wild	AP2
		Madanpur	Wild	AP3
		Kartala	Wild	AP4
		Saglawa	Wild	AP5
		Pasan	Wild	AP6
		Nakoya	wild	AP7
	Kawardha	Salewara	Wild	AP8
		Bhoramdeo	Wild	AP9
		Pandari	Wild	AP10
		Neur	Wild	AP11
	Surguja	Ambikapur	Wild	AP12
		Mainpat	Wild	AP13
		Partabpur	Wild	AP14
		Wadrefnagar	Wild	AP15
		Tattapani	Wild	AP16
	Raigarh	Sarangarh	Wild	AP17
		Kharisa	Wild	AP18
		Gharghoda	Wild	AP19
		Leonga	Wild	AP20
		Dharamjaygarh	Wild	AP21
	Bastar	Balenga	Wild	AP22
		Kondagaon	Wild	AP23
		Jagdalpur	Wild	AP24

distilled water instead of template genomic DNA. The amplified PCR products were resolved on 1.8% (w/v) agarose gels, in 1X TBE Buffer at 90 V for 2 h 10 min, visualized with ethidium bromide (0.5 µg/ml) staining and photographed under Gene Snap software of gel Documentation System (Gene Genius, Syngene, U.K.). Lambda DNA double digest ladder (1.5 Kb DNA ladder, Bangalore Genei) was used as molecular marker to know the size of the fragments. For each experiment, the reproducibility of the amplification products was tested thrice using similar reaction conditions at different times.

Screening of specific RAPD primers

Thirty (30) random primers (R-01 - R-30) from R-series, (purchased by Integrated DNA Technologies, Inc., U.S.A) were screened by RAPD for identification of specific markers. The screening of primers resulted in 16 R-series decamer primers which show polymorphisms with all 24 samples. The other 14 Integrated DNA primers could not amplify all the 24 samples.

RAPD data analysis

The amplified bands were scored from photographs into a binary data matrix as 1 (present) or 0 (absent). Based on presence/absence data, genetic similarity was calculated to estimate all pair wise differences in the amplification product for all isolates. The genetic associations between strains were computed based on Jaccard's similarity coefficient (Jaccard, 1908) using 'SAHN' (sequential, agglomerative, hierarchical nested clustering method) sub program of NTSYS-pc 2.20 (q). The program also generated a dendrogram on the basis of above index by unweighted pair group method with arithmetic averages (UPGMA) subprogram of NTSYC-pc 2.20 (q). The data generated from polymorphic fragments were analyzed according to the study of Nei and Li (1979) formula given below:

$$\text{Similarity (F)} = \frac{2\,Mx}{My + Mz}$$

Dissimilarity = 1 - F

Where, Mx is the number of shared fragments between genotypes y and z; My is the number of scored fragments of genotype y; Mz is the number of scored fragments of genotype z. Only clear and unambiguous bands were taken into account and the bands were not scored if they were faint or diffused. The RAPD data were also evaluated by principle component analysis (PCA).

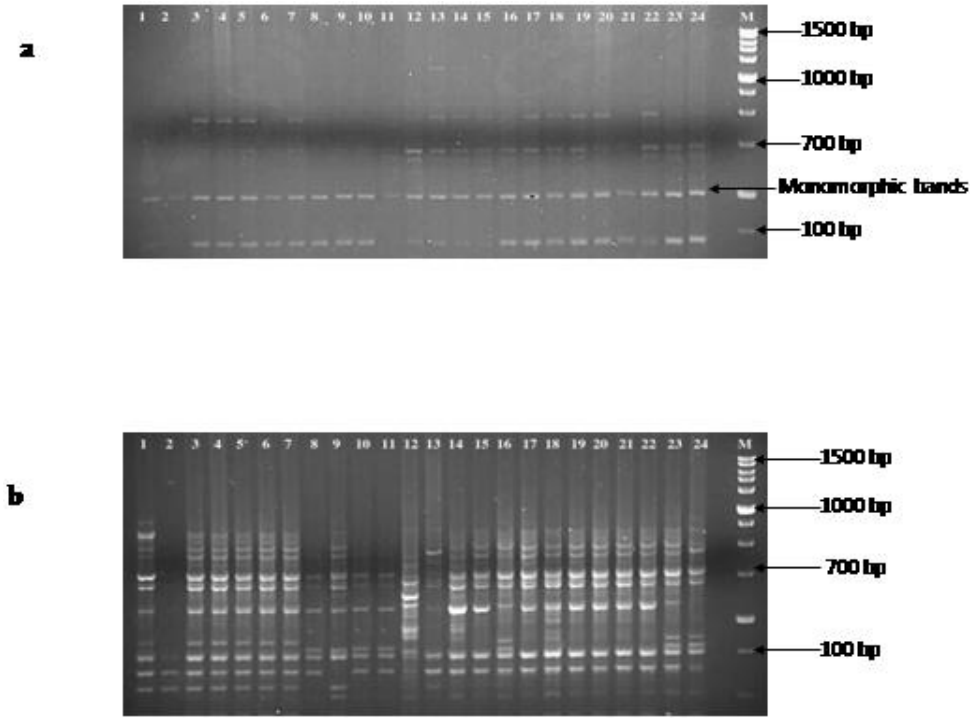

Figure 1. RAPD profiles of *A. paniculata* using R-series 10-decmer primers. a, R-09; b, R-17. The arrowheads indicate the precise size of RAPD markers. M represents 1 Kb molecular size marker, Lane numbers code for each accessions 1 to 7 Korba, 8 to 11 Kawardha, 12 to 16 Surguja, 17 to 21 Raigarh, 22 to 24 Bastar.

RESULTS AND DISCUSSION

RAPD analysis was used to evaluate the degree of polymorphism and genetic variability among 24 accessions of *A. paniculata* was further analyzed according to the resultant RAPD markers. The PCR reactions were repeated three times for each of the 24 accessions and the resultant DNA bands were highly reproducible. Results show that each accession collected from different localities showed genetic variability and dissimilarity in RAPD profiles by using different primers.

Of the 30 random primers screened, 16 R-series (= R) primers produced distinct, highly reproducible amplify-cation profile for all the screened samples (Figure 1a,b). These 16 primers were selected for further ana-lysis of the plant materials. The polymorphism exhibited by RAPD primers among 24 accessions of *A. paniculata* are presented in Table 2. A total 182 marker levels were amplified across the accessions, of which 159 (79. 95) were polymorphic bands.

A wide variation in the number of polymorphic bands ranging from 3 to 21 and monomorphic bands 1 to 3 was observed. The highest 22 bands were observed from primer R-17 and four bands for primers R-07 and R-09 (Figure 1a, b). The average number of polymorphic (9.93), monomorphic (1.43), unique bands (1.68) per primer and the percentage of polymorphism ranged from 50.0 (R-16) to 93.70 (R-13 and R-19) (Table 2). Primer R-17 amplified the highest number of polymorphic bands (21), while the lowest number (3) was observed with primer R-07, R-09 and R-16.

The size of amplified fragments varied with the different primers, ranging from 100 to 1500 bp. The monomorphic bands were observed in all primer whereas most of the primer shows monomorphic bands at positions less than 100 bp. Thus, the present results indicate the variable potentiality of the primers in resolving the variation in accessions studied.

A genetic distance matrix was computed based on Jaccard's similarity coefficient (Jaccard, 1908) using 'SAHN' (sequential, agglomerative, hierarchical nested clustering method) sub program of NTSYS-pc 2.20 (q) software package. The genetic dissimilarity matrix bet-ween accessions that ranged from 0.3635 and 2.0160 indicate that, the genetic distance among the accessions fall in medium range of variability (Table 3).

The highest genetic dissimilarity revealed by RAPD analysis (2.0160) detected between accessions AP10 and AP16 while the lowest dissimilarity (0.3635) between accessions AP3 and AP5. The dendrogram of *A. paniculata* was constructed based on RAPD data (Figure 2). All accessions initially fall under two major clusters which levels. Cluster I contains six accessions (AP1, AP2, AP8, AP10, AP11 and AP9) collected from Kusmunda,

Table 2. Polymorphism exhibited by RAPD primers.

Number	Primer	Sequence of primer	Amplified band	Number of monomorphic bands	Number of polymorphic bands	Unique bands	% of polymorphic bands
1	R-02	5'-GGCGCGTTAG-3'	10	1	9	1	90.0
2	R-03	5'-GGAACCCACA-3'	8	2	6	1	62.5
3	R-07	5'-ACCACCCGCT-3'	4	1	3	0	75.0
4	R-08	5'-AGTCGGCCCA-3'	13	3	10	1	84.6
5	R-09	5'-GGTCCTACCA-3'	4	1	3	0	75.0
6	R-11	5'-AACCGCGGCA-3'	9	1	8	3	77.7
7	R-13	5'-GGAGCGTACT-3'	16	1	15	3	93.7
8	R-14	5'-ACTGCCCGAC-3'	17	3	14	1	76.4
9	R-15	5'-GGTTACTGCC-3'	15	1	14	2	93.3
10	R-16	5'-GGGATGACCA-3'	6	3	3	1	50.0
11	R-17	5'-AAGCCCCCCA-3'	22	1	21	1	81.8
12	R-18	5'-ACGGCACGCA-3'	8	1	7	2	87.5
13	R-19	5'-GTGGCCGATG-3'	16	1	15	3	93.7
14	R-20	5'-AAGTGCACGG-3'	14	1	13	6	85.7
15	R-22	5'-GGTTGGAGAC-3'	9	1	8	0	88.8
16	R-28	5'-GTGCGCAATG-3'	11	1	10	2	63.6
Total			182	23	159	27	79.95
Average/primers			11.37	1.43	9.93	1.68	

Katghora, Salewara, Pandari, Neur and Bhoramdeo, respectively. Cluster I showed a high level of genetic variation among the accessions and was further sub-divided into two sub-clusters. Sub-Cluster Ia contains five accessions (AP1, AP2, AP8, AP10 and AP11) belonging to different areas such as Kusmunda, Katghora, Salewara, Pandari, Neur while Sub-Cluster Ib contained single genotype (AP9) belonging to Bhoramdeo. Cluster II was further divided in two subclusters (SC IIa and SC IIb). Sub Cluster (SC IIa) had a single accession 'AP24' collected from Jagdalpur, placing a separate position in dendrogram thus demonstrating the distinctiveness of the genetic background of these accession from all the other accessions.

The Subcluster (SC IIb) is further divided into two subcluster; II ba and II bb. Sub-Cluster SCII (ba) contained AP12 accessions and the subcluster SCII (bb) was further sub-divided into two subclusters; SCII (bbi) and (bbii). Sub-Cluster SCII (bbi) contained AP15 and sub-clusters SCII (bbii) is further divided two sub-clusters; SCII (bbii a) (bbii b) showing 90% dissimilarity level with all accessions. SCII (bbii a) comprised of two accessions (AP16 and AP23) and both of them belonged to a different area. SCII (bbii b) is further divided into two subclusters; SCII (bbii ba) and (IIbbii bb). SCII (bbii ba) has AP14 showing 97% dissimilarity level with all accessions and the subcluster SCII (bbii bb) is again divided into two subclusters; SCII (bbii bba) and SCII (bbii bbb). SCII (bbii bba) contained AP13 showing 89% dissimilarity levels. SCII (bbii bbb) was again divided two sub-clusters; SCII (bbii bbb x) and (IIbbii bbb y) showing 87% dissimilarity levels with all accessions. SCII (bbii Bbbx) contained six accessions (AP17, AP18, AP19, AP20, AP21 and AP22), five belongs to Sarangarh, Kharisa, Gharghoda, Leonga, were grouped into several subclusters. Dendrogram clusters can be divided into Clusters I (CI) and Clus-ters II (CII) at 1.36 dissimilarity Dharamjaygarh and one single accession belongs to Balenga. SCII (bbii bbb y) contained other accessions (AP4, AP7, AP3, AP5,

Table 3. Genetic dissimilarity matrix of 24 accessions of *Andrographis paniculata* obtained from RAPD markers.

	AP1	**AP2**	**AP3**	**AP4**	**AP5**	**AP6**	**AP7**	**AP8**	**AP9**	**AP10**	**AP11**	**AP12**	**AP13**	**AP14**	**AP15**	**AP16**	**AP17**	**AP18**	**AP19**	**AP20**	**AP21**	**AP22**	**AP23**	**AP24**
AP1	0																							
AP2	1.01	0																						
AP3	1.62	1.84	0																					
AP4	1.61	1.79	0.48	0																				
AP5	1.58	1.82	**0.36**	0.54	0																			
AP6	1.61	1.84	0.42	0.61	0.41	0																		
AP7	1.54	1.79	0.53	0.69	0.48	0.63	0																	
AP8	1.39	1.19	1.93	1.87	1.91	1.92	1.87	0																
AP9	1.32	1.38	1.62	1.61	1.62	1.64	1.59	1.73	0															
AP10	1.37	1.16	1.95	1.89	1.92	1.94	1.89	0.35	1.69	0														
AP11	1.33	1.21	1.67	1.61	1.68	1.67	1.65	1.59	1.25	1.51	0													
AP12	1.75	1.88	1.51	1.37	1.31	1.31	1.31	1.93	1.71	1.91	1.71	0												
AP13	1.49	1.68	1.13	1.13	1.07	1.07	1.02	1.76	1.52	1.77	1.57	1.18	0											
AP14	1.61	1.77	1.29	1.31	1.25	1.31	1.19	1.89	1.59	1.88	1.57	1.32	0.99	0										
AP15	1.47	1.51	1.31	1.23	1.27	1.32	1.28	1.65	1.48	1.61	1.38	1.37	1.11	1.23	0									
AP16	1.72	1.92	1.07	1.15	1.11	1.13	1.11	2.01	1.71	**2.01**	1.71	1.42	1.32	1.32	1.43	0								
AP17	1.61	1.84	0.89	0.93	0.88	0.88	0.93	1.91	1.65	1.92	1.63	1.29	0.97	1.11	1.22	1.14	0							
AP18	1.65	1.87	0.88	0.93	0.87	0.89	0.92	1.92	1.66	1.93	1.67	1.18	0.97	1.05	1.19	1.14	0.56	0						
AP19	1.64	1.85	0.95	1.02	0.97	0.99	0.89	1.91	1.63	1.91	1.62	1.21	0.89	1.11	1.22	1.09	0.67	0.63	0					
AP20	1.62	1.84	0.96	1.01	0.98	1.03	0.87	1.89	1.62	1.91	1.63	1.28	0.95	1.02	1.26	1.14	0.68	0.67	0.59	0				
AP21	1.66	1.87	0.98	1.02	0.97	0.97	0.99	1.91	1.68	1.93	1.65	1.34	1.03	1.12	1.27	1.17	0.51	0.66	0.72	0.64	0			
AP22	1.71	1.91	1.01	1.04	1.02	1.04	1.04	1.95	1.69	1.95	1.71	1.33	1.02	1.18	1.24	1.21	0.77	0.71	0.81	0.83	0.84	0		
AP23	1.78	1.97	1.14	1.21	1.12	1.19	1.12	1.98	1.81	2.00	1.86	1.46	1.41	1.49	1.46	0.91	1.24	1.21	1.19	1.22	1.22	1.26	0	
AP24	1.41	1.52	1.48	1.51	1.49	1.47	1.48	1.56	1.62	1.57	1.47	1.57	1.36	1.43	1.41	1.47	1.43	1.44	1.46	1.51	1.44	1.49	1.58	0

and AP6); all of them belonged to the same areas showing 48% dissimilarity level with all accessions. The cluster analysis with all the accessions tested showed a genetic variability between different accessions. According to the dendrogram, the accessions considered for this study did not cluster according to the geographical position of their sites. It might be possible that at the time of cross-pollination, these varieties get intermixed with other accession.

Genetic variability among *A. paniculata* was also visualized in detail; PCA was made for 182 bands produced by 16 decamer primers. It is evident from the data that *A. paniculata* were more dispersed on the PCA plot which reflects a wider genetic range. It shows clear- cut partition of 24 accessions into three groups; each group being distinct and showing dissimilarity from other group (Figure 3).

Group a contained AP24, AP13, AP12, AP15, AP14, AP16 and AP23; while group b contained AP8, AP10, AP11 and AP9; AP17, AP21, AP18, AP19, AP20, AP22, AP4, AP7, AP3, AP5, AP6 and AP1, AP2 were the part of group c.

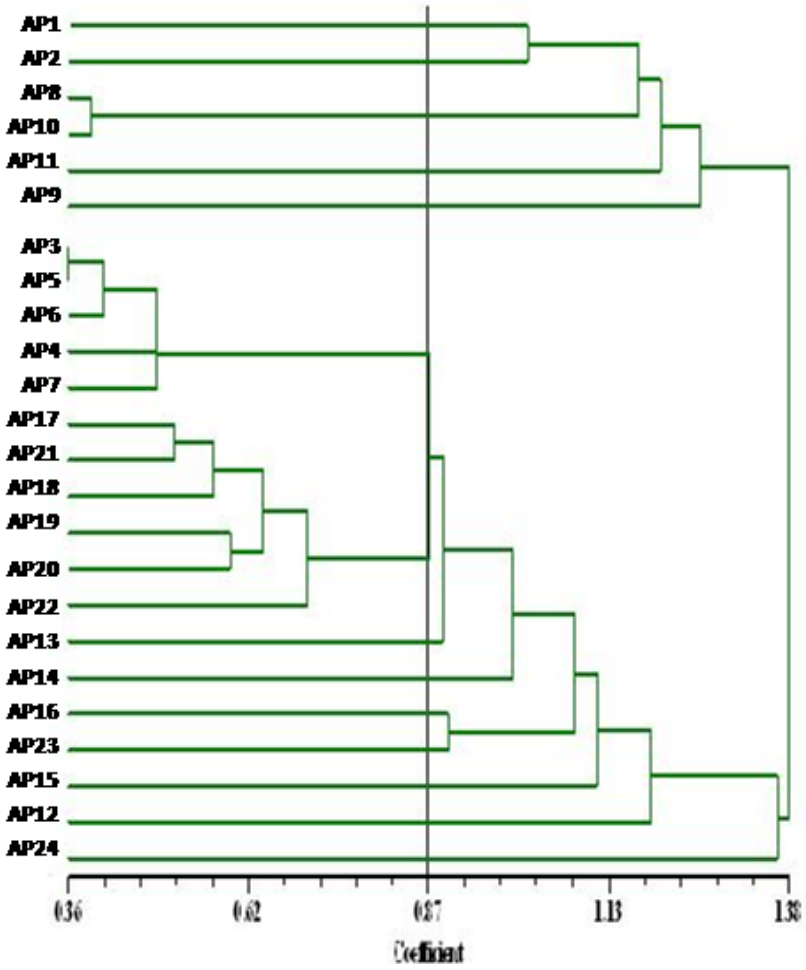

Figure 2. UPGMA dendrogram showing relationship (variation) among various *A. paniculata* accessions using 16 RAPD markers.

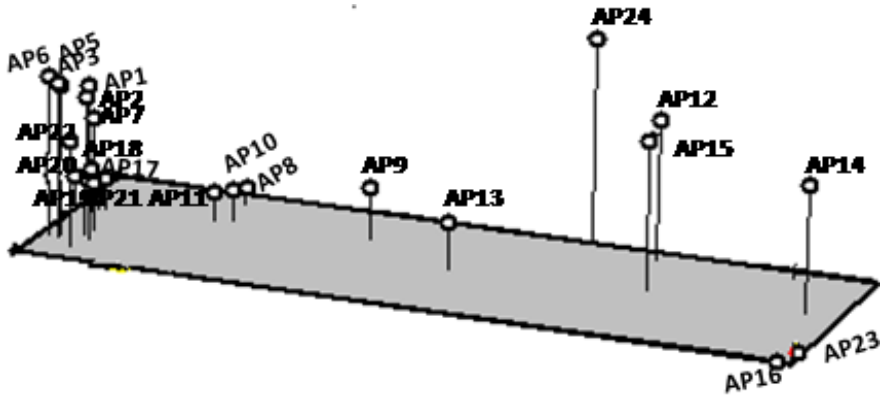

Figure 3. PCA of *A. paniculata* accessions generated by RAPD markers.

It might be possible that at the time of cross-pollination, these varieties get intermixed with other accession. In general, the data obtained from PCA are in concurrence with the dendrogram generated by UPGMA method which is a further evidence of the genetic variability delineated by cluster analysis.

In this study, we used RAPD markers to evaluate genetic variability and dissimilarity among 24 accessions of *A.*

paniculata and to select accession for further genetic improvement and conservation of genetic diversity for qualitative and quantitative traits. The selection of RAPD technique was motivated by the fact that no DNA sequence information is known about these species collected from five districts of different places of Chhattisgarh. In the present study, a high level of genetic variability and dissimilarity was found within 24 accessions of *A. paniculata* collected from various sites. The cluster analysis with all the accessions tested showed a genetic variability between different accessions.

According to the dendrogram, the accessions considered for this study did not cluster according to the geographical position of their sites. This result shows a large variation among accessions collected from different regions. It may have occurred due to the distribution of the species over a relatively small area and high ratio of cross-pollination. The plants collected for the studied were surrounded by natural environments, such as forest and mountains; there may be extensive chances of pollen and seed migration from one site to another. Our study shows a wide range of dissimilarity among 24 accessions belonging to different places; ranged from 0.3635 to 2.0160. Our results are also supported by those of Kumara and Anuradha (2011) who reported the genetic variability and highest dissimilarity among patchouli cultivars determined by using RAPD markers. El-kamali et al. (2011) determined the genetic variability of two *Sonchus* species collected from two locations in Khartoum State using RAPD technique.

Ambiel et al. (2010) studied the genetic dissimilarity among germ-plasm of six *Brachiaria* species and showed that dissimilarity indexes ranged from 0.262 to 0.907. El-Kamali et al. (2010) studied the genetic relationship and dissimilarity among *Pulicaria undulate* (UN) and *Pulicaria crispa* (CR) and their three putative hybrids (N1, N2 and HY) through RAPD makers. Highest dissimilarity was found between hybrid HY & N1 (0.50%) while the minimum dissimilarity was between *P. undulata* and hybrid N1 (0.22%). Subramanyam et al. (2010) analyzed *Jatropha curcas* germplasm accessions using RAPD markers and found that it is efficient for detecting genetic diversity and relationship of inter and intra populations. Several other reports are available on RAPD based molecular markers for diversity studies in *A. paniculata.* Sharma et al. (2009) reported genetic diversity among 15 genotypes of *A. paniculata* collected from different locations analysed using RAPD to discriminate molecular variability. Latto et al. (2008) compared diversity among 53 accessions collected from five different ecogeographic regions on the basis of morphometric and RAPD analysis for the purpose of genetic improvement and conservation of its variability.

Padmesh et al. (1999) studied the intraspecific genetic variability in Kalmegh by RAPD analysis. The RAPD-based marker also proved a useful technique for genetic diversity analysis in different plant species. Bharmauria et al. (2010) used eight random primers in the RAPD analysis of six samples of *Urtica dioica.* Out of 134 amplified fragments, 107 bands (20.2%) were polymorphic and 79.8% were monomorphic. Ikbal et al. (2010) used 50 arbitrary primers among them; 44 primers generated polymorphic profiles for RAPD analysis of *J. curcas.* The total number of bands was 328 of which 308 (93.90%) were polymorphic. Zou et al. (2011) studied genetic diversity among 33 accessions *Curcuma* species using 21 RAPD primers. The total number of amplified products was 115, including 106 polymorphic bands and represented a level of polymorphism of 92.17%. The present study of *A. paniculata* genetic variability generated a total of 182 reproducible and scorable amplification products by 16 primers across 24 accessions, of which 159 fragments (79.95%) were polymorphic. The highest number of bands generated by each primer varied from 04 (R-07 and R-09) to 22 (R-17). Our results are in agreement with those reported by Bharmauria et al. (2010) and Sharma et al. (2009). It has been shown by the result that RAPD could be effectively used for genetic diversity analysis in wild species as it is rapid, reliable and superior to those based on pedigree information. The RAPD analysis of different regions of *A. paniculata* revealed significant genetic polymorphism within each region. Perhaps, this is due to cross-pollination in the species. The analysis revealed that the species contains high level of genetic variation that enabled its wide distribution and formation of new varieties and species. This report describes the genetic variability in *A. paniculata* collected from five districts of different places in Chhattisgarh using RAPD markers. The result indicates that RAPD could be efficiently used for genetic diversity study in wild species of approaching value as it is quick, unswerving and superior to those on pedigree information.

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Full Length Research Paper

Determination of genetic polymorphism among indigenous and exotic maize inbreds using microsatellite markers

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Maize (*Zea mays* L.) is an important cereal crop of different countries of world. Undoubtedly, the concerted efforts of plant breeders and their breeding strategies have helped to increase the production and productivity to the tune of two to three folds in many crops including maize. Hybrid cultivars have played a vital role in increasing the acreage and productivity of maize. The success in identifying heterotic hybrid in maize hybrid breeding depends on the availability of genetically diverse maize inbred lines developed from different heterotic gene pool. Hence, generation of information on variability of inbreds at genotypic level become necessary. Molecular markers have proven to be a valuable tool for assessing the genetic diversity in many crop species. Simple sequence repeats (SSR) are currently considered as the molecular markers of choice and are rapidly being adapted by plant researchers for precise estimation of diversity. SSR based molecular diversity analysis of 27 maize inbred lines had produced 23 polymorphic alleles from 10 markers with an average of 2.3 alleles per locus and mean polymorphic information content (PIC) of 0.45. The dendrogram generated with hierarchical unweighted pair group method with arithmetic mean (UPGMA) cluster analysis revealed five major clusters at 0.62 similarity coefficient. The information on diversity of inbred lines generated in this study would be much useful in developing heterotic hybrids.

Key words: Maize, inbreds, marker diversity, simple sequence repeats (SSR).

INTRODUCTION

Maize (*Zea mays* L.) is a widely grown crop with a high rate of photosynthetic activity because of its C_4 pathway, leading to higher grain yield and biomass potential. It is predominantly cross-pollinated species, a feature that has contributed to its broad morphological variability and geographical adaptability. It has assumed greater significance due to its demand for food, feed and industrial utilization. The global production of maize is next to wheat and rice. The Food and Agriculture Organization (FAO) predicts that an additional 60 million tonnes of maize grain will be needed from the annual global harvest by 2030. The demand for maize as an animal feed will continue to grow faster than the demand for its use as a human food, particularly in Asia, where a doubling of production is expected from the present level of 165 million tonnes to almost 400 million tonnes in 2030 (Paliwal et al., 2000). The strength of heterosis breeding programme depends on the availability of diverse superior lines or inbreds. Hybrid cultivars have played a vital role in increasing acreage and productivity especially in maize. The high level of heterosis in a cross indicates that the parents are genetically diverse than those of crosses which show little or low heterosis (Mungoma and Pollack, 1988). Hence, information about genetic diversity of available inbreds is very important to develop heterotic hybrid combinations.

In many studies, genetic diversity among inbred lines or

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Abbreviations: SSR, Simple sequence repeats; **PIC,** polymorphic information content; **UPGMA,** unweighted pair group method with arithmetic mean

Table 1. Details of maize inbred lines taken for molecular diversity analysis.

Parent/inbred line	Kernel		Source
	Colour	Type	
UMI 1023-6, UMI 1044-7, UMI 1051-5, UMI 1053-6, UMI 1054-6, UMI 1690-6, CML 115, CML 357, CML 460, UMI 1093, UMI 1024-5, UMI 61	Yellow	Flint	India/CIM MYT
UMI 1114-10, CML 118, UMI 1025-10, UMI 1029-5, UMI 1131-5, UMI 2244-1, UMI 2128-1, UMI 1137-6	Yellow	Dent	India/CIM MYT
UMI 6143-16, UMI 285, UMI 1119	Orange	Flint	India
UMI 1266-7, UMI 1055-8, UMI 1265-6, UMI 1269-7	Orange	Dent	India

genotypes is being usually assessed based on morphological data, which do not reliably portray genetic relationship, due to environmental influence. Molecular markers have proven to be a valuable tool for genetic diversity analysis of many crop species during the past decade. Their expression, unlike morphological markers, is not influenced by environmental factors; hence, it reflects the actual level of genetic difference existing between genotypes. Genotyping techniques such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and simple sequence repeats (SSRs) have allowed assessment of the genetic diversity among maize inbred lines to synthesize most heterozygotic hybrid combinations (Trindade et al., 2010; Reif et al., 2006). Thus, attempts have been made to use molecular markers that directly evaluate genetic differences among maize inbred lines. Among the various types of markers, microsatellites or SSRs, which are short sequences containing tandemly repeated copies of one to six nucleotide fragments (Rafalski et al., 1996), are currently considered as the molecular markers of choice and are rapidly being adapted by plant researchers because of their simplicity, high levels of polymorphism (Fufa et al., 2005), high reproducibility and co-dominant inheritance patterns. Therefore, this study was conducted to investigate the genetic polymorphism and genetic relationships among 27 maize inbred lines sourced from India and CIMMYT origin.

MATERIALS AND METHODS

The genotypes used in this study consisted of 27 maize inbred lines including 24 University Maize Inbred lines (Tamil Nadu, India) and four CIMMYT lines (Mexico). The details on kernel colour and shape of inbreds used in the study was presented in Table 1.

DNA extraction

Genomic deoxyribonucleic acid (gDNA) was extracted using the cetyl trimethyl ammonium bromide (CTAB) method described by Dellaporta et al. *(*1983) with some modifications. The concentration of genomic DNA was determined on the basis of optical density readings and agarose gel electrophoresis. The final concentration of all the samples was adjusted to 25 ng/µl for amplify in polymerase chain reaction (PCR).

Primers and PCR amplification

10 SSR primers, based on their high polymorphism information content from previous studies (Smith et al., 1997; Ambika, 2010) and listed in the website maize genomic database (MaizeGD: http://www.agron.missouri.edu) were selected to determine the genetic variability in maize inbred lines (Table 2). PCR was performed in 15 µl reaction mixes consisting of 25 ng template DNA, 2.5 mM dNTP mix, 10 µM SSR primers (forward and reverse), 0.2 µl Taq polymerase and 10× PCR reaction buffer in a 1.5 ml microcentrifuge tube on ice. In a 15 µl PCR reaction volume, 13 µl of master mix was mixed with 2 µl of 25 ng DNA. The PCR profile was programmed with initial denaturation at 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min. This was followed by one final extension cycle at 72°C for 5 min, and an indefinite hold at 4°C.

Electrophoresis and fragment detection

Briefly, 5 µl of the final reaction product was mixed with 10 µl of electrophoresis loading buffer. After denaturation and immediate cooling, 2 µl of the sample was loaded onto a 6% polyacrylamide gel and electrophoresed at 400 V and 40 W till bromophenol blue reaches the bottom of the gel. The separated fragments were then visualized using a silver staining.

SSR statistical analysis

Gel photographs were scored manually and repeated twice to limit errors in scoring. The bands were binary coded by 1 or 0 for their presence or absence for each genotype, respectively. The binary matrix based on marker scores was subjected to cluster analysis. Cluster analysis was conducted with the unweighted pair group method based on arithmetic averages (UPGMA) to generate a dendrogram. The entire analysis was performed using NTSYS pc version 2.02 software (Rohlf, 1998).

RESULTS AND DISCUSSION

Genetic diversity is of prime importance for the survival, successful adaptation to certain agro-climatic conditions and improvement of any crop species. Without enough genetic diversity in the germplasm, it is practically

Table 2. SSR primer pairs used in the study and their sequences.

Primer	Sequence 5' to 3'
umc1166	Forward: CGATCAGATCATACACAACCTTGC Reverse: GAGGATCGATTCTTGGCGAGT
bnlg615	Forward: CTTCCCTCTCCCCATCTCCTTTCCAA Reverse: GCAACCTGTCCATTCTCACCAGAGGATT
phi299852	Forward: GATGTGGGTGCTACGAGCC Reverse: AGATCTCGGAGCTCGGCTA
bnlg1160	Forward: AATACTGGACCACCAGGCAC Reverse: CGTGGGTCACCAGGAGTC
phi037	Forward: CCCAGCTCCTGTTGTCGGCTCAGAC Reverse: TCCAGATCCGCCGCACCTCACGTCA
umc1484	Forward: GCGTACAGAACAGAGCAGTTACGA Reverse: ACTGAAGCTGCCTGCCTTCTATTT
phi079	Forward: TGGTGCTCGTTGCCAAATCTACGA Reverse: GATGTGGGTGCTACGAGCC
phi113	Forward: GCTCCAGGTCGGAGATGTGA Reverse: CACAACACATCCAGTGACCAGAGT
phi114	Forward: CCGAGACCGTCAAGACCATCAA Reverse: AGCTCCAAACGATTCTGAACTCGC
phi126	Forward: TCCTGCTTATTGCTTTCGTCAT Reverse: GAGCTTGCATATTTCTTGTGGACA

impossible to increase the yield and other desirable traits of any crop, since selection depends on the availability of genetic variability within the breeding material. Hence, characterization of the germplasm available would be the essential first step especially in case of heterosis breeding. Morphological characterization is easier and cheap but it does not reveal the genetic relationship reliably due to environmental influences. Therefore, the molecular markers are being employed now-a-days for precise assessment of variability at genome level. Of the 10 polymorphic SSR primer pairs used to determine the genetic diversity in 27 inbred lines, the number of alleles per locus in the lines ranged from two to four, for a total of 23 alleles. SSR marker profile of all the 27 genotypes with primer umc1166 is shown in Figure 1. The highest number of alleles was observed in loci phi126 (four alleles). Genetic diversity of the studied materials is the most important factor limiting average number of alleles identified per microsatellite locus during screening.

However, other factors such as (1) number of SSR loci and repeat types and, (2) the methodologies employed for detection of polymorphic markers have been reported to influence allelic differences. Wu et al. (2004) determined 5.4 alleles in pop corn landraces using 61 SSR loci; Liu et al. (2005) with 50 SSR loci found 4.1 alleles; Choukan et al. (2006) found 2 to 11 alleles with 4.9 alleles per locus with 46 SSR loci; Legesse et al. (2006) reported 3.85 alleles per locus using 27 SSR loci and Qi-Lun et al. (2008) found 6.4 alleles using 45 SSR loci. However, our value (2.3) closely agreed with the findings reported by Shah et al. (2009) and Aguiar et al. (2008) who reported 1.56 alleles using 10 SSR loci and 3.4 alleles with 28 polymorphic SSR loci. It is important to note that the total number of alleles reported in diversity studies is actually proportional to sample size. Therefore, the differences observed in the study may be attributable to sampling differences and lesser number of loci used for genetic diversity analysis.

Polymorphic information content

The polymorphism information content (PIC) demonstrates the informativeness of the SSR loci and their

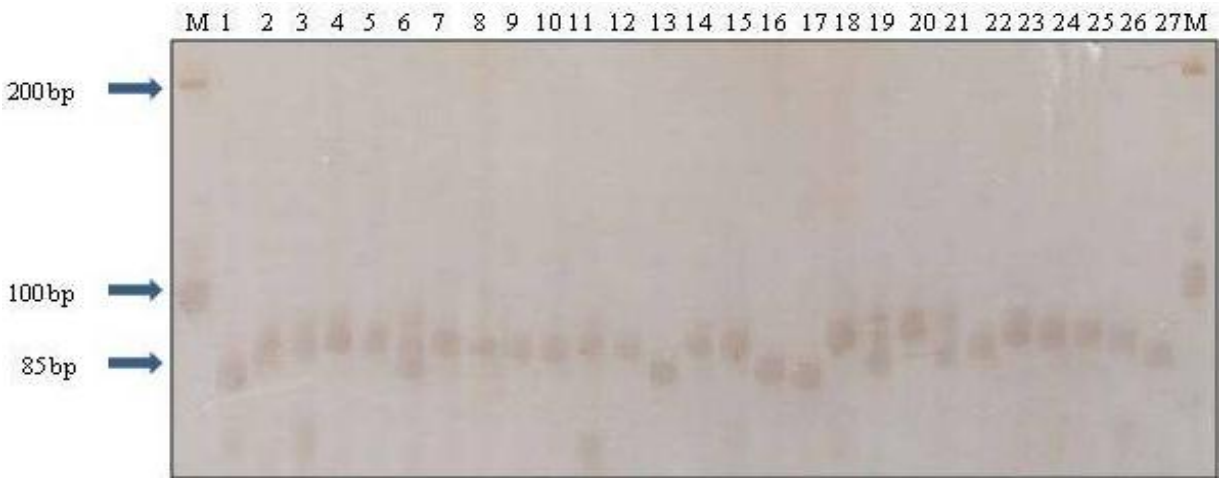

Figure 1. SSR marker profile of 27 maize inbreds produced by primer umc1166.

Table 3. Repeat types, bin number, number of alleles and PIC values of SSR markers used in the study.

SSR primer's code	Repeat type	Bin number	Number of alleles	PIC value
umc1166	CT	1.02	2	0.35
bnlg615	-	1.07	3	0.56
phi299852	AGC	6.07	2	0.47
bnlg1160	AG	3.06	2	0.48
phi037	AGC	1.08	2	0.34
umc1484	AGC	1.01	2	0.42
phi079	AGATG	4.05	2	0.25
phi113	GTCT	5.03	2	0.48
phi114	GCCT	7.03	2	0.49
phi126	AG	6.00	4	0.66
Mean	-	-	2.3	0.45

potential to detect differences among the inbred lines based on their genetic differences. In the present study, PIC values of the SSR loci ranged from 0.25 to 0.66 with mean of 0.45 (Table 3). The average PIC value determined in our investigation agrees with the earlier findings reported based on SSR marker in maize inbred lines. Choukan et al. (2006) found the PIC value of 0.54; Legesse et al. (2006) reported the PIC of 0.58 and Aguiar et al. (2008) with PIC value of 0.51.

Clustering of the inbred lines

The dendrogram constructed using the UPGMA clustering algorithm grouped the inbred lines into five clusters (Figure 2). The three yellow kernel type genotypes of Indian origin *viz.,* UMI 1023-6, UMI 1114-10 and UMI 1093 were grouped in cluster I, only two genotypes UMI 1025-10 and UMI 1119 were grouped into cluster II. Among the genotypes analysed, six and eight genotypes were grouped in cluster III and IV, respectively with one or two inbreds of CIMMYT origin. Fifth cluster contains eight genotypes which includes both yellow and orange type of grains with one line of CIMMYT origin (Table 4). It could be noted that the four genotypes from CIMMYT grouped into different cluster indicates the existence of good variability within the indigenous lines too. Even though the genotypes were grouped in different clusters, some of the flint and dent genotypes, yellow and orange kernel genotypes are grouped in same cluster.

This may be due to the reason that inbreds are developed from the same ancestral cross combining both flint and dent kernel genotypes. Further, the effects of selection drift and mutation or human error might be the cause of the aforementioned discrepancies (Warburton et al., 2002; Legesse et al., 2006). By utilizing the aforementioned genetic diversity information generated, 72 hybrid combinations were synthesised using these 27 genotypes by including genotypes viz., UMI 285, UMI 61 and UMI 1119 as testers and remaining 24 inbreds as lines, in a Line x Tester mating design. The resultant hybrids were found to be promising since testers selected in such a way that they occupied the different clusters. The parents of the hybrids which

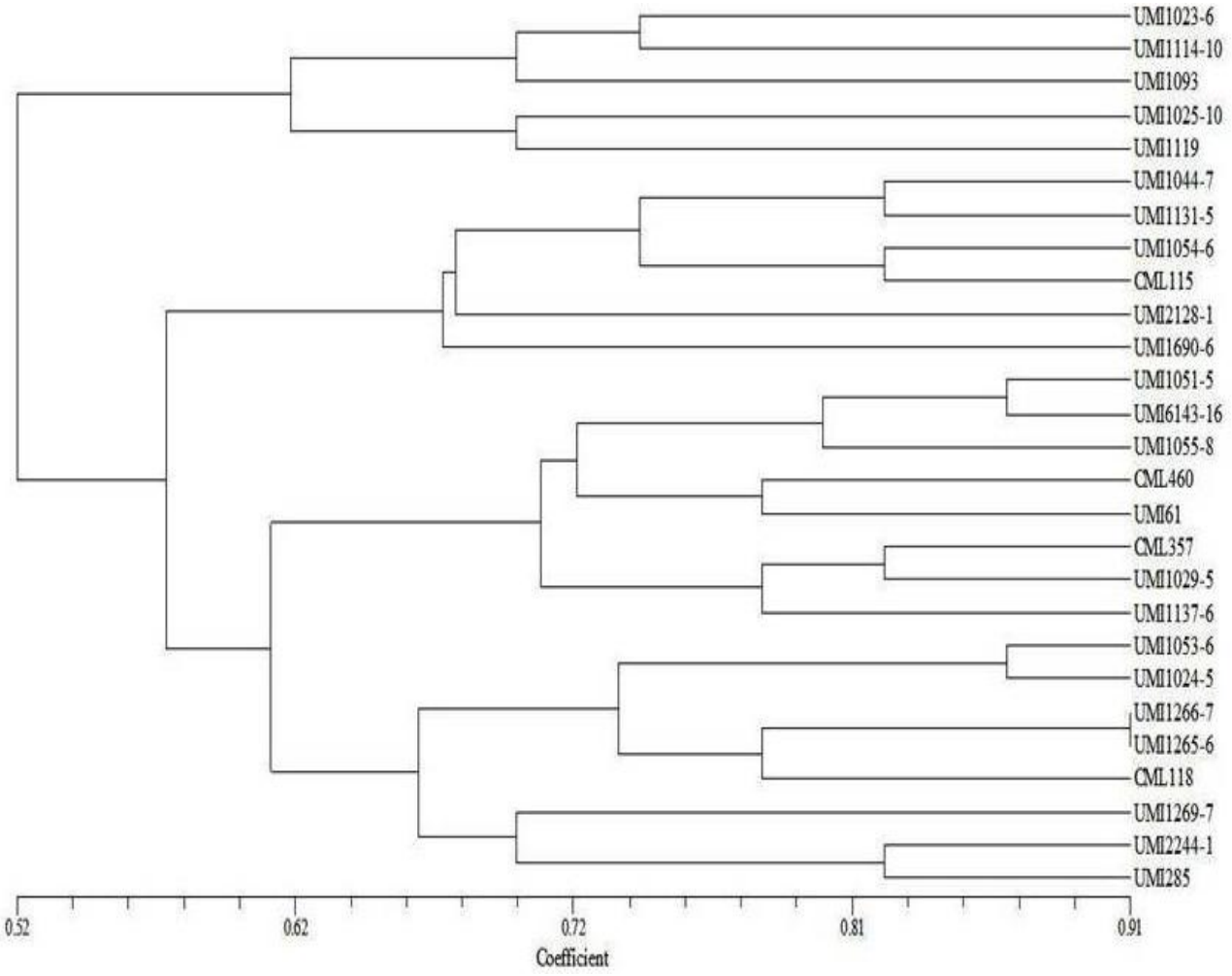

Figure 2. Dendrogram showing the relationship among 27 maize inbreds based on 10 SSR primer pairs.

Table 4. Clustering of maize inbred lines by SSR markers.

Cluster	Genotype/inbred
I	UMI1023-6, UMI1114-10, UMI1093
II	UMI1025-10, UMI1119
III	UMI1044-7, UMI1131-5, UMI1054-6, UMI2128-1, UMI1690-6, CML115
IV	UMI1051-5, UMI6143-16, UMI1055-8, UMI61, UMI1029-5, UMI1137-6, CML460, CML357
V	UMI1053-6, UMI1024-5, UMI1266-7, UMI1265-6, UMI1269-7, UMI2244-1, UMI285, CML118

show higher yielding ability and heterosis were also from different clusters (Table 5). Hence, these results further support that combining the diverse inbreds in heterosis would yield heterotic hybrids.

Conclusion

Present study indicates that SSR markers clearly separated the inbred lines into different clusters based on their genetic divergence and facilitated for the development of heterotic hybrid combination. Hence, whenever the inbred from indigenous and exotic origin is included in the development of hybrids, it is advisable to assess the genetic divergence in order to avoid narrow genetic base in resultant hybrids.

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Table 5. Hybrids combinations showing their parents cluster origin.

Hybrid	Grain yield per plant (G)	Heterosis over check variety (%)	Parent cluster
UMI 1044-7 × UMI 61	182.63	18.70	III × IV
UMI 1093 × UMI 61	182.35	18.51	I × IV
UMI 2244-1 × UMI 1119	177.87	15.61	V × II
UMI 1093 × UMI 1119	177.70	15.49	I × II
CML 460 × UMI 1119	172.49	12.11	IV × II
UMI 1044-7 × UMI 1119	170.78	11.00	III × II
UMI 1024-5 × UMI 1119	168.77	9.69	V × II
UMI 1266-7 × UMI 1119	165.59	7.62	V × II
UMI 1093 × UMI 285	162.29	5.48	I × V

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Molecular and functional diversity in *Capsicum* landraces of Andaman Islands

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The present study analyzed the diversity in 26 landraces of *Capsicum* from Andaman Islands using 20 morphological, 16 biochemical and 10 DNA markers. Significant differences were observed in tested landraces and 16 reference genotypes from mainland India. Biochemical markers grouped all the genotypes into eight clusters with inter-cluster distance of 0.5 to 1.9 while seven quantified morphological traits divided the test genotypes into three major clusters and seven sub-clusters with 0.1 to 1.6 inter-cluster distance value. The random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers assured the genetic nature of diversity in landraces. The similarity matrix from RAPD and ISSR markers revealed 48% diversity among 42 genotypes with polymorphism information content (PIC) values of 0.43 and 0.41, respectively. The correspondence in morphological and biochemical markers indicates their interdependence for observed traits. However, poor correlation between DNA profiles and functional markers suggest further screening of more number of markers.The study identified phytochemical rich landraces CA-334, SPG-7, CARI-1 and CCB-2. The information will be useful in chemo-taxonomic foot-printing of *Capsicum* landraces and devising apposite conservation and utilization strategies.

Key words: *Capsicum*, landraces, functional diversity, chemo-taxonomic diversity, DNA markers.

INTRODUCTION

The genus *Capsicum* belongs to family Solanaceae and have 27 species including five commonly cultivated species (*C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L. and *C. pubescens* Ruiz & Pav.). The most important species *C. annum* bears both pungent and sweet fruits having commercial value as spice and vegetable. Pungent chilli has diverse prophylactic and therapeutic uses such as antibacterial, antifungal, anticancer, anti-oxidant, anti-protozoal, hypocholesterolaemic, hypolipidemic, immunomodulatory and anti-mutagenic (Pawar et al., 2011). These properties are due to complex matrix of phytochemicals in fruits which includes flavonoids, phenolics and carotenoids. These compounds also acts as antioxidants and supplement the in built homeostasis mechanism of human body for inhibiting or neutralizing the free radicals (Nadhala et al., 2010). Some of these compounds showed strong correlation with antioxidant activity but, their concentration and capacity are influenced by genotypes, environment and estimation method (Singh et al., 2011). The screening of germplasm with these traits serves dual purpose of providing chemotaxonomic diversity (Goff and Klee, 2006) and information about phytochemical rich genotypes.

The archipelago of Andaman and Nicobar Islands (India) is consisted of 572 islands and located between 14° to 16° N and 92° to 94° E. It is recognized as one of the biodiversity hotspots (Mayers et al., 2000). The islands also have rich diversity of *Capsicum* with the existence of

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C. *annum*, *C*. *frutescens* and *C*. *chinense* (Abraham et al., 2008). It is presumed that these species were introduced during or after the second half of 19th century to meet up demand of settler communities. In period of 150 years, the introduced *Capsicum* germplasm faced natural evolutionary forces and got adapted to island conditions. This might have changed their genomic constitution at least to some extent but so far, no variety of *Capsicum* has been specifically bred for islands. Thus, assessment of diversity in the local germplasm was much needed to recognize the genetic relatedness in order to select the parents for breeding programme and also to avoid duplications in gene banks.

The limitation of morphological and biochemical markers can be reduced by the use of DNA markers as they are simple to use, cost effective, abundant in genome and independent to stage and environment. Thus, combined use of morphological, biochemical and DNA markers may generate sufficient information for authenticating the extent of diversity in germplasm. Among DNA markers, PCR based RAPDs and ISSRs are in common use for decoding the diversity in crop germplasm at preliminary stage when little is known about whole genome of the species (Singh et al., 2012). Therefore, the present study aimed to assess the extent of molecular and functional diversity in *Capsicum* landraces of Andaman Islands for understanding the extent of genetic distance between the landraces and identifying the potential genotypes for improvement of economic and phytochemical parameters.

MATERIALS AND METHODS

Collection of germplasm

Representative plant samples of 26 landraces of Capsicum were collected from different islands (Table 1). Sixteen elite genotypes were taken from all India Coordinated Research Project (vegetable Crops) as reference (Table 1). All the 42 sample genotypes were grown in randomized block design with three replications and managed with standard package of practices at Research Farm of Central Agricultural Research Institute, Port Blair during the dry season (December to April) of 2010-2011.

Chemicals and reagents

The analytical grade chemical reagents were used in the present study and 1,1-diphenyl 2 picrylhydrazyl (DPPH), gallic acid, anthrone reagent, aluminium chloride, formic acid, hexane, anthrone, dinitrosalicylic acid, 2, 6-dichlorophenol indophenols, ninhydrin, methyl orange, sulphuric acid, boric acid indicator, Davarda's alloy and leucine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tannic acid, Ascorbic acid, conc. HCl, sodium acetate buffer and sulphuric acid were purchased from Himedia (Himedia Laboratories Pvt. Ltd., Mumbai). Methanol, rutin, folin-ciocaulteu reagent, KCl, copper sulphate and sodium hydroxide were purchased from Merck (Merck, Darmstadt, Germany). Anhydrous sodium sulphate, potassium sodium tartarate, oxalic acid, sodium bicarbonate, citrate, potassium dichromate, ammonium thiocyanate, ferric chloride, ammonia, cal-cium chloride, potassium permanganate, magnesium oxide, sodium acetate, orthophosphoric acid, acetone and sodium carbonate solution were obtained from Rankem (RFCL Ltd., New Delhi, India).

Morphological parameters

Twenty (20) morphological characters including seven quantified characters viz., leaf size, leaf shape, leaf colour, growth habit, stem colour, seed colour, number of flower/ axil, flower colour, mature fruit colour, ripe fruit colour, fruit shape, fruit end shape and fruit surface were recorded from five random plants of Capsicum landrace/genotypes using standard procedures.

Biochemical analysis

Estimation of phytochemicals

The polyphenol content in green fruits was estimated by Folin-Ciocalteau reagent method (10%, v/v) (Singleton and Rossi, 1965) with some modifications. The absorbance from samples was measured at 765 nm using UV-spectrophotometer (Elico SL-164, Pvt Ltd, Hyderabad, India). Gallic acid was used as reference and the results were expressed as mg of gallic acid equivalent (mg/100 g fresh weight). Flavonoid content in test genotypes was determined spectrophotometrically using standard protocol as described by Chang et al. (2002) and expressed as mg rutin equivalent (mg/100 g fresh weight). Concentration of anthocyanin in chilli fruits was determined by pH differential method as described by Sadasivam and Manickam (1996), and results were expressed as C_3GE (cyanidine-3-glucoside) mg/100 g fresh weight (Fuleki and Francis, 1968). The ascorbic acid was estimated using standard colorimetric method (Sadasivam and Manickam, 1996) and concentration was expressed as mg/100 g fresh weight. Total carotenoid and chlorophyll content in chilli fruit were determined through procedure given by Lichtenthaler and Buschmann (2001). Tannin content was estimated using AOAC method (1990) with tannic acid as standard and expressed as TAE mg/100 g fresh weight.

DPPH antioxidant activity

The antioxidant activity of methanol extract of fruit of chilli genotypes was determined by 1,1-diphenyl-2- picrylhydrazyl (DPPH) method (Wong et al., 2006). Stock solution of extracts were diluted to 20, 40, 60, 80 and 100 µg/ml and incubated for 2 h in dark chamber and absorbance readings were taken at 517 nm at 10 min interval. Sample extract (0.1 ml) were added to 3 ml of methanol solution of DPPH (0.001 M). The antioxidant activity (%) was calculated as $[(A_0 – A_E)/A_0]$ x100 (A_0 = absorbance without extract; A_E = absorbance with extract), whilst IC_{50} values were estimated from percent inhibition of DPPH free radicals against concentration sigmoidal curve, using a non-linear regression analysis.

Proximate components

The total carbohydrate was estimated according to the modified method of Hedge et al. (1962) and reducing sugar by Nelson-Somogyi method (Sadasivam and Manickam, 1996). The non-reducing sugar was calculated by subtracting the quantity of reducing sugar and multiplying with a conversion factor (0.95). The absorbance for extractable colour value in mature fruits was observed with acetone solvent at 450 nm with $K_2Cr_2O_7$ solution as reference. The colour value was determined by the formula: Colour value (ASTA units) = (Absorbance of chilli extract at 450 nm × 200)/Absorbance of $K_2Cr_2O_7$ solution at 450 nm (Sadasivam and Manickam, 1996). The antinutritional factors like phytate, oxalate, nitrate and saponin content in chilli fruits were determined using the

Table 1. Capsicum landraces and reference genotypes used in the study.

Accession	Specie	Source
CHIVAR-1-I	*Capsicum annum* L.	AICRP/IET (Mainland India)
CHIVAR-3-I	*Capsicum annum* L.	AICRP/IET (Mainland India)
CHIVAR-4-I	*Capsicum annum* L.	AICRP/IET (Mainland India)
CHIVAR-5-I	*Capsicum annum* L.	AICRP/IET (Mainland India)
CHIVAR-6-I	*Capsicum annum* L.	AICRP/IET (Mainland India)
LCA-334-1	*Capsicum annum* L.	AICRP/IET (Gutur, A.P., India)
KA-2	*Capsicum annum* L.	AICRP/IET (Kerala, India)
CARI-1	*Capsicum annum* L.	Neil Island, South Andaman
CARI-2	*Capsicum annum* L.	Neil Island, South Andaman
M-1	*Capsicum annum* L.	Mangultan, South Andaman
M-2	*Capsicum annum* L.	Mangultan, South Andaman
M-3	*Capsicum annum* L.	Mangultan, South Andaman
N-1	*Capsicum annum* L.	Nayasagar, South Andaman
G-1	*Capsicum annum* L.	Girgutan, South Andman
G-2	*Capsicum annum* L.	Girgutan, South Andaman
H-1	*Capsicum annum* L.	Humphreygunj, South Andaman
H-2	*Capsicum annum* L.	Humphreygunj, South Andaman
CHIVAR-1-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-2-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-3-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-4-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-5-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-6-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-7-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CHIVAR-8-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
LCA-334-II	*Capsicum annum* L.	AICRP/AVT-II (Mainland India)
CCB-1	*Capsicum pubescens*	Choldhari, South Andaman
CCB-2	*Capsicum frutescens*	Choldhari, South Andaman
CCB-3	*Capsicum frutescens*	Diglipur, North Andaman
CCW	*Capsicum frutescens*	Diglipur, North Andaman
CCO	*Capsicum frutescens*	Okrabraj, South Andaman
CCR	*Capsicum frutescens*	Rangat, Middle Andaman
CCLG	*Capsicum frutescens*	Hut Bay, Little Andaman
CCG	*Capsicum frutescens*	Guptapara, South Andaman
SPG-1	*Capsicum frutescens*	Garacharma, South Andaman
SPG-2	*Capsicum frutescens*	Hut Bay, Little Andaman
SPG-3	*Capsicum frutescens*	Sippighat, South Andaman
SPG-4	*Capsicum annum* L.	Sippighat, South Andaman
SPG-5	*Capsicum annum* L.	Sippighat, South Andaman
SPG-6	*Capsicum annum* L.	Calicut, South Andaman
SPG-7	*Capsicum annum* L.	Collinpur, South Andaman
LMCF	*Capsicum annum* L.	Haddo, South Andaman

method described by Sadasivam and Manickam (1996).

Molecular markers

Genomic DNA isolation

The genomic DNA of 26 landraces and 16 elite genotypes of Capsicum were extracted leaves using the CTAB method with slight modifications. For this, the healthy leaves were collected, cleaned, surface sterilized and 3 g leaves were ground with 5 ml pre-warmed (65°C for 1 h) CTAB buffer. Further steps are similar to CTAB method of DNA isolation from plant tissues. Quantification and qualitative analysis of the DNA were performed using UV Spectrophotometer (ELICO Ltd., Hyderabad, India) and gel electrophoresis with 0.8% (w/v) agarose and ethidium bromide (3 µl/100ml gel

solution).

PCR analysis

The primer screening with 30 RAPD (Operon Technologies, Alameda, California; UB series, University of British Columbia, Vancouvur, BC Canada supplied by Bangalore Genei, Bangalore, India), and 37 ISSR markers (Sigma-Aldrich, St. Louis, Mo., USA) was done in 42 test genotypes. PCR analysis carried out in thermal cycler (G-STORM, Gene Technologies, United Kingdom) in a final volume of 20 µl containing 1 µl genomic DNA (25 ng), 1.5 µl dNTP mix (made up of 100 µM of each of the four dNTPs), 1 µl primer (RAPD/ISSR), 1.6 µl $MgCl_2$, 2 µl of 10X *Taq* buffer (10 mM Tris HCl pH 9.0, 50 mM KCl), 0.25 µl *Taq* DNA polymerase (0.5 U) (Banglore GeneI, Bangalore, India) and 12.65 µl sterile millipore water. A negative PCR was kept to test the PCR reactions.

The PCR programming for RAPD was done with steps of hot start (94°C, 5 min) and 39 cycles (denaturation at 94°C, 1 min; primer annealing at 36°C, 1 min and primer extension at 72°C, 1 min) followed by final extension (72°C, 10 min) and cooling (10°C, 1 h). The amplified products were taken out and kept at 4°C till electrophoresis. For ISSR markers, Touchdown PCR reaction (Don et al., 1991) was performed with minor modifications in PCR programming with steps of hot start (94°C, 5 min) and 8 down steps (94°C, 1 min; 45°C to 39°C, 1 min each; 72°C, 1 min) followed by 31 linear cycles (94°C, 1 min; 38°C, 1 min; 72°C, 1 min) followed by final extension (72°C, 10 min) and cooling (10°C, 1 h). Amplified PCR product (5 µl) were mixed in 6X bromophenol blue (5 µl) and separated by gel electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized with UVP MultiDoc-IT Digital Imaging System (UVP LCC, California).

Dendrogram construction and statistical analysis

The binary data of reproducible bands from RAPD and ISSR markers in 42 genotypes were subjected for construction of dendrogram through unweighted pair group method with arithmetic average (UPGMA) cluster analysis using software NTSYS-pc, version 2.02. Polymorphic information content (PIC) for each marker was calculated as PICi = 2fi (1-fi) as proposed by Roldan-Ruiz et al. (2000), where PICi is the polymorphic information content of i^{th} marker, fi is the frequency of the marker bands present, (1-fi) is the frequency of absent marker bands. While the dendrograms of test genotypes using seven morphological traits having quantitative data *viz*. plant height, seeds/fruit, fruit length, fruit width, pedicel length, fruits/plant and fruit yield/plant and 16 biochemical compounds were constructed using SAS 4.1 Enterprise software. The quantitative data for morphological and biochemical parameters were analysed for mean and standard deviation using Microsoft Excel 2007 software.

RESULTS AND DISCUSSION

Morphological diversity

The results from observations for 13 qualitative characters from test genotypes revealed that most genotypes had medium size (45.2%) and lanceolate leaves (40.5%) with horizontal orientation. Landraces had large leaves (53.0%) with intermediate growth habit (61.5%) while genotypes from mainland showed erect plant habit. The landraces showed variability in seed colour with predominance of cream (61.5%) and yellow (23.0%) while reference genotypes produced only cream colour seeds. Flower colour in germplasm was observed to be white, light white and purple. Only white flowers were observed in all reference genotypes while most of the landraces produced white (42.3%) and light white (42.3%) flowers and four landraces produced purple flowers.

Fruit colour showed great variation in landraces which was observed to be dark red (11), red (7), brownish red (4), light red (2) while one landrace (CCO) produced orange fruits. However, red fruit trait was predominant in reference genotypes. The fruit shape revealed great extent of diversity in landraces where small, medium, long, very long fruits were observed with fruit length of 1.1 to 7.5 cm length, maximum in CCB-1 while minimum in M-2. Two landraces CCB-1 and LMCF had round fruits while conical, conical long, curved, pointed, slightly curved, smooth, wrinkled, semi-wrinkled fruits were also observed.

The morphological traits having quantified observations showed significant variations between landraces and reference genotypes and also within them. Length of mature green fruits in investigated landraces ranged from 1.1 (CCB-1) to 7.5 cm (M-2) while in reference genotypes, it ranged from 5.8 (CHIVAR-1-I, CHIVAR-2-II) to 11.4 cm (CHIVARI-5-I). Notably, number of seeds per fruit showed great variation in *Capsicum* landraces with the range of 17 (CCW) to 78 (M-2) while narrow range for seeds per fruit was observed in reference genotypes that is between 24 (KA-2) to 47 (CHIVAR-2-II). The fruit diameter in landraces ranged from 0.3 (SPG-1) to 1.6 cm (M-1) while it varied from 0.9 to 4.4 cm in reference genotypes. Individual fruit weight in genotypes ranged from 2.14 to 19.56 g.

Dendrogram based upon seven morphological traits (Figure 1) revealed three major clusters and seven subclusters with 0.1 to 1.6 inter-cluster values. Cluster-I, cluster-IIIa and cluster-IIId consists of seven, five and two genotypes, respectively and all representing the AICRP (VC) entries from mainland while Cluster-II and IIIc had 10 and 3 landraces of islands. Cluster-IIIb had 13 genotypes, mostly from islands except only one genotype from AICRP (VC).

The present study revealed great diversity among *Capsicum* landraces from islands and also showed genetic distance from reference genotypes of mainland India. The variation in some of the adaptive traits like leaf orientation and leaf size indicate that landraces in islands have been evolved with adaptive mechanisms to transpire more in tropical humid conditions of islands. The flower colour is Capsicum helped in understanding the relationship among the different species. The white flowers are produced by *C. annum* or *C. chinense*, whitish green by *C. frutescens* and purple by *C*. *pubescens*. Similar observations were made in the present study though some of the landraces showed yellowish white pattern in flower colour. The findings support the reports of Abraham et al. (2008) for the presence of species diversity in *Capsicum* genus in islands. However, they did not report *C*. *pubescens* in islands which might have

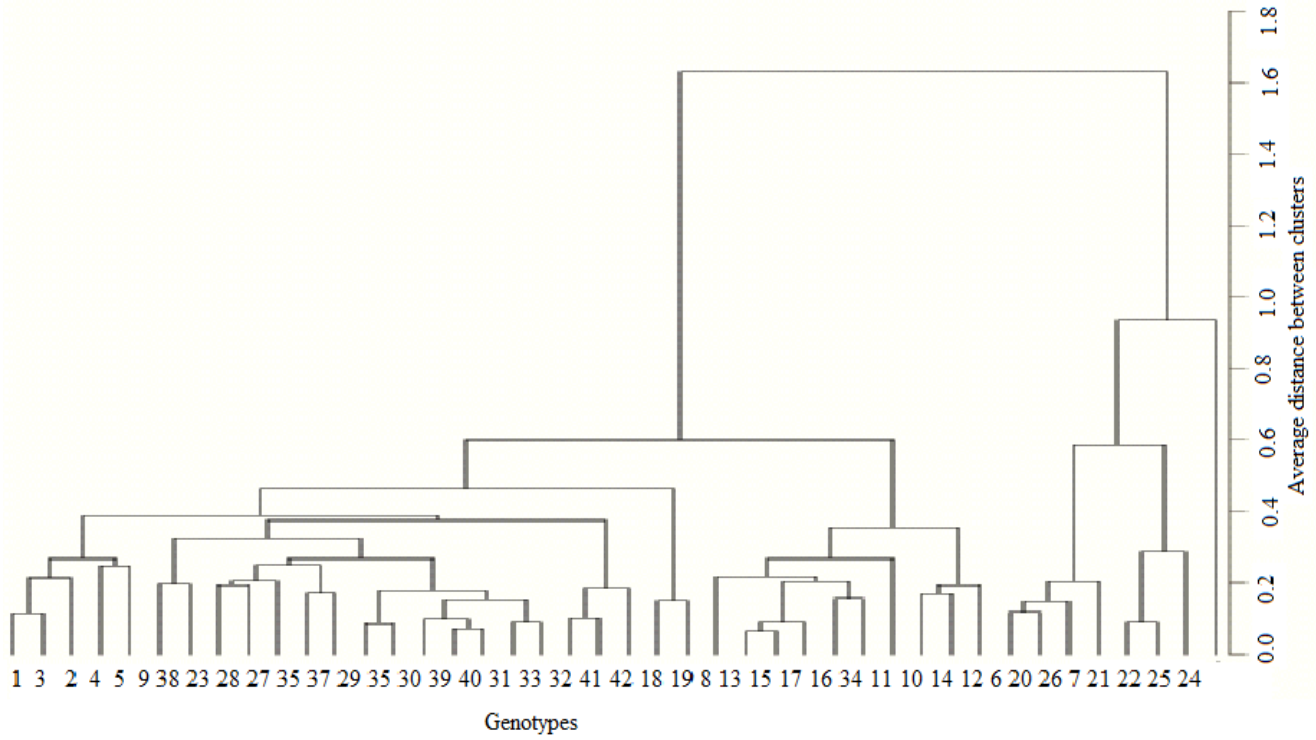

Figure 1. Cluster analysis of *Capsicum* genotypes with morphological parameters (S. No. 1-42 are genotypes as given in Table 1).

been brought after their survey period or remain unnoticed. The clustering pattern from morphological traits having quantitative observation showed distinctness of landraces from reference genotypes. This might be due to adaptive changes or 'in-house spread' of landraces among the islanders through their personal contacts or local seed vendors for thier homegardens or farms (Pandey et al., 2006).

Chemo-taxonomic diversity

The results for estimation of phytochemicals viz., polyphenol, flavonoid, tannin, anthocyanin, carotenoid, vitamin c and chlorophyll in 40 genotypes of *Capsicum* showed great extent of diversity, particularly in Islands landraces. Polyphenol content revealed significant variation among local landraces and reference genotypes of AICRP (VC). The highest polyphenol content was estimated in CA-334 (181.6 mg/100 g) while lowest in SPG-5 (53.1 mg/100 g). Some of the local landraces like CARI-1, CCO, M-1 and CCB-1 were also found to be rich in polyphenol content. The flavonoids content in test genotypes ranged from 41.01 to 791.0 mg/100 g, the highest was recorded in CARI-1 while lowest in M-1. The CHIVAR-6-I, CA-334, CHIVAR-5-I, CHIVAR-5-II and KA-2 of AICRP (VC) and CARI-1, G-2 and CCO from islands were found to be rich in flavonoids. The *Capsicum* landraces also showed variation for tannin content, the highest in CARI-1 (328.9 mg/100 g) and minimum in SPG-5 (113.3 mg/100 g). However, CHIVAR-4-II contained highest amount of tannin (415.6 mg/100 g) among the screened genotypes. Most of the genotypes were poor in anthocyanin content at mature green stage as its concentration was ranged from 0.9 to 41.7 mg/100 g with maximum in SPG-7 and minimum in CCB-1 and CCG. Capsicum genotypes were found to be rich in carotenoid content which was highest in SPG-7 (552.7mg/100 g) whilst lowest in H-1 (20.2 mg/100 g).

Chlorophyll content in mature green fruits ranged from 179.8 (CCB-3) to 579.5 µg/100 ml (CARI-1). The chlorophyll rich landraces were CARI-1, M-1, G-1, M-2, M-3, N-1 and CARI-2. The present study identified significant (p=0.05) differences among genotypes for ascorbic acid content which ranged from 43.3 (CHIVAR-7-II) to 140.0 mg/100 g (CHIVAR-1-I). The identified ascorbic acid rich genotypes were CHIVAR-1-I, CA-334, CHIVAR-4-I, CHIVAR-2-II, LCA-334 and SPG-6. The antioxidant activity of methanol extract of test genotypes ranged from 67.9 to 96.3%, the highest in H-1 and lowest in SPG-4.

The free amino acid content was highest in CCB-1 (747.1 mg/100 ml) while lowest in M-1 and SPG-7 (95.3 mg/100 ml). The Landraces CCB-2, G-2, CCW, CCG, G-1, CCLG and SPG-3 were identified to be rich in free amino acids. The level of sugar content also revealed diversity in Capsicum genotypes which ranged from 168.5 to 308.7 mg/100 g. Most of the genotypes rich in total sugar that is CHIVAR-3-I, CHIVAR-3-II, CHIVAR-5-II,

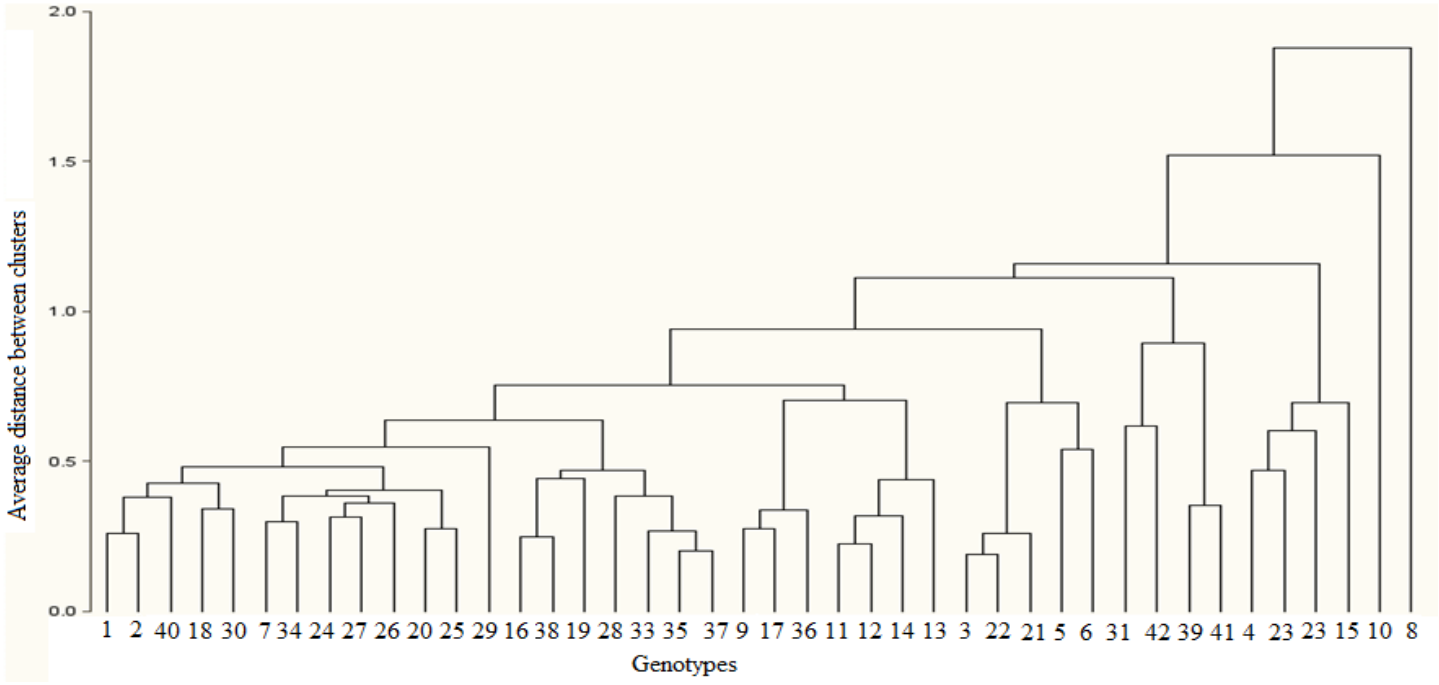

Figure 2. Cluster analysis of *Capsicum* genotypes with biochemical parameters (S. No. 1-42 are genotypes as given in Table 1).

CHIVAR-4-II and CHIVAR-2-II represented AICRP (VC) material while local landrace were low in total sugar content. Reducing sugar was estimated to be maximum in CCO (153.0 mg/100 ml) while the minimum was observed in M-3 (12.2 mg/100 ml). G-1, SPG-2, CCG, CCW, SPG-7 and CCO landraces were found to be rich in reducing sugar than AICRP (VC) genotypes. Non-reducing sugar content also showed significant (p=0.05) variations in capsicum genotypes which ranged from 61.1 (G-1) to 250.4 mg/100 g (CHIVAR-3-II). Among local landraces, the CCR (196.1 mg/100 g) and M-3 (188.9 mg/100 g) were found to be rich in non-reducing sugar. In the present study, significant (p=0.05) variations were also observed in capsicum genotypes for anti-nutrients such as nitrate content which ranged from 15.0 mg/100 g (SPG-2) to 83.8 mg/100 g (CHIVARI-2-II and CARI-1) (Figure 2). The phytate content was ranged from 25.9 mg/100 g (CARI-1) to 669.0 mg/100 g (LMCF). The highest oxalate content was observed in CHIVAR-3-I (10.1 mg/100 g) while minimum in CARI-1 (2.7 mg/100 g). The *Capsicum* genotypes were low in saponin content which ranged from 40.0 mg/100 g (M-1) to 95.0 mg/100 g (M-2). The methanol extract of green fruits of CHIVAR-1-I showed highest antioxidant activity (96.3 %) while lowest by another AICRP (VC) genotype CHIVAR-3-II (67.9 %). The highest colour values of mature green fruits *Capsicum* genotypes was observed for SPG-3 (214.0 ASTA Units) while the minimum in LMCF (14.9 ASTA Units).

The cluster analysis of 42 genotypes using 16 phytochemical parameters formed eight major clusters with intra-cluster similarly value of 0.5 to 1.9. Cluster-I had mixed representation from AICRP (VC) (CHIVAR-1-I, CHIVAR-3-I and CHIVAR-1-II) and landraces (CCW and SPG-6). Cluster-II represented AICRP (VC) genotypes from mainland while Cluster-III was constituted of local landraces. Cluster-IV, cluster-V, cluster-VII and cluster-VIII were predominated with local landraces while cluster-VI corresponded to the AICRP (VC) materials.

Chemosystematics helped in distinguishing the difference in capsicum varieties and species. Based on flavonoid content, Ballard et al. (1970) reported that *C. baccatum* var. *baccatum* and *C. baccatum* var. *pendulum* were representative of same species. The present study investigated the extent of diversity and characterized 26 *Capsicu* landraces from Islands by analyzing 16 phytochemicals in green fruits as markers. The green fruits of *Capsicum* are commonly used as vegetable, chutney, pickle or taste agent in various food items. The landraces were rich in carotenoids, chlorophyll, free amino acids, reducing sugar, nitrate, saponin while reference genotypes were comparatively rich in polyphenol, flavonoids, tannin, ascorbic acid, total sugar and non-reducing sugar contents. The findings for the phytochemicals in the present study were in parity with the findings of Ruanma et al. (2005) and Rodriguez-Burruezo et al. (2009). However, the slight differences might be due to variations in stage of samples, estimation methods, genotypes and environment. Now-a-days, the researchers are utilizing different set of markers to establish the relationship among the species or genotypes of crops (Singh et al., 2012). The study also identified phytochemical rich landraces/genotypes such as CCO, M-1, CCB-1, CCR and

Table 2. Amplification parameters of DNA markers in *Capsicum* genotypes.

DNA marker	Marker sequence	Amplicon size (bp)	Total amplicons in genotype	Amplicons per genotype	PIC value
OPA3	AGTCAGCCAC	700-1400	138	12.55	0.35
OPA6	GGTCCCTGAC	1200-7000	198	24.75	0.35
OPA8	GTGACGTAGG	250-1200	136	9.71	0.33
OPD10	GGTCTACACC	300-1250	199	15.31	0.40
OPD13	GGGGTGACGA	250-1150	249	20.75	0.43
GC-32	$(GA)_8T$	400-1400	226	18.83	0.41
GC-37	$(CA)_8A$	100-1050	139	13.90	0.38
GC-47	$(CT)_8RC$	300-1350	292	29.20	0.31
GC-48	$(TC)_8G$	450-1400	148	14.80	0.36
GC-50	$(AC)_8YT$	300-1250	343	28.58	0.36

CCB-3 landraces for polyphenol, SPG-7, CCR, SPG-6, CCB-3, CCW, SPG-4, CCO, CCB-2 and M-1 for anthocyanin and SPG-7, CARI-1, CCO, SPG-5, MCF, SPG-2, CARI-2, CCR and H-2 for carotenoids. The significant differences in genotypes for colour value, free amino acids, anti-nutrients like phytate, oxalate, nitrate and saponin and sugars in test genotypes can be used in breeding programme. Overall, the study identified CARI-1, CCO, SPG-7 and G-1 as promising landraces from islands for quality breeding program in *Capsicum*.

Molecular diversity

Considerable genetic diversity was observed among the genotypes based upon RAPD and ISSR markers (Figure 3). Out of the 36 RAPD markers, five showed amplification in all the genotypes and the results are presented in Table 2. A total of 920 amplicons were scored in all the genotypes. OPD-13 produced maximum number of amplicons (249) with polymorphism information content value of 0.43. The amplicon size from five RAPDs ranged from 250 to 1400 bp. A of total 1148 bands were generated by five ISSR markers in 42 genotypes with average PIC value of 0.36 (Table 2). The highest numbers of bands were produced with ISSR-GC-50 (343), while minimum with ISSR-GC-48 (148). The amplicon size ranged from 100 to 1400 bp. The average similarity matrix from pooled data of RAPD and ISSR markers was used for generating a tree (Figure 4) for cluster analysis by unweighted pair group method with arithmetic average (UPGMA) using NTSYS 2.0 software package. Analysis revealed the overall similarity coefficient of 48% in 41 test genotypes of Capsicum. Except SPG-3 which was outlier with 54% intra-cluster similarity, the remaining 41 genotypes were divided into five clusters. Cluster-I had nine genotypes representing island landraces except CHIVAR-8-II. Cluster-II consisted of mixed genotypes from mainland (CHIVAR-3-II, CHIVAR-4-II) and islands (N-1, G-1, CCB-2). Cluster-III represented 11 genotypes mainly from islands (nine landraces) and only two CHIVAR-6-I and CA-334-1 from mainland. Cluster-IV also had mixed representation from mainland (six) and islands (four). Cluster-V predominantly represented the AICRP (VC) material except G-2 from islands.

The RAPD and ISSR markers are PCR based random markers which have been used in various studies for estimating the diversity in germplasm (Singh et al., 2012). Thul et al. (2012) also reported genetic similarities in Capsicum genotypes in the ranges of 23-88% and 11-96% with the RAPD and ISSR markers, respectively. Though, RAPD markers have limitations of reproducibility but precise regulation for PCR temperature and aliquot constituents can improve the reproducibility (Meunier and Grimont, 2012). The ISSR markers are rapid, simple, inexpensive, and highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature. The study found good correspondence for diversity patterns generated with both DNA markers and functional markers viz. morphological and biochemical parameters within and between the species. Galvan et al. (2003) also reported good agreement between ISSR markers and morphological and biochemical. The polymorphic information content (PIC) value was measured to show the informativeness of used markers (Botstein et al., 1980). The amplified markers have considerable penetrance in the test genotypes which can be useful for further studies related to mapping of useful traits.

Correlation studies

The observation from different traits in three markers were assessed for correlation matrix and analyzed as pooled data. Three major set of markers showed significant diversity in *Capsicum* genotypes but no significant ($p>0.05$) correlation was observed among these markers. The r^2 value was 0.12 ($p>0.05$) for morphological and biochemi-cal markers while $r^2 = 0.08$ (p.0.05) for morphological and molecular markers. The biochemical and molecular markers showed $r^2=0.02$, indicating very poor correlation between them.

Similar analysis was conducted between biochemical traits and significant correlation was observed between

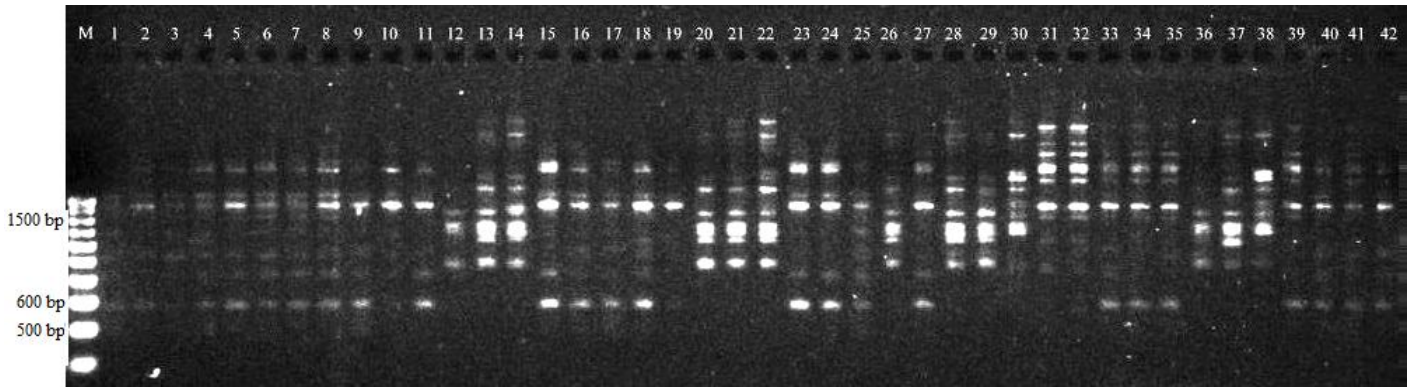

Figure 3. Diversity in 42 genotypes of *Capsicum* with RAPD-OPD10 (5' GGTCTACACC 3') marker. (S. No. 1-42 are genotypes as given in Table 1).

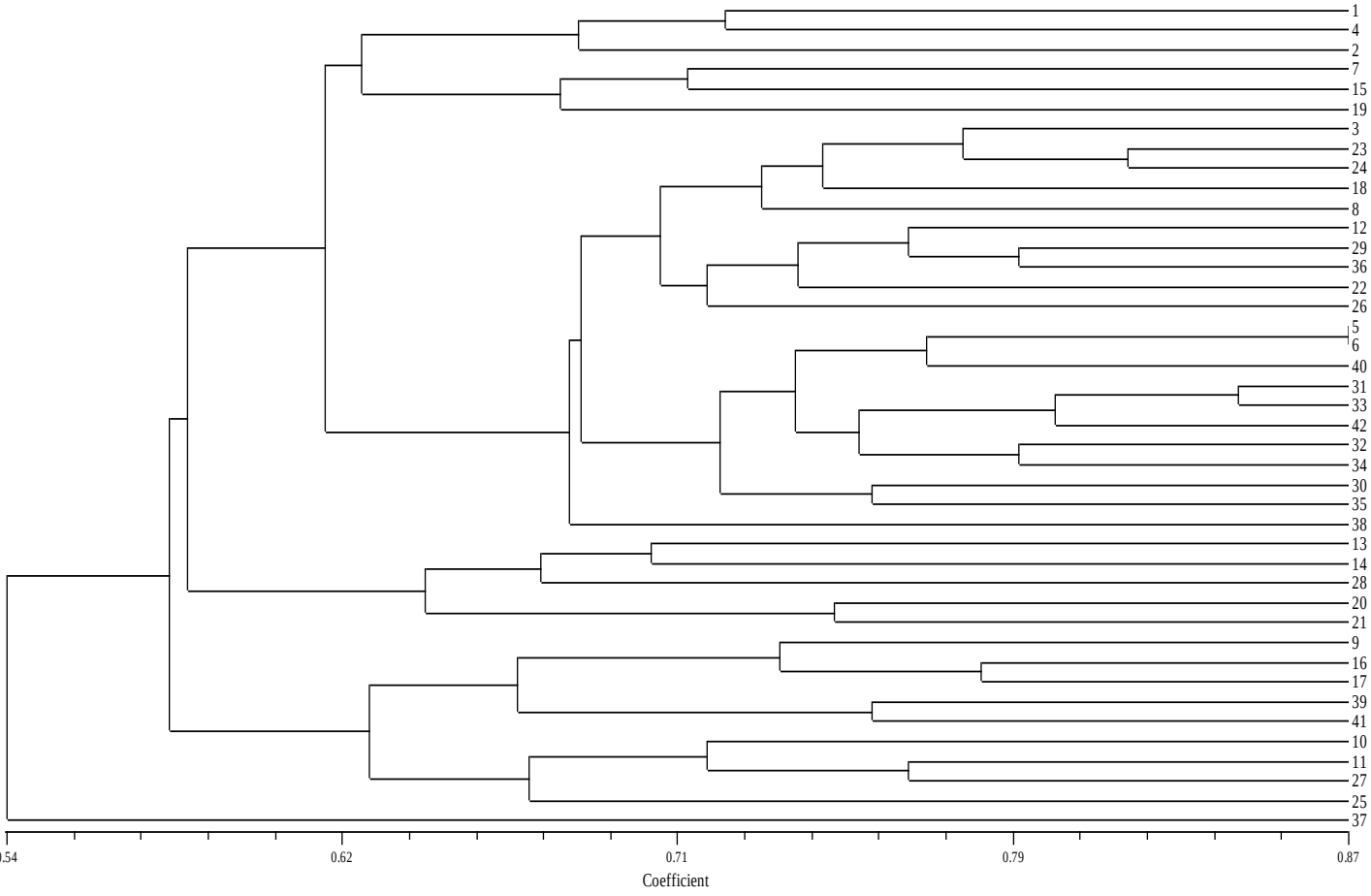

Figure 4. Dendrogram of *Capsicum* genotypes by UPGMA cluster analysis using DNA (RAPD and ISSR) markers similarity matrix (S. No. 1-42 are genotypes as given in Table 1).

antioxidant activity and polyphenol (r^2=0.33; p<0.05) and flavonoids (r^2=0.39; p<0.05). No correlation was observed between antioxidant and carotenoids, tannin, and ascorbic acid. However, positive correlation was observed between polyphenol and flavonoids (r^2 = 0.36; p<0.05) and flavonoids and tannin (r^2=0.570; p<0.01).

In conclusion, *Capsicum* landraces showed significant variation for morphological and biochemical parameters in islands. The local collections from islands showed significant difference over reference genotypes from

mainland India which may be due to adaptive changes in local collections. The morphological characteristics, bio-chemical and the molecular markers are found to be useful toward the delineation of the diversity in Capsicum landraces and identification of genetic stock. The distinct landraces Capsicum can be used in breeding program or collected and conservation in gene bank for further use.

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Full Length Research Paper

Plant regeneration protocol of *Andrographis paniculata* (Burm. f.) - an important medicinal plant

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Rapid direct plant regeneration of *Andrographis paniculata* was achieved from leaf and stem explants on Murashige and Skoog (MS) basal medium supplemented with 1.5 to 3.0 mg/l 6-benzyladenine (BA), 50 mg/l adenine sulfate (Ads) and 3% (m/v) sucrose. Inclusion of 1.0 mg/l 1-naphthalene acetic acid (NAA) in the culture medium along with BA + Ads promoted a higher rate of shoot bud regeneration. Maximum mean number of shoot bud per explant (28.6) was achieved on the MS medium supplemented with 3.0 mg/l BA, 50 mg/l Ads and 1.0 mg/l NAA after six weeks of culture. The percent of regeneration varied depending on the culture medium. The elongated shoots were rooted within 9 to 11 days on ½ strength MS medium supplemented with 0.5 mg/l indole-3-butyric acid (IBA) or 1-naphthaleneacetic (NAA) acid with 2% sucrose. Maximum percentage of rooting (76.24%) was obtained on medium having 0.5 mg/l IBA and 2% sucrose. Basal region of the micro-shoots became callusing when transferred to higher concentrations of either IBA or NAA. The rooted plantlets were survived in the greenhouse. The *in vitro* raised plantlets were grown normally under soil condition. This result will facilitate the conservation and propagation of the important medicinal plant.

Key words: *In vitro*, shoot bud regeneration, growth regulators, medicinal plants.

INTRODUCTION

Andrographius paniculata (Burm. f.) is a valuable medicinal plant which belongs to the family Acanthaceae. The plant is distributed throughout tropical India and Sri Lanka and is commonly known as Kalmegh. It is used as laxative and to overcome difficulty in breathing, cough, edema, skin diseases, syphilitic eachexia, syphilitic ulcers, acidity and liver complaints (Sivarajan and Balachandran, 1994). The leaves and roots are used for the treatment of diabetes, malaria, cholera, dysentary, pneumonia, tuberculosis and scabies (Kirtikar and Basu, 1981). A number of secondary metabolites have been isolated from various parts of the plant namely, carvacrol, eugenol, myristic acid, hentriacontane, tritriacontane, oroxylon A and diterpenoids like andrograpanin, andropanoside, andrographolide and neoandrographolide (Rastogi and Mehrotra,1993; Sharma, 2003). Two flavonoids 2', 4', 6', 2, 3, 4-hexamethoxychalcone and 5-hydroxy-7, 2, 5-trimetrhoxyflavone together with a known flavones glycoside, echioidinin 5-0-beta-D-glucopyranoside were isolated from the whole plant of *Andrographis neesiana* (Pawar et al., 2011). The bitter principle of a diterpene lactone, Andrographolide which was originally extracted from *A. paniculata*, has a high demand to pharmaceutical industries, is largely met by

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Abbreviations: MS, Murashige and Skoog; **BA,** 6-benzyladenine; **Ads,** adenine sulfate; NAA, 1-naphthalene acetic; **IBA,** indole-3-butyric acid.

extraction of this compound from wild population. Conventional vegetative propagation is very difficult and propagation from seed is unreliable due to poor germination and deaths of many young seedlings under natural conditions (Anonymous, 1992) are some of the major hurdles to meet the ever growing demand of the industries. Thus, plant regeneration from different explants through *in vitro* is an alternative method for conservation and propagation of this species, but a few reports on such investigations on *A. paniculata* are available in literature (Prathanturarug et al., 1996; Martin, 2004).

The influence of plant growth regulators on plant regeneration has been reported in several medicinal plants by different investigators (Rout et al., 2000, Gopi et al., 2006; Faridah et al., 2011). The present investigation aimed to describe an efficient protocol for direct plant regeneration from leaf and stem explants of *A. paniculata*, an important medicinal plant.

MATERIALS AND METHODS

Plant material

Elongated shoots (2 to 4 cm long) were collected from field-grown plants of *A. paniculata* and brought to the laboratory with cut ends dipped in distilled water and subsequently, washed in a 2% (w/v) (Teepol, Qualigen, India) detergent solution. Further, surface sterilization was performed in a 0.1% (w/v) aqueous mercuric chloride solution for 5 min. After rinsing four to five times with sterile distilled water, stem (10 to 12 mm long) as well as leaf segment (0.25 cm^2) were used as explants source.

Culture medium and culture conditions

Both stem and leaf explants were placed on semi-solid Murashige and Skoog (MS, 1962) mineral salts plus 555 µM myo-inositol, 4.06 µM nicotinic acid, 2.43 µM pyridoxine-HCl, 0.296 µM thiamine-HCl supplemented with various concentrations of cytokinins, that is, 6-benzylaminopurine (BA: 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l), kinetin (Kn: 0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/l), adenine sulfate (Ads: 0, 25, 50 and 100 mg/l) and auxin like indole-3-acetic acid (IAA: 0.0, 0.25, 0.5, 1.0 and 2.0 mg/l), 1-naphthaleneacetic acid (NAA: 0.0, 0.25, 0.5, 1.0 and 2.0 mg/l) and 2,4-dichlorophenoxyacetic acid (2,4-D: 0.0 0.25, 0.5, 1.0 and 2.0 mg/l) for callus induction and shoot bud regeneration.

The pH of the medium was adjusted to 5.8 prior to autoclaving. Each treatment was represented by 10 cultures and the experiment was repeated three times. The cultures were incubated under a 16-h photoperiod having a light intensity of 55 µE/m^2/s from cool, white fluorescent lamp at 25 ± 2°C.

Induction of rooting and acclimatization

Elongated shoots (2 to 3 cm long) were excised from the culture and transferred to ½ strength semisolid MS medium supplemented with different concentrations of IBA and/or NAA (0, 0.1, 0.25, 0.5, 1.0 and 1.5 mg/l) and 2% (w/v) sucrose for root induction. One excised shoot was cultured in each tube (25 × 150 mm) with 15 ml of the culture medium. All the cultures were incubated at 25 ± 2°C under 16 h photoperiod with cool white fluorescent lamps. The percentage of shoots forming roots and the number of roots per shoot were examined periodically up to 3 weeks of culture. Rooted micropropagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm earthen pots containing a mixture of soil, sand and dry cow-dung manure (1:1:1, w/v) and kept in the greenhouse for acclimatization. The plants were watered at every 2-day intervals. The survival rate was recorded one month after the transfer into pots.

Scoring of data and statistical analysis

All the cultures were examined periodically, and the morphological changes were recorded on the basis of visual observations. There were 10 cultures per treatment for organogenic calli production and plant regeneration. Then each experiment was repeated three times and subculture was carried out in a 6-week interval. The mean percentage of cultures producing shoot regeneration and mean number of shoots per culture were recorded after 6 weeks. The percentage of rooting and the average number of roots per shoot were recorded after six weeks. The data were analyzed statistically by the Duncan's multiple range test (Duncan, 1955). Means followed by the same letter within columns were not significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Green calli were initiated from leaf and stem explants of *A. paniculata* on the MS medium supplemented with different concentrations of BA or Kn alone or in combination with either NAA or 2,4-D. The initiation of callus and the subsequent proliferation could not be achieved on the medium without growth regulators. The media containing BAP alone or in combination with Ads showed a low frequency of orgonogenic calli initiation as compared to kinetin (Kn) alone or Kn + Ads (Table 1). The combination of Kn plus 2,4-D or NAA did not influence the shoot bud regeneration.

The medium having 3.0 mg/l BAP + 50 mg/l Ads + 1.0 mg/l NAA favoured the development of organogenic calli within six weeks of initial culture (Figure 1A). Initially, the organogenic calli were initiated in the upper surface of the explants and subsequently it proliferated into shoot buds. The medium containing BAP + Ads + NAA produced a significant number of shoot buds regeneration in leaf and stem derived calli as compared with BA + Ads (Table 1). The medium having 2,4-D or NAA alone had no effect on shoot bud regeneration (data not shown).

The regeneration of shoot buds from organogenic calli was varied on the basis of the culture medium composition. About 75.3% in case of leaf-derived calli and 63.4% in case of stem-derived cultures showed shoot bud regeneration in the medium having 3.0 mg/l BA, 50 mg/l Ads and 1.0 mg/l NAA after six weeks of first subculture (Figure 1B). The increase of NAA concentration higher than 2.0 mg/l suppressed the rate of shoot bud regeneration and slow growth of the organogenic calli.

The maximum number of shoot buds (28.6) was obtained in the medium containing 3.0 mg/l BA, 50 mg/l Ads and 1.0 mg/l NAA after four weeks of culture initiation

Table 1. Effect of different concentrations of cytokinins and auxins on shoot bud regeneration from leaf (A) and stem (B) derived calli of *A. paniculata* after 6 weeks of culture.

MS + growth regulators (mg/l)					Percent of organogenic calli development (mean ±S.E)*		Average number of shoot buds per culture (mean ±S.E)*	
BAP	Kn	Ads	NAA	2,4-D	A	B	A	B
0	0	0	0	0	0	0	0	0
1.0	0	0	0	0	12.6±0.8^{b}	8.36±0.6^{a}	3.32±0.5^{c}	3.28±0.3^{b}
1.5	0	0	0	0	18.5±0.7^{c}	12.5±0.7^{b}	4.66±0.6^{d}	4.12±0.5^{c}
2.0	0	0	0	0	24.2±0.8^{d}	20.2±0.8^{f}	10.1±0.4^{h}	8.32±0.8^{f}
2.5	0	0	0	0	26.8±0.4^{e}	20.8±0.9^{f}	18.4±0.6^{k}	11.6±0.7^{h}
3.0	0	0	0	0	28.5±0.7^{f}	21.6±0.7^{g}	20.2±0.8^{l}	13.2±0.6^{j}
0	1.0	0	0	0	11.8±0.6^{a}	14.2±0.8^{c}	1.8 ±0.4^{a}	2.4±0.3^{a}
0	2.0	0	0	0	12.6±0.9^{b}	17.6±0.5^{d}	3.22±0.7^{c}	2.9±0.6^{a}
0	3.0	0	0	0	24.4±0.5^{d}	18.8±0.4^{e}	8.45±0.6^{f}	6.5±0.4^{e}
2.0	0	25.0	0	0	30.2±0.8^{g}	32.2±0.7^{k}	14.5±0.5^{j}	12.8±0.2^{i}
3.0	0	50.0	0	0	52.7±1.1^{k}	40.5±0.9^{n}	22.6±0.3^{m}	14.6±0.7^{k}
0	2.0	25.0	0	0	28.6±0.8^{f}	26.8±1.0^{j}	8.88±0.5^{f}	10.4±0.2^{g}
0	3.0	50.0	0	0	32.2±1.0^{h}	26.6±0.8^{j}	10.2±0.7^{h}	12.4±0.3^{i}
2.0	0	25.0	1.0	0	65.8±1.3^{l}	52.7±1.0o	24.8±0.6^{n}	15.9±0.7^{l}
2.0	0	25.0	0	1.0	25.6±1.1^{d,e}	24.2±0.8^{i}	9.7 ±0.3^{g}	6.8±0.4^{e}
3.0	0	25.0	1.0	0	72.8±1.2^{n}	61.8±1.0q	18.8±0.6^{k}	12.6±0.5^{i}
2.5	0	50.0	1.5	0	68.8±1.3^{m}	55.2±1.1p	22.7±0.8^{m}	15.5±0.6^{l}
3.0	0	50.0	1.0	0	75.3±2.1o	63.4±1.2r	28.6±0.8o	16.2±0.8^{m}
0	2.0	25.0	1.0	0	34.8±0.8^{i}	32.2±0.9^{k}	12.3±0.4^{i}	8.2±0.6^{f}
0	3.0	50.0	1.0	0	38.2±1.2^{j}	35.1±1.1^{l}	14.6±0.7^{j}	12.5±0.5^{i}
2.0	0	50.0	0	2.0	30.6±0.8^{g}	38.2±1.2^{m}	6.33±0.6^{e}	5.6±0.4^{d}
0	2.0	25.0	0	1.5	28.6±0.7^{f}	20.4±0.7^{f}	3.36±0.3^{c}	2.8±0.6^{a}
0	3.0	50.0	0	1.5	31.4±0.9^{g}	22.4±0.5^{h}	2.56±0.7^{b}	2.9±0.8^{a}

*Means of 10 replicates/treatment; repeated thrice; Mean followed by different letters are significantly different at the 5% level.

(Table 1). It indicates that cytokinin/auxin ratio being the principal players in the induction of shoot multiplication from explants. Similar observations indicating the effect of cytokinin and auxin on shoot multiplication were previously reported in *Clerodendrum colebrookianum* (Mao et al., 1995), *Plumbago* (Rout et al., 1999), and *Ocimum gratissimum* (Gopi et al., 2006). The present findings suggest a high frequency of shoot production from organogenic calli could be obtained by manipulating the growth regulators and culture condition. There were differences between treatments both in the percentage of cultures with response and in the mean number of shoot buds per culture.

Many authors reported that cytokinin is required in optimal quantity for shoot proliferation in many genotypes but an inclusion of a low concentration of auxin along with cytokinin increases the rate of shoot bud proliferation (Sharma et al., 1993; Sharma and Singh, 1997; Shasany et al., 1998; Rout et al., 2000; Rout, 2005). However, the molecular mechanisms through which auxin-cytokinin crosstalk act in concert to exert the shoot meristem induction are still poorly understood. A lower concentration of BA (< 3.0 mg/l) in the culture medium inhibited the growth of the shoot buds. The number of shoot buds per culture varied from 1.8 to 28.6 in case of leaf and 2.4 to 16.2 in case of stem in different treatments.

The rate of shoot bud regeneration increased as the number of subcultures increased. Similar observations were reported for *Gentiana kurroo* (Sharma et al., 1993) and *Plumbago* species (Rout et al., 1999).

Induction of rooting and acclimatization

Elongated shoots were excised and placed in half/ full strength MS medium supplemented with various concentrations of IBA or NAA for root induction. Full strength MS medium without growth regulators did not promote root induction; roots were observed in media containing ½ strength MS medium supplemented with NAA or IBA with 2% sucrose.

However, optimal rooting and growth of micro shoots were observed in medium containing 0.5 mg/l IBA or NAA with 2% sucrose after 9 to 11 days of culture without intervening callus (Table 2). The maxi-mum percentage of rooting (76.2%) was obtained in the medium containing

Figure 1. Direct plant regeneration from leaf and stem explants of *Andrographis paniculata.* A) Organogenic calli development from leaf explant on medium having 3.0 mg/l BAP + 50 mg/l Ads + 1.0 mg/l NAA after 6 weeks of initial culture. B) Shoot bud regeneration (arrows) from rganogenic calli on medium having 3.0 mg/l BAP + 50 mg/l Ads + 1.0 mg/l NAA after 6 weeks of subculture. C) Root initiation from micro shoots of *Andrographis paniculata* after 3 weeks of culture of ½ strength MS medium supplemented with 0.5 mg/l IBA + 2% sucrose. D) Plantlets established in the pot.

0.5 mg/l IBA within three weeks of culture (Figure 1C). Root development was; however, slow at higher concentrations of IBA or NAA. The rooted plantlets were transferred into pots for acclimatization. About 60% of the rooted plantlets survived in the pot one week after the transfer. The plants were grown normally (Figure 1D).

Table 2. Effect of different concentrations of NAA and IBA on root induction from excised shoots of *Andrographis paniculata* after three weeks of culture.

½ MS + auxin concentrations (mg/l)		Days to rooting	Percent of rooting (mean ±SE)*
NAA	IBA		
0	0	0	0
0.25	0	11 - 12	25.42±1.2[a]
0.50	0	10 - 11	62.24±1.3[f]
1.0	0	12 -13	42.12±1.4 +[c]
0	0.25	10 -11	52.24±1.2[e]
0	0.50	9 - 10	76.24±2.1[g]
0	1.00	12-13	46.28±1.4+[d]
0.25	0.50	13 -14	40.16±1.5+[c]
0.50	0.25	12-13	36.22±1.1+[b]

*10 micro-shoots/treatment; repeated thrice; + - callusing at the basal end. Mean followed by different letters are significantly different at the 5% level.

In conclusion, a successful production of shoot bud regeneration from leaf and stem explants and induction of roots from excised root were dependent on the nutrient medium and the culture conditions. This study might provide new opportunities for mass propagation and conservation of an important medicinal plant, *A. paniculata.*

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Full Length Research Paper

In vitro anti-inflammatory and phytochemical properties of crude ethyl acetate extract of *Baliospermum montanum* Leaf (Muell – Arg)

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Baliospermum montanum **(Muell – Arg) which belong to Euphorbiaceae family is a well known perennial herb in Indian medicine used to treat various disorders like asthma, bronchitis, purgative, anthelmintic, diuretic, diaphoretic, rubefacient and tonic. The anti-inflammatory activity of four different solvent extracts of *B. montanum* leaf was investigated in Phytohaemagglutinin (PHA) induce peripheral blood mononuclear cells (PBMC) cells using MTT assay. Among the samples, ethyl acetate extract revealed good anti-inflammatory response comparatively with other extracts such as hexane, acetone, methanol and the preliminary screening of phytochemical test was investigated. The results of phytochemical screening revealed the presence of flavonoids, tannin, steroids, glycosides, amino acid and carbohydrates. Our study demonstrates that the ethyl acetate extracts of *B. montanum* leaves contains effective anti-inflammatory agents, which could ultimately be used as functional material and traditional remedy against inflammation.**

Key words: *Baliospermum montanum*, anti-inflammatory, MTT assay, phytochemical.

INTRODUCTION

Baliospermum montanum (Muell – Arg) an aromatic medicinal plant belonging to the family Euphorbiaceae includes 280 genera with 730 species with the largest genes Euphorbia (Husain et al., 1980). Euphorbia plants are widespread in nature ranging from herbs and shrubs to trees in tropical and temperate regions all over the world (Johnson et al., 2003). Root, leaf and seeds of *B. montanum* are used medicinally and are documented from Asian countries including Nepal, Burma, Malaya and India (Mali et al., 2008). Phorbol esters, include montanin, baliospermin, 12 - deoxyphorbol – 13 – palmitate, 12 – deoxy -16 – hydroxypherbol - 13-palmitate and 12 – deoxy - 5β - hydroxyphorbol - 13 myristate. Leaves contain 8-sitosterol, and 8-D-glucoside and hexacosamol was observed from roots and 11, 13-dihydroxytetracos-trans-9-enoic acid was reported from seeds of *B. montanum* (Johnson et al., 2010; Husain et al., 1980). The prelimnary phytochemical analysis revealed the presence of flavonoids, glycosides, steroids and absence of alkaloids, saponins and terpenoids in the root and glycosides and terpenoids in the seeds of the plant (Mail et al., 2008; Johnson et al., 2010). *B. montanum* is known for its ethanobotanical and traditional use (Mali and Wadekar, 2008).

Inflammation is the protective mechanisms of local microcirculation responsible to fight against tissue injury caused by physical and chemical factors; immunological reactions, microbial infections, and tissue damage (Mahesh et al., 2011). Redness, swelling, heat, pain and loss of function are considered as symptoms of inflammation

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and are responsible for interruption and resolution of the infectious diseases. Persistence of inflammation leads to various diseases associated with chronic inflammation, including arthritis, atherosclerosis, and even cancer (Schett, 2006; Libby et al., 2002; Karin et al., 2005). Adverse effect of available anti-inflammatory drugs cause leads to search of novel curative agents of plant origin. Natural products are rich in novel bioactive secondary metabolites and it is important to identify natural products with pharmacological or biological activity for use in pharmaceutical drug discovery and design (Jang et al., 2013).

Roots of *B. montanum* are considered as purgative, anthelmintic, diuretic, diaphoretic, rubefacient, febrifuge and as tonic. Additionally, they are also reported to be useful in the treatments of dropsy, constipation, jaundice, leprosy and skin disease. The roots have long been used as Ayuvedic remedy for Jaundice (Ogura et al., 1978). The leaves are found to be good for asthma and bronchitis (Wadekar et al., 2008). The seeds of the plant are drastic, purgative, rubefacient, hydragogue and stimulant.

Based on the above review of available literature, it was noticed that there is need for considerable pharmacological research on the medicinal herbs *B. montanum*. Thus, in the present study, the anti-inflammatory potential of crude extract and phytochemical screening from *B. montanum* leaves were evaluated and the results were discussed in details.

MATERIALS AND METHODS

Chemical and reagents

RPMI 1640 medium, fetal bovine serum (FBS), trypan blue, Histopaque-1077, penicillin G, streptomycin, gentamycine, amphotericine B, 3-(4,5-Dimethylthiazlo-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethylsulfoxide (DMSO), Trypsin and phytohaemagglutinin (PHA) were purchased from Sigma. All other chemicals and solvents were purchased from Merck.

Collection of plant materials

The plant tissue material taken for investigation on anti-inflammatory studies was shade dried leaf of *B. montanum* (Muell – Arg). The plants were collected from their natural habitats in Pondicherry, India. The voucher specimen is available for reference (BST/WC/Tech 277).

Extraction with organic solvent

The dried plant leaf powders (100 g) of *B. montanum* (Muell – Arg) were extracted with different solvent with increasing polarity viz hexane, ethyl acetate, acetone, methanol, at room temperature. The extract was filtered with Whatman No 1 filter paper. Each of the extract was concentrated in a rotary evaporator under reduced pressure and temperature to prevent the extract. The compound thus obtained was re-suspended in appropriate volume of DMSO for the treatment of cells (Bhakuni et al., 1971).

Cell culture

PBMC was cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with glutamine (100 U/ml), streptomycin (0.75 µg/ml), penicillin (120 U/ml), amphotericin B (3 µg/ml) and gentamycine (160 µg/ml) and 10% FBS was maintained at 37°C with a humidified atmosphere of 5% CO_2.

Isolation of PBMC

PBMC were isolated from heparinized venous blood by Histopaque-1077 (Sigma) gradient centrifugation. The cells were suspended in RMPI-1640 medium containing 1% penicillin, streptomycin and amphotericin B, supplemented with 10% fetal bovine serum. Ten milliliter (10 ml) of blood collected aseptically in a syringe was mixed gently with heparin and carefully layered over 5 ml of Ficoll gradient (2:1 ratio) and centrifuged at 1800 rpm for 30 min at room temperature (Souza-Fagundes et al., 2002). PBMC identified as a buffy layer at the interface were collected and washed twice with the RPMI medium without serum and centrifuged at 1500 rpm for 15 min. The pellet was suspended in RPMI with serum and 10 µl of the suspension was mixed with trypan blue and loaded in RPMI in the Neubauer's chamber to check the viability. 0.2 x 10^6 cells were dispensed in 200 µl of each well of 96-well plate (Bignold et al., 1987; Selvakkumar et al., 2007).

Cytotoxic studies by MTT assay

The isolated PBMCs (0.2 x 10^6 /100 µl) were seeded into a 96 well plate. 10 µl of phytohaemagglutinin (PHA) (0.4 µg/ml) was to each well and appropriate control of the cells were incubated for 2 to 3 h at 37°C, 5% CO_2 and 90% humidity. Compounds in the crude extract were added (2 µl) in various concentrations (0.1 to 100 µg/ml) to the wells. Negative control and positive control (Triton X was used as in case of MTT), were also maintained, un-induced control and a solvent control was used along with it. The cells were then incubated overnight at 24 h for 37°C, 5% CO_2 and 90% humidity. Medium from the wells were removed and 10 µl of MTT (5 mg/ml re-supended in PBS) was added to each well. Plates were incubated for 4 h at 37°C, floating cells were carefully removed and 100 µl of DMSO was added to each well to lyses the cells and the absorbance was measured at 570 nm. Finally, the percentage of cell viability was calculated using the formula: Cell viability (%) = (Absorbance of test sample/Absorbance of control) x 100 (Ashalatha et al., 2010).

Phytochemical screening

Chemical tests were carried out on the solvent extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowora (1993), Trease and Evans (1983) and Harborne (1998).

Test for alkaloids

Test sample (1 ml) was mixed with few drops of Mayer's reagent and the formation of orange brown precipitate was recorded as indicator for the presence of alkaloids.

Test for anthraquinones

The Borntrager test was used for the detection of anthraquinones. Two milliliter (2 ml) of test sample with 4 ml of hexane was added and shaken well. The upper lipophilic layer was separated and treated with 4 ml of dilute ammonia. If the lower layer changed from violet to pink, it indicated the presence of anthraquinones.

Table 1. Inhibitory concentration of crude extracts from *Baliospermum montanum* leaf against PBMC cells.

Concentration (µg/ml)	Anti-inflammatory activity (% Inhibition) (24 h)			
	Hexane	Ethyl acetate	Acetone	Methanol
0.1	6.277±0.18	8.5319±0.25	11.27338±0.33	5.2451±0.157
1	7.658±0.22	15.295±0.45	20.85575±0.62	10.381±0.311
10	9.838±0.29	55.012±1.65	37.12529±1.11	15.368±0.461
50	15.06±0.45	64.452±1.93	45.17038±1.35	17.140±0.514
100	18.08±0.54	80.245±2.40	49.01358±1.47	21.265±0.637

Test for flavonoids

Three methods were used to determine the presence of flavonoids in the plant sample (Sofowora, 1993; Harbrone, 1998). Five milliliter (5 ml) of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by the addition of concentrated H_2SO_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing. Few drops of 1% aluminum solution were added to a portion of each filtrate. A yellow colouration was observed indicating the presence of flavonoids. A portion of the powdered plant sample was in each case heated with 10 ml of ethyl acetate over a steam bath for 3 min. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow coloration was observed indicating a positive test for flavornoids (Obianime and Uche, 2007).

Test for cardiac glycosides (Keller-Killani test)

5 ml of each extracts was treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was underlayer with 1 ml of concentrated sulphuric acid. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer (Ekhaise et al., 2010).

Test for phlobatanins

Deposition of a red precipitate in extracts boiled with 1% aqueous hydrochloric acid was taken as the evidence for the presence of phlobatanins.

Test for phenolic compound

1 ml of test solution was treated with 10% ethanolic ferric chloride. Phenolic compounds were considered present when a colour change to blue green or dark blue was observed.

Test for saponin

About 2 g of the powered sample was boiled in 20 ml of distilled water in a water bath and filtered. Ten milliliter (10ml) of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion (Edeoga et al., 2005).

Test for steroids

200 µl of acetic anhydride was added to 0.5 ml ethyl acetate of each sample with 2 ml H_2SO_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids (Akinpelu et al., 2008).

Test for tannins

About 0.5 g of the dried powdered samples was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and observed for brownish green or a blue – black coloration (Kubmarawa et al., 2007).

Test for terpenoids (Salkowski test)

5 ml of each extract was mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface was formed to show positive results for the presence of terpenoids.

Statistical analysis

All values were expressed as mean ± standard. The statistical significance was evaluated by one – way analysis of variance (ANOVA) using SPSS version. When there was a significant difference, Tukey's multiple comparisons were performed by fixing the significance level at $p \leq 0.05$.

RESULTS

The leaves of *B. montanum* were collected and shade dried and used for extraction of its active ingredients. A total of four different solvent extracts of *B. montanum* were tested with *in vitro* model for studying its anti-inflammatory activity using MTT assay. The optimum concentration of crude leafs extract was evaluated with varying doses using PHA induced PBMC for 24 h and the inhibitory effect was studied (Table 1). Our study revealed that among the solvents used to prepare the crude extract, ethyl acetate provided the ingredients with notable anti-inflammatory activity. It was also noted that the ingredients of crude extracts from hexane and methanol were not effective even at higher dose. In hexane extract we noticed just 18.08±0.54% anti-inflammatory activity at the highest dose (100 µg/ml). Similarly, in methanol extract also, the highest dose used revealed just 21.26±0.64% anti-inflammatory activity. However, the crude extract with acetone revealed 49.01±1.47% anti-inflammatory activity at its higher dose (100 µg/ml). The ethyl acetate extract of *B. montanum* showed highest anti-inflammatory

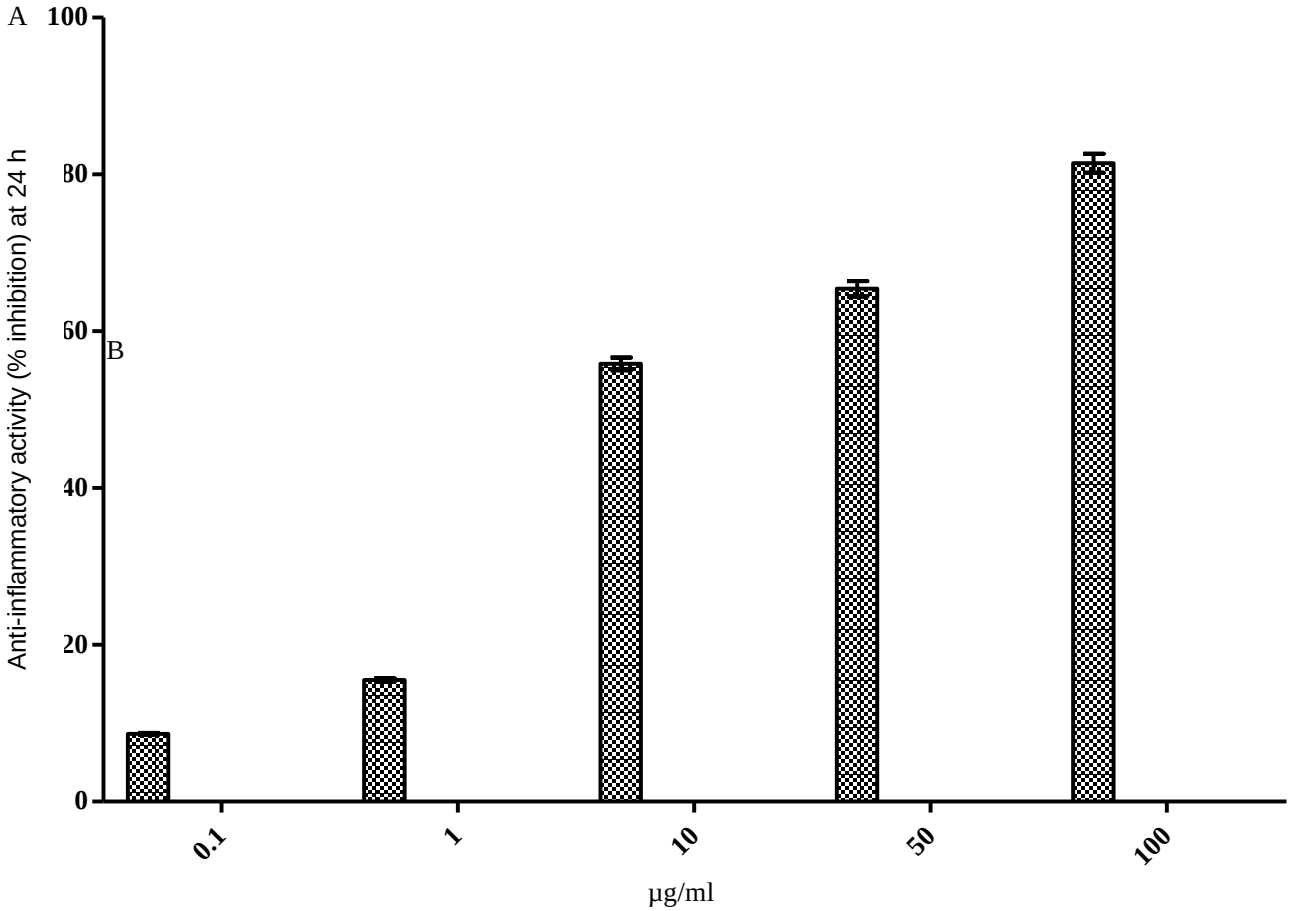

Figure 1. (A) Inhibitory effect of crude ethyl acetate leaf extracts from *Baliospermum montanum* on mitogen induced PBMCs with different concentrations (0.1, 1, 10, 50 and 100 µg/ml). The IC50 value was found to be 9.08 µg/ml.

activity against PBMC even at low dose with IC_{50} values of 9.08 µg/ml (Figure 1A). However, on considering the ethyl acetate extract, inhibitions of proliferation were low and not significant in other crude extracts even at higher concentrations (Figure 1B).

Further phytochemical constituents were analysed for the active constitutents involved in the anti-inflammatory activity from all four solvent extracts. Our phytochemical analysis revealed that ethyl acetate and acetone crude extracts showed the presence of flavonoids, tannins, and steroids. Hexane and methanol crude extracts did not show remarkable phytochemical activity (Table 2). Phytochemical constituents such as flavonoids, steroids, tannins, amino acids and carbohydrates were observed in the extract of ethyl acetate. Similarly, all these phytochemicals were observed in acetone. Our study reveals that the glycosides are exclusively observed only in acetone extract.

DISCUSSION

The present study revealed the anti-inflammatory activity of crude extracts like hexane, ethyl acetate, acetone, and methanol of *B. montanum* leaf. Our study revealed notable anti inflammatory effect in crude extracts with ethyl acetate and acetone. It was also observed that the flavornoids detected in both extracts are known to be good anti-inflammatory agents. Studies of Raju et al. (2005) on anti-inflammatory potential of *Cassia fistula* revealed the responsibility of flavonoid and alkaloids in anti-inflammatory reactions. Similarly, flavonoid with anti-inflammatory potential are reported from *Morindatinctoria roxb,* and *Vernonia amygdalina* (Sivaraman and Muralidharan, 2010; Udeme et al., 2009). Inspite of flavonoids, steroids were noticed in both the extracts (ethyl acetate and methanol) and studies of Neto et al. (2005) reported the presence of steroids with anti-inflammatory potential in *Pafaffia glomerata*.

The ethyl acetate extract showed good anti-inflammatory response comparatively with other extracts. The preliminary phytochemical test suggested the presence of flavonoids, steroids, tannins, glycosides, amino acids and carbohydrates in the ethyl acetate and acetone extracts. Hexane and methanol crude extracts did not show remarkable phytochemical activity. Results of the present investigation are directly correlated with previous observa-

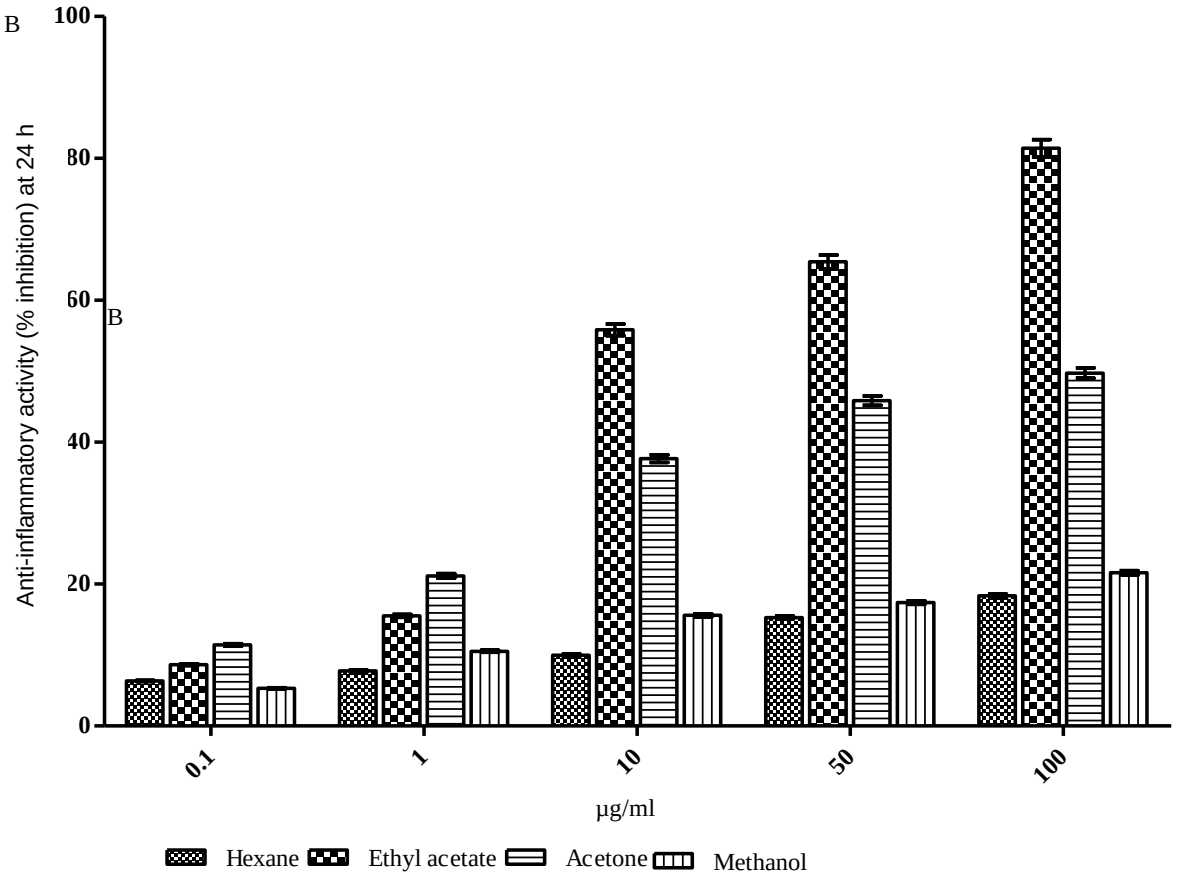

Figure 1. (B) Inhibitory effect of crude leaf extracts from *Baliospermum montanum* on mitogen induced PBMC with different concentrations (0.1, 1, 10, 50 and 100 µg/ml): increasing polarity viz Hexane, Ethyl acetate, Acetone, Methanol.

Table 2. Preliminary phytochemical screening of various extracts of *Baliospermum montanum* leaf.

Phytochemical	Hexane	Ethyl acetate	Acetone	Methanol
Alkaloids	–	–	–	–
Anthraquinones	–	–	–	–
Flavonoids	–	+	+	–
Glycosides	–	–	+	–
Phlobatannins	–	–	–	–
Phenolic compound	–	–	–	–
Saponins	–	–	–	–
Steroids	–	+	+	–
Tannins	–	+	+	–
Terpenoids	–	–	–	–
Amino acid	–	+	+	–
Protein	–	–	–	–
Carbohydrates	–	+	+	–

+ = Present; – = absent.

tions (Johnson et al., 2010). So, this study first reported the anti-inflammatory potential of leaf ethyl acetate extract from *B. montanum.* Results from our study demonstrated that the ethyl acetate extract of *B. montanum*

leaves contains effective anti-inflammatory agents, which could ultimately be used as functional material and traditional remedy against inflammation. Future studies are required for isolation of bioactive compounds for analysis of the molecular mechanisms responsible behind its anti-inflammatory potential.

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Full Length Research Paper

Surface sterilization method for reducing microbial contamination of field grown strawberry explants intended for *in vitro* culture

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An effective disinfection method for strawberry (*Fragaria x ananassa* Duch.) *cv.* Senga Sengana micropropagation using runner tips and nodal segments as explants was developed. The explants were surface sterilized with different sterilants for different durations. The present studies on the effect of different regimes of sterilization revealed that maximum aseptic cultures were obtained from both explants runner tips and nodal segments when treated with 1.5% sodium hypochlorite for 20 min plus ethyl alcohol 70% for 30 s, but the surviving percentage was less because this treatment resulted in necrosis and tissue injury of explants. However, mercuric chloride (0.1%) for 4 min resulted in less percentage of aseptic cultures but gave highest percentage of surviving explants as most of researchers have found that a single sterilant is more effective than the combination. Surface sterilization with mercuric chloride (0.1%) for 4 min was the optimum duration which resulted in highest percentage of explant survival.

Key words: *In vitro*, senga sengana, strawberry, sterilization.

INTRODUCTION

Microbial contaminations present a major challenge to the initiation and maintenance of viable *in vitro* cultures. These contaminants are particularly dangerous when they are plant pathogens. The problem is further exacerbated when explants material is sourced directly from field grown plants. Contamination in this paper refers to fungi or bacteria naturally present on the surface and natural openings on the explants material, which become manifested after initiation and can either, be overt or covert. Overt refers to contamination that can be identified by visible inspection, whereas covert refers to latent contamination, which requires special indexing and/or assaying techniques for identification. The cultivated strawberry (*Fragaria x ananassa* Duch.) a member of Rosaceae is the most important soft fruit worldwide (Hancock, 1990). They are valued for delicious flavour and fragrance and for health resorting qualities. These qualities have ensured that the economic importance of this crop has increased throughout the world and nowadays, it remains a crop of primary interest for both research and crop production. It offers quicker return on capital investment than any other fruit crop. Since, under special methods of cultivation, a crop can be picked as early as first summer after planting.

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Table 1. Different sterilents and their combination for varying time duration.

Sterilants and their combination	Time duration
Mercuric chloride (0.1%)	2 min
Mercuric chloride (0.1%)	3 min
Mercuric chloride (0.1%)	4 min
Sodium hypochlorite (1.5%)	10 min
Sodium hypochlorite (1.5%)	15 min
Sodium hypochlorite (1.5%)	20 min
Mercuric chloride (0.1%) + ethyl alcohol (70%)	2 min + 30 s
Mercuric chloride (0.1%) + ethyl alcohol (70%)	3 min + 30 s
Mercuric chloride (0.1%) + ethyl alcohol (70%)	4 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	10 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	15 min + 30 s
Sodium hypochlorite (1.5%) + ethyl alcohol (70%)	20 min + 30 s

The majority of strawberry cultivars are generally propagated by runners (Gautam et al., 2001). Vegetative propagation by runners produced from stolons of established plants, though perpetuates all the characters of mother plant, viral diseases can be frequently transmitted through the runners and the rate of multiplication through conventional method is too slow. The strawberry plants propagated vegetatively are often infected by virus and mycoplasma diseases (Biswas et al., 2007). These diseases result in significant reduction in yield. Healthy stocks used for propagation through conventional methods are not available. Micropropagation of strawberry plants were introduced in 1974 (Boxus, 1974). Tissue culture techniques allow rapid multiplication of plantlets obtained from different explants through direct or indirect morphogenesis. The division of offshoots and runners of strawberry are not always suitable for this type of cultivation due to their vulnerability and susceptibility to pathological agents. Several studies have attested the tissue cultured plants being more advantageous than those by conventional propagation in terms of fruit yield (Moore et al., 1991), pest resistance (Rancillac et et al*.,* 1987), vigor, yield per plant, the number of runners and leaves per plant (Zebrowska et al., 2003). Micropropagation of strawberry from runners for initiation has been reported and may be applied to efficiently generate a large number of disease free plants (Adams, 1972; Boxus, 1974). However, they are often limited in certain season because the strawberry only produces runners during the vegetative development phase. If we can obtain explant materials from offshoot, this problem will be overcome. But the offshoot larger than runner size is also more difficult for disinfection. In addition the browning at initial establishing stage of *in vitro* culture is the main cause leading to explant death (Zaid, 1984; Pirtilla, 2008). According to Paredes and Lavin (2005), explants of wild strawberry were surface sterilized with 70% ethanol, use of an antioxidant and sodium hypochlorite (25%) for 15 min and rinsed in sterile and distilled water. An effective method of disinfection and micropropagation with enhanced survival rate of explants and reduced phenol induced browning in strawberry was developed (Ko et al., 2009) in which the surface sterilization of the explants was done in sodium hypochlorite (0.5%) containing a few drops of Tween 20 for 7 min. However, in the present study, effect of mercuric chloride (0.1%) and sodium hypochlorite (1.5%) alone and in combination with ethyl alcohol (70%) for varying time duration was studied on disinfecting the explants. To avoid the problems of microbial contamination in *in vitro* cultures, it becomes imperative to develop a protocol for disinfecting the field grown explants intended for *in vitro* culture. Keeping in view the problems of microbial contamination in *in vitro* cultures, an efficient and simple disinfection protocol to increase survival of explants was developed in this study.

MATERIALS AND METHODS

Runner tips and nodal segments were used as explant for *in vitro* culture. They were collected from field grown strawberry plants *cv.* Senga Sengana planted at Division of Fruit Science Farm Sher-e-Kashmir University of Agricultural Science and Technology Kashmir (J&K). The explants were washed with tween 20 detergent for 5 min then rinsed with water for 4-5 times. After washing, the explants were reduced in size by removing tissues of size (0.5-1.0 cm) with the help of surgical blade and forceps before inoculation. After washing the explants, they were brought to laminar flow cabinet and were subjected to surface sterilization. The explants were subjected to different sterilants and their combinations for varying time durations as shown in Table 1, followed by a 5 min rinse in sterile distilled water under aseptic conditions in the laminar flow chamber. The explants were put on medium in such a manner that conformed to the original polarity and exposed above the surface of medium. MS basal medium (Murashige and Skoog, 1962) was used during the study. The composition and preparation of stock solutions for MS (1962) medium is given in Table 2. Appropriate quantities of various stock solutions and plant growth regulators were pipetted out and stirred with 400 ml distilled water. After adding sucrose at a concentration of 3%, pH was adjusted to 5.7 with 0.1 N NaOH and 0.1 HCl. Lastly agar agar at concentration of 0.7% was added and the final volume was made to 1 L with distilled water. The medium was sterilized in an autoclave at 15 psi (121°C for 15 min). The table surface of laminar flow cabinet was first swabbed with 95% ethanol and all the required materials except

Table 2. Composition and preparation of stock solutions for MS (1962) medium.

Stock solution designation	Ingredient	Weight of ingredient (mg)	Volume of water used (ml)	Volume of stock solution taken for making 1 lt of medium (ml)	Final conc. of the ingredient in the medium (mg l^{-1})
I	**Macronutrient**				
A	$NH_4 NO_3$	16500	500	50	1650
	KNO_3	19000			1900
B	$MgSO_4 7H_2O$	3700	500	50	370
	$KH_2 PO_4$	1700			170
C	$CaCl_2 2H_2O$	4400	500	50	440
II	**Micronutrient**				
	$H_3 BO_3$	620			6.20
	KI	83			0.83
D	$Na_2 MoO_4 2H_2O$	25	500	50	0.25
	$CoCl_2 6 H_2O$	2.5			0.025
	$CuSO_4$	2.5			0.025
E	$ZnSO_4$	860	500	50	8.6
	$MnSO_4$	2230			22.30
F	Na_2 EDTA $2H_2O$	373	200	20	37.3
	$FeSO_4 7H_2O$	278			27.8
	Glycine	40			2.0
	Nicotinic acid	10			0.5
G	Thiamine HCL	2	200	20	0.1
	Pyridoxine HCL	10			0.5
	Myo-inositol	2000			100
H	Sucrose	30000			30000
	Agar				8000

living plant tissues were kept inside the chamber and exposed to UV light for 60 min. The laminar flow was switched on 10 min prior to inoculation or sub culturing. The culture room used for incubating the culture was maintained at temperature of 24±1°C by regulating the room air conditioner or thermostatically controlled heater as per requirement. For maintaining light, flourecent light tubes of 3000-3200 lux were fixed to maintain 16 h photoperiod.

Observations were percentages (%) of aseptic cultures, necrotic cultures and explant survival and were made within three weeks of inoculation. Each treatment combination was assigned to 10 explants with one explant per test tube and replicated three times. The data generated was subjected to ANOVA in complete randomized design using R- software at 5% level of significance. To satisfy model, assumptions of experiments were subjected to arc sine and square root transformations. The significant difference among treatments was compared by critical difference.

RESULT AND DISCUSSION

Strawberry explants (runner tips and nodal segments) were subjected to 12 different sterilization regimes using MS (Murashige and Skoog, 1962) as the basal medium. The effect of various sterilization regimes and explants on culture asepsis, necrosis and explant survival (Table 3) was highly significant. The highest percentage of aseptic cultures (78.33%) was obtained by treating the explants with 1.5% sodium hypochlorite for 20 min + 70 % ethyl alcohol for 30 s. The aseptic frequency of runner tips explants was significantly higher (53.05 %) than the nodal segments (47.22 %). Interaction studies showed that the maximum culture asepsis was 80.00% when the runner tips were treated with 1.5% sodium hypochlorite 20 min + 70 % ethyl alcohol for 30 s. The highest necrotic cultures (58.32%) was obtained when explants were surface sterilized with 1.5% sodium hypochlorite 20 min + 70 % ethyl alcohol for 30 s .The lowest mean (8.88%) of necrotic cultures was obtained by treating the explants with mercuric chloride 0.1% for 4 min sterilization regime. The percentage of necrotic cultures of runner tip explants was lower (28.69%) than the nodal segments (31.64%).

Table 3. Influence of different sterilants on per cent aseptic cultures, explant survival and necrotic culture in strawberry *cv.* Senga Sengana.

Sterilants (time duration)	*Aseptic cultures (%)			**Explant survival (%)			**Necrotic cultures (%)		
	Runner tip	Nodal segment	Mean	Runner tip	Nodal segment	Mean	Runner tip	Nodal segment	Mean
Mercuric chloride (0.1%) (2 min)	30.00 (33.20)	26.66 (31.08)	28.33 (32.14)	21.11 (4.69)	17.77 (4.32)	19.44 (4.51)	11.11 (3.47)	14.44 (3.92)	12.77 (3.69)
Mercuric chloride (0.1%) (3 min)	36.66 (37.25)	33.33 (35.25)	35.00 (36.25)	24.44 (5.04)	17.77 (4.32)	21.10 (4.68)	14.44 (3.92)	17.77 (4.32)	16.01 (4.12)
Mercuric chloride (0.1%) (4 min)	43.33 (41.16)	40.00 (39.23)	41.67 (40.20)	37.77 (6.22)	31.11 (5.66)	34.44 (5.94)	6.66 (2.76)	11.11 (3.47)	8.88 (3.12)
Sodium hypochlorite (1.5%) (10 min)	43.33 (41.16)	36.66 (37.26)	40.00 (39.21)	26.66 (5.26)	17.77 (4.32)	22.21 (4.79)	17.77 (4.32)	20.00 (4.58)	18.88 (4.45)
Sodium hypochlorite (1.5%) (15 min)	50.00 (45.00)	40.00 (39.16)	45.00 (42.08)	23.33 (4.93)	11.00 (3.47)	17.16 (4.20)	27.77 (5.36)	31.11 (5.66)	29.44 (5.51)
Sodium hypochlorite (1.5%) (20 min)	53.33 (46.91)	43.33 (41.16)	48.33 (44.04)	20.00 (4.58)	7.77 (2.95)	13.88 (3.76)	34.44 (5.95)	37.77 (6.22)	36.10 (6.08)
Mercuric chloride (0.1%) + ethyl alcohol (70%) (2 min + 30 s)	50.00 (45.00)	43.33 (41.16)	46.67 (43.08)	27.77 (5.36)	18.88 (4.45)	23.32 (4.90)	23.33 (4.93)	26.66 (5.26)	24.99 (5.09)
Mercuric chloride (0.1%) + ethyl alcohol (70%) (3 min + 30 s)	53.33 (46.91)	46.66 (43.08)	50.00 (45.00)	31.11 (5.66)	18.88 (4.45)	24.99 (5.06)	24.44 (5.04)	34.44 (5.95)	29.44 (5.49)
Mercuric chloride (0.1%) + ethyl alcohol (70%) (4 min + 30 s)	60.00 (50.79)	56.66 (48.83)	58.33 (49.81)	34.44 (5.95)	28.88 (5.46)	31.66 (5.70)	27.77 (5.36)	31.11 (5.66)	29.44 (5.51)
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (10 min + 30 s)	66.66 (54.73)	56.66 (48.83)	61.66 (51.78)	17.77 (4.32)	14.44 (3.92)	16.10 (4.12)	51.11 (7.21)	45.33 (6.65)	48.22 (6.93)
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (15 min + 30 s)	70.00 (56.80)	66.66 (54.73)	68.33 (55.77)	23.33 (4.93)	17.77 (4.32)	20.55 (4.63)	47.77 (6.98)	51.11 (7.21)	49.44 (7.10)
Sodium hypochlorite (1.5%) + ethyl alcohol (70%) (20 min + 30 s)	80.00 (63.44)	76.66 (61.12)	78.33 (62.28)	22.22 (4.81)	21.11 (4.79)	21.66 (4.75)	57.77 (7.66)	58.88 (7.73)	58.32 (7.70)
Mean±SD	53.05±14.31 (46.86±8.53)	47.22±14.4 (43.41±8.54)		25.82±6.0 (5.15±0.56)	18.59±6.49 (4.36±0.74)		28.69±16.26 (5.25±1.51)	31.64±14.84 (5.55±1.30)	

Values in the parenthesis are *arc sine and **square root transformed.

Interaction studies show maximum percentage of necrotic cultures (58.88%) when the runner tips and nodal segments were treated with 1.5% sodium hypochlorite for 20 min + 70% ethyl alcohol for 30 s, while the lowest necrotic percentage (6.66%) was obtained when explants were surface sterilized with mercuric chloride 0.1% for 4 min.

The highest percentage of surviving cultures (34.44%) was obtained by treating the explants with mercuric chloride (0.1%) for 4 min. The response of runner tips explants was significantly higher (25.82%) than the nodal segments (18.59%). Interaction studies showed that maximum percentage of surviving explants was (37.77%) when the runner tips were treated with mercuric chloride (0.1%) for 4 min. Surfaces of plant carry wide range of microbial contaminants. To avoid these sources of infection, the tissues must be surface sterilized before planting on nutrient medium. The present studies on the effect of different regimes

of sterilization revealed that maximum aseptic cultures were obtained from both the explants (runner tips and nodal segments) when treated with 1.5% sodium hypochlorite for 20 min plus ethyl alcohol 70% for 30 s, but the surviving percentage was less because this treatment resulted in necrosis and tissue injury of explants. However, mercuric chloride (0.1%) for 4 min resulted in less percentage of aseptic cultures but gave highest percentage of surviving explants as most researchers have found that a single sterilant is more effective than the combination. These results are in close conformity with those of Dalal et al. (1992) in grape, Modgil et al. (1994) in apple and Peer (2008) in cherry. Our results are in line with those of Rattanpal et al. (2011) who micropropagated strawberry through meristem culture and found that treating explants with mercuric chloride (0.1%) for 4 min was the most effective surface sterilization procedure for maximum survival of explants with minimum tissue injury. Likewise, Gautam et al. (2001) also found that treating the explants of strawberry with 0.1% mercuric chloride for 3 min gave minimum contamination with maximum culture establishment.

Conclusion

Various sterilization treatments yielded aseptic cultures but the highest percentage of aseptic cultures were achieved by treating the explants with sodium hypochlorite (1.5%) for 20 min plus ethyl alcohol (70%) for 30 s. The maximum percentage of explant survival was achieved when explants were surface sterilized with 0.1% mercuric chloride for 4 min. The use of sodium hypochlorite (1.5%) for 20 min plus ethyl alcohol (70%) for 30 s gave the highest aseptic cultures but resulted in higher necrotic cultures. So it is concluded from the above study that sterilization treatment of 0.1% mercuric chloride for 4 min is effective for disinfecting the field grown strawberry explants intended for *in vitro* culture. This treatment resulted in maximum percentage of explant survival.

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Full Length Research Paper

Regeneration of plantlets from unpollinated ovary cultures of Ethiopian wheat (*Triticum turgidum* and *Triticum aestivum*)

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An *in vitro* culture protocol was established for direct regeneration of plantlets from unpollinated ovary cultures of four Ethiopian wheat varieties. Unpollinated ovaries were excised from durum wheat (Yerer and Ude varieties) and bread wheat (Simba and Galama varieties). Analysis of variance (ANOVA) has shown that genotypes, types of media, concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D) and kinetin (KIN) and durations of cold pretreatment at 4°C significantly (P≤0.05) affected direct formation of embryonic tissues independently. Stage II of wheat spikes, MS medium containing 1 mg/l of each of 2,4-D and KIN and 15 days of cold pretreatment were found to be the best conditions for direct formation of embryonic tissues. The highest frequency of shoots were regenerated from the cultured embryonic tissues of Yerer (41.6%) and Simba (41.3%) on medium containing 0.1 mg/l 2,4-D. From a total of 14,524 cultured unpollinated ovaries, 1,100 embryonic tissues (7.6 %) and 75 regenerants were obtained. The average percentage of embryonic tissues and regenerants were 9.0 and 1.1% from 3,444; 9.8 and 0.55% from 4,732; 5.6 and 0.17% from 2,988; 4.7 and 0.12% from 3,360 cultured unpollinated ovaries for varieties Yerer, Simba, Ude and Galama, respectively.

Key words: Embryonic tissues, *Unpollinated ovaries*, regenerants, wheat varieties.

INTRODUCTION

Wheat is predominantly a selfing and annual crop plant that originated in the Fertile Crescent of South East of Turkey (Waines and Hegde, 2003). In Ethiopia, wheat is traditionally grown by small scale farmers on heavy black clay soils at altitudes ranging from 1800-2800 m above sea level (masl) (Tesemma and Belay, 1991). Some of the major production constraints of wheat crop that pose a serious threat to global food security are fungal and viral diseases, insect pests and increasing human population (Jauhar, 2006; Getahun et al., 2012). *In vitro* regeneration of plants is one of the pre-requisites for successful genetic transformation, the fastest and the only easy methods for producing homozygous lines (Smale et al., 1996) which are very helpful to mitigate these constraints.

In vitro culture of unpollinated ovaries and ovules have

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxy acetic acid; **KIN,** kinetin; **NAA,** naphthaline acetic acid; **BAP,** benzyl amino purine; **IAA,** indole acetic acid; **DZARC,** Debre Zeit Agricultural Research Center; **HARC,** Holetta Agricultural Research Center; **PGRs,** plant growth regulators; **ET,** embryonic tissues.

been successfully applied to many plant species that are not amenable to androgenesis and to overcome many of the problems associated with androgenesis such as albinism,
inviability and recalcitrant (Shivanna and Mohan, 2005; Jauhar et al., 2009). Successful results of *in vitro* gynogenesis to produce haploids and double haploids have been reported in tef (*Eragrostis tef*) (Gugsa et al., 2006).

Potential use of wheat in tissue culture studies have been reported using various explants such as immature embryos (Sears and Deckard, 1982), anthers (Dogramaci et al., 2001), microspores (Liu et al., 2002) and unpollinated ovaries (Slama and Slim, 2007). However, tissue culture works have not been reported in any of the explant types using Ethiopian wheat genotypes. The objectives of this study were to regenerate plantlets from unpollinated ovary cultures of four Ethiopian wheat varieties and optimize the concentrations of plant growth regulators essential for the formation of embryonic tissues and regenerations of plantlets.

MATERIALS AND METHODS

Plant materials

Two bread wheat varieties (*Triticum aestivum*) varieties namely, Simba, and Galama and two durum wheat (*Triticum turgidum*) varieties (Ude and Yerer) were used in this study. Seeds of these varieties were obtained from Debre Zeit Agricultural Research Center (DZARC) and Holetta Agricultural Research Center (HARC). Seeds were sown in 30 cm diameter pots filled with black soil and grown in a glasshouse. Five seeds were sown per pot and two pots were used for each variety. Seeds were sown every two weeks interval for one year to get continuous source of explants.

Surface sterilization and culture initiation

Immature spikes were sterilized with 70% ethanol for 1 min, followed by 20% sodium hypochlorite of the original stock 5.25% and four drops of Tween 20 for 10 min, and then rinsed with sterile double distilled water. Unpollinated ovaries were excised aseptically using sterilized scissors, forceps and scalpel blades under stereomicroscope. Twenty ovaries were placed in each sterilized Petri-dish (15 x 100 mm) containing 20 ml of MS medium containing 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) and 30 g/l sucrose. Before autoclaving the medium was adjusted to pH of 5.8 and 0.8% agar was added. The Petri-dishes were kept in growthroom incubated under 16 h photoperiod and a light intensity of 1032-1557 lux flourescent intensity and 8 h dark at 24 ± 4°C.

Identifying the appropriate stages of unpollinated ovary explant

Spikes taken from variety Simba were categorized into three stages and unpollinated ovaries were excised from each stage. These were before the emergence of own from the boot (stage I), spike length of 5-9 cm which might be equivalent to uninucleate stage of wheat anther. When the owns emerged out from the boot, stage II (spike length of 10-14 cm) which might be equivalent to late uninucleate to binucleate stage of wheat anther. When the owns completely emerged out from the boot, stage III (spike length of 15- 18 cm) which might be equivalent to the trinucleate stage of wheat anther.

Effect of cold pretreatment durations on the formation of embryonic tissues

Spikes at stage II of the four varieties were cold pretreated at 4°C for 5, 10, 15, 20 and 25 days. Unpollinated ovaries were plated in a disposable Petri-dish containing 20 ml MS medium supplemented with 1 mg/l of 2,4-D. Unpollinated ovaries from spikes of all varieties without cold pretreatment were cultured in the same way as a control.

Effect of culture media on induction of embryonic tissues

Three types of media MS (Murashige and Skoog, 1962), N_6 (Chu, 1978) and B_5 (Gamborg et al*.,* 1968) were used as embryo induction media. Each medium was supplemented with 1.0 mg/l 2,4-D and 30 g/l maltose.

Effect of 2,4-D and kinetin (KIN) on the formation of embryonic tissues

Unpollinated ovaries from stage II of the four varieties were cold pretreated at 4°C and cultured in Petri-dishes containing MS medium supplemented with 12 different concentrations and combinations of 2,4-D and KIN and 30 g/l maltose.

Effect of plant growth regulators (PGRs) on regeneration of shoots

Embryonic tissues of all varieties were transferred into MS regeneration medium supplemented with different concentrations and combinations of PGRs. All treatments were supplemented with 60 g/l sucrose.

Acclimatization of plantlets

Plantlets of varieties Yerer and Simba were removed from Majenta jar using forceps. The roots were washed with distilled water to remove the gelrite and placed into pots filled with 3:2:1 ratios of black soil, compost and sand, respectively.

Data collections and analyes

Each independent experiment had 3 replications and repeated 2-3 times. In induction media, 20 ovaries and in MS regeneration medium, 5 embryonic tissues were used as a unit of replication. Subculturing was carried out every 2-3 weeks. The frequency of responsive unpollinated ovaries was assessed in terms of percentage of developed ovaries and formation of embryonic tissues. All the experiments were carried out in complete random design (CRD). The analysis of variance (ANOVA) was conducted using SAS computer software. The possible pairs of treatment means were compared using LSD/Duncan test.

RESULTS

Determination of appropriate harvesting stages of spikes

Ovaries (Figures 1a and b) taken from stage II induced maximum percentage of embryonic tissues (25%) (Figure 1c) followed by stage I (11.6%), however, overies taken from stage III could not sprouted out to give amenable

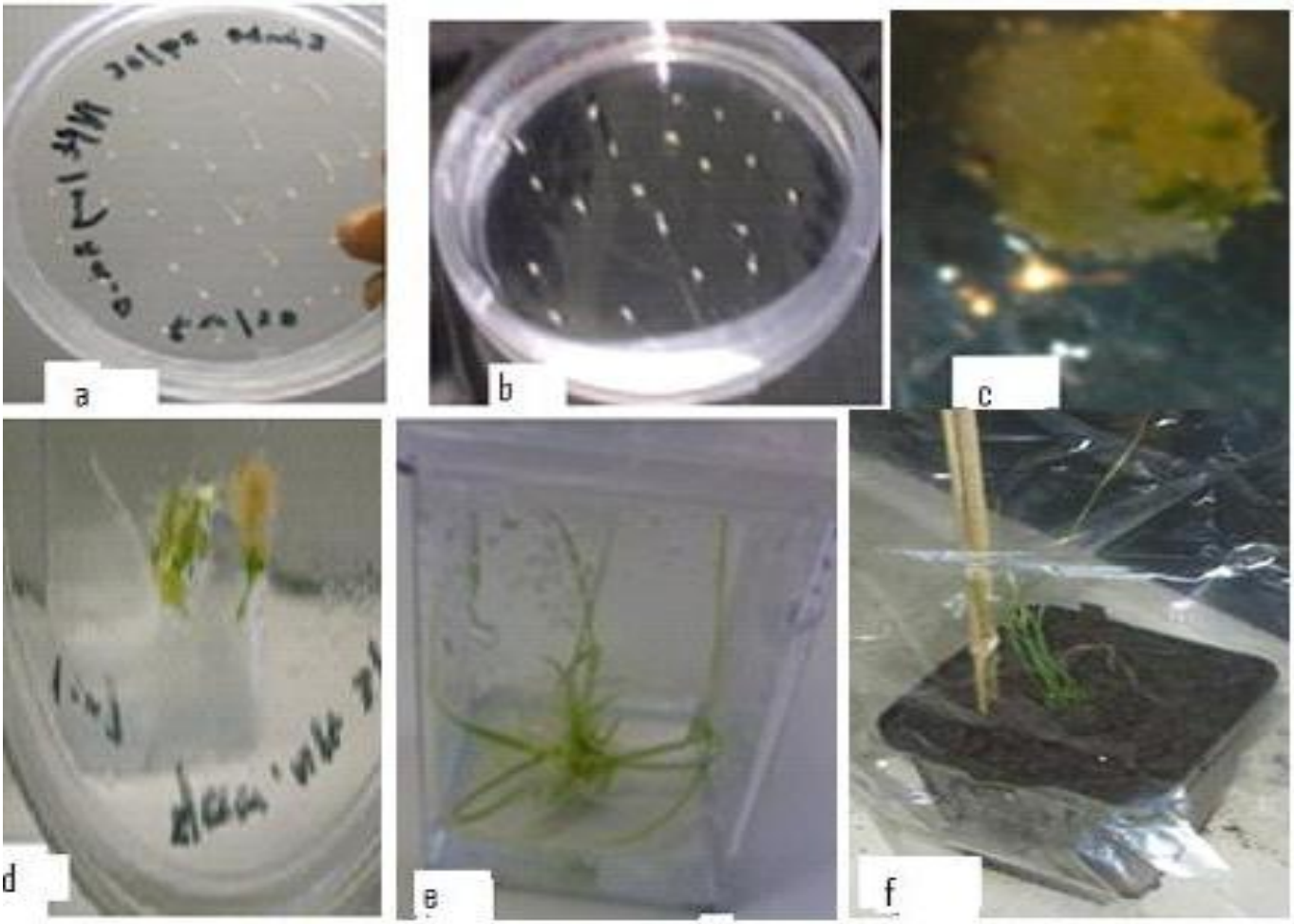

Figure 1. Embryonic tissues and plantlets of varieties Simba and Yerer.**a,** Ovary culture of variety Simba; **b,** ovary culture of variety Yerer; **c,** embryonic tissues of variety Simba; **d,** embryonic tissues of variety Yerer; **e,** plantlets of variety Yerer; **f,** acclimatization of plantlet of variety Yerer.

Table 1. Determination of appropriate harvesting stages of spikes for formation of embryonic tissues.

Stages of spike	Stage I	Stage II	Stage III
Cultured ovaries	120	120	120
ET	14	30	6
%ET±SD	11.6±4.7	25.0±9.6	5.0±2.9

ET, Embryonic tissues; SD, standard deviation.

amount of embryonic tissues and were found to be the least responsive (5.0%) (Table 1).

Effect of cold pretreatment durations on the formation of embryonic tissues

The highest percentage of embryonic tissues (8.3-20.0%) was obtained from unpollinated ovaries of all varieties that were pretreated at 4°C for 15 days followed by 10 days (5.8-10.8%). There was significant difference among all pretreatment durations for all varieties (Table 2).

Effect of culture media on the formation of embryonic tissues

The formation of embryonic tissues of variety Simba indicated that there was significant difference (P≤0.05) among MS, N6 and B5 media on the percentage of induced

Table 2. Effect of cold pretreatment durations on the percentage of embryonic tissues.

Variety	Percentage of embryonic tissues						
	0	5[p]	10	15	20	25	Mean of the means
Simba	6.0±3.5	8.3±2.4	10.0±4.0	20.0±4.0	9.2±1.9	5.8±1.9	9.2[a]
Yerer	8.3±4.4	9.2±4.2	10.8±2.5	16.6±4.5	3.3±2.4	0.8±2.5	8.6[a]
Ude	5.0±2.9	5.8±2.4	5.8±2.4	8.3±2.4	4.2±2.5	1.6±2.4	5.6[b]
Galama	3.3±2.4	6.0±2.4	6.0±2.4	13.3±2.4	1.7±3.2	0.0±0.0	4.7[b]
Mean of the means	4.2[c]	7.9[b]	8.8[b]	14.6[a]	4.6[c]	2.9[c]	

ANOVA for the effect of cold pretreatment on % of ET formation

Source of variation	DF	MS	F-value	P
Variety	3	87.4	6.6	0.0006**
Pretreatment	5	192.0	14.5	<0.0001**
Rep	2	1.4	0.1	0.9006
Error	61	13.2		
Total	71	294.0		

[p] Pretreatments (days).
Means with the same letter along the row and column are not significantly different at p≥0.05 using Duncan multiple range test. It is shown as mean of % ET±SD. **, Significant difference at p ≤0.1.

Table 3. Analysis of variance for the effect of different media on the formation of embryonic tissues from ovary cultures of variety Simba.

Source of variation	DF	MS	F-value	P
Media	2	304.2	24.3	0.04*
Rep	2	18.8	1.5	0.4
Error	2	12.5		
Total	6	335.5		

*, Significant at p≤0.05.

induced embryonic tissues (Table 3). The highest percentage of induced embryonic tissues were better in MS medium (20.0 %) followed by N_6 medium (5.0%) (data not shown).

Effect of PGRs on the formation of embryonic tissues

Maximum percentage of embryonic tissues were induced in MS medium supplemented with 1.0 mg/l 2,4-D combined with 1.0 mg/l of KIN for all varieties except variety Galama (Table 4). In this treatment, the highest percentage (35%) and the second highest (26.6%) percentage of embryonic tissues were obtained from varieties Yerer and Simba, respectively. Moreover, these varieties produced high quality embryonic tissues that could be easily differentiated into shoots (Figures 1c and d). Analysis of variance has shown that there was significant difference among varieties and treatments on the formation of embryonic tissues (Table 4).

Effect of PGRs on shoot and plantlet regeneration

A total of 462, 158, 168 and 312 embryonic tissues for varieties Simba, Galema, Ude and Yerer were induced on induction media under different experimental conditions, respectively (Table 6). Eleven combinations of auxins and cytokinins were selected and continuously supplemented in the regeneration medium for the regeneration of shoots (Table 5 and Figure 1e). Maximum responsive embryonic tissues, 41.6 and 41.3% were obtained in MS medium supplemented with 0.1 mg/l 2,4-D without cytokinin (Table 5) from varieties Yerer and Simba, respectively.

Variety Yerer gave better percentage of regenerants (1.1%) from a total of 3,444 cultured ovaries. Variety Simba produced the second highest percentage of regenerants (0.55%) and the maximum percentage of embryonic tissues (9.8%) from a total of 4,732 cultured unpollinated ovaries followed by variety Yerer (9.0%) (Table 6). Using all varieties from a total of 14,524 cultured unpollinated ovaries, 1100 embryonic tissues (Table 6) were produced and 75 regenerants were obtained (data not shown).

Acclimatization

Two plantlets of variety Simba and 16 plantlets of variety Yerer were successfully transferred into pots containing

Table 4. Effect of 2,4-D and KIN on the formation of embryonic tissues using all varieties.

2,4-D (mg/l)	KIN (mg/l)	Percentage % of embryonic tissues				
		Simba	Galama	Ude	Yerer	Mean
0.5	0.0	6.7±2.3	1.7±2.3	2.5±2.5	10.0±0.0	5.2^{d}
0.5	1.0	6.7±2.3	2.5±2.5	3.3±1.3	11.6±2.0	6.0^{d}
0.5	2.0	9.2±3.4	5.8±1.9	4.2±0.8	11.6±4.7	7.7^{cd}
1.0	0.0	20.0±4.0	14.2±1.9	8.3±2.3	16.6±2.4	14.8^{ab}
1.0	1.0	26.6±4.6	8.3±2.9	13.3±3.3	35.0±9.4	20.8^{a}
1.0	2.0	14.2±5.2	5.8±1.8	9.2±0.8	15.8±3.5	11.3^{bc}
1.5	0.0	11.7±4.7	5.0±2.8	8.3±2.4	14.2±4.6	9.8^{cd}
1.5	1.0	8.3±3.2	5.0±2.8	8.3±2.4	10.8±1.9	8.1^{cd}
1.5	1.0	7.5±2.5	7.5±3.8	6.7±2.4	6.7±2.4	7.1^{cd}
2.0	0.0	5.8±1.8	9.2±1.9	7.5±2.7	5.8±2.5	7.1^{cd}
2.0	1.0	5.0±1.3	7.5±2.5	5.8±2.5	5.0±2.9	5.8^{d}
2.0	2.0	7.5±2.5	5.0±2.8	5.8±2.5	3.3±1.7	5.4^{d}
Mean		10.8^{ab}	6.5b	6.9b	11.2^{a}	

ANOVA for the effect of 2,4-D and KIN on the formation of ETs

Source of variation	DF	MS	F-value	P
Variety	3	202.5	3.11	0.0134 *
Pretreatment	11	278.5	5.11	<0.0001**
Rep	2	25.5	0.47	0.6274
Error	127	54.5		
Total	143	561.0		

Means with the same letter along the row and column are not significantly different at p≥0.05 using Duncan multiple range test. ET, Embryonic tissues; *, significant at p≤0.05; **, very significant at p≤0.01. Mean values are shown as ± SD.

Table 5. Effect of PGRs on the percentage of responsive embryonic tissues. Their effect is shown as mean value of % RET±SD.

Treatment		Variety				Mean of the means
		Simba	Yerer	Ude	Galama	
0.0 2,4-D	0.0 KIN	5.1±1.2	0.0±0.0	5.8±2.4	0.0±0.0	2.7^{b}
0.05 mg/l 2,4-D	2.0mg/l KIN	3.3±1.9	23.8±5.5	0.0±0.0	0.0±0.0	6.8^{b}
0.05 mg/l 2,4-D	3.0 mg/l KIN	0.0±0.0	29.2±4.1	0.0±0.0	0.0±0.0	7.3^{b}
0.1 mg/l 2,4-D	0.0 mg/l KIN	41.3±13.3	41.6±14.4	0.0±0.0	0.0±0.0	20.7^{a}
0.1 mg/l 2,4-D	1.0 mg/l KIN	20.6±10.6	0.0±0.0	0.0±0.0	0.0±0.0	5.2^{b}
0.1 mg/l 2,4-D	2.0 mg/l KIN	0.04±0.0	0.06±0.0	0.0±0.0	0.0±0.0	0.0^{b}
0.1 mg/l 2,4-D	2.0 mg/l BAP	0.042±0.0	9.0±5.0	5.6±2.4	0.0±0.0	3.7^{b}
0.1 mg/l NAA	0.0 mg/l KIN	0.0±0.0	4.3±1.1	0.0±0.0	0.0±0.0	1.1^{b}
0.1 mg/l NAA	1.0 mg/l KIN	28.0±11.0	25.9±9.8	17.6±6.7	26.6±13.3	24.5^{a}
0.1 mg/l NAA	2.0 mg/l KIN	4.2±2.1	0.06±0.0	0.0±0.0	0.0±0.0	2.1^{b}
0.5 mg/l IAA	1 mg/l BAP	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0^{b}
Mean of the means		9.4^{ab}	12.2^{a}	2.6^{b}	2.4^{b}	

3:2:1 proportions of soil, compost and sand (Figure 1f). Each pot was covered with transparent plastic bags and kept in the growthroom for acclimatization. After 2 weeks, 11 plantlets of variety Yerer were transferred to glasshouse.

DISCUSSION

Determination of appropriate harvesting stage of spike

Sibi et al. (2004) have reported that better gynogenic res-

Table 6. The effect of genotypes on the percentage of responded embryonic tissues and regenerants from the total cultured ovaries of each genotype.

Variety	Total cultured unpollinated ovary	Number of embryonic tissue	Embryonic tissue (%)	Regenerant (%)
Simba	4732	462	9.8	0.55
Yerer	3360	158	4.7	0.12
Ude	2988	168	5.6	0.17
Galama	3444	312	9.0	1.10
Total	14, 524	1100	7.6	0.50

ponse was found when the spikes were harvested at bi- or trinucleate stage of microspores of wheat. Slama and Slim (2007) reported the highest regeneration frequency of gynogenic response from unpollinated ovary cultures of durum wheat. This was obtained when the microspore population was in late uninucleate to binucleate stages. These reports may be in consistent with stage II of the present study.

Effect of cold pretreatment durations on the formation of embryonic tissues

Seven days of cold pretreatment were taken as the optimum for the formation of embryonic tissues (Sibi et al., 2004). Slama and Slim (2007) have also reported that in durum wheat, 14 days pretreatment at 4°C gave the highest response of ovary development and callus induction. The above reports were in agreement with the present study. For all varieties, better number of gynogenic embryos were responded at 15 days of cold pretreatment (4°C) followed by 10 days.

Effect of culture media on the formation of embryonic tissues

In the present study, three types of induction media, MS, N_6, and B_5, were compared. From a total of 60, 40 and 40 ovaries cultured in MS, N6 and B5 media, the percentage of induced embryonic tissues were highest in MS medium (20.0 %) followed by N6 medium (5.0 %). In B5 medium, none of the enlarged ovaries induced embryonic tissues (0.0 %). MS medium supplemented with different combinations and concentrations of growth regulator has been commonly used as induction and regeneration medium in wheat crops. Immature embryo culture of durum wheat (Sears and Deckard, 1982; Satyavathi et al., 2004), spike and immature embryo cultures of bread wheat (Lin et al., 2006), and somatic embryo clulture of F1 hybrids of teff (Getahun et al., 2012) were some of them which are in agreement with the present study.

Effect of PGRs on the formation of embryonic tissues

The combination of kinetin and 2,4-D was evaluated for callus induction of immature embryo cultures of wheat (Jones et al., 2005). In immature embryo cultures of bread wheat, callus was initiated by 1 mg/l 2,4-D (Sears and Deckard, 1982). In seedlings, roots and stem explants of common wheat, callus growth was also vigorous when 0.5-2.0 mg/1 2,4-D was added (Shimada et al., 1969). In the present study, MS medium containing 1 mg/l 2,4-D in combination with KIN was the most effective PGR combination for all genotypes except for variety Galema.

The response of genotypes on the formation of embryonic tissues and regeneration

Unpollinated ovary cultures of durum wheat (Sibi et al., 2004), immature embryo cultures of durum wheat (Satyavathi et al., 2004), spike and immature embryo cultures of bread wheat (Lin et al., 2006) and unpollinated ovary cultures of durum wheat (Slama and Slim, 2007) and somatic embryo cultures of F1 hybrids of teff with its wild relatives (Getahun et al., 2012) revealed that plantlet regeneration was influenced by genotypes. In the present study, the embryonic tissue formation and regeneration of plants were affected by genotypes.

In anther cultures of durum wheat, from a total of 86,400 cultured anthers, 324 (0.38%) plants were obtained (Doramaci et al., 2001). Out of 324 plantlets, 248 were green plants (0.29 %) and 76 were albino plants (0.09%). In microspore cultures of bread wheat, 27.2%, were green and 72.8% were albino plants (Kim et al., 2003). In the present study, ovary cultures of three genotypes from the four wheat genotypes (75%) induced green plants which were better than the anther cultures of durum wheat (Doramaci et al., 2001). They induced a minimum and a maximum regenerants of 0.12 and 1.1% from ovary cultures of varieties Galema and Yerer, respectively. From a total of 14,524 cultured ovaries using four varieties, 75 regenerants (0.50 %) were obtained which were better than the work of Doramaci et al. (2001). All the plantlets were green and no albino plants were obtained. This is considered as an advantage over androgenic cultures reported by Doramaci et al. (2001) and Kim et al. (2003).

The effect of plant growth regulators on regeneration

In immature embryo cultures of wheat, shoots were initiated by reducing the 2,4-D from 1 to 0.1 mg/l. Complete plants were regenerated by transferring to 2,4-D free medium (Sears and Deckard, 1982) and somatic embryos of F1 hybrids of teff regenerated into plantlets in MS medium without plant growth regulators (Getahun et al., 2012). In the present study, the best response of plant regeneration was obtained from Simba and Yerer varietis on MS medium containing 0.1 mg/l 2,4-D. To some extent, these results are in agreement with the works of Sears and Deckard (1982) and Getahun et al. (2012).

Growth conditions of regenerants

Out of 18 green plantlets that were transferred into pots, 68.8% of the regenerants survived and 32.2% of the regenerants died in the growthroom. Out of 11 plantlets of variety Yerer, four plantlets (36.4%) survived in the glasshouse.The death of plantlets in the glasshouse was mainly due to high temperature of the glasshouse (38-40°C) as the glasshouse did not have temperature regulation system. As reported by Leone et al. (2006) the temperature of the glasshouse for wheat should have been between 21-25°C in Ethiopia.

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Effects of ten years treated wastewater drip irrigation on soil microbiological properties under Mediterranean conditions

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Water shortage in most countries of the southern Mediterranean basin has led to the reuse of municipal wastewater for irrigation. Despite numerous advantages for soil fertility and crop productivity, recycling wastewater in the soil also has several ecotoxicological and sanitary problems. To evaluate the chronic soil contamination and the cumulative impact of wastewater, we compared two plots, all under orange-grove that had been drip irrigated for 10 years. The first plot was irrigated with treated wastewater; the second one was irrigated with groundwater. No negative effects of treated wastewater drip irrigation treatment were observed on the measured soil parameters (pH, organic matter and cation exchange capacity). A slight increase in the concentration of soil enteric bacteria and soil fungal densities was recorded in the wastewater plot reaching a maximum value in the first soil layer (0 to 20 cm). This result was recorded essentially around the emitters. Groundwater plots and wastewater plots exhibited similar repartitions of soils DNA quantity with depth, with the highest values in the upper layer and a progressive decrease with soil depth. For both soils, DNA quantity was positively affected by soil organic matter content. This work confirms that, under suitable conditions, treated wastewater use in irrigation can have positive effects, not only in the aspects of soil quality, but also in social terms, as it allows the maintenance of irrigated agriculture in areas where groundwater has been polluted by seawater intrusion.

Key words: Treated wastewater, groundwater, drip irrigation, enteric bacteria, soil DNA.

INTRODUCTION

The scarcity of conventional water resources constitutes a social, agricultural and economic problem in most countries of the southern Mediterranean Basin. Water shortage in these countries is a result of a combination of arid climatic conditions and an increase in water requirements due essentially to population growth and the development of tourism. There is thus an urgent need to make alternative water sources available for agriculture to replace the high quality water required for human consumption (Angelakis et al., 1999). In this context, the use of municipal wastewater for irrigation could provide a realistic alternative water supply for agriculture, as has been proved in many countries in the Mediterranean region, such as Israel, Cyprus, Jordan and Tunisia (Angelakis et al., 1999).

With per capita, freshwater of about 450 m^3, Tunisia is one of the most drought-stressed countries in the Middle East and North Africa region. In this country, the reuse of treated wastewater in irrigation is considered as a strategic approach to preserve fresh water resources. This process date back, in fact, to 1965. Treated wastewater currently represents approximately 5% of Tunisia's total

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Table 1. Irrigation volume during the years of trials.

Year	Irrigation volume (mm)	
	Site 1	Site 2
1998	248	122
1999	245	125
2000	240	145
2001	250	120
2002	265	140
2003	270	135
2004	300	140
2005	370	130
2006	385	128
2007	380	135

available water; this planned to increase to 11% by 2030 (Shetty, 2004). Treated wastewater can be suitable for a large variety of applications. Among the most common reuse applications are irrigation; residential uses; urban and recreational uses; groundwater recharge; bathing water; aquaculture; industrial cooling water; and drinking water production (Huertas et al., 2008). Water reuse for irrigation has been largely applied to agriculture due to the advantages related to nutrient recovery possibilities, socio-economic implication, decline of fertilizer application and effluent disposal (Candela et al., 2007). However, scientific and technical treated domestic wastewater application for irrigation or aquifer recharge is mainly reduced to countries with a high scientific and technical development and water scarcity (Sheng, 2005).

Water quality criteria being generally applied for agricultural reuse have been mainly based on microbiological aspects, focusing on the existence of potential pathogens (viruses, bacteria and protozoa), which may cause sanitary problems (WHO, 1998), total dissolved solids (TDS) and salinity aspects (Martinez-Beltran, 1999). Haruvy (2006) presents more specific water quality parameters related to water reclamation and reuse. Salinity level of wastewater is generally high, and regular treatment processes do not get rid of salinity unless combined with rather expensive desalination processes and increase of water supply costs (Appelo and Postma, 1993). Research studies have focused on sanitary effects from reused domestic treated wastewater to evaluate the risk of edible crops by sprinkler irrigation (Haas, 1996). The presence of pathogenic microorganisms and NO_2 and CO_2 production for perched aquifers has been mentioned in the literature (Campos, 2008). Possible risk of pesticide leaching on the golf courses application has been evaluated by Cohen et al. (1999). Weber et al. (2006) have evaluated human risk of the organic contaminants in reclaimed wastewater used for irrigation. Candela et al. (2007), Dère et al. (2006) and Rusan et al. (2007) have mentioned the long-term effect of wastewater irrigation on soil and plant quality parameters. In this context, the aim of our study was to evaluate the effects of treated wastewater irrigation, of 10 years' duration, on the abundance soil microbial communities. Experiments were conducted in the "Nabeul-Hammamet" region of northern Tunisia. This is one of the most drought-stressed countries in the Middle Eastern and North African region, with freshwater per capita of around 450 m^3/years. Treated wastewater currently represents approximately 5% of Tunisia's total available water but is expected to increase to 11% by 2030 (Shetty, 2004).

We compared the microbial quality in two soils that had been irrigated for 10 years, in the same experimental field. The first soil was irrigated with secondary treated wastewater, the second was irrigated with groundwater. The bacterial and fungal abundance in each plot was assessed from counts on synthetic culture media. Enteric bacteria (*Escherichia coli*, fecal coliform and fecal streptococcus), indigenous to the soils irrigated for the same durations, were assessed by using the most probable number (MPN) method.

MATERIALS AND METHODS

Study area and sampling strategy

The valley of Nabeul (North East of Tunisia, 36° 29' N, 10° 42' E) is irrigated since 1980. The climate is temperate and semi arid with mild and rainy winters. The mean mensal temperature range is between 12 and 27°C. Evapo-transpiration (10.3 mm/d) was recorded in July and August, whereas rainfall occurred mainly from September to March. Annual precipitation was around 470 mm. The middle length of sunstroke is between 5.4 and 12.9 h/day. Soils were usually sampled in triplicate at two different sites, all under an orange - grove that had been irrigated for 10 years: Plot 1: soils irrigated with secondary treated wastewater (WWP); plot 2: soils irrigated with groundwater (GWP).

A drip irrigation system was used, with lines running along the citrus tree rows and two droppers situated 50 cm on both sides of each tree; flux per dipper was around 4 l/h. Annual application rates ranged from 240 to 410 mm for the plot 1 and from 120 to 145 mm for the plot 2. Details on the irrigation scheduling are provided in Table 1.

Sampling was carried out using a drill at the end of the dry season extending between the 30 and 31 October, 2007. Each site was divided into three locations or blocks, and composite soil sample from 0-20, 20-40 and 40-60 cm soil depth was taken from each block. Soil samples were collected at three points along a 150 cm transect that was perpendicular to the direction of the drip irrigation system (Figure 1). Soil was sieved (2 mm) in order to remove rocks and root fragments, placed inside plastic vented bags and stored at 5°C for 15 days for later analysis. Physico-chemical soil characteristics were measured at each site for each treatment by the Laboratory of Soil Analysis (INRA-Arras, France, http://www.arras.inra.fr/), using standard methods. The main results are given in Table 2.

Microbial enumeration

Bacteria and fungi were extracted by blending soil samples with 0.8% (w/v) sterile NaCl solution and the homogenous soil suspension was serially diluted tenfold with sterile saline solution. Indirect counting of bacteria was carried out by spreading 100 µl of appropriate dilutions on plate count agar (aerobic plate count agar) (Al-Lahham et al., 2003). Bacterial colonies were counted after 48 h

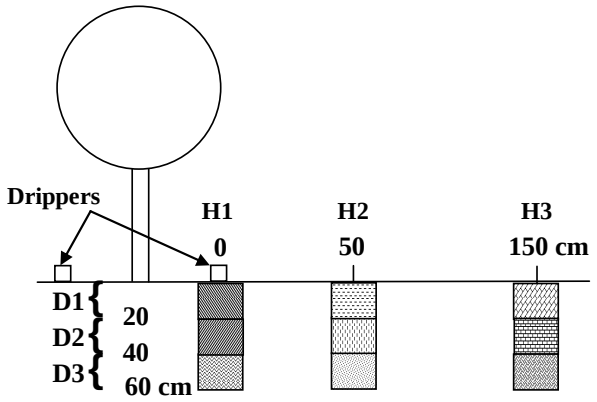

Figure 1. Soil sampling mode. D1: depth 1 (0-20 cm), D2: depth 2 (20-40 cm), D3: depth 3 (40-60 cm), H1: Horizon 1 (0 cm/dripper), H2: Horizon 2 (50 cm/dripper), H3: Horizon 3 (150 cm/dripper).

of incubation at 28°C. Only plates with between 10 and 100 colonies per plate were counted. For fungi, the appropriate soil dilution was spread on malt extract agar (30 g/l malt extract, 3 g/l protease peptone, 1.5% agar, pH 5.6). The number of developing colonies was counted after 7 days of incubation at room temperature and was expressed as the number of colony forming units (CFU) per gram dry weight of soil.

Pathogenic contamination

Soil examination

The mass of 10 g of soil was dispersed in 90 ml of sterile distilled water. They were then submitted to a mechanical shaking for 2 h (Edmond Buhler, type KI-2), in order to remove bacteria from their organo-mineral substrates. Finally, soil suspensions were used for *E. coli*, fecal coliform and fecal streptococcus determination using the most probable number (MPN) method and following the 3 replications × 5 dilution scheme (APHA, 1998).

Water examination

All the glassware used was cleaned with hot water and a suitable detergent, rinsed with hot water to remove all traces of the detergent used, and finally rinsed with distilled water. The sampling glass bottles WEre sterilized in an autoclave at 121°C for 15 min (APHA, 1998).

Samples of treated wastewater and conventional water are collected in sterilized glass bottles from the wastewater treatment plant and from the Nabeul groundwater. 10% of sodium thiosulfate ($Na_2S_2O_3$) was added to the samples that have been treated with chlorine, as a de-chlorinating agent to neutralize any residual chlorine and to prevent the continuation of its action on bacteria thereafter. Solution of $Na_2S_2O_3$ at the rate of 0.1 ml of 10% and 0.1 ml of 3% to 100 ml sample bottles are added to the two water samples. Wastewater samples were collected and transported directly to the laboratory at +4°C and kept in the refrigerator for later analysis. The samples were examined within 24 h for the presence of coliforms and streptococci bacteria group, and the total bacterial count was done in accordance with APHA (1998).

Soil DNA extraction

Microbial DNA was extracted from independent triplicates of soils sampled at each site, according to the method described by Ranjard et al. (2003). Briefly, 1 g from each soil sample was mixed with 4 ml of a solution containing 100 mm Tris (pH 8.0), 100 mm EDTA (pH 8.0), 100 mM NaCl and 2% (wt/Vol) sodium dodecyl sulfate. 2 g of 106 µm-diameter glass beads and 8 glass beads of 2-mm diameter were added to the mixture in a bead-beater-tube. The samples were then homogenized for 30 s at 1600 RPM in a mini bead-beater cell disruptor (Mikro-dismembrator S. B. Braun Biotech International). The samples were incubated for 20 min at 70°C, then centrifuged at 14 000 *g* for 1 min at 4°C. The collected supernatants were incubated for 10 min on ice with 1/10 volume of 3 M potassium acetate (pH 5.5) and centrifuged at 14 000 *g* for 5 min. After precipitation with one volume of ice-cold isopropanol, the nucleic acids were washed with 70% ethanol. DNA was separated from the residual impurities, particularly humic substances, by centrifuging through two types of minicolumn. Aliquots (100 µl) of crude DNA extract were loaded onto polyvinyl polypyrrolydone minicolumns (BIORAD, Marne-la-Coquette, France) and centrifuged at 1000 *g* for 2 min at 10°C. The collected eluate was then purified with the Geneclean turbo kit (Q-Biogene, Illkirch, France). Purified DNA was quantified by spectrophotometry (Bio-Rad Smart Spec^{TM} Plus, France) (Leckie et al., 2004).

Statistical analysis

The effect of irrigation on soil physicochemical properties and microbial abundance was tested by the SPSS statistical program (SPSS 10.05 for Windows; SPSS Inc., Chicago, IL, USA) and differences between means were tested with the Student-Newman-Keuls test.

RESULTS AND DISCUSSION

Water characteristics

Treated wastewater

This study was conducted at the SE4 Wastewater Treatment Plant (Nabeul -north east Tunisia-) which was set up in May 1979. The SE_4 wastewater treatment plant is an activated sludge-extended aeration system that involves a mechanical screen, grit removal tanks, primary sedimentation tanks, extended aeration tanks and finally sedimentation tanks. The characteristics of the waste water used for irrigation varied both within and between the years of application. The wastewater was, on average, alkaline with a basic pH value of 7.8 and had a moderate level of total dissolved solids (TDS) of 1556 mg/l. It contained considerable amounts of nitrate (31 mg/l), ammonia (53 mg/l) phosphate (17.8 mg/l) and potassium (53.3 mg/l). It presented an electrical conductivity of 3.27 (mmhos cm^{-1}), a chemical and biochemical oxygen demand of 95 and 17.4 mg/l, respectively.

On the other hand, the concentrations of micronutrients and heavy metals in the wastewater were relatively low with 0.0007 mg L^{-1} of Cd, 0.02 of Co, 0.01 of Cu, 0.05 of Mn, 0.19 of Fe, 0.05 of Ni, 0.03 of Pb, 0.03 of Zn and 0.03 of Cr.

Microbiological contamination and organic matter in the wastewater can produce detrimental effects on human health (Al-Shammiri et al., 2003). A recent WHO report concluded that crop irrigation with untreated wastewater

Table 2. Physico-chemical soil characteristics.

Sample	Clay (%)	Silt (%)	Sand (%)	pH	Tot org. C (mg. g^{-1})	N tot. (mg. g^{-1})	Org. M. (mg.g^{-1})	CEC (Cmol^{+}. kg^{-1})
WWH1D1	14	8.7	77.3	7.07^{a} (±0.85)	18.07^{a} (±3. 47)	1.88^{c} (±0.36)	29.80^{a} (±5.78)	9.57^{gh} (±1.42)
WWH1D2	10.4	6.1	83.5	7.97^{cd} (±0.38)	5.6^{bc} (±1.79)	0.65^{ab} (±0.20)	9.70^{bc} (±3.12)	5.82^{abcd} (±0.76)
WWH1D3	11.07	7.03	81.9	8.25^{cd} (±0.14)	3.01^{c} (±0.50)	0.37^{a} (±0.04)	5.21^{c} (±0.87)	4.91^{abcd} (±0.14)
WWH2D1	12.53	8.07	79.4	7.69^{b} (±0.38)	15.03^{a} (±3.87)	1.66^{c} (±0.41)	26.07^{a} (±6.69)	8.38^{efg} (±1.34)
WWH2D2	8.8	6.3	84.9	8.24^{cd} (±0.30)	4.77^{c} (±0.99)	0.53^{a} (±0.12)	8.24^{c} (±1.70)	4.63^{abc} (±0.17)
WWH2D3	7.83	4.17	88	8.40^{cd} (±0.30)	2.15^{c} (±0.50)	0.27^{a} (±0.06)	3.73^{c} (±0.88)	3.73^{a} (±0.70)
WWH3D1	10.87	6.2	82.93	7.99^{cd} (±0.18)	10.06^{b} (±3.11)	1.10^{b} (±0.36)	17.43^{b} (±5.40)	5.83^{abcd} (±0.84)
WWH3D2	8.77	4.87	8637	8.50^{d} (±0.14)	2.70^{c} (±0.37)	0.33^{a} (±0.03)	4.68^{c} (±0.63)	3.88^{ab} (±0.21)
WWH3D3	9.3	4.67	86.03	8.55^{d} (±0.16)	2.58^{c} (±1.08)	0.31^{a} (±0.09)	4.47^{c} (±1.87)	4.37^{abc} (±0.72)
GWH1D1	22.83	18.5	58.7	8.61^{d} (±0.09)	14.2^{a} (±1.93)	1.53^{c} (±0.22)	24.57^{a} (±3.36)	8.97^{fgh} (±1.15)
GWH1D2	20.03	16.4	63.33	8.77^{d} (±0.03)	7.10^{bc} (±1.19)	0.68^{ab} (±0.12)	12.29^{bc} (±2.08)	6.94^{efg} (±0.65)
GWH1D3	23.43	17.7	58.8	8.79^{d} (±0.02)	4.77^{c} (±1.35)	0.48^{a} (±0.07)	8.25^{c} (±2.34)	6.25^{cde} (±0.26)
GWH2D1	22.63	21.5	55.87	8.25^{cd} (±0.08)	18.17^{a} (±4.10)	1.91^{c} (±0.39)	31.43^{a} (±7.10)	10.48^{h} (±1.18)
GWH2D2	19.23	16.9	63.83	8.67^{d} (±0.05)	6.49^{bc} (±0.36)	0.71^{ab} (±0.02)	11.2^{bc} (±0.53)	7.05^{efg} (±0.78)
GWH2D3	20.60	15.7	63.7	8.71^{d} (±0.15)	4.82^{c} (±0.69)	0.48^{a} (±0.04)	8.33^{c} (±1.19)	6.29^{cde} (±1.23)
GWH3D1	18.60	19.2	62.2	8.31^{cd} (±0.14)	16.17^{a} (±1.12)	1.69^{c} (±0.08)	27.97^{a} (±1.94)	8.55^{fg} (±0.05)
GWH3D2	19.53	17.6	62.87	8.54^{d} (±0.14)	6.15^{bc} (±0.87)	0.68^{ab} (±0.02)	10.64^{bc} (±1.49)	7.05^{efg} (±1.05)
GWH3D3	20.23	15.6	64.10	8.61^{d} (±0.05)	4.19^{c} (±0.55)	0.499^{a} (±0.08)	7.24^{c} (±0.961)	6.09^{bcd} (±0.92)

(a, b, c, ...): for each property, means followed by the same letter are not significantly different according to the Student-Newman-Keuls test at $P < 0.05$. WW, wastewater; GW, groundwater, horizontal transect (H1: 0 cm/drip, H2: 50 cm/drip and H3: 150 cm/drip), vertical transect (D1: 0-20 cm, D2: 20-40 cm and D3: 40-60 cm). Each value is the mean of three replicates. Tot org. C, total organic carbon; N tot, total nitrogen; Org. M., organic manure; CEC, cation exchange capacity.

causes significant excess intestinal nematode infection in crop consumers and field workers, while irrigation with adequately treated wastewater does not (Al-Shammiri et al., 2003). And there is no actual health risk from using wastewater to irrigate crops (Takashi, 1994). WHO recommended that treated wastewater intended for crop irrigation should contain less than 1 viable intestinal nematode egg per liter and less than 10^3 fecal coliform bacteria (FCB) per 100 ml. In the effluent from preliminary treatment, the number of fecal coliforms, of fecal streptococci bacteria and of *E. coli* are respectively 1.6×10^5, 5.4×10^4 and 9.1×10^4 MPN/100 ml. The secondary treatment reduced concentrations of fecal coliform bacteria by about 1 log units and concentrations of *E. coli* and fecal *streptococci* bacteria by about 2 log units. In the pond, the average concentrations of fecal coliforms, streptococci bacteria and *E. coli* is respectively 1.1×10^3, 9.0×10^2 and 9.1×10^2 MPN/100 ml.

According to World Health Organization (1998), wastewater reclaimed by the SE_4 Wastewater Treatment Plant could be used for fruit tree irrigation. Irrigation should be stopped 2 weeks before harvest and no fruit should be picked off the ground.

Groundwater

The groundwater was, on average, alkaline with a basic pH value of 7.5 and had an electrical conductivity of 3.42 (mmhos/cm). It contained considerable amounts of nitrate (24 mg/l), ammonia (53 mg/l), phosphate (1.58 mg/l) and potassium (55.6 mg/l) which are considered essential nutrients for improving plant growth together with soil fertility and productivity levels. It presented a chemical and biochemical oxygen demand of 14.8 and 11.4 mg/l, respectively.

The concentrations of micronutrients and heavy metals in the groundwater were relatively low with 0.005 mg/l of Cd, 0.02 of Co, 0.005 of Cu, 0.019 of Mn, 0.05 of Fe, 0.036 of Ni, 0.039 of Pb, 0.028 of Zn and 0.03 of Cr. It is evident from the data that the heavy metal content was greater in the treated wastewater than groundwater. However, the level of all the heavy metals was under the Tunisian standards for wastewater reuse irrigation (N. A. W. M., 2001).

In the groundwater, the number of fecal coliform, fecal streptococci bacteria and of *E. coli* is respectively 4.3×10^2, 2.5×10^2 and 3.1×10^2 MPN/100 ml. The presence of enteric bacteria in the groundwater could be attributed to the anthropogenic influence.

On the whole, chemical proprieties of the water used in this study satisfy the standards for wastewater reuse irrigation of Tunisian (N. A. W. M., 2002). These kinds of waters could thus be considered of good quality.

Soil characteristics

On the whole, soil texture was sandy clayey for the GWP and only sandy for the WWP.

Impact of water quality on soil physicochemical properties

Soil pH

Soil pH ranged from 7.07 to 8.55 pH units for the field irrigated with treated wastewater for 10 years and from 8.25 to 8.77 pH units for the field irrigated with groundwater (Table 1). In the two studied fields, pH value exhibits the same pattern all over vertical and horizontal transects. Student-Newman-Keuls statistical test ($p \leq 0.05$) reveals a significant decrease in soil pH value due to the treated wastewater irrigation. This result agrees with those reported by Mohamed and Mazahreh (2003) who found a decrease in the pH value as a result of the wastewater irrigation. This fact is due to the oxidation of organic compounds and nitrification of ammonium. Tarchouna et al. (2010) found that soil pH increased as the result of successive several years of wastewater irrigation and they attributed this pH increase to the chemistry and high content of alkaline cations such as Na, Ca and Mg in the wastewater used for a long period of irrigation. Bicarbonate and carbonate ions combined with calcium or magnesium will precipitate as calcium carbonate ($CaCO_3$) or magnesium carbonate ($MgCO_3$). This will cause an alkalizing effect and will increase slightly the pH level. Therefore when a water analysis indicates high pH level, it may be a sign of a high content of carbonate and bicarbonate ions.

Soil organic matter (SOM) and cation exchange capacity (CEC)

SOM contents as affected by the type of water used for irrigation along the studied transects are shown in Table 2.

In the WWP, SOM values along the studied transect varied from 1.73 to 2.98% for the superficial first soil layer (0 to 20 cm), from 0.47 to 0.97% for the second soil layer (20 to 40 cm) and from 0.45 to 0.52% for the third soil layer (40 to 60 cm). The highest values were recorded at the beginning point of the studied transect (0 cm/dripper). These results show the positive effect of treated wastewater content on soil organic matter content (Rusan et al., 2007). On the whole, SOM was lower in the treated wastewater irrigated soil as compared to the groundwater irrigated one. Such a diminution, despite the organic matter supplied by the TWW, has been observed elsewhere (Gloaguen et al., 2007) and it is likely related to an intensification of microbial activity due to labile C and N supplied by the TWW (Tarchouna et al., 2010). In this case, this result may be due to soil texture that was sandy clayey for the GWP and only sandy for the WWP.

Along the studied transects, SOC and soil total nitrogen (STN) exhibited the same variations as compared to soil organic matter values.

Weak but significant variations in CEC values were observed between the two studied sites. This result was true for the horizontal and for the vertical transects (Table 2). In the WWP, a good correlation was found ($R^2 = 0.99$) for the first (0-20 cm) soil layer between the CEC values and the soil horizons.

Impact of water quality on soil microbial properties

Soil microbial abundance

The microbial density in the different soils of the studied site was evaluated by counting the bacteria and fungi on culture media (Table 3). The average numbers of bacteria in the WWP ranged from 29.72 (± 0.57) x 10^4 to 31.35 (± 2.64) x 10^5 CFU g^{-1} soil and from 3.53 (± 0.30) x 10^4 to 9.16 (± 0.25) x 10^4 in the GWP (Table 3). Similar significant differences were recorded for fungi, both between the different soils and between the studied transects. Altogether, these data revealed that irrigation with wastewater induced a significant increase in soil microbial abundance. This growth of microorganisms might be explained by the ready source of easily degradable compounds in the oligotrotrophic soil environment brought about by wastewater irrigation (Ramirez-Fuentes et al., 2002).

Soil DNA content

Soil DNA content as affected by the type of the water used for irrigation along the studied transects is shown in Figures 2 and 3.

In the WWP, soil DNA content values along the studied transect varied from 2.03 to 3.77 $\mu g.g^{-1}$ dry weight for the first soil layer (0-20 cm), from 0.89 to 2.63 µg/g dry weight for the second soil layer (20-40 cm) and from 0.92 to 1.65 µg/g dry weight for the third soil layer (40-60 cm). The highest values were recorded at the beginning point of the studied transect (0 cm/dripper). The weak significance of the positive relationship ($r = 0.55$, $p = 0.05$) between the increase in C_{org} contents and soil DNA content could be partly explained by the sandy texture of the soils, that would promote greater and a more rapid organic matter mineralization. This fact would lead to a transitory increase in C with a significant resiliency due to the rapid decrease in the stock of fresh organic matter (Lejon et al., 2007).

In the GWP, soil DNA content values along the studied transect varied from 5.24 to 5.50 µg/g dry weight for the first (0-20 cm), from 2.68 to 4.28 µg/g dry weight for the second soil layer (20-40 cm) and from 0.90 to 1.90 µg/g dry weight for the third soil layer (40-60 cm). The highest values were recorded at the upper soil layer (0-20 cm). The observed stratification of soil DNA content with soil depth generally corresponded to the decrease in C_{org} and N_{org} contents, as classically observed by Lejon et al. (2005) and Lejon et al. (2007).

Altogether, our results show that there is more DNA in soil irrigated with ground water as compared to soil irriga-

Table 3. Bacterial and fungal counts in soils from the studied sites.

Sample	Heterotrophic bacteria (cfu.g^{-1} soil)	Filamentous fungi (cfu.g^{-1} soil)
WWH1D1	31.35^{a} (± 2.64) x 10^{5}	3.42^{a} (± 0.43) x 10^{5}
WWH1D2	91.18^{c} (± 2) x 10^{4}	9.44^{b} (± 0.11) x 10^{4}
WWH1D3	32.99^{e} (± 2.08) x 10^{4}	3.49^{c} (± 0.25) x 10^{4}
WWH2D1	29.45^{b} (± 1) x 10^{5}	9.08^{b} (± 0.35) x 10^{4}
WWH2D2	65.69^{d} (± 4) x 10^{4}	7.98^{b} (± 0.51) x 10^{4}
WWH2D3	29.72^{e} (± 0.57) x 10^{4}	3.07^{cd} (± 0.05) x 10^{4}
WWH3D1	87.4^{c} (± 4) x 10^{4}	8.04^{b} (± 0.30) x 10^{4}
WWH3D2	57.23^{d} (± 1) x 10^{4}	7.82^{b} (± 0.61) x 10^{4}
WWH3D3	92.77^{f} (± 3.05) x 10^{4}	3.00^{cd} (± 0.11) x 10^{4}
GWH1D1	9.16^{f} (± 0.25) x 10^{4}	8.96^{de} (± 0.30) x 10^{3}
GWH1D2	6.13^{f} (± 0.30) x 10^{4}	6.52^{de} (± 0.20) x 10^{3}
GWH1D3	4.16^{f} (± 0.35) x 10^{4}	2.02^{e} (± 1.79) x 10^{3}
GWH2D1	8.40^{f} (± 0.5) x 10^{4}	8.49^{de} (± 0.15) x 10^{3}
GWH2D2	6.46^{f} (± 0.83) x 10^{4}	6.14^{de} (± 0.40) x 10^{3}
GWH2D3	3.96^{f} (± 0.20) x 10^{4}	1.96^{e} (± 1.73) x 10^{3}
GWH3D1	6.8^{f} (± 0.20) x 10^{4}	8.27^{de} (± 0.20) x 10^{3}
GWH3D2	4.53^{f} (± 1) x 10^{4}	5.82^{de} (± 0.30) x 10^{3}
GWH3D3	3.53^{f} (± 0.30) x 10^{4}	ND

(a, b, c, ...): For each count, means followed by the same letter are not significantly different according to the Student-Newman-Keuls test at P = 0.05. WW, wastewater; GW, groundwater; H1: 0 cm/drip, H2: 50 cm/drip and H3: 150 cm/drip, horizontal transect; D1: 0-20 cm, D2: 20-40 cm and D3: 40-60 cm; vertical transect. Each value is the mean of 3 replicates; ND = non-detectable.

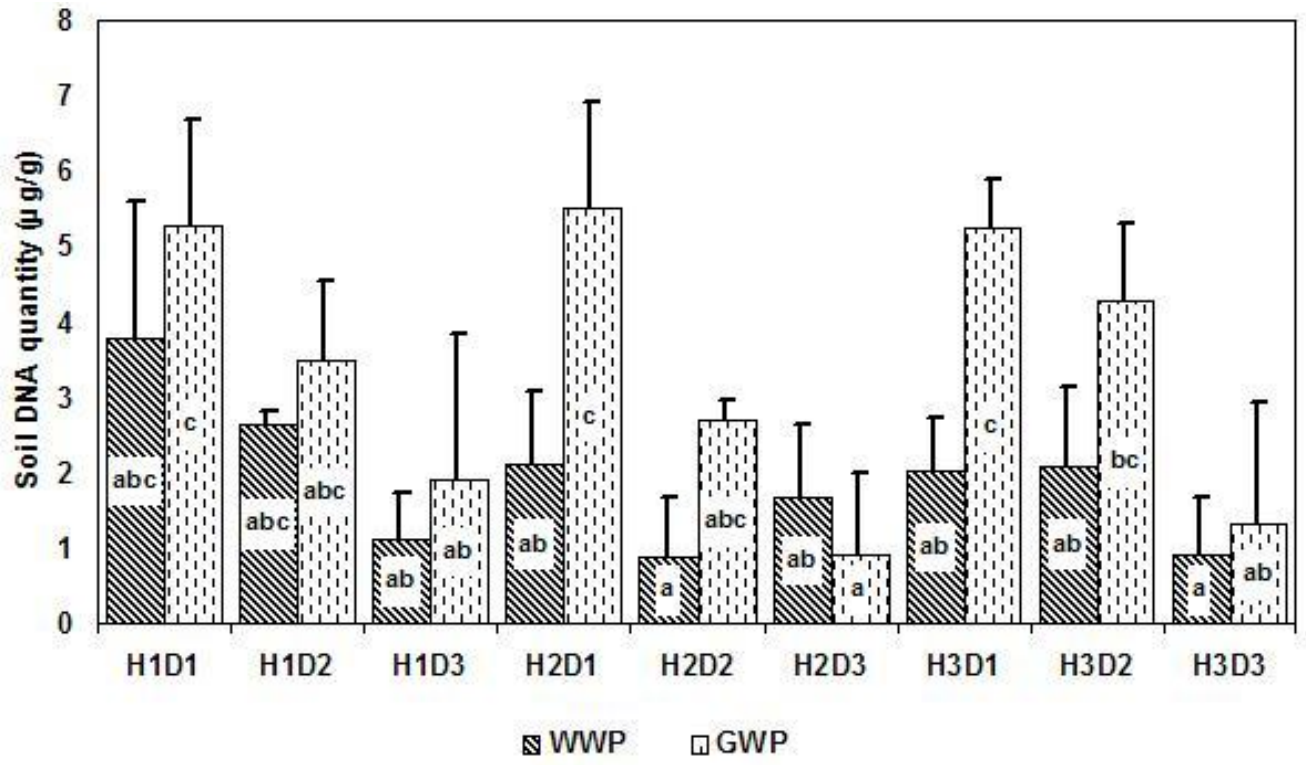

Figure 2. Soil DNA content in the wastewater plot as affected by the vertical (D1: 0-20 cm, D2: 20-40 cm and D3: 40-60 cm) and the horizontal (H1: 0 cm/drip, H2: 50 cm/drip and H3: 150 cm/drip) soils transects, WWP: wastewater plot, GWP: groundwater plot. Vertical bars represent standard deviation (*n* = 3 replicates for soil sample), (a, b, c ...): For each count, means followed by the same letter are not significantly different according to the Student-Newman-Keuls test at *P < 0.05*, WW: wastewater.

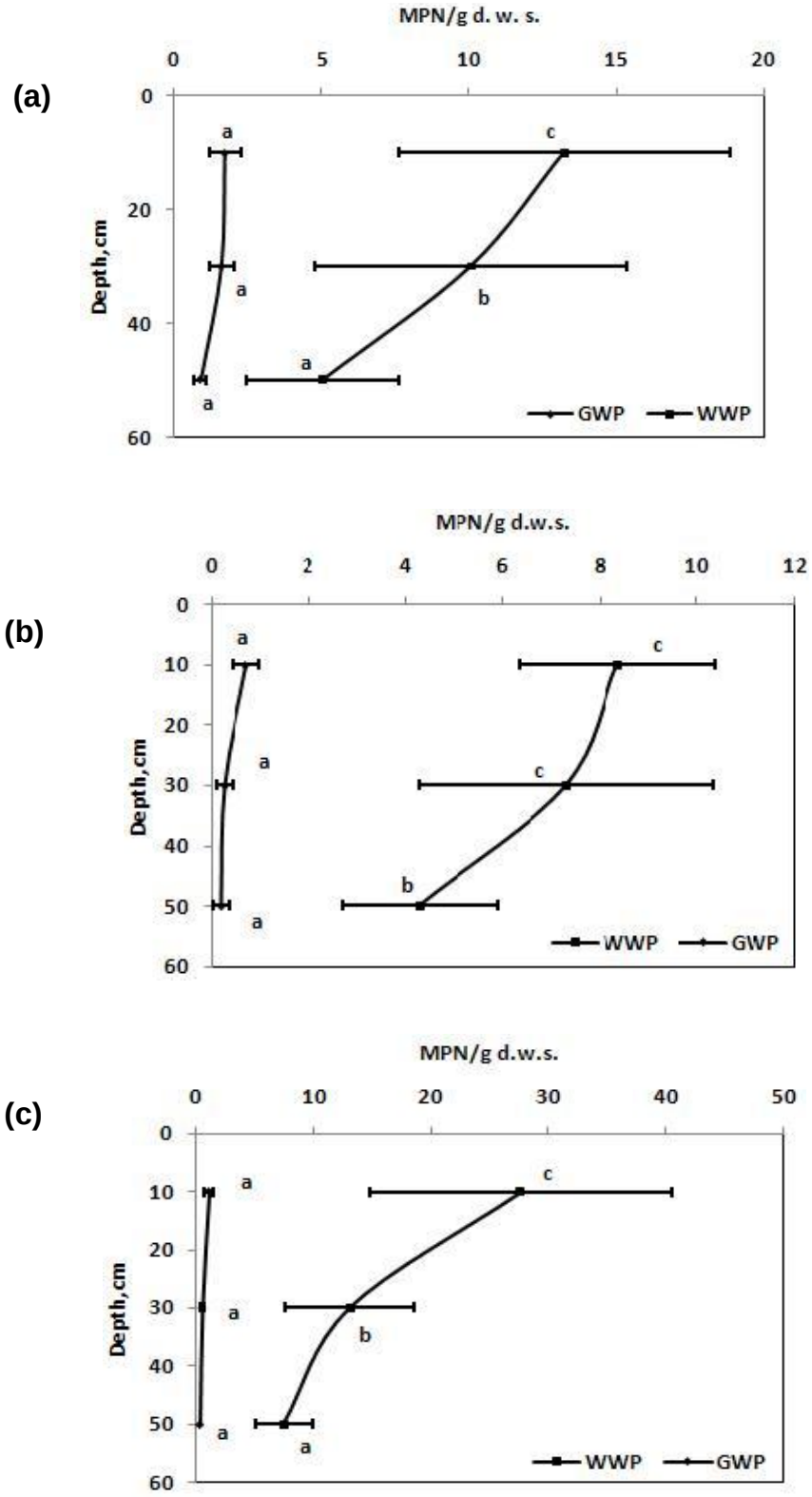

Figure 3. Fecal coliforms (a), *Escherichia coli* (b) and fecal Streptococci (c) concentrations as affected by the vertical (D1: 0-20 cm, D2: 20-40 cm and D3: 40-60 cm) and the horizontal (H1: 0 cm/drip, H2: 50 cm/drip and H3: 150 cm/drip) soils transects. Horizontal bars represent the standard deviation (n = 3 replicates for soil sample), (a, b, c ...): For each count, means followed by the same letter are not significantly different according to the Student-Newman-Keuls test at $P < 0.05$, WWP: wastewater pilot, GWP: groundwater plot.

ted with wastewater for the same period. This unexpected result could be partly explained, in the WWP, by the soil's sandy texture, which would lead to weak accumulation of organic matter in the topsoil due to the significance of mineralization process. Results published by Hidri et al. (2010) show that long-term (26 years) irrigation with wastewater significantly stimulated microbial growth by providing a nutrient source.

Soil microbiological examination for fecal coliforms, Escherichia coli and fecal streptococci

Soil microbial contamination was assessed by measuring the number of fecal coliforms, of *E. coli*, and of fecal *Streptococci* on samples taken at depths of 0-20, 20-40 and 40-60 cm, after seasonal irrigation. Results show that WWP soil was significantly ($p \leq 0.05$) more microbial polluted than GWP soil (Table 4).

A slight increase in the concentration of fecal coliforms bacteria along the studied transect (150 cm) was recorded in the WWP and reaching a maximum value in the first (0-20 cm) soil layer (from 3.78 to 22.8 MPN/g dry weight (Figure 3). Taking into account the quality of the treated wastewater (fecal coliform bacteria concentration was equal to 1.1 10^3 MPN/100 ml) and that water was distributed to the orange grove daily during the irrigation period (an average of 2.46 mm/day), the measured contamination should be considered as very slight. As reported by Campos et al. (2000) 1 day after irrigation, fecal coliforms contamination in the soil will be considerably reduced (an abatement up to four logarithmic units) depending on the quality of the wastewater and the type of irrigation system. In addition, the soil seems to be able to reduce human bacteria contamination and their associated health risk following wastewater irrigation. From this point of view, land application could be considered as an efficient-means of wastewater disposal (Campos et al., 2000). On the other hand in GWP, soil fecal coliforms concentration was negligible (ranging from 1.3 to < 1 MPN/g dry weight). This low contamination could be ascribed to grazing, common in the experimental site, and considered as a non-point (diffuse) source of contamination together with roaming wild animals and birds and runoff from agriculture areas (Palese et al., 2009)

Fecal coliforms prevalence was detected especially in the upper soil layer (0-20 cm) particularly at the beginning point of the studied transect (0 cm/ dripper) (Figure 3a). Decreasing concentrations were observed according to soil depth and values measured at the deepest levels were negligible (ranging from 5 to < 1 MPN/g dry weight that is equivalent to no contamination). We infer this result in considering that soil matrix act as a filter, and so reducing the bacterial concentration in the deeper soil layers. These findings are in agreement with the results of Palese et al. (2009) and Oran et al. (2001). In particular, Oran et al. (2001) observed a gradual reduction of fecal coliform concentration through the soil profile, a silty clay type, and a complete disappearance of contamination beyond the limit of 25 cm, when the raw wastewater bearing 1000 CFU of coliforms per 100 ml was used for irrigation.

E. coli contamination followed the same trend in both wastewater and ground water used for irrigation soils even if in the former they showed higher contents (Figure 3b). Such enrichment was clearly due to the distribution of wastewater which, during the experimental period, equal to 9.1 10^2 MPN/100 ml. A very slight *E. coli* contamination (ranging from 1.3 to < 1 MPN/g dry weight soil) was recorded in soils sampled from the ground water plot (GWP).

As reported for fecal coliforms, *E. coli* was present particularly in the upper soil layers (0-20 cm) peaking at the beginning point of the studied transect (0 cm/dripper); in the other layers (20-40 and 40-60 cm) *E. coli* concentration tended to decrease with depth close to negligible values (always < 1 MPN/g dry weight in the deepest levels). Straining, depending on the soil pores and bacterial size, and adsorption onto soil particles are the most important factors influencing bacteria transport through the soil (Campos et al., 2000; Oran et al., 2001). Furthermore, the presence of channels due to plant root systems and earthworm burrows can strongly influence vertical migration of pathogens through the soil profile (Joergensen et al., 1998). On the other hand, the correct irrigation management (low water volumes distributed daily by a drip irrigation system according to soil hydrological and physical parameters and climatic pattern) and the intense water absorption by roots of both trees and cover crops, active in the wetted soil volume, excluded water logging by runoff and percolation to deeper soil layers avoiding aquifer pollution by fecal bacteria (Palese et al., 2009).

A significant increase ($p = 0.01$) in the concentration of fecal *Streptococci* was recorded in the WWP reaching a maximum at the beginning point of the studied transect (Table 4). This result probably reflects the high numbers of fecal bacteria present in the wastewater used for irrigation. It is believed that the use of less contaminant irrigation methods or better quality effluents might further reduce the risk of transmission of fecal bacteria pathogens (Al-Lahham et al., 2003).

Fecal *Streptococci* prevalence was detected especially in the upper soil layer (0-20 cm). Decreasing concentrations were observed according to soil depth and fecal *Streptococci* values measured at the deepest levels which is negligible (Figure 3c). The observed stratification of microbial biomass according to soil depth corres-ponded and correlated generally to the decrease in C and N organic contents, as classically observed Lejon et al. (2007).

Conclusion

After more than 10 years of treated wastewater drip irrigation, and compared to soils irrigated with increase

Table 4. Fecal coliforms, *Escherichia coli* and fecal streptococci counts in soils from the studied sites.

Sample	Fecal coliforms (MPN .g $^{-1}$ dry weight soil)	*Escherichia coli* (MPN .g $^{-1}$ dry weight soil)	Fecal *Streptococci* (MPN .g $^{-1}$ dry weight soil)
WWH1P1	19±4.35^{a}	9.69±1.48^{a}	36.73±12.06^{a}
WWH1P2	15.52±4.45^{b}	8.73±2.52^{a}	30.39±12.32^{a}
WWH1P3	7.58±1.43^{cd}	6.63±0.62^{ab}	15.68±4.49^{bc}
WWH2P1	13.3±1.65^{b}	9.5±4.11^{a}	19±4.35^{b}
WWH2P2	9.89±1.51^{c}	7.50±1.42^{ab}	12.61±1.68^{bcd}
WWH2P3	3.00±1.10^{e}	4.9±1.19^{bc}	7.58±1.43^{cd}
WWH3P1	7.35±1.39^{cd}	5.42±1.15^{bc}	8.26±0.99^{cd}
WWH3P2	4.72±1.30^{de}	4.85±1.18^{bc}	9.31±2.20^{cd}
WWH3P3	4.48±2.61^{de}	2.61±1.05^{cd}	4.77±1.32^{d}
GWH1P1	2.26±0.34^{e}	0.88±0.38^{d}	1.54±0.30^{d}
GWH1P2	1.79±0.23^{e}	0.74±0.11^{d}	0.93±0.11^{d}
GWH1P3	0.95±0.23^{e}	0.49±0.17^{d}	0.75±0.11^{d}
GWH2P1	1.75±0.30^{e}	0.31±0.05^{d}	0.72±0.11^{d}
GWH2P2	1.89±0.40^{e}	0.13±0.22^{d}	0.48±0.17^{d}
GWH2P3	1.01±0.11^{e}	0.33±0.06^{d}	0.33±0.06^{d}
GWH3P1	1.19±0.30^{e}	0.22±0.19^{d}	0.47±0.16^{d}
GWH3P2	1.15±0.17^{e}	0.10±0.17^{d}	0.32±0.06^{d}
GWH3P3	0.69±0^{e}	0.23±0.20^{d}	0.29±0^{d}

(a, b, c, ...): For each count, means followed by the same letter are not significantly different according to the Student-Newman-Keuls test at P = 0.05. WW, wastewater; GW, groundwater; H1: 0 cm/drip, H2: 50 cm/drip and H3: 150 cm/drip, horizontal transect; D1: 0-20 cm, D2: 20-40 cm and D3: 40-60 cm; vertical transect. Each value is the mean of 3 replicates; ND = non-detectable.

groundwater, no negative changes have been observed in the evaluated soil properties with the exception of an in soil microbial biomass (heterotrophic bacteria and filamentous fungi) and in soil fecal indicator bacteria (coliforms, *Escherichia coli* and *Streptococci*) essentially around the emitter. This growth of microorganisms might be explained by the ready source of easily degradable compounds in the oligotrophic soil environment brought almost certainly by wastewater irrigation. Indeed, microorganisms are mainly heterotrophic and carbon-limited in soil and the observed differences could be due to a higher availability and quality of the carbon source supplied by wastewater irrigation. This would lead to a transitory increase in soil microorganisms with a significant resiliency due to the rapid decrease in the stock of fresh organic matter (Lejon et al., 2007).

Thus, treated wastewater use in irrigation could have positive effects, not only in aspects of soil quality (organic content), but also in social terms, as it allows the maintenance of irrigated agriculture in areas where groundwater has been polluted by seawater intrusion. In these sites, treated municipal wastewater seems to be an alternative water resource for citrus tree irrigation with a correct salts management. However, studies of different types of wastewater and soils are needed before these results can be generalized, because changes in microbial community are also considerably influenced by soil type and certain agricultural practices.

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Full Length Research Paper

Fermentation process for alcoholic beverage production from mahua (*Madhuca indica* J. F. Mel.) flowers

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Mahua flowers are rich in sugar (68-72%), in addition to a number of minerals and one of the most important raw materials for alcohol fermentation. The present investigation was for the development of a non-distilled alcoholic beverage from Mahua flowers. Eighteen (18) treatment combinations consisting of two temperatures (25 and 30°C), three pH (4.0, 4.5 and 5.0) and three period of fermentation (7, 14 and 21 days) were used in the fermentation conditions. The maximum yield of ethanol (9.51 %) occurred at 25°C with pH 4.5 after 14 days of fermentation of Mahua flower juice. The fermented non-distilled alcoholic beverage contained total sugar (8.83 mg/ml), reducing sugar (0.82 mg/ml), total soluble solids (6.37°Brix) titrable acidity (0.65 %), and volatile acidity (0.086%). Methanol was not detected at any stage of fermentation. The developed fermented alcoholic beverage had characteristic flavor and aroma of Mahua flowers with about 7 to 9% alcohol.

Key words: *Madhuca indica*, ethanol, reducing sugar, fermentation.

INTRODUCTION

Mahua (*Madhuca indica* J. F. Mel syn *Madhuca latifolia* Macb.) is a common tree in deciduous forests of India, quite prominent in states of Andhra Pardesh, Bihar, Gujarat, Karnataka, Madhya Pradesh., Orissa, Rajasthan, Uttar Pradesh and West Bengal. Mahua flowers are in dense fascicles near end of the branches having 1.5 cm long fleshy cream coloured corolla tube and are scented. Flowering period of Mahua is from the month of March to May. Flower induction starts from the top portion to lower branches and also from more illuminated part to shaded part of the tree. Flowers mature in about 32-35 days. One to two good flowering is expected every three years, that is, it has an alternate bearing habit. Mahua flowers are rich in total sugars out of which maximum proportion is of reducing sugars. Sugars identified are sucrose, maltose, glucose, fructose, arabinose and rhamnose. When flowers are mature and ready to fall, there is maximum total sugar content in the flowers. Fructose is present in a greater proportion than glucose and in the ripe stage the quantities are almost equal. Sucrose increases in amount up to shedding of corollas and is latter converted into invert sugars. Mahua flowers are rich in total sugars (68-72%), out of which maximum proportion is of reducing sugars. Sugars

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identified are sucrose, maltose, glucose, fructose, arabinose and rhamnose. When flowers are mature and ready to fall, there is maximum total sugar content in the flowers (Sikarwar, 2002). However, in spite of being a rich source of nutrition, a major portion of dried Mahua flowers are being used in the preparation of country liquor (Patel and Naik, 2006). Wine is an alcoholic beverage typically made of fermented fruit juice and any fruit with good proportion of sugar may be used in producing wine and the resultant wine is normally named after the fruit. The type of wine to be produced dictates the fruit and strain of yeast to be involved (Idise, 2012). Therefore, the present investigation was carried out to study fermentation conditions for production of non-distilled alcoholic beverage that is wine from mahua flower juice extract.

MATERIALS AND METHODS

Mahua juice

The mahua flowers obtained were cleaned and dried in hot air oven at 60°C temperature for 5 h. 1 kg of mahua flowers was soaked in 1 L distilled water containing 1.5 g potassium meta bisulphite (KMS) for 12 h to prevent the growth of other contaminants. For preparation of juice from the flowers, additional water was added to facilitated the easy grinding. The mixture was ground with the help of electric mixture grinder and filtered through muslin cloth, stored in screw capped bottles and kept in a refrigerator at 4°C. The obtained juice had total soluble solids (TSS) 20°Brix, pH- 5.3 and volume about 3.5 L from 1 kg of dried mahua flowers.

Inoculum of yeast culture

The yeast strain obtained from the Department of Dairy and Food Microbiology, College of Dairy and Food Science Technology, Udaipur (*Saccharomyces cerevisiae*) was cultured on MGYP broth (Himedia, India) at 25°C for 48 h in a biological oxygen demand (BOD) incubator. The MGYP broth contained malt extract (0.75 g), peptone (1.25 g), yeast extract (0.75 g) and dextrose (5 g) in 250 ml distilled water. The yeast inoculum was had 4.8×10^4 cfu/ml.

Fermentation conditions

For optimization of fermentation conditions, the following treatment combinations were used: Temperature of 25 and 30°C; pH of 4.0, 4.5 and 5.0; period of fermentation of 7, 14 and 21 days. The pH of juice extract was adjusted at pH 4.0, 4.5 and 5.0 with help of citric acid. The yeast extract was added as a nitrogenous source at the rate of 0.1% (w/v) in the mahua juice. Each Erlenmeyer flask containing 100 ml of juice was inoculated with 1 ml (1% v/v) of 48 h old yeast culture under aseptic conditions. Then transferred in a BOD incubator for fermentation and maintained at 25 or 30°C for different periods of incubation. The samples were analysed after 7, 14 and 21 days of alcoholic fermentation. All experiments were conducted in triplicates and a control (un-inoculated) was also taken for each treatment.

Biochemical analysis of fermented samples

The total sugar was estimated by anthrone reagent method as described by Hedge and Hofreiter (1962). Reducing sugars in samples were estimated by using dinitrosalicylic acid (DNS) reagent method as described by Miller (1972). The total soluble solids in the samples were determined with the help of hand refractometer (Erma) and expressed in terms of ^{o}Brix and pH of the extract was measured by using hand held pH meter (Eutech). Titrable acidity of the samples was determined by titration with 0.1 N sodium hydroxide and was expressed in terms of anhydrous citric acid (Ranganna, 1986).Volatile acidity of the fermented samples was determined after distillation. The distillate was titrated with 0.01 N sodium hydroxide and was expressed in terms of acetic acid (Ranganna, 1986). The ethanol in the fermented sample was determined by using the specific gravity method (AOAC, 2000). The methanol in the fermented sample was determined with the help of quantitative colorimetric micro determination method (Boos et al., 1948).

RESULTS AND DISCUSSION

The optimization study was done by using different combination of temperature, pH and duration of fermentation. The alcoholic beverage obtained after fermentation was analysed for total sugar, reducing sugar, total soluble solids, titrable acidity, volatile acidity and ethanol. In the present investigation, the total sugar were found maximum (96.47 mg/ml) at 25°C with pH 5.0 after seven days of fermentation whereas, minimum total sugar (0.45 mg/ml) were found at 30°C with pH 4.5, after 21 days of fermentation (Table 1). The total sugar content decrease with the increasing period of fermentation in all the treatments irrespective of pH and temperature. Yeast cells require carbon and nitrogen sources for their growth and development. During fermentation, sugar is utilized for energy production and ethanol is produced as a by-product of the fermentation process.

With increase in fermentation period, there was decrease in total sugar content because sugar was being utilized for ethanol production. At higher temperature the metabolic reaction of yeast cells increase which resulted into faster decrease in the total sugar content of the mahua flower juice extract during the fermentation studies. Rivera-Espinoza et al. (2005) have reported that the fermen-tation of cane sugar juice at 30°C temperature is faster than that at 25 and 28°C temperature. Similar observa-tions were reported by Singh and Kaur (2009) in case of litchi wine production where total sugar decreased from 85.25 to 3.5% (w/v) after five days fermentation. Yan et al. (2012) observed that many factors can affect the ethanol content and volatile acid content of blueberry wine and suggested that the maximum ethanol content and minimum volatile acid production of blueberry wine fermentation with *Saccharomyces cerevisiae* AS2.316 commercial wine yeast could reach 7.63% and 0.34 g l^{-1} under the optimal condition of temperature, 22.65°C; pH, 3.53; inoculums size, 7.37%.

Total reducing sugar content of fermented alcoholic beverage decreased during fermentation period. The reducing sugar were found maximum (57.68 mg/ml) at

Table 1. Total sugars (mg/ml) during fermentation of Mahua flowers extract.

Fermentation medium	pH of fermentation medium	25°C			30°C			Mean
		Period of incubation (days)						
		7	14	21	7	14	21	
Sample	4.0	92.62	8.77	1.10	79.17	12.71	0.46	32.47
Control	4.0	219.31	218.35	217.63	218.94	219.31	218.15	218.61
Simple	4.5	88.17	8.83	0.74	65.75	11.53	0.45	29.25
Control	4.5	218.15	217.53	217.33	216.84	216.44	216.25	217.09
Sample	5.0	96.47	9.60	0.60	79.97	10.95	0.49	33.01
Control	5.0	217.13	216.54	215.54	218.22	217.92	217.03	217.07
Mean		155.31	113.27	108.82	146.48	114.81	108.80	124.58
Interaction			SEM±			CD (0.0 5)		
Temperature			0.49			1.39		
Days			0.60			1.70		
pH			0.60			1.70		
Temp.× Days× pH			1.48			NS*		

*NS stands for non-significant.

Table 2. Reducing sugars (mg/ml) during fermentation of Mahua flowers extract.

Fermentation medium	pH of fermentation medium	25°C			30°C			Mean
		Period of incubation (days)						
		7	14	21	7	14	21	
Sample	4.0	57.68	0.90	0.48	53.60	0.86	0.46	18.99
Control	4.0	187.56	186.53	185.66	185.37	185.51	184.47	185.85
Simple	4.5	43.96	0.82	0.46	52.05	0.75	0.43	16.41
Control	4.5	184.49	184.05	183.18	184.91	183.03	182.30	183.66
Sample	5.0	49.80	0.84	0.46	52.32	0.71	0.44	17.43
Control	5.0	184.64	184.06	184.20	183.91	182.87	181.68	183.56
Mean		118.02	92.87	92.41	118.69	92.29	91.63	100.98
Interaction			SEM±			CD (0.0 5)		
Temperature			0.44			1.26		
Days			0.55			1.55		
pH			0.55			1.55		
Temperature× Days × pH			1.34			NS*		

*NS stands for non-signifcant.

25°C with pH 5.0 after seven days of fermentation. The reducing sugar content decreased gradually with the increasing period of fermentation. After 21 days of fermentation, the lowest reducing sugar (0.43 mg/ml) was recorded at 30°C with pH 4.5 (Table 2). Reducing sugar is a fermentable sugar and decreases with increasing period of fermentation. Reducing sugar is most important sugar for fermentation as it is easy to metabolize by yeast. Similarly, Reddy and Reddy (2009) reported that reducing sugar was decreased (from 185 to 2.0 g/litre) after alcoholic fermentation of mango juice. Yadav et al. (2009) observed reducing sugar decrease up to 0.08% in mahua wine at 30°C temperature after 15 days of fermentation. Chowdhury and Roy (2007) reported that the reducing sugar content decrease from an initial value of 6.48 ± 0.06 g/100 ml in jamun must to 0.49 ± 0.04 g/100 ml in wine prepared from jamun.

The total soluble solids content of fermented alcoholic beverage decreased with increase of fermentation period up to the end of experimentation. The highest TSS content (7.87^{o}Brix) was observed at 25°C with pH 5.0 after seven days of fermentation and lowest TSS content (5.50^{o}Brix) were found at 30°C with pH 5.0 after 21 days of fermentation (Table 3). The decrease in TSS content of mahua flower alcoholic beverage indicates the utilisation of sugar present in the mahua flower juice

Table 3. Total soluble solids (^{o}Brix) during fermentation of Mahua flowers extract.

Fermentation medium	pH of fermentation medium	25°C			30°C			Mean
		Period of incubation (days)						
		7	14	21	7	14	21	
Sample	4.0	7.27	6.37	6.07	6.97	6.23	5.80	6.45
Control	4.0	19.87	19.73	19.67	19.90	19.73	19.60	19.75
Simple	4.5	7.60	6.37	6.00	7.07	6.23	5.67	6.49
Control	4.5	19.80	19.77	19.67	19.93	19.87	19.73	19.79
Sample	5.0	7.87	6.63	6.00	7.20	6.27	5.50	6.58
Control	5.0	19.80	19.77	19.63	19.87	19.83	19.60	19.75
Mean		13.70	13.11	12.84	13.49	13.03	12.65	13.14
Interaction			SEM±			CD (0.0 5)		
Temperature			0.01			0.04		
Days			0.02			0.06		
pH			0.01			0.06		
Temperature× Days × pH			0.05			NS*		

*NS stands for non-signifcant.

extract during the fermentation process. It is obvious from the data that, with the increased temperature, the rate of fermentation also increased which in turn decreased the TSS of mahua flower extract during the fermentation. Similarly, Ukwuru and Awah (2013) observed that the purified yeasts from palm wine showed highly viable cells and good metabolic activity during grape must fermentation. Grape must fermentation resulted in increase in temperature (28 to 32°C) and reduction in pH (4.3 to 3.1) and total solid concentration in the wines decreased consistently during fermentation (21 to 5%). Titratable acidity increased during fermentation from 0.44 to 0.82%. Akubor et al. (2003) observed the decrease in TSS of banana juice from 18 to 4.8 ^{o}Brix at the end of 14 days fermentation at 30 ± 2°C temperature. The trend of TSS decrease during various conditions of mahua extract fermentation is similar to those reported by Chowdhury and Ray (2007) in jamun wine fermentation process. Similarly, Ezeronye (2004) have reported the reduction of TSS in cashew apple juice from 24 to 6.0 ^{o}Brix after 14 days fermentation at 20°C during cashew apple wine fermentation.

The titrable acidity of fermented alcoholic beverage exhibited increasing trends till the end of experimentation in all the treatments. There was positive effect of fermentation and treatment combinations on titrable acidity during entire fermentation period as shown in Table 4. After fermentation, maximum acidity (1.11%) was observed at 30°C with pH 5.0 after 21 days of fermentation, whereas, minimum titrable acidity (0.55%) was found at 25°C with pH 4.5 after seven days of fermentation. Similar observations were made by Chowdhery and Roy (2007) when they reported an increase in titrable acidity (from 0.51 to 3.30%) during the alcoholic fermentation. However, Vaidya et al. (2009) reported decrease in titrable acidity (from 1.07 to 0.52%) after fermentation of kiwi fruit juice into wine at 22 ± 1°C. Titrable acidity is an important characteristic of wines and it depends on the biochemical composition of fruit juice used in the alcoholic fermentation and process parameters of fermentation. The titrable acidity of fruit wines vary between 0.5-1.0% ((Joshi, 1998).

The change in volatile acidity of fermented alcoholic beverage during fermentation under different treatment combinations was assayed for quality of alcoholic beverage. The volatile acidity of fermented alcoholic beverage exhibited increasing trends till the end of experimentation in all the condition of fermentation. The maximum volatile acidity (0.134%) was observed at 30°C with pH 5.0 after 21 days of fermentation, whereas, minimum volatile acidity (0.070%) was found at 25°C with pH 4.5 after seven days of fermentation (Table 5). The concentration of total volatile compounds increased during fermentation. Volatile acidity may result from the coupled oxidation of wine phenolics to yield peroxide which in turn oxidized ethanol to acetaldehyde and subsequently to acetic acid (Zoecklein et al., 1995). Volatile acidity of strawberry fruit wine may vary from 0.027-0.030% acetic acid equivalent (Joshi et al., 2005; Joshi et al., 2006).

The ethanol content of fermented alcoholic beverage was estimated at various intervals of fermentation under different treatment combinations. The ethanol content of fermented alcoholic beverage increased with advancement of fermentation period in all the treatments. The treatments had significant effect on ethanol content of fermented alcoholic beverage during entire fermentation periods (Table 4). The maximum ethanol (9.51%) content was observed at 25°C with pH 4.5 after 14 days of fermentation and minimum ethanol (6.70%) was found

Table 4. Titrable acidity (%) during fermentation of mahua flowers extract.

Fermentation medium	pH of fermentation medium	25°C			30°C			Mean
		Period of incubation (days)						
		7	14	21	7	14	21	
Sample	4.0	0.58	0.69	0.80	0.68	0.75	0.94	0.74
Control	4.0	0.39	0.41	0.42	0.39	0.41	0.42	0.41
Sample	4.5	0.49	0.65	0.76	0.65	0.85	1.01	0.74
Control	4.5	0.35	0.42	0.46	0.38	0.41	0.45	0.41
Sample	5.0	0.55	0.78	1.00	0.65	0.89	1.11	0.83
Control	5.0	0.34	0.42	0.45	0.35	0.46	0.42	0.41
Mean		0.45	0.56	0.65	0.51	0.63	0.73	0.59
Interaction			SEM±			CD (0.0 5)		
Temperature			0.006			0.01		
Days			0.008			0.02		
pH			0.008			0.02		
Temperature× Days × pH			0.019			NS*		

*NS stands for non-signifcant.

Table 5. Volatile acidity (%) during fermentation of mahua flowers extract.

Fermentation medium	pH of fermentation medium	25°C			30°C			Mean
		Period of incubation (days)						
		7	14	21	7	14	21	
Sample	4.0	0.072	0.088	0.108	0.074	0.100	0.106	0.091
Sample	4.0	0.070	0.086	0.108	0.078	0.108	0.126	0.096
Sample	4.5	0.086	0.104	0.130	0.092	0.124	0.134	0.112
Mean		0.076	0.093	0.115	0.081	0.111	0.122	0.100
Interaction			SEM±			CD (0.0 5)		
Temperature			0.002			0.013		
Days			0.002			0.005		
pH			0.002			0.005		
Temperature × Days× pH			0.005			NS*		

*NS stands for non-signifcant.

at 25°C with pH 4.0 after seven days of fermentation. There was an increase in ethanol content up to 14 days of fermentation and it decreased in all the treatments combinations after 21 days of fermentation (Table 6). Thornton and Rodriguez (1996) reported that juices should be fermented at lower temperature (often 15°C) to retain their fruity character. Chowdhury and Ray (2007) made red wine from jamun (*Syzgium cumini* L.) fruit with low alcohol (6%) concentration after six days of fermentation at 32 ± 2°C. They reported that low alcohol content in jamun wine was probably due to low TSS (16.5 ^{o}Brix) in jamun must in comparison to grape must TSS (usually 22 to 24^{o}Brix) which yields wine with 8 to 10%t alcohol. The percent ethanol obtained in present investigation is in accordance with several workers who reported that wine made from fruit juice fermentation contains about 8 to 10 (v/v)% ethanol (Yadav et al., 2009). Similarly, Reddy and Reddy (2005) observed that pH 5.0 and temperature 30°C were optimum for highest ethanol production (7-8%) in case of mango wine. However, Yadav et al. (2009) observed that mahua wine fermented at 16°C had higher content of alcohol (9.9%) compared to that at 20 and 25°C after 15 days of fermentation. This difference in ethanol content may be attributed to nutritional composition of mahua flower and yeast strain used for fermentation. Soni et al. (2009) had reported highest alcohol content (10%) of amla (*Amblica officinalis*) wine at 25 and 30°C but fermentation efficiency decreased at higher than 30°C. Most of the yeast strains grow best at a temperature less than 35°C. Changes in membrane fluidity of the mesophilic yeast lead to a retarded or no growth at higher temperature

Table 6. Ethanol (%) during fermentation of mahua flowers extract.

Fermentation medium	pH of fermentation medium	25°C			30°C			Mean
		Period of incubation (days)						
		7	14	21	7	14	21	
Sample	4.0	6.70	9.40	7.20	7.25	8.44	6.94	7.65
Sample	4.0	7.51	9.51	7.53	8.09	9.29	7.46	8.23
Sample	4.5	6.91	9.16	6.96	7.64	9.18	6.85	7.78
Mean		7.04	9.36	7.23	7.66	8.97	7.08	7.89
Interaction			Sem±			CD (0.0 5)		
Temperature			0.21			0.61		
Days			0.08			0.20		
pH			0.08			0.20		
Temperature × Days× pH			0.21			NS*		

*NS stands for non-signifcant.

(Banat et al., 1998). Methanol is a harmful content of alcoholic beverage. It is always hazardous for human health. In our study, the methanol content was not detected at any stage of fermentation process. Similarly, Rivera-Espinoza et al. (2005) reported that methanol was not detected in the course of alcoholic fermentation from sugarcane juice. Okunowo and Osuntoki (2007) reported that fermentation of the orange juice by *S. cerevisiae* from yam and *S. cerevisiae* from sugarcane molasses resulted in products with different concentrations of alcohol types despite the fact that the fermenting organisms are of the same species. This indicates that the source of the yeast may influence the alcohol profile of the wine produced. It is therefore concluded that the source of the yeast is thus an important factor in the determination of the quality of the wine.

Though the level of ethanol production was higher after 14 days of fermentation at both temperature, seven days of incubation at 25°C was selected as optimized condition for fermentation to retain the characteristics aroma of mahua flowers because at 30°C there was increase in total titrable acidity.

It may be concluded that elaboration of alcoholic beverage with acceptable characteristics of wine that may be produced by using mahua flower juice extract as a substrate, is technically feasible and a good alternative use for raw material. It is our hope that these optimized conditions can be an incentive for an eventual commercial production of non-distilled alcoholic beverage from mahua flowers in an economical way.

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Full Length Research Paper

Decolourization of remazol brilliant blue R by enzymatic extract and submerged cultures of a newly isolated *Pleurotus ostreatus* MR3

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A local white-rot fungus basidiomycete *Pleurotus ostreatus* MR3 was isolated from MacRitchie Reservoir Park, Singapore. Among all the ligninolytic activities, laccase was the only enzyme detected in the supernatant when the fungus was grown in liquid culture. This newly isolated white rot fungus was able to completely decolourise remazol brilliant blue R (RBBR) *in-vivo* on agar plates within five days and in the liquid culture (in the presence of inducers) within three days. The addition of inducers was able to enhance laccase production and therefore enhanced *in-vivo* RBBR decolourisation. Veratryl alcohol was shown to be the best inducer for laccase production with the maximum laccase activity reaching about 5.99 U/mL. Cu^{2+} also had a positive effect on laccase production, the laccase activity being enhanced to 5.24 U/mL. *In-vitro* RBBR decolourisation using the laccase from *P. ostreatus* MR3 was much comparable to that using the commercial laccase from *Trameters versicolor*.

Key words: Dyes, remazol brilliant blue R, *Pleurotus ostreatus* MR3, decolourisation, inducers, laccase activity.

INTRODUCTION

Among industrial wastewaters, the treatment of dye wastewater from textile and dyestuff industries is one of the most challenging. Over 10,000 dyes with an annual production in excess of 7×10^5 metric tonnes worldwide are commercially available, and typically 5 to 10% of this amount is discharged in industrial effluents (Fu and Viraraghavan, 2001). As a wide range of structurally diverse dyes may be used within a short duration in a typical textile manufacturing process, the effluent can be highly variable in composition. Moreover, the discharge of those colored wastewaters into rivers leads to a reduction of sunlight penetration in natural water bodies, which in turn decreases both photosynthetic activity and dissolved oxygen concentration. Several of these dyes are very stable to light, temperature, and microbial attack, making them recalcitrant compounds (Rodriguez et al., 1999). This highlights the need for a non-specific dye treatment process for textile dye effluent.

The dye used in the biodegradation studies reported in the present study is one of the most importantly industrial dyes, Remazol brilliant blue R (RBBR), which is frequently used as a starting material in the production of polymeric dyes. It is an anthracene derivative and represents an important class of often toxic and recalcitrant organo-pollutants. The various treatment technologies currently available for dye removal primarily depend on physical and/or chemical principles. Most of these technologies suffer several shortcomings, including high

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Abbreviations: RBBR, Remazol brilliant blue R; **WRF,** white rot fungi; **PCR,** polymerase chain reaction; **SmF,** submerged fermentation; **ABTS,** 2,2′-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid]; **LiP,** lignin peroxidase; **MnP,** manganese peroxidase.

amounts of chemical usage and/or sludge generation, costly infrastructure requirements and/or high operating expenses (Nigam et al., 2000). Therefore, new methods must be developed, in particular, by using biotechnological approaches for the degradation of the recalcitrant compounds. Specific microorganisms were proven to be potentially effective in treatment of this pollution source in an eco-efficient manner (Willmott et al., 1998; McMullan et al., 2001; Zissi and Lyberatos, 2001). The white rot fungi (WRF) are, so far, the most efficient microorganisms in degrading synthetic dyes, with basidiomycetous fungi that are able to depolymerize and mineralize lignin. This WRF's property is due to the production of extracellular lignin-modifying enzymes. Due to their low substrate specificity, lignin-modifying enzymes are also able to degrade a wide range of xenobiotic compounds (Barr and Aust, 1994; Pointing, 2001) including dyes (Glenn and Gold, 1983; Spadaro et al., 1992). Most of the previous studies reported, focused on the lignin-degrading enzymes of *Phanerochaete chrysosporium* and *Trametes versicolor*. Lately, there has been a growing interest in studying the lignin-modifying enzymes of a wide array of WRF searching for better lignin-degrading systems (Munari et al., 2007).

Pleurotus ostreatus is the third most important cultivated mushroom for food purposes and it is also a well studied WRF. The genus *Pleurotus* is often associated with the bioconversion of agricultural wastes into valuable food products through the use of their ligninolytic enzymes for biodegradation of organo-pollutants, xenobiotics and industrial contaminants (Cohen et al., 2002). The ligninolytic system of *Pleurotus* sp. has been extensively studied, and it appears to be an effective alternative for the bioremediation of resistant pollutants (Barr and Aust, 1994; Cohen et al., 2002). Submerged cultures of *Pleurotus sajorcaju* has been shown to bring about effective degradation of total polyphenols from paper mill effluents (Munari et al., 2007). Laccases were identified as the main agents of wastewater decolourization by *P. ostreatus* (Faraco et al., 2009). Laccases are multicopper enzymes, which catalyse the oxidation of phenolic compounds including a range of dyes with concomitant reduction of oxygen (Eggert et al., 1996; Chivukula and Renganathan 1995; Muñoz et al., 1997). The findings that the substrate range of laccase can be expanded to non-phenolic dyes, even in the presence of suitable mediators (Bourbonnais and Paice, 1990), boosted recent interest in laccase. *P. ostreatus* laccases have been extensively studied; one of these, POXC, is the most abundantly produced under all the growth conditions examined (Giardina et al., 1996). Moreover, three other laccase isoenzymes secreted by the mycelium have been purified and characterised (POXA1w, POXA1b and POXA3) (Palmieri et al., 1997; Giardina et al., 1999; Palmieri et al., 2003).

In this study, *P. ostreatus* MR 3 was primarily screened for RBBR decolorization in agar dye plates. The fungus was also cultivated in media formulated with inducers to increase laccase production. The capability of the extracellular enzyme in decolorization of RBBR solution was assessed. Moreover, decolourization of industrial dyes was performed in liquid cultures of *P. ostreatus* MR3.

MATERIALS AND METHODS

Isolation of microorganism and its maintenance

Wild strains *P. ostreatus* MR3 and *Lentinula edodes* MR6 were isolated from MacRitchie Reservoir Park, Singapore using potato agar agar plates containing 200 mg L^{-1} RBBR. Identification of the isolated fungi was carried out by 18S rDNA sequencing using a polymerase chain reaction (PCR) primers of NS1 (5′-GTAGTCATATGCTTGTCTC-3′) and NS-4 (5′-CTTCCGTCAATTCCTTTAAG-3′). The PCR fragments were sequenced and the results were compared with the database retrieved from GenBank. It was identified that the isolated microbes were closely related to *P. ostreatus* (99%) and *L. edodes* (98%), so they were designated the name of *P. ostreatus* MR3 and *L. edodes* MR6. *T. versicolor* ATCC 20869 was obtained from the American Type Culture Collection (ATCC). The above fungal strains were maintained in potato dextrose agar (Merck, Germany) plates at room temperature for 10 days before inoculation.

Medium and cultural conditions for submerged fermentation (SmF)

The basal medium used for laccase production had the following composition (g/L): 2 g of glucose, 2 g of ammonium tartrate, 2 g of malt extract, 0.26 g of KH_2PO_4, 0.26 g of Na_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CuSO_4 \cdot 5H_2O$, 0.0066 mg of $CaCl_2 \cdot 2H_2O$, 0.005 mg of $FeSO_4$, 0.5 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of Na_2MoO_4, 0.09 mg of $MnCl_2 \cdot 4H_2O$, 0.07 mg of H_3BO_3. The pH was adjusted to 5.5 before autoclaving at 121°C for 20 min. Adequate aeration was provided by agitation at 150 rev/min at 30°C for four days. The culture grown under the same conditions for 48 h was used as the inoculum (10%) for enzyme production.

Determination of enzyme activities

Enzyme activities were determined through colorimetric assays at 30°C in a total volume of 1 mL. Absorbance changes were measured through a spectrophotometer (UV-1601 OC, Shimadzu, Japan). Laccase activity was determined according to Xin and Geng (2010) using 2,2′-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS; Boehringer) as the substrate with some modifications. The reaction mixture contained 100 µL enzyme extract and 900 µL ABTS solution (1 mM) in sodium acetate buffer pH 5.0 (50 mM) for 10 min. Oxidation was followed via the increase in absorbance at 420 nm ($\varepsilon=3.6\times10^4$ $cm^{-1}M^{-1}$). Manganese peroxidase was determined using phenol red as a substrate. Concentration of the reaction product was measured at 610 nm ($\varepsilon=2.2\times10^4$ $cm^{-1}M^{-1}$) (Glenn and Gold, 1985). The reaction mixture contained 50 mM succinate buffer, pH 4.5, 0.01% phenol red and the appropriate volume of enzyme solution. The addition of H_2O_2 (0.1 mM final concentration) initiated the reaction. Lignin peroxidase (LiP) activity was determined by oxidation of veratryl alcohol to veratryl aldehyde in 0.1 sodium tartrate buffer, pH 3.0, 2 mM veratryl alcohol, 0.4 mM H_2O_2 Oxidation reaction was followed measuring optical absorbance at 310 nm ($\varepsilon=9.3\times10^6$ $cm^{-1}M^{-1}$). One enzyme unit (U) was defined as 1.0 µmol of product formed per

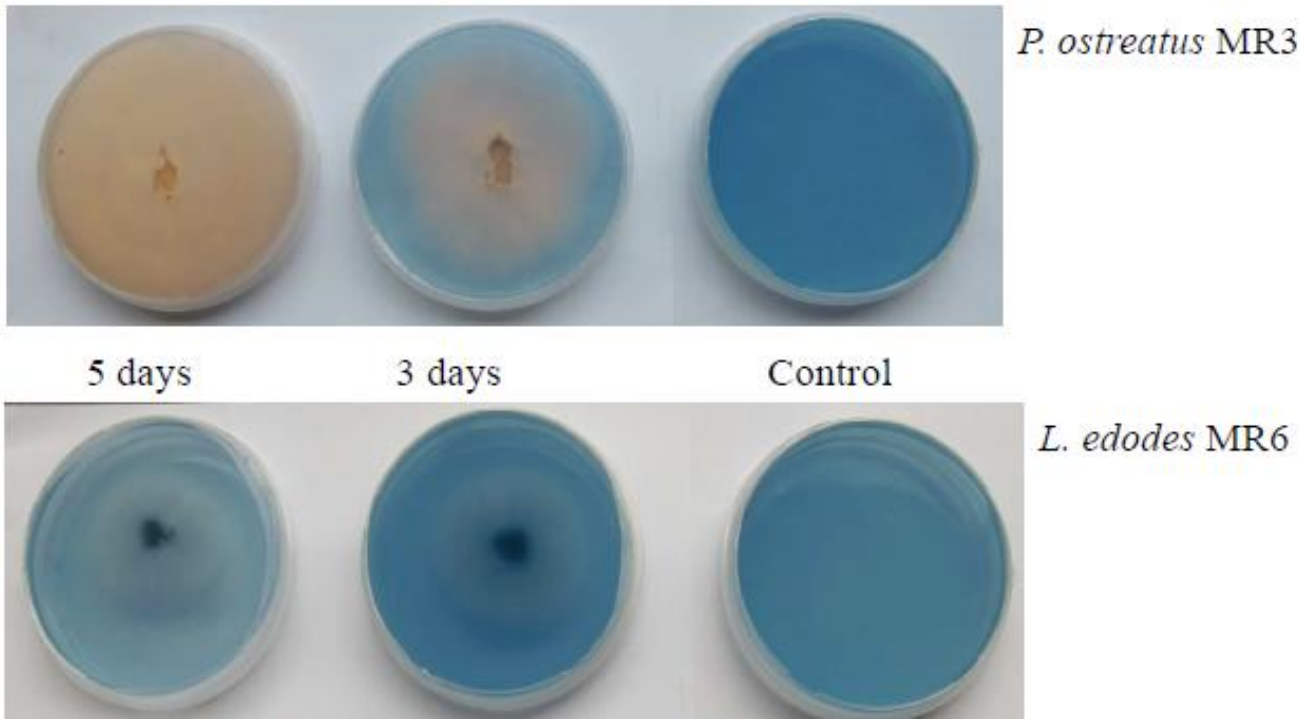

Figure 1. Decolourization of media containing 200 mg/L RBBR by local isolates *P. ostreatus* MR3 and *L.edodes* MR6. Controls without inoculums.

minute under the assay conditions. All activity assays were carried out in duplicates.

In vitro decolorization of RBBR by the enzyme extract from *P. ostreatus* MR3

Unless otherwise indicated, all the experiments were performed using 3 mL-disposable cuvettes with 2 mL final reaction volume. The reaction mixture was composed of 100 mM acetate buffer pH 5.0, 200 mg L^{-1} RBBR and 0.2 U mL^{-1} laccase. The reaction was initiated with addition of laccase and further incubated in the dark at 30°C. The decolourization of the RBBR was followed by recording the spectra of the reaction mixture every 15 min with OD at 595 nm. All experiments were performed in duplicate and controls were performed by using heat inactivated enzymes after incubation at 100°C for 10 min. Decolourization of RBBR by the enzyme broth was also investigated using the same enzyme dosage. Dyes contents were monitored at 595 nm, which is the maximum visible absorbance for RBBR. The assays were done in duplicates. The decolourization degree was calculated according to Xin and Geng (2010) by means of the formula:

$$D = 100(A_{ini} - A_{obs}) / A_{ini}$$

Where, D is the decolourization (in %), A_{ini}, the initial absorbance, and A_{obs}, the observed absorbance.

RESULTS AND DISCUSSION

Laccases were firstly described in 1883 from the Japanese lacquer tree *Rhus vernicifera* (Fu and Viraraghavan, 2001). Since then, several laccases have been studied with respect to their biological function, substrate specificity, copper binding structure, and Industrial applications (Barr and Aust, 1994; Bourbonnais and Paice, 1990; Giardina et al., 1999). *Pleurotus* sp. is a wood rotting basidiomycetes and laccase is the dominant ligninolytic enzyme synthesized by this species. The newly isolated *P. ostreatus* MR3 could completely decolorize RBBR at day 5 on potatoes dextrose agar (PDA) agar plates containing RBBR (Figure 1). When grown in liquid culture, only laccase activity was detectable in the supernatant. The other two ligninolytic activities (manganese peroxidase (MnP) and lignin peroxidase (LiP) could not be detected.

Data reported in Figure 2A and B show that decolorizing ability of RBBR was strongly correlated to laccase production. *P. ostreatus* MR3 produced laccase activity of 1.56 IU/mL on day 3, while the maximum acivity of 2.82 IU/mL was obtained on day 5. Pattern of enzymatic activity production in control cultures (without any added dye) is similar to that monitored in the presence of RBBR (data not shown). Dye decolourization at 200 mg/L RBBR concentration (Figure 2B) occurred at a rapid rate and only three days are needed to obtain the complete transformation of the dye to colourless compounds (95%). Moreover, the blue colour disappearance was not due to any protonation/deprotonation of the dye, since the pH of cultures was not altered during fungal growth, remaining constant at about 5.0. Absorbance spectra of samples withdrawn from fungal culture at different growth times

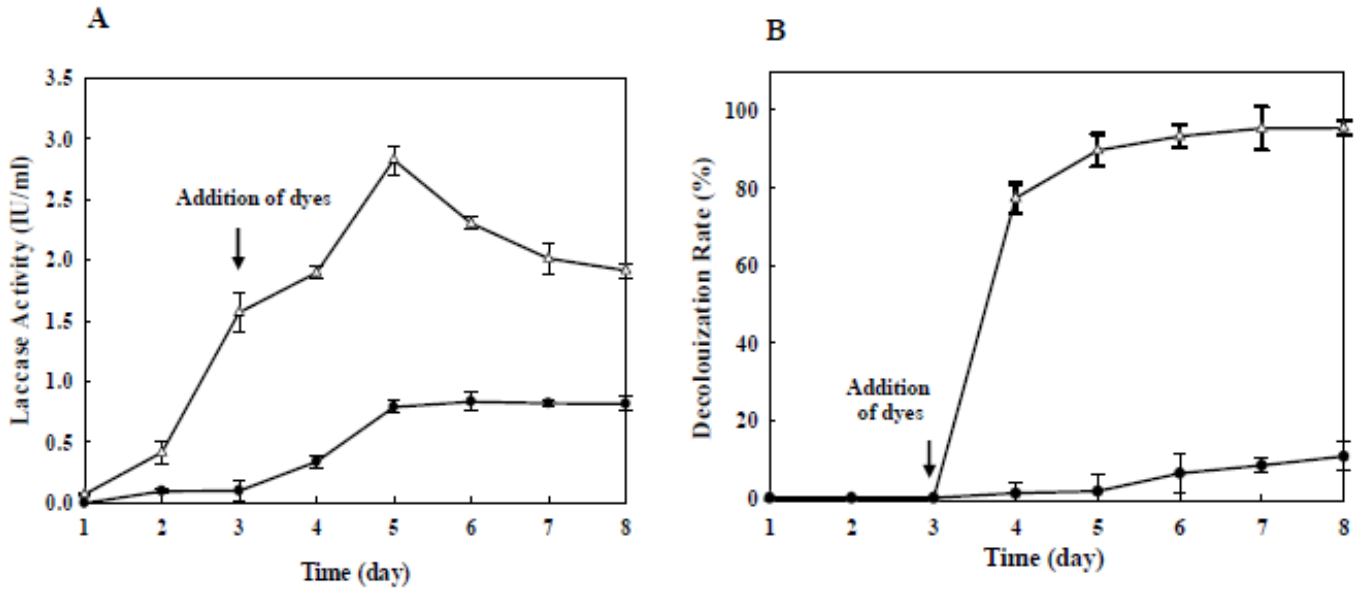

Figure 2. Time course of laccase production (A) and RBBR decolourization percentage (B). ∆, *P. ostreatus* MR3; ●, *L.edodes* MR6.

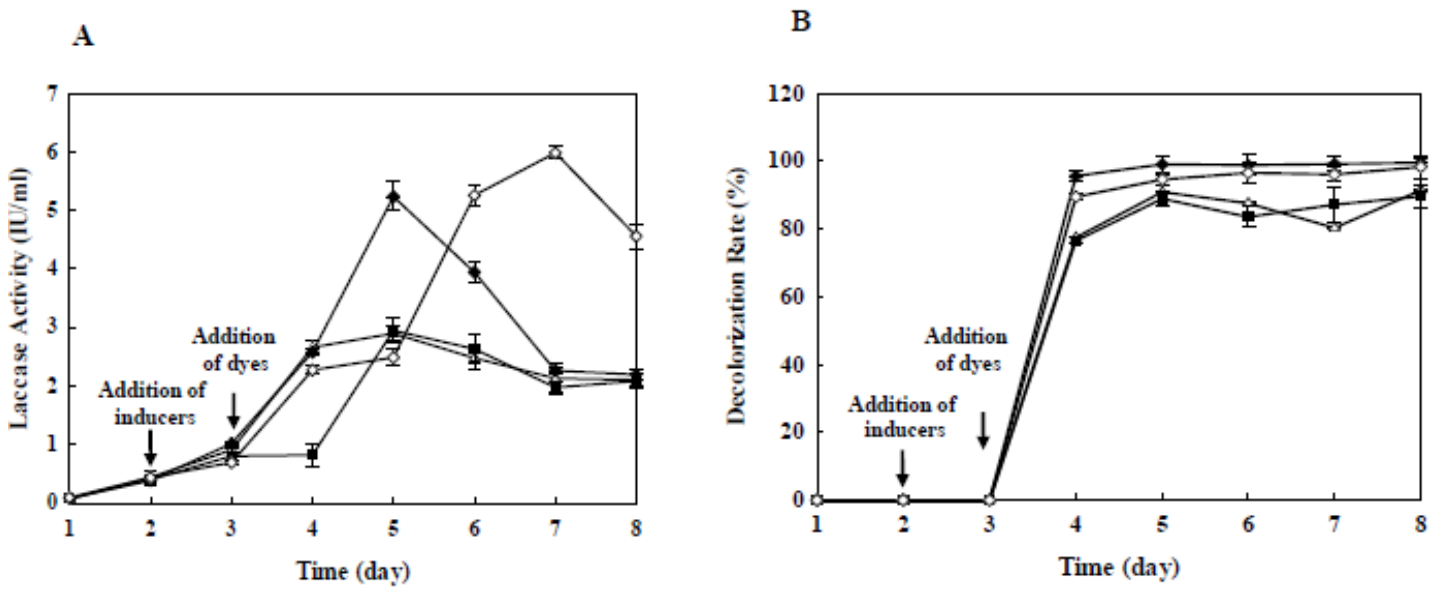

Figure 3. Time course of laccase production by *P. ostreatus* MR3 with different inducers (A) and decolouirization rate (B). ∆, Control; ♦, copper sulphate; ■, ABTS; ◊, veratryl alcohol.

showed a disappearance of the 592 nm absorbance peak concomitantly with changes in absorption in the 350 to 400 nm regions.

One of the most effective approaches to increase the yield of ligninolytic enzymes is the supplementation of the nutrient medium with an appropriate inducer. Aromatic and phenolic compounds have been widely used to elicit enhanced laccase production by different organisms (Chivukula and Renganathan, 1995) and the nature of the compound that induces laccase activity differs greatly with the species. Among the various laccase inducers reported in the literature, copper sulfate, veratryl alcohol, and ABTS were selected in this study and a control culture with no inducer addition was also performed (Chivukula and Renganathan, 1995; Xin and Geng, 2010; Giardina et al., 1996). The final inducer concentration in the basal medium was kept at 1 mM and added to the medium at day 2 (Figure 3). The results of our studies using different laccase inducers indicate that all the inducers included in the study were capable to enhance laccase production by *P. ostreatus* MR3. It is notable that the highest laccase activity obtained in the presence of

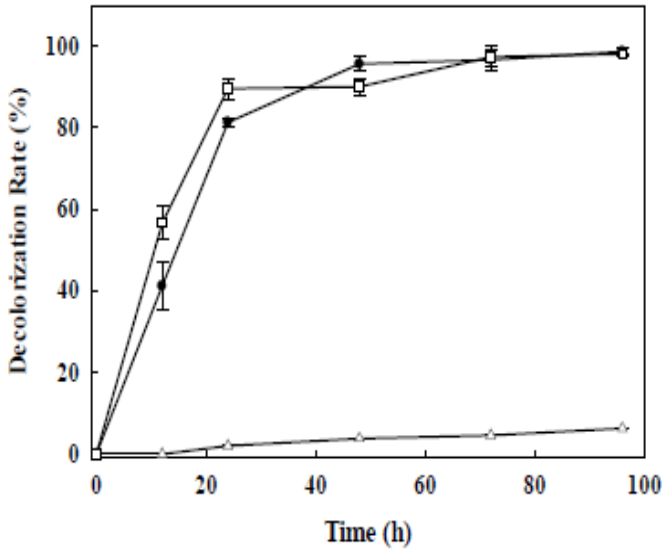

Figure 4. Decolorization potentials of the prepared laccase samples and the commercial laccase. ∆, Control; ●, samples; □, commercial laccase.

veratryl alcohol was 2.07-fold higher than that of the control followed by copper sulfate (1.81-fold) and ABTS (1.02-fold). Copper sulfate and veratryl alcohol have been reported to be strong laccase inducers for many fungal species (Giardina et al., 1996). According to Giardina et al. (1999), the presence of inducers, their chemical nature, the amount added, and the time of their addition influenced laccase production by many fungal species (Myrna et al., 2012).

Some fungal laccases as well as laccase mediator systems are efficient for dye decolorization. Different dyes are decolorized by different laccases at different rates. The decolorization rate depends on the structure and the redox potential of the enzyme as well as the structure of the dye. Some studies showed that the laccase alone could decolorize RBBR (Wong and Yu, 1999; Schliephake and Lonergan, 1996). However, Soares et al. (2001) found that the laccase alone did not decolorize RBBR and a small molecular weight redox mediator was necessary for decolorization to occur. The difference in fungal species from which the laccase was obtained might be a reason for this discrepancy. The redox potential of the laccase varied depending on the source of the laccase. In order to test the decolourization potentials of the laccase produced by *P. ostreatus* MR3, crude enzyme mixture was prepared. The fungus was grown in cultures which were supplemented with or without Cu^{2+} as laccase inducer. After 12 days of incubation, the cultures were filtered through Whatman filter paper, RBBR was injected from a stock solution into the culture filtrates (final concentration 100 mg L^{-1}) and then the OD was measured at 431 nm. The decolourization results are displayed in Figure 4. No decolourization was observed in the control, indicating that the decolourization was biological and it was caused by laccase in the samples since no activities of other ligninolytic enzymes such as LiP and MnP were detected in the enzyme extract. *T. versicolor* is one of the best studied white rot fungi. The most active of the enzymes from *T. versicolor* is laccase. Laccase from *T. versicolor* can directly decolorize RBBR and the decolourization rate decreased with the increase of dye concentration (Guo et al., 2008). For both the prepared laccase sample and the laccase from *T. versicolor*, the decolourization degree increased with the increase of the prepared laccase samples and the com-mercial laccase. The initial decolourization rate for com-mercial laccase was higher, indicating that commercial laccase has better decolourization performance than our own enzyme samples. However, such difference became insignificant when the decolorization time reached 48 h. The highest decolorization degree was reached at 72 h for both enzyme samples, being 97.41% for the commercial laccase and 96.67% for our sample. This result suggests that laccase produced by *P. ostreatus* MR3 with the commercial laccase, having a high capability in decolourization.

Conclusion

The indigenous strain of *P. ostreatus* MR3 isolated in Singapore was found to be effective in *in-vivo* treatment of RBBR. A significant increase in the *in-vivo* decolourization rate by *P. ostreatus* MR3 was observed on day 5, corresponding well to the high laccase production at that point of time. *In-vitro* dye decolourization by *P. ostreatus* MR3 compared favorably with the extensively studied laccase from *T. versicolor* strain, ATCC 20869. Veratryl alcohol was effective in inducing laccase production by *P. ostreatus* MR3 and therefore enhancing RBBR *in-vivo* decolourisation. Strain *P. ostreatus* MR3 is therefore a potential white rot fungus that can be used for laccase production and dye decolourization.

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Full Length Research Paper

Response of haco-cockerels fed graded levels of toasted bambara nut offal and supplementary enzyme

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Eighty 4 weeks old spotted cockerels (Harco strain) were used to determine the effect of toasted bambara nut offal (TBNO) and supplementary enzyme on growth performance, haematology and organ weights of birds. The birds were randomly divided into 8 groups of 10 birds each. The groups were randomly assigned to 8 energetic (11.30 MJ/kg Me) and nitrogenous (20.02 to 20.06% crude protein) diets in a 4 × 2 factorial arrangement involving four levels (0, 10, 15 and 20%) of TBNO and 2 enzyme levels (0 and 0.02%). Each treatment was replicated 2 times with 5 birds per replicate. Average final body weight (AFBW), average daily weight gain (ADWG), feed conversion ratio (FCR) and feed cost per kg weight gain were similar, while average daily feed intake (ADFI) was different (P<0.05) among treatments. Inclusion TBNO and supplementary enzyme in the diets of cockerels had significant (P < 0.05) effect on the apparent retentions of dry matter (DM), nitrogen, crude fibre (CF), ether extract (EE) and nitrogen-free extract (NFE). While there were significant interactions (P< 0.05) between TBNO and enzyme levels on DM, nitrogen, CF, EE and NFE retentions. Enzyme supplementation significantly (P < 0.05) increased DM, nitrogen, EE and NFE retentions at all the TBNO inclusion levels. Enzyme supplementation also increased (P < 0.05) CF retention at the 10, 15 and 20% TBNO inclusion levels. It is concluded that 20% TBNO can be included in the diet of growing cockerels without any deleterious effect on their growth performance.

Key words: Toasted bambara nut waste, enzyme, effect, cockerels, growth performance.

INTRODUCTION

The challenges of acute protein shortage in the diets of most Nigerians occasioned by high cost of poultry meat due to high cost of production inputs are indisputable (Babatunde and Hamza, 2005). The astronomical cost of cereal grains like maize and wheat and sources of protein ingredients like soya bean meal, groundnut meal, etc vis-à-vis their acute shortage and competitiveness between man and monogastric animals especially poultry over them calls for a paradigm shift from competitive, costly conventional feed ingredients (foodstuff) to alternative, cheap, non-conventional ingredients with less competetion. The use waste /by- products and residues as replacement for stable energy and protein feedstuff without deleterious effect on animals stand as a major breakthrough in solving the problems of competition of humans with animals over staples such as grains and the high cost of feed inputs which limits production. The by-product being considered in the present study is toasted

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Abbreviations: TBNO, Toasted bambara nut offal; **AFBW,** average final body weight; **ADWG,** average daily weight gain; **ADFI,** average daily feed intake; **DM,** dry matter; **CF,** crude fibre; **EE,** ether extract; **NFE,** nitrogen free extract.

bambara offal [*Vigina subterranean (L) verdc*] while the animal species under focus is the cockerel. Cockerels are good sources of protein meat in Nigeria and command high demand because they possess special flavour and toughness. However, the major problems with cockerels are their slow rate of growth, high feed intake, poor feed conversion efficiency and consequently high cost of production (Ojewola and Ozuo, 2006). The use of local by -products like Bambara nut offal can help to overcome high cost of production. Interestingly, Bambara nut is widely cultivated in both northern and southern Nigeria with a very high yield under good management. The seeds are processed into flour and consumed as moi moi (Enwere, 1998).

The young fresh seeds may be boiled and eaten as a snack in a manner similar to boiled peanuts. Bambara offal is the ultimate discard from the milling of Bambara nut. It has no direct feeding value for humans and in most places it is dumped indiscriminately thus constituting environmental problems/hazards. Bambara nut waste contains up to 16.40% crude protein (Okeke, 2000). It has been used in the feeding of poultry and rabbits (Ani and Okafor, 2004; Ani, 2006). The antinutritional factors in the raw beans such as protease inhibitors, haemaglutinin, tannins, cyanogenic glycosides and flatulence factors limit their nutrient potentials as feed ingredients (Doku and Karikari, 1971; Ensminger et al., 1996; Enwere, 1998). However, the toasting of the Bambara nut offal and the supplementation with Enzyme-Roxazyme G are known to counter the effects of the anti-nutritional factors. This study was therefore conducted to determine the effect of graded levels of toasted Bambara nut offal and supplementary enzyme (Roxazyme) on the growth performance of cockerel chicks.

MATERIALS AND METHODS

The study was carried out at the Poultry Unit of the Department of Animal Science Teaching and Research Farm, University of Nigeria, Nsuskka. Toasted Bambara waste and other feed ingredients used in the study were purchased at Nsukka and Enugu, in Enugu State, Nigeria.

Animal and experimental diets

The experiment was carried out in accordance with the provisions of the Ethical Committee on the use of animals and humans for biomedical research of the University of Nigeria, Nsukka (2006). Eighty 4- week old spotted cockerel chicks (Harco strain) weighing 252.50 to 276.50 g on the average were randomly divided into 8 groups of 10 birds each. The groups were randomly assigned to 8 energetic (11.30 MJ/kg Me) and nitrogenous (20.02 to 20.06% crude protein) diets in a 4 × 2 factorial arrangement involving four level (0, 10, 15 and 20%) of TBNO and 2 enzyme levels (0 and 0.02%). The percentage composition of diets is shown in Table 1. Each treatment was replicated 2 times with 5 birds per replicate placed in 2.6 × 3 m deep litter pens of fresh wood shavings. Feed and water were supplied *ad libitum* to the birds. The birds were properly vaccinated against Newcastle disease and gumboro disease. Prophylactic treatment with oxytetracyline based drugs was administered against bacterial infection and sulfaquinoxaline drugs against coccidioses. The experiment lasted for a period of 5 weeks during which feed intake, weight gain, feed conversion ratio and protein efficiency ratio were monitored. Feed intake was determined daily by the weigh back technique.

Live weights were recorded weekly for each bird. Feed conversion ratio was then calculated from these data as quantity (gram) feed consumed per unit (grams) weight gained over the same period.

Apparent nutrient retention by cockerel chicks

During the 5th week of experimental period, a seven-day excreta collection was made from one bird per replicate to determine the apparent retention of proximate components. Within that period, birds were housed individually in metabolism cages and weighed quantity of feed (90%) of the daily feed intake) was offered to birds daily. The birds were allowed two days to adjust to the cage environment before droppings were collected. Daily feed consumption was recorded as the difference between the quantity offered and the quantity left after 24 h. Faecal droppings were collected from separate cages in detachable trays placed beneath the wire mesh floor of the cages. Collected faecal samples were oven-dried and weighed over the seven days period. At the end of the collection period all faecal samples from each bird were bulked and preserved for analysis.

Proximate and statistical analyses

Feed and excreta samples were assayed for proximate composition by the method of AOAC (1990). Data collected were subjected to analysis of variance (ANOVA) in a completely randomized design (CRD) as described by Steel and Torrie (1980). Significantly, different means were separated using Duncan's New Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Growth performance of cockerels

Table 2 shows the proximate composition of the experimental diets while data on growth performance of cockerels fed diets containing graded levels of toasted Bambara nut offal (TBNO) and supplementary enzyme are presented in Table 3. There were no significant ($P < 0.05$) differences among treatments in average final body weight (AFBW), average daily weight gain (ADWG), feed conversion ratio (FCR) and feed cost per kg weight gain. However, there were significant differences ($P < 0.05$) among treatments in average daily feed intake (ADFI). Birds on treatments 1 and 7 had comparable ($P > 0.05$) average daily feed intake values with those on treatments 2, 5, 6 and 8 and these were significantly ($P < 0.05$) higher than the ADFI values of birds on treatments 3 and 4. Birds on treatment 8 had comparable ($P > 0.05$) average daily feed intake values with those on treatments 2, 4, 5 and 6. Similarly, birds on treatment 3 had comparable ($P > 0.05$) average daily feed intake values with those on

Table 1. Percentage composition of experiment diets.

Ingredient	Diet*							
	1	2	3	4	5	6	7	8
Maize	47.50	47.50	44.00	44.00	41.20	41.20	39.00	39.00
Wheat offal	5.60	5.60	4.60	4.60	3.00	3.00	2.00	2.00
Soya bean meal	25.40	25.40	25.00	25.00	24.70	24.70	24.4	24.4
Bambara nut offal	0.00	0.00	10.00	10.00	15.00	15.00	20.00	20.00
Palm kernel cake	12.00	12.00	6.40	6.40	5.60	5.60	3.60	3.60
Fish meal	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Palm oil	0.00	0.00	0.50	0.50	1.00	1.00	1.50	1.50
Bone meal	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Oyster shell	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Salt	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Methionine	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Lysine	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Vit/min premix**	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Enzyme	0.20	0.00	0.20	0.00	0.20	0.00	0.20	0.00
Total	100	100	100	100	100	100	100	100
Calculated composition								
Energy (Mcal/KgME)	2.70	2.70	2.70	2.70	2.70	2.70	2.70	2.70
Crude protein (%)	21.13	21.02	20.32	20.45	19.96	19.89	20.00	20.72
Crude fibre(%)	4.33	4.33	5.48	5.48	6.55	6.55	6.77	6.77

Diets*1(0% TBNO and 0% Enzyme); 2(0% TBNO and 0.02% Enzyme); 3(10% TBNO and 0% Enzyme); 4(10% TBNO and 0.02% Enzyme); 5(15% TBNO and 0% Enzyme); 6(15% TBNO and 0.02% Enzyme); 7(20% TBNO and 0% Enzyme); 8(20% TBNO and 0.02% Enzyme); ******Viamin Premix (2.5 kg/1000 kg): vitamin A (15,000 U.I); vitamin D_3(3,000.000 I.U) and vitamin E (30,000 I.U) vitamin k (2,500 I.U), Thiamin B, (2,000 mg), Riboflavin B_2 (6,000 mg). Pyridoxine B_0 (4000 mg), Niacin (40,000 mg), vitamin B_{12} (20 mg), panthothenic B_6 (10,000 MG), folic acid (1,000 mg), Biotin (80 mg), chlorine chloride (500 mg), antioxidant (12 g). managaness (96 g), zinc (60 g), iron (24 g), copper (69), lodine (1.4 g), selenium (24 g) and cobalt (12 g).

Table 2. Proximate composition of experiment diets.

Component	Diet*							
	1	2	3	4	5	6	7	8
Dry matter (%)	88.78	91.00	88.48	89.90	89.40	89.57	90.93	92.88
Nitrogen (%)	21.13	21.02	20.32	20.45	19.96	19.90	20.00	20.72
Ash (%)	12.02	12.65	10.40	11.25	10.20	12.10	12.42	12.98
Crude fibre (%)	4.95	4.10	5.35	5.25	4.62	4.80	4.62	5.20
Ether extract (%)	2.54	3.03	2.09	3.54	3.04	2.42	2.26	3.01
Nitrogen- free extract (%)	48.14	50.21	50.31	49.41	51.56	50.35	51.62	50.97

Diets*1(0% TBNO and 0% Enzyme); 2(0% TBNO and 0.02% Enzyme); 3(10% TBNO and 0% Enzyme); 4(10% TBNO and 0.02% Enzyme); 5(15% TBNO and 0% Enzyme); 6(15% TBNO and 0.02% Enzyme); 7(20% TBNO and 0% Enzyme); 8(20% TBNO and 0.02% Enzyme).

treatments 2, 4, 5 and this was significantly ($P < 0.05$) lower than the ADFI values of birds on treatments 1, 7 and 8. There were no significant interactions ($P > 0.05$) between toasted bambara nut offal and enzyme levels in ADFI. There was no incidence of chicks' mortality throughout the period of the experiment. As shown in Table 3, the average daily feed intake (ADFI) was significantly influenced by the inclusion of toasted Bambara nut offal in the diets. The ADFI varied from 39.06 to 39.69 g. However, the feed intake result obtained in the present study does not agree with the reports of Apata and Ojo (2000) and Ani and Omeje (2007) which showed that

Table 3. Performance of cockerels fed graded levels of toasted Bambara nut wastes and supplementary enzyme.

Parameter	Treatment*								
	1	2	3	4	5	6	7	8	SEM
Average initial body weight (g/bird)	285.00	293.50	261.00	269.00	242.00	263.00	279.00	284.00	16.63
Average final body weight (g/bird)	684.00	685.00	707.00	680.00	664.00	689.00	681.00	664.00	13.83
Average daily weight gain (g/bird/day)	11.40	11.19	12.74	11.74	12.06	12.17	11.49	11.43	0.52
Average feed intake (g/bird/day)	39.60^{a}	39.33^{abc}	39.06^{c}	39.13^{bc}	39.41^{abc}	39.40^{abc}	39.69^{a}	39.53^{ab}	0.18
Feed conversion ratio	3.48	3.52	3.08	3.34	3.27	3.24	3.46	3.48	0.16
Feed cost/kg gain (₦)	291.33	295.510	259.40	281.87	274.53	272.48	391.41	293.69	40.84
Cost of total feed intake (₦)	115.53	115.72	114.94	115.53	115.72	116.07	116.96	116.93	0.69
Protein	8.32	8.31	7.99	8.00	7.84	7.88	8.22	7.91	026
Protein efficiency ratio	1.37	1.35	1.59	1.47	1.54	1.54	1.40	1.45	0.26
Mortality (%)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	-

^{abc} Rows with different superscripts are significantly different ($P < 0.05$); SEM: standard error of mean; Treatments*1(0% TBNO and 0% Enzyme); 2(0%TBNO and 0.02%Enzyme); 3(10% TBNO and 0% Enzyme); 4(10% TBNO and 0.02% Enzyme); 5(15% TBNO and 0% Enzyme); 6(15% TBNO and 0.02% Enzyme); 7(20% TBNO and 0% Enzyme); 8(20% TBNO and 0.02% Enzyme).

feed intake in broiler chicks fed diets containing cowpea testa and raw bambara nut waste, respectively increased with increasing levels of cowpea testa and raw bambara nut waste in the diets.

The result also contradicts the report of Nnamani (2010) which showed that feed intake in growing cockerels decreased significantly beyond 10% RBO inclusion level in the diets of the cockerels. Perhaps, toasting of the bambara nut offal might have influenced the feed intake pattern obtained in the present study. The comparable ADFI values observed amongst the TBNO dietary treatments contradicts earlier report (Apata and Ojo, 2000) which showed that feed intake decreased with increase in cowpea testa level in the diets of broiler chicks. Reduction in feed intake had also been reported in chicks fed diets containing processed sesame seed meal and dehulled pegion pea seed meal (Amaefule and Obioha, 2001; Akanji et al., 2003). The reduction in feed intake was attributed to the non-palatable nature of the diets containing those legume seed meals. Perhaps, the heat treatment applied to the raw Bambara nut prior to its inclusion in the diets helped to improve its texture, palatability and nutritive value by destroying or inactivating the heat – labile toxic compounds and anti-nutritional factors such as protease inhibitors, haemaglutinins, tannins, cyanogenic glycosides and flatulence factors present in the raw Bambara nut (Enwere, 1998; Ensminger et al., 1996; Liener, 1986; Liener and Kakade, 1980). Earlier report (Enwere, 1998) had shown that heat treatment applied to legume foods improved their texture, palatability and nutritive value by destroying or inactivating heat – labile toxic compounds and other enzyme inhibitors. Palatability in particular had been shown to influence feed intake and hence the overall performance of animals (Jurgens, 2002; Holness, 2005). Evidently, the palatability of the control diet was not superior to that of the diets containing toasted Bambara nut offal (TBNO) considering the comparable feed intake values of birds fed diets containing the heat-treated Bambara nut offal and those fed the control diets. It does appear that it is more beneficial to include processed toasted Bambara nut offal in the diet of chicks as this would not help birds fed dietary Bambara nut offal to have comparable feed intake with those fed the control diet. This would also help to conserve feed and consequently lead to reduction in the cost of feed.

The results also contradict the report of Ani and Omeje (2007) which showed that the feed intake of chicks increased as the level of raw Bambara nut waste in the diet increased to 20%. Incidentally, enzyme supplementation did not have any significant effect on feed intake (Table 2). The present observation is not in consonance with the reports of Ranade and Rajmane (1992), Samarasinghe et al. (2000), Ani and Omeje (2007) and Nnamani (2010) which showed that enzyme supplementation resulted in decreased feed intake at all the levels of raw Bambara nut waste inclusion in the diets. The non-significant effect of supplementary enzyme on feed intake could be as a result of the use of processed (toasted) Bambara nut waste in the present study as against the raw Bambara nut waste used by Ani and Omeje (2007) and Nnamani (2010). Since feed intake was not adversely influenced by the inclusion of toasted Bambara nut offal in the chicks' diets, therefore, the inclusion of enzyme in some of the diets did not play any significant role in regulating the amount of feed

Table 4. Effect of toasted Bambara nut offal and supplementary enzyme on apparent retention of nutrients by cockerels.

Parameter	Treatment*								
	1	2	3	4	5	6	7	8	SEM
Dry matter	40.26^{b}	41.30^{a}	39.52^{d}	40.02^{c}	36.81^{f}	37.15^{e}	35.50^{h}	36.52^{g}	0.51
Nitrogen	57.19^{b}	57.42^{a}	55.72^{f}	56.00^{d}	54.92^{h}	55.76^{e}	55.01^{g}	56.56^{c}	0.25
Crude fibre	54.22^{a}	54.22^{a}	52.56^{d}	53.22^{b}	52.28^{e}	52.99^{c}	51.51^{g}	51.74^{f}	0.25
Ether extract	58.69^{b}	60.20^{a}	55.76^{d}	56.95^{c}	52.77^{f}	54.81^{e}	50.51^{h}	50.67^{g}	0.86
Nitrogen- free extract	49.39^{b}	49.45^{a}	48.13^{e}	48.80^{c}	47.19^{g}	48.17^{d}	47.19^{h}	48.03^{f}	0.29

$^{ab,...,h}$Rows with different superscripts are significantly different ($P < 0.05$); SEM: Standard error of mean; Treatments*1 (0% TBNO and 0% Enzyme); 2(0% TBNO and 0.02% Enzyme); 3(10% TBNO and 0% Enzyme); 4(10% TBNO and 0.02% Enzyme); 5(15% TBNO and 0% Enzyme); 6(15% TBNO and 0.02% Enzyme); 7(20% TBNO and 0% Enzyme); 8(20% TBNO and 0.02% Enzyme).

consumed by the birds.

Effect of toasted Bambara nut offal and supplementary enzyme on apparent retention of nutrients by cockerels

Data on Table 4 show the effect of toasted Bambara nut offal and supplementary enzyme on apparent retention of nutrients by cockerels. Dry matter (DM) apparent digestibility (%), nitrogen (N) apparent retention, crude fibre (CF), apparent digestibility (%), ether extract (EE) apparent digestibility (%) and nitrogen-free extract (NFE) apparent digestibility (%) were significantly ($P < 0.05$) affected by treatments. Birds on treatment 2(0% TBNO diet with enzyme) had significantly ($P < 0.05$) higher DM apparent digestibility (%) than birds on other treatments. Similarly, birds on treatment 1(0% TBNO diet without enzyme) had significantly ($P < 0.05$) higher DM apparent digestibility (%), than birds on treatments 3 to 8. Birds on treatment 7 had the least DM apparent digestibility (%) ($P < 0.05$). The nitrogen retention of birds on treatment 2 was significantly ($P < 0.05$) higher than that of birds on other treatments. Similarly, birds on treatment 1(0% TBNO diet without enzyme) had significantly ($P < 0.05$) higher nitrogen retention than birds on treatments 3 to 8. The least nitrogen retention was observed in treatment 5 ($P < 0.05$). Birds on treatments 1 and 2(0% TBNO diet with and without enzyme, respectively) had significantly ($P < 0.05$) higher apparent CF digestibility (%) than birds on other treatments. Birds on treatments 7 and 8 (20% TBNO diet with and without enzyme) had significantly ($P < 0.05$) lower apparent CF digestibility (%) than birds on other treatments. Birds on treatment 7 had the least apparent CF digestibility (%) ($P < 0.05$).

The EE apparent digestibility (%) of birds on treatment 2 was significantly ($P < 0.05$) higher than those of birds on other treatments. Similarly, birds on treatment 2(0% TBNO diet with enzyme) had significantly ($P < 0.05$) higher EE apparent digestibility (%) than birds on treatments 3 to 8. Birds on treatment 7 had the least EE apparent digestibility (%) ($P < 0.05$). Birds on treatment 2(0% TBNO diet with enzyme) had significantly ($P < 0.05$) higher NFE apparent digestibility (%) than birds on other treatments. Similarly, birds on treatment 1 had significantly ($P < 0.05$) higher NFE apparent digestibility (%) than birds on treatment 3 to 8. Birds on treatment 4 (10% TBNO diet with enzyme) had significantly ($P < 0.05$) higher NFE apparent digestibility (%) than birds on treatments 3, 5, 6, 7 and 8. Birds on treatment 7 had the least NFE apparent digestibility (%) ($P < 0.05$). There were significant ($P < 0.05$) interactions between TBNO and enzyme levels on DM apparent digestibility (%), nitrogen apparent retention, and CF, EE and NFE apparent digestibility (%). Enzyme supplementation significantly ($P < 0.05$) increased DM apparent digestibility (%), nitrogen apparent retention, and EE and NFE apparent digestibility (%) at all the TBNO inclusion levels. Enzyme supplementation also increased ($P < 0.05$) CF apparent digestibility (%) at the 10, 15 and 20% TBNO inclusion levels.

As shown in Table 4, the apparent digestibility (%) of dry matter, apparent retention of nitrogen, and crude fibre, ether extract and nitrogen-free extract apparent digestibility (%) decreased with the increase in the levels of TBNO in the diets. The observed reduction in the apparent digestibility (%) and retention of these nutrients could have been as a result of the high fibre content of the TBNO containing diets. Decrease in nutrient digesti-bility with increasing dietary fibre level has been earlier documented in poultry (Babatunde and Hamzat, 2005) and in rabbits (Jokthan et al., 2006). It has been shown by Deaton et al. (1997) that insoluble dietary fibre exerts certain physiological effects on the gastrointestinal tract of animals by inhibiting digestive enzymes and by combining with mucin layer covering the villi of the small intestine to affect nutrient absorption. Besides, Choct and Annison (1992) reported that the increased bulk and viscosity of the intestinal content led to a decrease in the

rate of diffusion of substrate and thereby hindered the effective interaction at the mucosal surface. Reduction in apparent nutrient digestibility had been attributed to higher rate of passage of digester in animals that were fed high fibre diets (Kass et al., 1980; Fielding, 1991). Kung and Grueling (2000) had reported that high level of dietary fibre tends to limit the amount of intake and the retention of the available energy by birds and contributes to excessive nutrient excretion. However, supplementation of some of the diets with enzyme resulted in great improvement in the apparent digestibility (%) and retention; and in the utilization of these nutrients due to enhancement in their digestion and absorption.

Evidently, the performance of birds that were fed the enzyme-supplemented diets was not inferior to that of birds on the control diets. This agrees with earlier reports (Bedford, 1997; Annison and Choct, 1991; Acromovic, 2001; Toibipont and Kermanshashi, 2004) that enzymes increase digestibility of feed ingredients by reducing the viscosity of the gut contents. This results in increased nutrient absorption and in the reversal of the adverse effects of NSPs on growth of birds.

Conclusion

It is evident from the results obtained in the present study that up to 20% toasted Bambara nut offal can be included in the diet of growing cockerels without any deleterious effect on their growth performance.

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Full Length Research Paper

Ten (10) M Ammonium acetate is an efficient molecular concentration for the extraction of genomic DNA from small insects used for rolling circle amplification

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Most of the published methods for DNA isolation from vectors (insects) are not always effective in mealy-bugs, aphids and leafhoppers because they have high amount of polyphenols in their body systems. A simple, rapid, reliable labor-effective and carcinogenic compound-free salting out method for isolation of high molecular DNA from these insects is described. The ratio of OD_{260}/OD_{280} ranged between 1.8 to 2.0. The method was successfully applied to Rolling Circle Amplification and in restriction analysis, indicating the removal of the common inhibitors.

Key words: *Pseudococcidae*, *Aphididae*, *Auchenorryncha*, DNA extraction, rolling circle amplification, restriction analysis.

INTRODUCTION

Insects species that directly feed on plants play important functional roles in agricultural ecosystems, as they contribute to nutrient recycling. Moreover, most species can be serious pests and vectors of crop diseases, having economically important consequences for the agricultural industries. Due to their ecological and economical importance, research on systematic, phylogenetics, ecological genetics and molecular methods for detection and control of vector transmitted diseases of insect species are needed. Nonetheless, the isolation of high quality DNA for molecular studies and diagnosis of the disease from the vectors has been hindered by lack of simple, efficient and cheap molecular tools for DNA isolation. A fundamental requirement for molecular biology is the rapid DNA isolation and amplification of the specific DNA sequences. Typically, many DNA isolation protocols from the class insecta have been developed but none of the protocols can be used to extract the DNA from two or more species separately (Philips and Simon, 1995; Chen and Ronald, 1999; Starks and Peters, 2002; Strauss and Zangerl, 2002; Calderon et al., 2010). These protocols differ in the extraction buffer components and the pH of the buffer. Most of them use proteinase K digestion and chelex extraction especially while dealing with fresh specimens, but give poor quality DNA extracts (Strauss and Zangerl, 2002) due to phenolics and tannins in the insects' gut.

Phenolics are the major contaminants in DNA preparations from plant pests (Couch and Fritz, 1990; Lodhi et al., 1994; Kim et al., 1997). Phenolics, as powerful oxidizing agents, can reduce the yield and purity of DNA; inhibiting enzymatic modifications of the DNA such as

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restriction endonuclease digestion and poly-merase chain reaction (PCR) (Lodhi et al., 1994; Horne et al., 2004; Friar, 2005; Padmalatha and Prasad, 2006; Arif et al., 2010). Other additives such as polyvinylpyrroli-done (PVP) (Deobagkar, 1982; Strauss and Zangerl, 2002) and citrate have been used as antioxidant in the extraction buffer to inhibit oxidases in mealy-bugs (Aljanabi and Martinez, 1997). High concentration of ammonium acetate inhibits competitively the covalent binding of the phenolic compounds to the isolated nucleic acids through the formation of ionic bond. More so, the salt concentration enhanced the cell lysis due to the crenation effects. However, salting out protocol (Wahl, 1984) has been used to isolate DNA from small insects (aphids and whiteflies), but the salting agent was not included in the extraction buffer. This makes the protocol to be long and time consuming and the nucleic acids gets degraded by nucleases. In addition, most of the des-cribed methods are laborious, costly and are not amenable to isolation of high quality DNA. Most of the known DNA extraction buffers used in insect and other animal tissues does not employ the crenation effect to lyse the cells and nuclear membranes. Most of the isola-tion procedures usually involve marceration of the insect tissue in a boiling potassium hydroxide (KOH) solution (Rose et al., 1994; Knolke et al., 2005). These methods sclerotise structures of the abdomen and genitalia while soft tissues are discarded during preparation leading to a loss of a considerable amount of DNA. In addition, the high temperatures used during the boiling process in KOH can lead to the nicking of the DNA.

DNA extraction is invasive and causes damage to the specimens; although, efforts have been made to minimize the destruction by using small portions of the insects in other species (Mitchell et al., 1997; De Verno et al., 1998; Shneider et al., 1999; Cruickshank, 2002). Nondestructive DNA extraction methods have been reported (Philips and Simon, 1995; Rung et al., 2009). However, the method described by the Rung et al. (2009), is destructtive since the specimens are cut into half, whereas that of Philips and Simon (1995) can be considered semi-destructive since the abdomen is perforated several times with insect pins and submerged in the fluid to wash them. In case of the mealy-bug, aphids and leafhoppers, these protocols may not apply since the insects are very small in size especially where instars DNA is required. Several DNA isolation commercial kits such as DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA), a FastDNA kit (Qbiogene, Inc., Carlsbad, CA, USA) are being used to extract DNA from leafhoppers, aphids and mealybugs (Zidani et al., 2005). These commercial kits are expensive and tend to produce low DNA yields with short storage life from tissues rich in polyphenols (Lodhi et al., 1994; Fire and Xu, 1995; Calderon et al., 2010) imposing barriers to diagnose disease caused by insect vectors. In most cases, PCR is used to amplify defined sequences but the fidelity of the Taq DNA polymerase is low and the technique is limited to amplification of short (20 to 30 bp) DNA segments (Lodhi et al., 1994). The use of PCR to detect disease causative agents in insect vectors has been complicated by the lack of quick and easy DNA extraction protocols and by the inhibition of PCR by components in insect extracts (Lodhi et al., 1994; Fire and Xu, 1995).

Linear rolling circle amplification is the prolonged extension of the oligonucleotide primer annealed to the circular template DNA (Inoue-Nagata et al., 2004; James et al., 2011). The rolling circle amplification has the advantage of not requiring a thermal cycling instrument. The technique has been used in assaying banana streak virus (BSV) (James et al., 2011) and begomovirus in plants (Reagin et al., 2003). In this work, 'rolling circle amplification' was used to amplify the isolated DNA since the *Phi29* DNA polymerase is known to be more sensitive to the inhibitory compounds in the DNA samples compared to *Taq* DNA polymerase (Liu et al., 1996; Reagin et al., 2003; Inoue-Nagata et al., 2004; James et al., 2011).

We evaluated and compared the effectiveness of the developed method and Michele et al. (2002) method for isolation of DNA from three different insect species belonging to the order Hemipteran. The protocol circumvented the deleterious carcinogenic chemicals such as phenol and chloroform compounds that may contaminate the DNA from the procedure. In this study, we developed an inexpensive and rapid DNA isolation method by incorporating 10M ammonium acetate in the extraction buffer [sodium chlorides-tris- EDTA (STE)]. We evaluated the quality of the DNA isolated using the developed extraction buffer by electrophoretic and biophotometric analysis, uncut unmethylated λ standard method, restriction endonucleases digestion and rolling circle amplification.

The isolated DNA was suitable for these molecular applications, confirming that the method can be applicable to other insect vectors.

MATERIALS AND METHODS

Sample collection

Insects were collected during a short-rains season from a banana plantation at the Kenya Agricultural Research Institute (KARI) - Kisii in Western Kenya. Direct searching method was used to collect the mealy-bugs. Leafhoppers were collected using a black cage and an aspirator was used to suck them into 50 ml vials. Banana aphids were collected using a camel hair brush and put in the vials containing 70% ethanol as described by Rung et al. (2009). The insect samples were stored at -20°C until DNA extraction.

Protocol development and nucleic acid extraction

To develop and evaluate the protocol, samples were selected to include different haplotypes of *Pseudococcidae*, *paracoccidae*, *Aphididae* and *Auchenorryncha*, and placed in an Eppendorf tube

In the laboratory, specimens were transferred to vials containing absolute ethanol and stored in a -80°C freezer until DNA extraction. The insect samples were cleaned using distilled H_2O containing 2 mM EDTA or TE by vortexing for 30 s, after which the dH_2O-EDTA solution was removed with a pippete. Four hundred micro liter (400 µL) of extraction buffer [0.5M sodium chloride, 10 mM tris (pH 8.0), 36 Mm EDTA (pH 8.0), 0.2% sodium dodecyl-sulfate (SDS), 25 µL proteinase K and 10 M ammonium acetate; the overall pH of the buffer was adjusted to 7.6] was added to the Eppendorf tube. The insects were ground with a sterile teflon Epperdorf grinder (Kontes) and the DNA was isolated as described by Liu et al. (1996). The mixture was incubated in a heating block at 55°C for 2 h (1 h is adequate for this step, but it has been shown that the more the incubation time, the better the yields). The Eppendorf tubes were centrifuged in a non-refrigerated microcentrifuge at 14000 rpm for 5 min to pellet the cell debris and precipitate proteins. This step eliminates the use of the phenols and chloroform to dissolve the proteins. Then 2 µL of RNases (10 mg/ml) were added to the supernatant in a fresh Eppendorf tube and incubated for 1 h at 37°C. This step was meant to digest all the RNA in the total nucleic acid isolated.

2 m^3 of ice-cold isopropanol were added to precipitate the DNA and the mixture was gently mixed by inverting the tubes. The tubes containing the DNA were placed at -20°C for 30 min to allow the DNA to precipitate. The tubes were centrifuged at 14000 rpm for 15 min. The supernatant was removed and the same volume of cold 70% ethanol was added to the pellet. The tubes were spun at 14000 rpm in a non-refrigerated micro centrifuge for 5 min. The ethanol from the Eppendorf tube was poured off and the tubes were air dried completely. The tubes must be air dried for at least 15 min. The DNA pellet was resuspended in 50 µL of distilled H_2O and incubated at 37°C for 30 min or at 4°C overnight. The detection of the virus was done using rolling circle amplification. In addition, the sodium tris-EDTA (STE) based DNA extraction protocols described by Michele et al. (2002) was tested on the samples and analyzed using electrophoresis and bio-photometer.

Electrophorectic analysis of the genomic DNA

Electrophoresis of DNA from mealybug, aphids and leafhopper samples isolated using the developed and the STE based (Michele et al., 2002) protocols was carried out at 100 V for 1 h in 1% agarose gels in tris-(hydroxyl-methyl)-aminomethane (tris) (40 mM)-acetic acid (20 mM)-EDTA (2 mM) at pH 8.1. Gels were stained with SYBR safe (5 µg/ml) for 30 min. DNA bands were visualized using ultra-violet illumination Gel Doc (Bio-RAD) software (USA) and photographed. Lambda DNA was used as a molecular marker (positive control).

Biophotometric analysis

DNA was quantified using OD260/280 nm methods and fully expressed as yields per gram of starting material. Fifty (50) folds dilutions (in sterilized water) were made for each sample prior to OD reading. DNA concentration was extrapolated from the OD readings. Pure preparations of DNA have OD260/280 ratio ranging from 1.8 to 2.0.

Uncut unmethylated lambda DNA standards method

DNA yield and quality (integrity) was made by visual estimation using the mini-gel method (Wachira, 1996). Two microliter (2 µL) of DNA was drawn from the test samples, 3 µL of the gel loading dye (50% of the glycerol, 250 mM EDTA pH 8.0, 0.01% bromophenol blue) was added to it, and the mixture run on a 1.5% agarose gel in 1x TBE buffer (89 mM Tris-HCl pH 8.3, 89 mM boric acid, 2.5 mM EDTA) at 50 V, alongside standards of uncut unmethylated (100, 250 and 500 ng) Lambda DNA. The mini-gel was stained with the SYBR-safe dye and visualized under UV-light (312 nm) Gel Doc (Bio-RAD) USA. The dye was intercalated between the DNA molecules which ultimately fluoresced under UV-light. Comparisons of the band size and staining intensity were made between the test samples and the standards. DNA concentration was inferred from the comparisons. The mini-gel was also used to give the indication of the intactness of the DNA samples. Photography of the gels was done on UV-light (312 nm) Gel Doc (Bio-RAD) USA.

Rolling circle amplification of circular genomes (mitochondria DNA and banana streak virus genome)

The rolling circle amplification (RCA) technique was carried out using the standard protocol of Sambrook and Russell (2001) as modified by James et al. (2011). Amplification of BSV circular DNA was performed using a TempliPhi™ Kit (GE Healthcare, UK) following the manufacturer's protocol. The templiphi kit contained sample buffer, reaction buffer (salt and dNTPs) and enzyme mix (*Phi29* DNA polymerase and random primers in 50% glycerol). Rolling circle amplification degenerate primer mix was also added as described by Wambulwa et al. (2013). Twenty nanogram (20 ng) of total nucleic acid was dissolved in sample buffer, denatured for 3 min at 95°C and cooled down in ice for 3 min. After adding 5 µL of reaction buffer and 0.2 µL of enzyme mix, the reaction was run for 18 to 20 h at isothermal temperature of 30°C. The reaction was stopped by the incubation for 10 min at 65°C to inactivate the *Phi29* DNA polymerase. Aliquots corresponding to 250 ng nucleic acids in 10.2 µL volume of rolling circle amplification product were digested using the restriction enzyme (*Stu*1) for 2 h according to the manufacturer's protocol. Restriction products were resolved on a gel SYBR-safe green stained 1% agarose gel, using TBE as running buffer, and visualized under UV-light transillumination (AlphaDigiDoc, Cambridge, UK). DNA molecular weight marker (Hyperladder™ 1, Bioline) was loaded for band size comparison. To estimate unknown fragment sizes, migration distances were compared to those of reference fragments from the molecular marker (Hyperladder™ 1, Bioline).

Restriction enzyme digestion of the rolling circle amplification and gel electrophoresis

Ten microlitre (10 µL) of the TempliPhi reaction product from each of the insects' samples were incubated separately with the restrictiton enzyme *Stu 1* (Gibco BRL, Eggenstein) for 2 h. A 20 µL aliquot of the digested TempliPhi product was mixed with 2 µL of 5x gel loading dye (Biolabs) and electrophoresed for about 20 min at 100 V on a 1% SYBR safe stained agarose gel using 1x TAE as the running buffer. The gel was visualized under ultra violet (UV) illumination with Gel Doc (Bio-RAD) software (USA). Internal standards (positive controls) for banana streak virus (BSV) isolates generated using *Stu*1 (New England BioLabs*)* were used to identify the isolates present on each sample (James et al., 2011).

Genomic DNA restriction enzymes analysis

Restriction enzymes, *Kpn*1 and *Stu*1 were obtained from New England Biolabs, USA. Digestion was carried out according to the conditions specified by the manufacturer. Two microgram (2 µg) of the mealybug, aphids and leafhopper DNAs were digested for 6 h at 37°C with four units of the enzyme. Electrophoresis of DNA was carried out at 50 V for 8 h in 1% agarose gels in tris-(hydroxyl-methyl)-aminomethane (tris) (40 mM)-acetic acid (20 mM)-EDTA (2

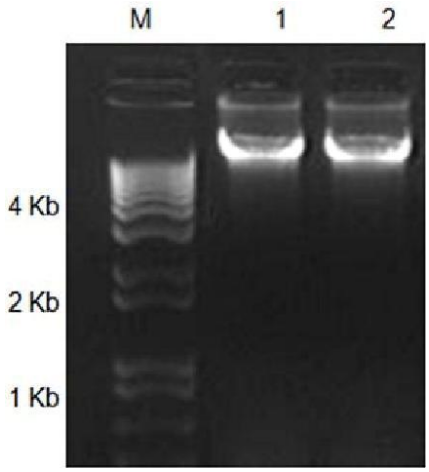

Figure 1. Agarose gel analysis of mealy-bugs' gDNA isolated using the new protocol. Lane M represent molecular marker (Invitrgen, Carlsbad, CA, USA), lane 1 represent mealy-bugs' gDNA and lane 2 aphids' gDNA.

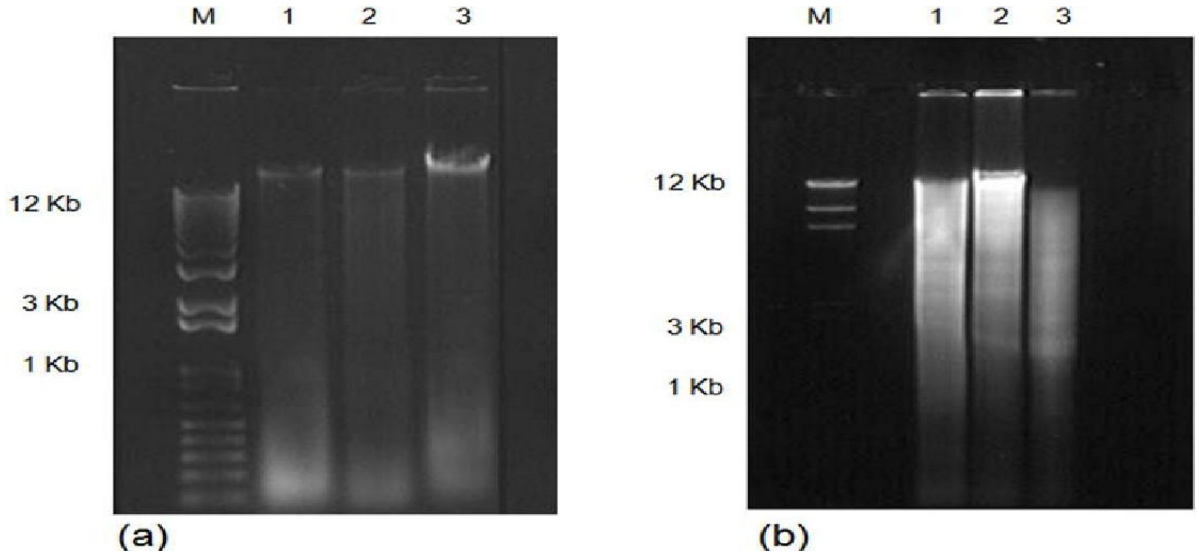

Figure 2. Agarose gel analysis of DNA prepared from mealy-bugs, aphids and leafhoppers using Michele et al. (2002) protocol. Lane M represent molecular marker (Invitrogen, Carlsbad, CA, USA), lane 1 represent mealy-bugs' gDNA, lane 2 represent aphids' gDNA and lane 3 represent leafhoppers' DNA.

mM) at pH 8.1. Gels were stained with SYBR safe (5 µg/ml) for 30 min. DNA bands were visualized using ultra-violet illumination Gel Doc (Bio-RAD) software (USA) and photographed. Lambda DNA digested with both *Kpn* 1 and *Stu* 1 was used as a molecular marker (positive control).

RESULTS

Protocol development and nucleic acid extraction

The results from the new protocol indicated high molecular weight genomic DNA (Figure 1). However, the results from the study of Michele et al. (2002) protocol were undesirable; the DNA was degraded (Figure 2).

Biophotometric analysis

In this study, the ratio of the OD260/280 nm obtained from the isolated DNA samples ranged between 1.8 to 2.0 which was certainly due to nucleic acids. Yields obtained using the OD260 readings were variable amongst the insects' DNA samples. The quantity of the DNA ranged from 2 ng/µl for leafhopper DNA to 7.2 ng/µl for

Table 1. UV-quantification of DNA from the three types of insects using the new protocol.

Sample number	DNA concentration (ng/µl) at OD260	OD260/280	X50 dilution factor (ng/µl)
Mealy-bugs			
1	7.2	2.00	360
2	7.2	1.86	360
3	2.6	1.95	130
Aphids			
1	5.4	1.82	270
2	5.3	1.89	265
3	4.2	1.93	210
Leafhoppers			
1	2.0	1.87	100
2	2.8	1.85	140
3	3.5	1.87	165

Table 2. UV-quantification of DNA from the three insects using the Michele et al. (2002) protocol.

Sample no.	DNA concentration (ng/µl) at OD260	OD260/280	X50 dilution factor (ng/µl)
Mealy-bugs			
1	2.0	1.21	100
2	1.9	1.19	95
3	1.8	1.22	90
Aphids			
1	1.7	1.25	85
2	1.4	1.30	70
3	1.6	1.19	80
Leafhoppers			
1	2.4	1.48	120
2	2.7	1.39	135
3	3.6	1.29	180

OD260/OD280 ratio results obtained using Michele et al. (2002) protocol ranged from 1.19 to 1.48 indicating high level of proteins in the nucleic acids. The concentration of the DNA was below that of the new protocol (ranged from 1.4 ng/µl (aphids) to 3.6 ng/µl (leafhoppers) as indicated in Table 2.

Uncut unmethylated lambda DNA standards method

The results obtained by the method revealed that the DNA isolated was of good quality, relatively intact and of high molecular weight as indicated in Figure 3.

Rolling circle amplification test

The results reveal that the organellar DNA was isolated by the new protocol and were amplified by the non-specific random primers in the reaction mix. However, the results confirmed the absence of the banana streak virus (BSV) in the insects gut as shown in Figure 4.

Endonuclease digestion of the genomic DNA

The results reveal that the nucleic acids isolated were free from phenolic compounds. The DNA was restritable by *Kpn* 1 and *Stu* 1 and restriction enzymes as shown in Figures 5 and 6.

DISCUSSION

The type of contaminations arising in DNA isolated from biological material varies according to its origin such as organism, tissue and life stage (Aljanabi and Martinez, 1997). Therefore, the type and condition of specimens and tissues are key factors in selecting a DNA isolation

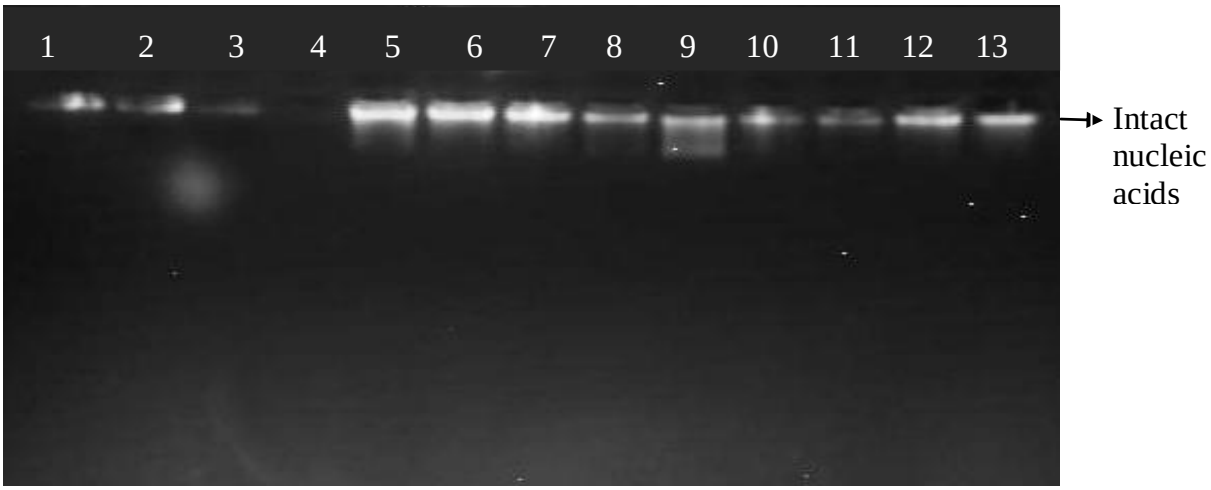

Figure 3. Quantity and quality of isolated DNA from the three insects using new protocol. Lanes 1 to 3 represent uncut unmethylated λ DNA standards (750, 500 and 250 ng, respectively), lane 4 represent negative control (SDW), lanes 5 to 7 represent aphid DNA, lanes 8 to 10 represent leafhopper DNA, Lanes 11-13 represent mealy-bugs DNA.

method. Tissues in the digestive tracts of vectors of plant diseases are rich in phenolics and tannins. These secondary metabolites must be removed to obtain DNA free from contaminants. Phenolics and other secondary metabolites cause damage to DNA and inhibit restriction endonucleases and DNA polymerases (Lodhi et al., 1994; Friar, 2005; Padmalatha and Prasad, 2006; Arifa et al., 2010). The widely used methods occasionally fail to remove all phenolics from DNA preparations. To tests the effects of inclusion of 10M ammonium acetate in our DNA isolation method, we compared the developed method with the traditionally used methods described by Michele et al. (2002). The results indicated good yields of high molecular weight DNA using the developed protocol. The ratio of the OD260/OD280 obtained for the isolated DNA samples ranged from 1.8 to 2.0 which was certainly due to nucleic acids (Table 1). A ratio less than 1.8 could indicate the presence of proteins and/or other metabolites in the DNA samples, in which case it would be necessary to re-precipitate the DNA (Honeycutt et al., 1992; Draper and Scott, 1998; Simon et al., 2003).

A DNA extract contaminated by chloroform and/or phenol normally gives a ratio higher than 2.0 in which case the extract should be re-precipitated with isopropanol (Brondmann, 2008). All the samples in this study indicated a ratio that was within the range which was attributed to relatively pure DNA. Like any other antioxidant, the ammonium acetate forms complex ionic bonds with proteins and other secondary metabolites and co-precipitate with cell debris upon cell lysis (Lodhi et al., 1994; Couch and Fritz, 1990). The acetate complexes accumulate at the interface between the organic and aqueous phases and can be eliminated from the DNA preparations (Reineke et al., 1998). High concentration of the ammonium acetate helps to reduce the browning of the DNA preparations produced by the oxidation of the secondary metabolites (Horne et al., 2004; Li et al., 2007). However, there is one complexity of the mealy-bug system and other vectors; this pertains to the presence of certain yeast and bacteria-like symbionts in both sexes of the insects. They are transmitted by the mother to the egg. The symbionts invade certain polyploid cells which form a small organ called the mycetome whose function is not known. When DNA is isolated from whole insects, the possibility of “contamination” of mealy-bug DNA by the symbiont DNA cannot be avoided (Deobagkar et al., 1982). This may become important, particularly when one is studying differences between DNA isolated from males and females, because the two sexes are vastly different in size. At least, in the stock that was used in this study, it would have been difficult to demonstrate symbionts in adult mealy-bug females, whether virgin or gravid (Aljanabi and Martinez, 1997). However, in any kind of study, specific primers are used to amplify only the sequences of interest BSV and therefore solving a problem of contamination by nucleic acids from symbionts.

Yields obtained using the OD260 readings were variable amongst the insects that the DNA was isolated. These results were attributed to the variation of the hardness of the vectors cuticle; mealy-bugs have the softest cuticle while leafhoppers have the hardest cuticle. This quantity of DNA obtained was adequate for PCR and RCA techniques which require about 0.3 ng/µl for amplification to occur. Indeed, it has long been known that polysaccharides like other contaminants are impossible to detect by non-degradative analytical techniques and usually they interfere with quantification of the nucleic acids spectrophotometrically and may even cause anomalous hybridization kinetics (Kim et al., 1997). However, the OD260/280 nm ratio for the Michele et al. (2002) protocol (1.19 to 1.48) indicated a higher level of contamination in the DNA preparations (Table 2). The

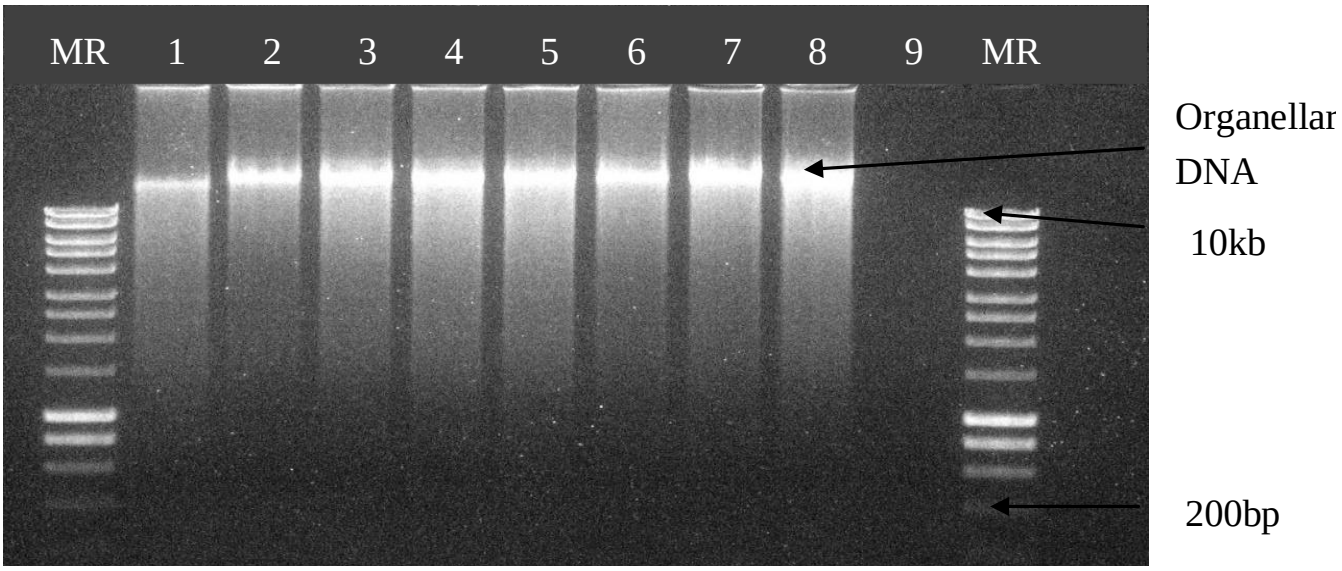

Figure 4. Rolling circle amplification of the mitochondrial circular DNA from the three insects. Lane MR represent molecular marker (Hyperladder^{TM} 1, Bioline), lanes 1 to 3 represent mealy-bugs DNA, lane 4 to 5 represent aphids DNA, lanes 6 to 8 represent leafhopper DNA and lane 9 represent negative control (SDW).

results also indicated low amounts of DNA than the new developed protocol. Running the isolated DNA against different concentrations of uncut unmethylated lambda (λ) phage DNA (Figure 3), was a realistic approach used in this study to determine the intactness of the isolated DNA. Although, the results obtained by this method revealed that the DNA isolated was of good quality, relatively intact and of high molecular weight, degradation of part of the isolated DNA is always inevitable. For adequate resolution of PCR, or RCA, and restriction fragment length polymorphism (RFLPs), it is known that native DNA should migrate as a tight band of high molecular weight (Brondmann, 2008).

The isolated DNA using high concentrated amount of ammonium acetate was tested for amplification reactions (Figure 4). The rolling circle amplification technique was used to amplify a DNA sample to confirm whether the sample of DNA contained inhibiting compounds or not. The technique is more sensitive to the inhibitory compounds in the isolated DNA (CIMMYT, 2005) than the PCR. The high sensitivity of the technique is due to the nature of the enzymes (*Phi29* DNA polymerase in RCA) used. *Phi29* DNA polymerase is known to recognize and amplify as little as 1 picogram of the template DNA (Reagin et al., 2003). In this study, the amplification products of the organellar DNA were obtained using RCA technique which was proof that DNA were free from inhibitory substances such as phenolic compounds that are known to form complexes with the DNA. Most common contaminants in DNA preparations are polysaccharides, RNA and phenolics compounds (Lodhi et al., 1994; Fire and Xu, 1995; Kim et al., 1997; Reineke et al., 1998; Michiels et al., 2003; Rung et al., 2009; Arif et al., 2010). Polysaccharides and phenolics produce highly viscous and brown coloured solutions (Henry et al., 1990; Couch and Fritz, 1990; Puchooa and Venkatasamy, 2005). They also reduce the storage lifespan of the DNA preparation (Lodhi et al., 1994). Given that RNA contamination is normally removed by treatment with RNase (Puchooa and Venkatasamy, 2005), and the isolated DNA was not viscous, it is likely that phenolics are the contaminants present in the Michele et al. (2002) isolated DNA. In addition, the inclusion of 10M ammonium acetate cleared the DNA solutions. This suggests DNA isolated by the developed protocol had lower concentrations of phenolics compared with the Michele et al. (2002) method.

The isolated DNA sample was further validated for its quality by determining its digestability by restriction enzymes. Indeed, the restrictability of the DNA is essential often before setting up large-scale digestion experiments. The *Stu*1 and *Kpn*1digest results obtained in this study with the DNA of the developed isolation protocol suggested that the DNA had no phenolic compounds that are known to inhibit the restriction enzymes (Figures 5 and 6) and DNA polymerase even without addition of the antioxidant (citrate) as described in other protocols (Aljanabi and Martinez, 1997). Phenolic compounds usually are formed during the isolation procedure and these bind firmly to DNA. Since many of these phenolic compounds contain methyl groups (Aljanabi and Martinez, 1997), they can end up hindering the restrictability of the isolated DNA. The production of these phenolic compounds is particularly catalyzed by the polyphenolic oxidases (Aljanabi and Martinez, 1997) in insects. Since many factors can cause a restriction digestion to fail or succeed, a single digestion should not be the decisive factor to immediately make conclusions. Other factors such as

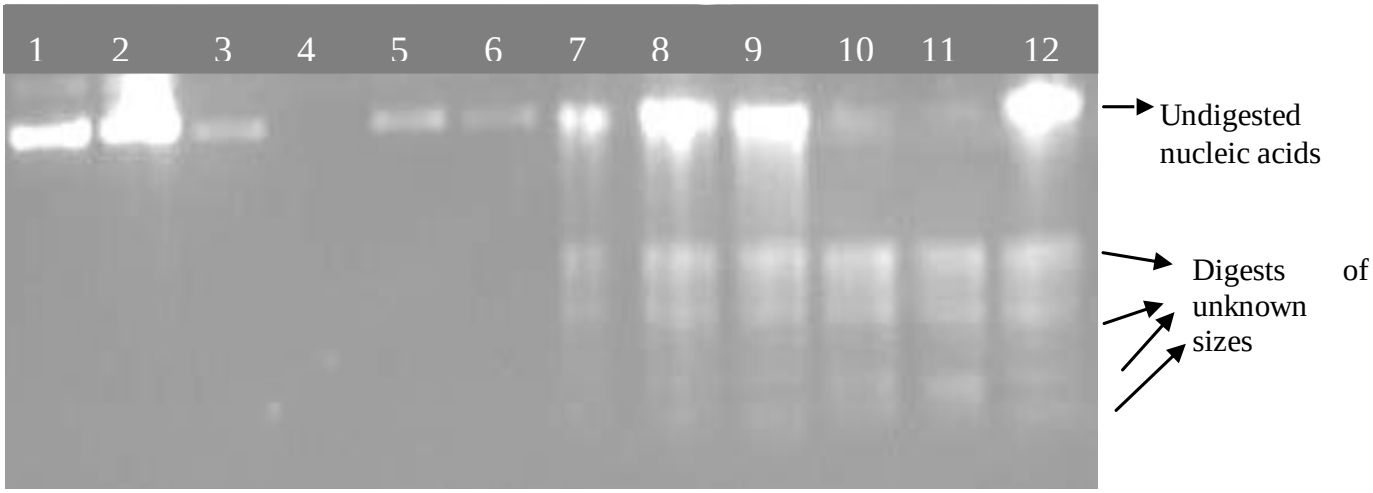

Figure 5. Analysis of non-digested and digested DNA from the three insects and lambda phage DNA using Kpn1. Lanes 1 to 6 represent undigested DNA while lanes 7 to 12 represent digested DNA. Lanes 1 and 2 represent undigested λ DNA, lanes 3, 5, 6 represent undigested mealy-bugs, leafhoppers and aphids DNA, lane 4 is sterilized distilled water, lanes 7 and 8 represent mealy-bug DNA, lanes 9 and 10 represent leafhopper DNA, lane 11 represent aphids DNA and lane 12 represent λ DNA.

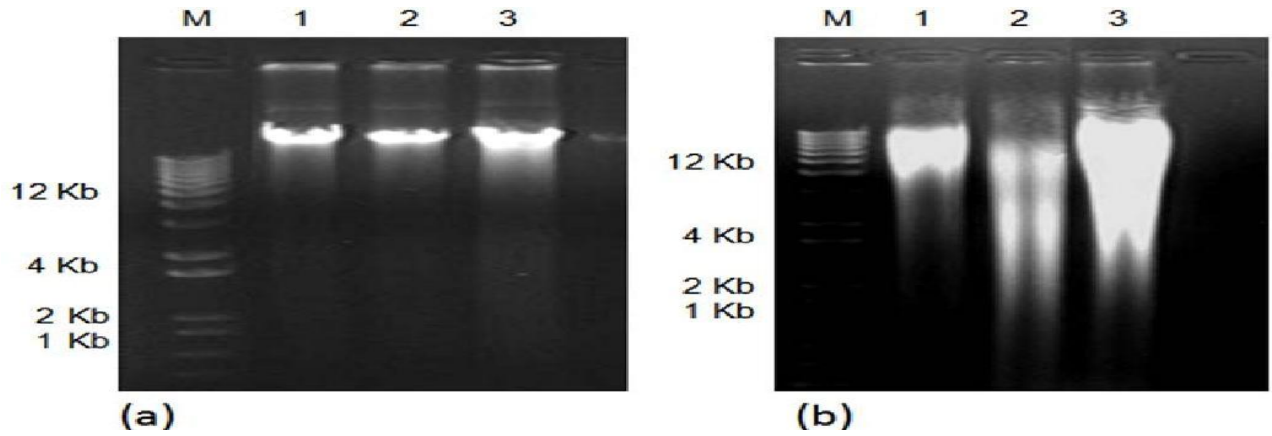

Figure 6. Digestability of DNA from the three insects using *Stu*1 (biolabs): Lane M represent molecular marker, lane 1 represent mealy-bugs' DNA, lane 2 represent aphid DNA and lane 3 represent leafhopper DNA.

poor reaction conditions could account for these results.

Conclusions

Ten molar (10M) ammonium acetate is an efficient molecular concentration for extraction of genomic DNA from small insects used for rolling circle amplification. The developed protocol from the modified sodium chloride-ris-EDTA based buffer isolated suitable DNA for rolling circle amplification (RCA) and restriction analysis. The method is simple, rapid and labor effective for DNA isolation from the three mentioned insects. Additionally, the method circumvents the use of the potentially carcinogenic compounds that may be deleterious to the users.

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Full Length Research Paper

Spasmogenic, Spasmolytic and Chemical Screening of Cigarettes

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The aqueous and ethanolic extracts derived from cigarettes (Morven Gold) were screened for chemicals, spasmogenic and spasmolytic activities. Aqueous extract showed strong relaxant activity that is, 92% against KCl induced contractions while ethanolic extract was found to be moderately spasmolytic (70%). Ethanolic extract was also found to have a strong spasmogenic activity, while aqueous extract depress the spasmogenic activity of pilocarpine induced contractions. Thus, the ethanoilc extract was found to be more efficient for spasmogenic activity while aqueous extract was noted to be more efficient for spasmolytic activity. The chemicals found in sufficient quantity in both the extracts were saponin and glycosides. It was also noted that tannins were present only in ethanolic extract in excess quantity. The research indicated clearly that cigarette is a good spasmolytic agent while the ethanolic extract has spasmogenic activity. Further studies are necessary to elucidate its exact mechanism of action.

Key words: Sapsmogenic, spasmolytic, chemical screening, cigarette.

INTRODUCTION

Cigarette is a type of smoking tobacco produced and used all over the world. It is a small roll of paper mostly about 7 cm of length and 5 to 7mm of width, filled with a finely, pieced tobacco leaves and may have some additives. At one end of the cigarette, a filter is attached mostly made up of cellulose acetate and of about 2 cm of length. Presently, the use of tobacco is the leading cause of death worldwide (Brundtland, 2000) and is estimated that by 2030 it would be over 10 million annual deaths globally (Warnakulasuriya et al., 2005; John, 2005), 70% of which will be in the developing world (WHO, 2000). It has also been reported that all forms of tobacco carry serious health consequences, most importantly is oral and pharyngeal cancers (Gupta and Ray, 2003; Mack, 2001; IARC, 1985; Merchant et al., 2000; Avon, 2004). In Pakistan, oral cancer is the second most common cancer in women and third most common in men (Jafarey and Zaidi, 1987). Smoking (in form of cigarette) of tobacco has also been reported as a well-known cause of oral sqamous cell carcinoma (Gupta et al., 1982; Jayant and Deo, 1986; Brennan et al.,1995; Choi and Kahyo, 1991; Negri et al.,1993). Smoking during pregnancy may cause low birth weight, pre-mature birth and infant death (U.S. Dept. of Health and Human Services, 2001), and also increase the neonatal health care costs (Adams et al., 2002). Furthermore, cigarettes contain carcinogens that not only stimulate genetic damage, but also result in the production of atypical cells, mutations and eventually

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cancer, they also impair the function of the p53 gene which, when functioning normally, prevent mutations from developing into cancer (Langdon and Partridge, 1992). It has also been reported that cigarette smoke contains carcinogens that alter biochemical defense systems that lead to deleterious effects on the respiratory tract, heart, pancreas, reproductive tract and other organs (Ostergaard, 1977), and also has a link to common causes of death and disability in elderly aged persons associated with chronic illnesses (Bratzler et al., 2002). But smoking has also been observed to reduce the incidence of various diseases for example, endometrial cancer, and ulcerative colitis, hypertension in pregnancy, Alzheimer's disease and Parkinson disease (English et al., 1995; Graves et al., 1991; Van Duijin and Hofman, 1991). Several other epidemiological studies have also found a beneficial effect of smoking in Parkinson disease (Fratiglioni and Wang, 2000; Checkoway et al., 2002).

In the current study, we present the spasmogenic, spasmolytic and chemical screening of aqueous and ethanolic extract of cigarette. This study was designed with a view to confirm and explore the pharmacological activity of cigarette which contains tobacco and may other ingredients and is used by a large number of people throughout the world.

MATERIALS AND METHODS

Sample material

Two packs of Morven Gold containing 40 cigarettes were purchased from the local market of Abbottabad, Pakistan. A sample pack, marked with a number 1327 was deposited in the Pharmacy Museum, University of Malakand Pakistan.

Preparation of extracts

The materials were withdrawn from each cigarette and were pulverized into fine powder and weighed 13.300 g in duplicate. Each was then extracted in distilled water of 60 ml and ethanol (70%) of 60 ml separately for about 3 weeks. Both the extracts were separately filtered and evaporated under reduced pressure to yield a gum (1.5 to 02 gaqueous and 1.5 to 02 gethanolic) by using Rotary Evaporator and Freez Dryer.

Drugs and standards

Analytical grade chemicals were used in the bioassay technique and chemical screening. All the solutions were freshly prepared in distilled water on the same day of experiments.

Animals and data recording

Rabbits of either sex were bred locally. Their average weight was in the range of 1.5 to 2.0 kg. They were maintained at the "Animal House of Frontier Medical College Abbottabad" as per Byelaws of Scientific Procedures. Animals were given free access to standard diet along with fresh water. Before the start of experiments, animals were given only water and were kept fasted overnight. Intestinal responses were recorded using Organ bath and kymograph.

Spasmogenic activity

The extracts were screened for possible cholinomimetic and spasmolytic activities as per procedure mentioned. Tyrode's solution was prepared having the following concentration (mM): KCl, 2.68; NaCl, 136.9; $MgCl_2$, 1.05; $NaHCO_3$, 11.90; NaH_2PO_4, 0.42; $CaCl_2$, 1.8 and glucose 5.55. The animals were then slaughtered and their abdomens were opened. Rabbit's jejunum portion(s), of about 1.5 to 2 cm length, was isolated and mounted in the tissue bath containing 10 ml of Tyrode's solution maintained at 37°C and supplied with carbogen gas (5% carbon dioxide and oxygen mixture). These portion(s) were kept in Tyrode's solution previously aerated with the carbogen gas (Qayum, 2004). Earlier, the tissues were stabilized for normal activity for a period of about 25 to 40 min. For possible pharmacological screening on the tissues through series of experiments, aqueous and ethanolic extracts of cigarette were tried at doses of 2 and 5 mg/ml. All the doses were applied in cumulative manner and the results were recorded (Farre et al., 1991). The spasmogenic and spasmolytic activity was recorded as given in Figure 1.

Spasmolytic activity

We used the procedure described by Farre et al. (1991) to screen spasmolytic activity. Contractions in the intestine portions were produced by high KCl (80 mM) to depolarize the intestine portions (Farre et al., 1991). The extracts were then applied in similar fashion to relax the tissues and percent relaxation response on KCl induced contractions were recorded as given in Table 1 and shown in Figure 1. The following formula was used for calculations:

$$\% \text{ Inhibition/stimulation} = 100 - \frac{Average height\ of\ contractions\ after\ extract\ (mm)}{Average height\ of\ normal\ contraction\ (mm)} \times 100$$

Chemical screening

The aqueous and ethanolic extracts of cigarette were evaluated for the presence of alkaloides, glycosides, terpenes, saponins, tannins, flavonoids and carbohydrates using simple qualitative methods of Sofowora (1993) and Evans (1998). Also the pH of both extracts was recorded.

RESULTS AND DISCUSSION

According to Figure 1, moderate spasmogenic activity of ethanolic extract of cigarette was noted while aqueous extract showed a depressant activity against Pilocarpine induced contractions. By this, it had been confirmed that the cholinomimetic activity of ethanolic extract of cigarette, may be because of the presence of nicotine which may act on any mechanism as discussed. According to Gillespie and Mackenna (1960), the response to nicotine of intestinal preparations *in vitro* is usually a contraction due to stimulation of the parasympathetic cholinergic neurons in Auerbach's plexus. Same was the result recorded in the current study for cigarette. Also, the nature of the medium had been studied which was found to be acidic in both the aqueous and ethanolic extracts.

In another series of experiments, tissues were depolarized with high potassium level (80 mM bath concentration) that produced a sustained contraction (Farre et al., 1991). The test samples were then tried in cumulative

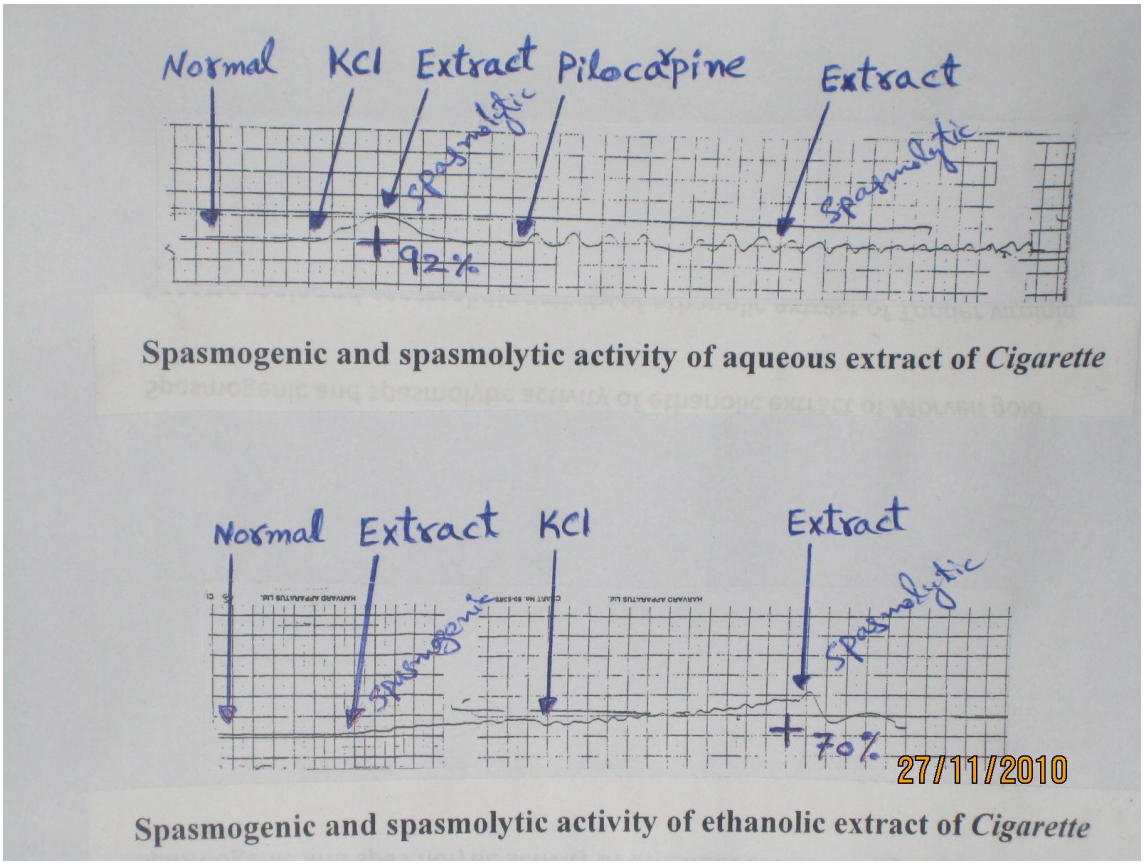

Figure 1. Spasmogenic and spasmolytic activity of crude extracts of cigarette. In the first portion of the graph, aqueous extract of cigarette showed a strong spasmolytic activity, while in the next portion, ethanolic extract showed significant spasmogenic and spasmolytic activity.

manner to observe the spasmolytic effect on the tissues. As it has been postulated that contractions produced by potassium are mediated through calcium channels via influx of calcium from extra cellular fluid and a substance which will inhibit the contraction produced by KCl is considered to have calcium channel blockade (Bolton, 1979). Hence, the extract produced a dose-dependent spasmolytic response on the KCl-induced contractions and is considered to have calcium channel blocking activity. According to Figure 1, the extract produced a spasmolytic effect on the KCl depolarized tissues in dose dependant manner; with a maximum dose of 5.0 mg/ml, KCl-induced contractions (80 mM) were relaxed by the extract in the similar doses. As in the current study, the aqueous extract of cigarette was found to have strong spasmolytic effect, noted as 92%, and for ethanolic extract, it was measured as 70% as given in Table 1 and Figure 1. Positive relaxing effects on KCl induced contraction are mostly referred to calcium channel blocking activity (Gilani et al., 2005). Hence, the spasmolytic activity of cigarette may be mediated through calcium channel blocking activity. On another point of view, Ambache (1946) and Feldberg (1951) obtained evidence that barium could excite ganglian cells in the intestine for spasmogenic effect and Ambache (1949) showed that barium excited ganglian cells. Douglas et al. (1961) reported that the spasmogenic effect of barium in the intestine or superior cervical ganglian were depressed by hexamethonium or nicotine. So from these above discussed mechanisms, may be one would be responsible for the spasmolytic effect of cigarette. Further studies are necessary to elucidate the exact mechanism of action for spasmolytic and spasmogenic activity of cigarette.

The qualitative chemical screening of cigarette revealed the presence of alkaloids and carbohydrates in minute quantity, saponins and glycosides in moderate amount in both the aqueous and ethanolic extracts, while tannins were found only in ethanolic extracts in large amount as given in Table 2.

Conclusion

In the current study, a moderate spasmogenic activity of ethanolic extract of cigarette was found. It was also noted that both extract have a relaxant activity against KCl induced intestinal contraction, which may be due to any of the above discussed mechanism. The spasmolytic activity of the aqueous extract was found to be more effi-

Table 1. Spasmogenic and spasmolytic activity of crude extracts of cigarettes.

Extraction medium	Spasmogenic activity	Spasmolytic activity (%)	Nature
Ethanol	Moderate	70	Acidic
Distilled water	Negative	92 %	Acidic

Aqueous extract were found to be more effective for spasmolytic activity and ineffective in the case of spasmogenic activity while the ethanolic extract were noted to be moderately effective both for spasmogenic and spasmolytic effects.

Table 2. Chemical screening of aqueous and ethanolic extracts of cigarettes.

Test	Observation	Inference	
		Et-Ext	Aq-Ext
Alkaloids :Extract + 10 % tannic acid solution	Turbidity/precipitation	+	+
Saponins: Extract vigorously shaken in a test tube for 2 minutes	Frothing less than 1 cm	++	++
Flavonoids: (Shinoda test) Ethanolic extract + magnesium fillings + conc HCl	Pink or red color	-	-
Tannins: Extract + Few drops of $FeCl_3$	An immediate green precipitate formed	+++	-
Terpenes: Decolorized Extract residue + Chloroform + acetic anhydride+conc H_2SO_4	Brown precipitate formed	-	-
Carbohydrates: Extract + Molisch's reagent + conc H_2SO_4	Purple precipitate	+	+
Glycosides: Extract + Fehlings reagent and boild for 2 min	Brick red color	++	++

+, Mild; ++, moderate; +++, excess; -, absent.

cient then the spasmolytic activity caused by ethanolic extract against KCl induced contraction. Further studies are necessary to elucidate its proper mechanism of action. The results also showed the acidic nature of the extracts and the presence of alkaloids, carbohydrates, saponins and glycosides in high and low concentration as given in Table 2.

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Full Length Research Paper

Identification of proteins similar to AvrE type III effector proteins from *Arabidopsidis thaliana* genome with partial least squares

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Type III effector proteins are injected into host cells through type III secretion systems. Some effectors are similar to host proteins to promote pathogenicity, while others lead to the activation of disease resistance. We used partial least squares alignment-free bioinformatics methods to identify proteins similar to AvrE proteins from *Arabidopsidis thaliana* genome and identified 61 protein candidates. Using information from Genevestigator, Arabidopsidis GEB, KEGG, (GEO: accession number GSE22274), and AraCyc databases, we highlighted 16 protein candidates from Arabidopsidis genome for further investigation.

Key words: Partial least squares, Type III effectors, AvrE, and Arabidopsis.

INTRODUCTION

Plant pathogens deliver small molecules referred to as effectors, by type III secreting systems (T3SS) directly into plants (Abramovitch et al., 2006; Block et al., 2008; Zhou and Chai, 2008). The injected effectors target different cellular compartments and subvert numerous signaling pathways for the benefit of the bacteria. Through the resistance (R) proteins, plants evolved to gain the ability to recognize directly or indirectly effectors. Several T3SS effectors contribute to virulence by sup-pressing Pathogen-Associated Molecular Patterns (PAMPs) (Hauck et al., 2003), and other effectors suppress hypersensitive cell death elicited by various Avr proteins (Abramovitch et al., 2003). Some effectors mimic plant proteins (Bender et al., 1999; Weiler et al., 1994), while others mimic plant molecules (Janjusevic et al., 2006; Rosebrock et al., 2007). AvrE are type III effectors proteins with very low sequence identity (Ham et al., 2009).

Plant genomics and many agriculturally important crops are resulting in a rapidly increasing database of genomic and sequences. These databases have proved to be rich resources for several genes of importance agronomic traits, such as, virus and insect resistance, bacterial resistance, abiotic stress tolerance, and novel genetic markers for crop improvements. Silverstein et al. (2005) searched Arabidopsidis genome using profile hidden Markov model (HMM) (Durbin et al., 1998) and Basic Alignment Search Tools (BLAST) (Altschul et al., 1990) to identify defensin-like sequences (DEFLs) in *Arabidopsidis* genome. They identified 317 DEFLs in *Arabidopsidis* including 15 known defensins. Thus, bioinformatics has become an integral aspect of plant and crop science research. The objective of the study was to identify proteins that are similar to AvrE-family effector proteins from Arabidopsidis genome with partial least squares (PLS) alignment-free methods (Opiyo and Moriyama, 2007).

Alignment-based methods have limitations because alignments are known to become unreliable when sequence similarity drops below 40% (Petsko and Ringe,

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2003). Some proteins such as AvrE are highly divergent and have low sequence identity (WtsE and AvrE have 27.1% amino acid identity) even though they still share similar structures, biochemical properties, and functions. In such cases, obtaining reliable alignments among these protein sequences is extremely difficult, and alignment-based methods such as BLAST, position specific iterative BLAST (PSI-BLAST) (Altschul et al., 1997), and profile HMMs would fail to identify these proteins from databases. Using PLS alignment-free methods, we predicted 61 protein candidates from Arabidopsidis genome as similar to AvrE effectors. Using information from Genevestigator v3 (Hruz et al., 2008), Arabidopsidis Gene Expression Browser (GEB) (Zhang et al., 2010), KEGG: Kyoto Encyclopedia of Genes and Genomes (Kanehisa and Goto, 2000), (GEO: Gene Expression Omnibus (Edgar et al., 2002); accession number GSE22274 (Wang et al., 2011), and AraCyc (Mueller et al., 2003), we highlighted 16 protein candidates for further investigation.

MATERIALS AND METHODS

Dataset sources

Training dataset

Twelve (12) AvrE proteins (positives) from study by Ham and associates (Ham et al., 2009) and non-AvrE proteins (negatives) were downloaded from National Center for Biotechnology Information (NCBI) websites (http://www.ncbi.nlm.nih.gov/), and were used for training the PLS methods.

Databases

Arabidopsidis thaliana: 35 386 proteins from the release 10 (November, 2010) of The Arabidopsidis Information Resource (TAIR) database (http://www.arabidopsidis.org/).

Sequence descriptors used for PLS alignment-free methods

Amino acid composition: From each protein sequence, frequencies of 20 amino acids were calculated. In this study, amino acid composition was used as descriptors for a PLS classifier (PLS-AA).

Dipeptide composition: Dipeptide composition represents all 400 frequencies of consecutive amino acid pairs in a protein sequence and corresponds to a 400 (20 × 20) feature vector. It can encapsulate information on composition of amino acids, as well as, their local order. We used dipeptide composition as descriptors for a PLS classifier (PLS-DIP).

Physicochemical properties of amino acids: We developed five descriptors (PC1- PC5) using the principal component analysis (PCA) of 12 physicochemical properties of amino acids (mass, volume, surface area, hydrophilicity, hydrophobicity, isoelectric point, transfer of energy solvent to water, refractivity, non-polar surface area, and frequencies of alpha-helix, beta-sheet, and reverse turn) (Opiyo and Moriyama, 2007). The five descriptors were used in this study.

Auto/cross covariance transformation: Auto/cross covariance (ACC) transformation method discussed in Opiyo and Moriyama (2007) was used to transform each amino acid sequence using the five physicochemical property based descriptor set (PC1-PC5). ACC with the maximum lag of 30 residues yielded 775 descriptors for each sequence. The calculation of ACC was performed using the R implementation (version 2.12.0; http//www.R-project.org; 2010).

Partial least squares

Partial least squares [PLS; (Geladi and Kowalski, 1986)] is a projection method similar to principal component analysis (PCA) where the independent variables, represented as the matrix X, are projected onto a low dimensional space. PLS uses both independent variables X (sequence descriptors such as amino acid composition) and dependent variables Y (positive or negative label). PLS using descriptors transformed by ACC (PLS-ACC) was used in (Opiyo and Moriyama, 2007). PLS discriminate analysis is performed to separate groups of observations. It consists of a classical PLS where the response variable is a categorical one (replaced by the set of dummy variables describing the categories, e.g., 0 and 1) expressing the class membership of the statistical units. In this study, each of a training sample, a response variable was a signed 1 for the positive sample (AvrE) and 0 for a negative sample (non-AvrE). The group membership, AvrE or non-AvrE of a new sequence was predicted based on descriptors and y-value. Predicted y-value closer to 1 was considered to be AvrE candidate and closer to 0 WAS considered to be non-AvrE candidate. PLS analysis was performed using an R implementation; the PLS package was developed by Wehrens and Mevik (version 1.2.1) (Wehrens and Mevik, 2007).

Performance analysis

Cross-validation analysis (leave-one-out) was performed for all the 24 sequences used for training the methods. One sequence in the training dataset was left out and the learning algorithm was trained on the rest of the sequences. The trained model was used to predict the class (AvrE or non-AvrE) of the earlier left out. For the 24 sequences, the process was repeated 24 times leaving each of the 24 sequences out and creating a model from the remaining 23 sequences.

Predictions were grouped as follows: i) True Positives (TP): the number of actual AvrEs that were predicted as AvrEs; ii) False Positive (FP): the number of actual non-AvrEs that were predicted as AvrEs; iii) True Negative (TN): the number of actual non-AvrEs that were predicted as non-AvrEs and iv) False Negative (FN): the number of actual AvrEs that were predicted as non-AvrEs.

Minimum error point

The minimum error point (Karchin et al., 2002) was used to determine threshold values of PLS methods. The sequences are ranked based on the values. The threshold value where the minimum number of errors (FN + FP) occurs is the minimum error point (MEP) and the number of false positives and false negatives are assessed at this point. The minimum error point tells us the best case accuracy of a method. The minimum error points for PLS-AA, PLS-DIP and PLS-ACC were 0.94, 0.96 and 0.94, respectively. The upper cut-off point for all methods was set at 1.00 to further reduce the number of false positives. To be selected as a candidate, a protein has to be identified by all the three methods (PLS-AA, PLS-DIP and PLS-ACC) as positive.

Goodness of Prediction of PLS methods

The goodness of prediction, Q^2 equation 2, describes how well the

Table 1. The number of PLS components and the predictive abilities of PLS-AA, PLS-DIP, and PLS-ACC, respectively from the leave-one-out cross validation procedures.

Method	Number of PLS components	Q^2
PLS-AA	4	0.72
PLS-DIP	3	0.67
PLS-ACC	4	0.78

method can predict a data.

$$Q^2 = 1\text{-}PRESS/SS_Y \quad (2)$$

Where SS_Y is the total sum of squares, PRESS is the predictive residual sum of squares, which is calculated from the difference between observed and predicted Y values. $Q^2 > 0.50$ is considered good. In this study, the leave-one-out cross-validation procedure was used for the Q^2 calculation. Detailed results of PLS analyses are given in Table 1 for PLS-AA, PLS-DIP, and PLS-ACC, respectively.

RESULTS AND DISCUSSION

Mining *A. thaliana* proteome using three PLS methods

Our objective was to identify proteins similar to AvrEeffector proteins from Arabidopsidis genome. PLS methods trained using 12 AvrE-family effector proteins predicted 61 protein candidates from Arabidopsidis genome. Thirty-eight proteins (62%) were enzymes, and they included kinases, hydrolases, and proteases. Other proteins predicted were F-box family protein, unknown protein, transcription factor, auxin-responsive family protein, and other proteins. In order to further study the predicted proteins, we used Genevestigator, Arabidopsidis GEB, KEGG, (GEO: accession number GSE22274), and AraCyc databases to analyze the proteins.

Expression patterns of the predicted proteins in Genevestigator and Arabidopsidis Gene Expression Browser databases

We utilized the server of Genevestigator and Arabidopsidis GEB databases to study expression patterns of the predicted proteins. Of the 61 proteins predicted, three (AT2G44280, AT3G59590, and ATMG00140) had no expression data in the Genevestigator database. In this study, only responses with expression levels altered by more than two-fold under the biotic stress are presented. Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein (AT2G13820), unknown protein (AT2G17850), and receptor protein kinase-related (AT3G46270) were down-regulated by both *Cryphonectria arabidopsidis,* and *Pseudomonas syringae*. Mean while, phosphoglycerate kinase (AT1G79550), cyclin-dependent kinase B1; 2 (AT2G38620), and exopolygalacturonase / galacturan 1, 4-alpha-galacturonidase / pectinase (AT3G07850) proteins were specifically down-regulated by *P. syringae*. In addition, invertase/pectin methylesterase inhibitor family protein (AT2G47340) was mainly up-regulated by *P. syringae.* From Arabidopsidis GEB, glycosyl hydrolase family 17 proteins / beta-1, 3-glucanase (AT3G55430), lipase class 3 family protein (AT3G62590), Senescence-Associated Gene 101 (AT5G14930), and glutathione transferase (AT5G62480) were up-regulated by *P. syringae, Golovinomyces orontii, and Botrytis cinerea*. These data reveal that AvrE proteins might mimic *Arabidopsidis* proteins that are up-regulated or/and down-regulated by both fungi and bacteria.

Metabolic pathways identified from KEGG and AraCyc databases

Out of the 61 proteins predicted, 13 were linked to KEGG metabolic pathways. The KEGG pathways linked to the predicted proteins are Typtophan metabolism (ath00380), biosynthesis of secondary metabolites (ath01110), starch and sucrose metabolism (ath00500) and others. The AraCyc software (http:// www.arabidopsidis.org/tools/aracyc) provides a good starting point to paint expression data on metabolic pathways. AraCyc metabolic pathways linked to the predicted proteins were oxidative ethanol degradation and superoxide radicals degradation (AT1G20620), abscisic acid glucose ester biosynthesis (AT2G29740), gibberellin biosynthesis III (AT1G80330), choline and phosphatidylcholine biosynthesis (AT2G32260), trehalose biosynthesis (AT4G12430), and photorespiration (AT5G47760). AT2G29740 protein *syringae* as shown in Genevestigator involved in abscisic acid glucose ester biosynthesis was up-regulated by *P.* database. These results show that AvrE proteins might mimic proteins that involve metabolomics pathways related to biosynthesis of secondary metabolites, steroid and trahalose biosynthesis, abscisic and gibberellin biosynthesis as shown from both KEGG and AraCyc databases. Based on the information from Genevestigator, Arabidopsidis GEB, KEGG, and AraCyc, we highlighted 16 protein candidates as priorities for further investigation (Table 2). We predicted protein subcellular localizations by WoLF PSORT (Horton et al., 2007); and the predictions show that 50% (8 proteins) of the 16 proteins candidates are located in Cytosol. These protein candidates were identified by computational predictions; experiments are ultimately needed to determine if they are mimic by AvrE effector proteins.

Conclusions

In this study, we predicted 61 proteins from *Arabidopsidis*

Table 2. Sixteen protein sequences highlighted for further investigations.

Accession number	Length (aa)	TAIR Description
AT1G20620	485	SEN2, CAT3 CAT3 (CATALASE 3); catalase chr1:7143132-7146183 FORWARD
AT1G79550	401	PGK PGK (PHOSPHOGLYCERATE KINASE) chr1:29929240-29931188 REVERSE
AT1G80330	355	ATGA3OX4, GA3OX4 ATGA3OX4 (GIBBERELLIN 3-OXIDASE 4); gibberellin 3-beta-dioxygenase chr1:30202953-30204429 REVERSE
AT2G13820	129	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein chr2:5782887-5783361 REVERSE
AT2G29740	474	UDP-glucoronosyl/UDP-glucosyl transferase family protein chr2:12713824-12715248 FORWARD
AT3G07850	444	exopolygalacturonase / galacturan 1,4-alpha-galacturonidase / pectinase chr3:2505819-2507444 REVERSE
AT3G25070	211	RIN4 RIN4 (RPM1 INTERACTING PROTEIN 4); protein binding chr3:9132465-9133754 FORWARD
AT3G45640	370	MPK3, ATMPK3 ATMPK3 (MITOGEN-ACTIVATED PROTEIN KINASE 3); MAP kinase/ kinase/ protein kinase chr3:16767903-16769461 FORWARD
AT3G53250	109	auxin-responsive family protein chr3:19753946-19754275 FORWARD
AT3G55430	449	glycosyl hydrolase family 17 protein / beta-1,3-glucanase, putative chr3:20560783-20562981 REVERSE
AT3G62590	649	lipase class 3 family protein chr3:23158949-23161145 REVERSE
AT4G04740	520	CPK23 CPK23 (calcium-dependent protein kinase 23); calmodulin-dependent protein kinase/ kinase chr4:2405404-2408491 REVERSE
AT4G12720	282	NUDT7, GFG1, AtNUDT7 AtNUDT7 (ARABIDOPSIDIS THALIANA NUDIX HYDROLASE HOMOLOG 7); hydrolase/ nucleoside-diphosphatase chr4:7487713-7489554 FORWARD
AT5G14930	239	GENE101, SAG101 SAG101 (SENESCENCE-ASSOCIATED GENE 101) chr5:4828757-4830168 FORWARD
AT5G48870	88	SAD1 SAD1 (SUPERSENSITIVE TO ABA AND DROUGHT 1) chr5:19830633-19831588 FORWARD
AT5G62480	240	GST14, GST14B, ATGSTU9 ATGSTU9 (GLUTATHIONE S-TRANSFERASE TAU 9); glutathione transferase chr5:25106001-25106792 REVERSE

genome as proteins that are similar to AvrE proteins using PLS alignment-free bioinformatics method. Furthermore, we used information from gene expression data, and metabolomics pathways to highlight 16 proteins for further investigations. This study suggests that using different PLS alignment-free bioinformatics methods com-

bined with information from available databases offers a promising approach to predict proteins that are similar to AvrE proteins. Such approaches may address a challenging issue of effector target discovery.

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Full Length Research Paper

Effect of paclobutrazol on three different aquatic macrophytes under *in vitro* monoculture or polyculture conditions

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Three aquatic plants, coontail (*Ceratophyllum demersum* L.), hydrilla [*Hydrilla verticillata* (L. f.) Royle] and giant duckweed [*Spirodela polyrhiza* (L.) Schleiden], were successfully surface sterilized and cultured on liquid basal MS (Murashige and Skoog, 1962) medium under aseptic conditions. Shoot explants obtained from these plants were transferred to basal MS medium supplemented with 0, 0.25 and 0.5 mg/l paclobutrazol (PBZ) under *in vitro* monoculture or polyculture conditions. There were some differences in the patterns of fresh weight increases of the three aquatic plants under monoculture and polyculture conditions. Among the three macrophytes studied, coontail was the most sensitive to 0.25 or 0.5 mg/l PBZ as its fresh weights did not increase at these PBZ concentrations during eight weeks under both monoculture and polyculture conditions. Giant duckweed were relatively more sensitive than hydrilla in response to addition of PBZ to the growth medium under both monoculture or polyculture conditions suggesting that PBZ might not be an effective aquatic pest control agent for hydrilla. The dominance of giant duckweed over hydrilla was effectively overturned with the addition of 0.5 mg/l PBZ to the polyculture medium.

Key words: Aquatic plants, coontail, giant duckweed, hydrilla, plant growth retardant.

INTRODUCTION

Coontail (*Ceratophyllum demersum* L.) and hydrilla [*Hydrilla verticillata* (L. f.) Royle] are submergent perennial fresh-water plants that are well known for being used as decoration and oxygen production in a fish aquarium. The giant duckweed [*Spirodela polyrhiza* (L.) Schleiden] is a free-floating macrophyte found in natural fresh waters. Under natural conditions, these plants provide many beneficial ecological services, but they could also be problematic weedy species; particularly, hydrilla is known to be highly invasive and difficult to control (Sousa, 2011). More research is needed to help better management of these aquatic weedy plants in the natural environments. Gibberellin synthesis inhibitors including paclobutrazol (PBZ) have been suggested as promising herbicides for limiting excessive stem growth of submerged aquatic weeds without reducing plant viability

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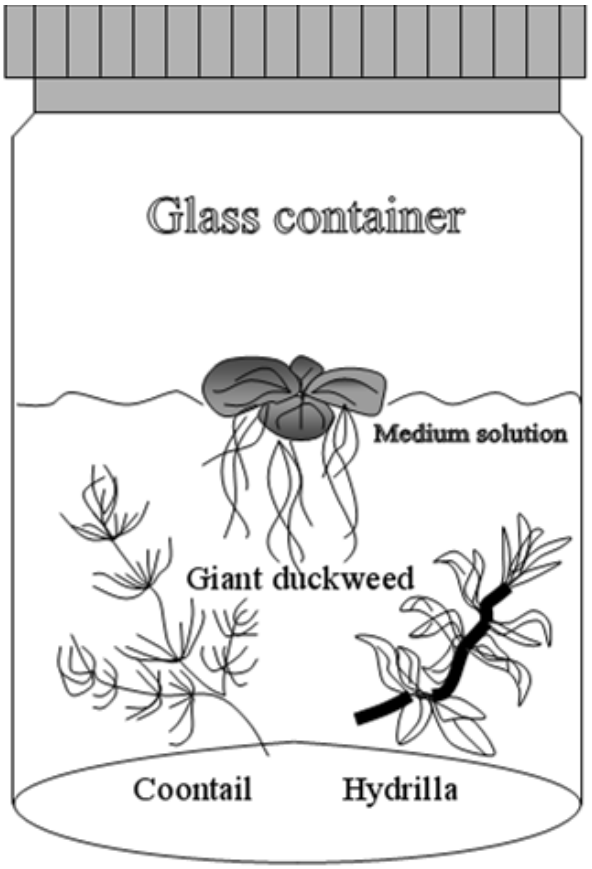

Figure 1. A schematic drawing showing the concept of *in vitro* polyculture of three aquatic macrophytes.

(Lembi and Chand, 1992; Van, 1988). Many factors including rainfall and degradative activities of micro-organisms could complicate the interpretation of the results obtained from trials of these herbicides under natural conditions. Plant tissue culture has been used in various investigations with a range of objectives including micropropagation (Thorpe, 2007) and application to aid assessment of some ecological questions (Hughes, 1981; Kauth and Kane, 2009).

The main objective of the present study was to compare growth (fresh weight changes) of the three different aquatic macrophytes when cultured singly (*in vitro* monoculture) in the absence or presence of PBZ as this had not been investigated before under highly controllable and aseptic environmental conditions. In addition, the relative sensitivity (differences in growth or fresh weight changes) of these three plants to different concentrations of PBZ was also investigated under *in vitro* polyculture conditions (Figure 1) as response of aquatic macrophytes with different genetic propensities placed within the same environment to PBZ was not known. This was the first study to grow different types of plants in the same culture vessel *in vitro*; although the principles and practice of polyculture of crops in cultivated fields are not new (Geno and Geno, 2001).

MATERIALS AND METHODS

Establishment of *in vitro* stock plant cultures

Coontail and hydrilla plants were purchased from the Chatuchak market in Bangkok, Thailand while giant duckweed plants were collected from natural waters in the Trat province, Thailand. In the laboratory, the plants were washed with clean running tap water for 15 min to remove unwanted matters. Then, all the leaves were removed from the long stem before it was cut into small pieces as explants (each about 3 cm length with 3 nodes) for establishing *in vitro* stock cultures. All these explants were rinsed briefly 3 times in distilled water. Surface sterilization began by immersing 50 pieces of explants from each species in 15% (v/v) Clorox (a commercial bleach solution containing 5.25%, w/w, sodium hypochlorite as available chlorine) to which 2 to 3 drops of Tween-20 were added and washed in distilled water and when required, immersed in Clorox again as shown in Table 1. After surface sterilization, nodal explants of coontail, hydrilla or whole giant duckweed plants were cultured separately in culture vessels containing basal MS medium (Murashige and Skoog, 1962) without addition of agar or any plant growth regulator for 4 weeks to establish stock plant cultures. All the culture media used in this study were adjusted to pH 5.7 before they were autoclaved at 121°C and 15 psi for 20 min.

Glass containers (4.5 cm diameter × 8.5 cm height) were used as culture vessels. All cultures were kept in a growth room at 25±2°C under 16 h of illumination with white fluorescent lamps (47.31 μmol m^{-2} s^{-1} light intensity) and 8 h of darkness.

Monoculture and polyculture experiments

The new shoots developed from coontail and hydrilla nodal explants of the stock cultures were excised into 3 cm long pieces. The three macrophytes (1 excised shoot of coontail, hydrilla or 3 giant duckweed plants) from their respective stock cultures were placed separately in a culture vessel containing 35 ml of basal MS medium supplemented with 0, 0.25 or 0.5 mg/l PBZ (monoculture experiment) or all placed together in a culture vessel (polyculture experiment) containing 100 ml of basal MS medium supplemented with the same range of PBZ concentrations. There were four replicate culture vessels for each concentration of PBZ.

Data analysis

Fresh weights of the plant materials during culture were determined at 0, 2, 4, 6 and 8 weeks of the monoculture and polyculture experiments. Mean percentages of fresh weight of the three macrophytes were analysed and 1-way ANOVA was first performed at the significance level of $P < 0.05$. After this, when appropriate, Duncan comparison of means was carried out at $P < 0.05$.

RESULTS AND DISCUSSION

Surface sterilization and preliminary observations

An objective of this study was to use plant tissue culture techniques to aid the study of the effects of PBZ on the three selected aquatic plants and not to micropropagate them. There was no prior report on *in vitro* culture of coontail and hydrilla and the previous tissue culture protocol of giant duckweed (Li et al., 2004) was not appropriate for the present purpose. Therefore, some preliminary tissue culture investigations were necessary.

Table 1. Time and sequence of steps (from top to bottom) for surface sterilization of shoot explants of the three aquatic macrophytes to establish *in vitro* stock cultures.

Steps Involved	Time for each step (min)		
	Coontail	Hydrilla	Giant duckweed
15% (v/v) clorox	2	2	1.30
Distilled water	3	3	-
15% (v/v) clorox	2	2	-
Distilled water	3	3	-
15% (v/v) clorox	2	2	-
Distilled water	3	3	3
Distilled water	3	3	3
Distilled water	3	3	3

In particular, surface-sterilization of explants from the three aquatic plants was a challenging problem. It was found that the plant parts of the three macrophytes changed into transparent or white-pale structures following surface sterilization based on a protocol previously used in our laboratory (Bodhipadma et al., 2010). Thus, to minimize this from occurring, the time of immersing the explants in a bleach solution was reduced and this step had to be repeated several times, particularly as far as coontail and hydrilla were concerned (Table 1). In addition, it was also important to remove all the leaves from the explants.

Once the stock plants were free of any contamination, surface-sterilization of experimental materials taken from the stock plants was unnecessary anymore. All three macrophytes when cultured separately were able to grow on liquid basal MS medium with 3% sucrose.

Monoculture experiment

Under monoculture conditions in the absence or presence of PBZ, there were little or no changes in the fresh weights of the three macrophytes in the first two weeks (Figure 2A). Giant duckweed exhibited a different pattern of increase in fresh weight from those of hydrilla and coontail. The main period of increase in the fresh weight of giant duckweed was between weeks 2 and 4 before the rate of increase started to slow down. In contrast, that of hydrilla and coontail was between weeks 6 and 8. In response to medium supplemented with 0.25 mg/l PBZ, the main period of increase in the fresh weight of giant duckweed was delayed to between weeks 4 and 6 while that of hydrilla was not changed compared to culture in the absence of PBZ (Figure 2B). In response to medium supplemented with 0.5 mg/l PBZ, both giant duckweed and hydrilla exhibited close to linear increases in their fresh weights between weeks 2 to 8 and weeks 4 to 8, respectively (Figure 2C). Among the three macrophytes, coontail was most sensitive to PBZ as the growth of coontail was inhibited in the medium supplemented with 0.25 or 0.5 mg/l PBZ (Figure 2B and C). Giant duckweed was more sensitive to 0.25 mg/l PBZ than hydrilla. The fresh weight of giant duckweed after 8 weeks of culture in the absence of PBZ was more than double than in the presence of 0.25 mg/l PBZ (compare Figure 2A and B). By contrast, the fresh weight of hydrilla at the end of experiment (8 weeks of culture) was the same in the absence or presence of 0.25 mg/l PBZ (Figure 2B).

In response to 0.5 mg/l PBZ, the fresh weights of both giant duckweed and hydrilla were lower than those at 0.25 mg/l PBZ (Figure 2C).

Polyculture experiment

Under polyculture conditions without any added plant growth regulator, the most notable change was that the fresh weight increase of giant duckweed was already evident at week 2 and the increase continued almost linearly throughout the experiment (Figure 2D). In contrast, the increase in the fresh weight of hydrilla or coontail was only evident from week 4 and then leveled off after week 6 (Figure 2D). This suggested that giant duckweed was more successful than hydrilla and coontail when all three species were under *in vitro* polyculture conditions. This might be related to the observation that giant duckweed plants exhibited an inconspicuous adjustment (lag) period following subculture onto fresh medium than the other two plants. This could give giant duckweed a competitive advantage under *in vitro* polyculture over the other two aquatic plants in the present study. The fresh weight increases of giant duckweed, when cultured under polyculture conditions and in the presence of 0.25 mg/l PBZ, were severely curtailed (Figure 2E) compared to polyculture in the absence of PBZ (Figure 2D). Most of the fresh weight increase of giant duckweed occurred between weeks 2 and 4 in the absence of any plant growth regulator (Figure 2D) but that in the presence of 0.25 mg/l PBZ occurred between 4 and 6 weeks instead (Figure 2E).

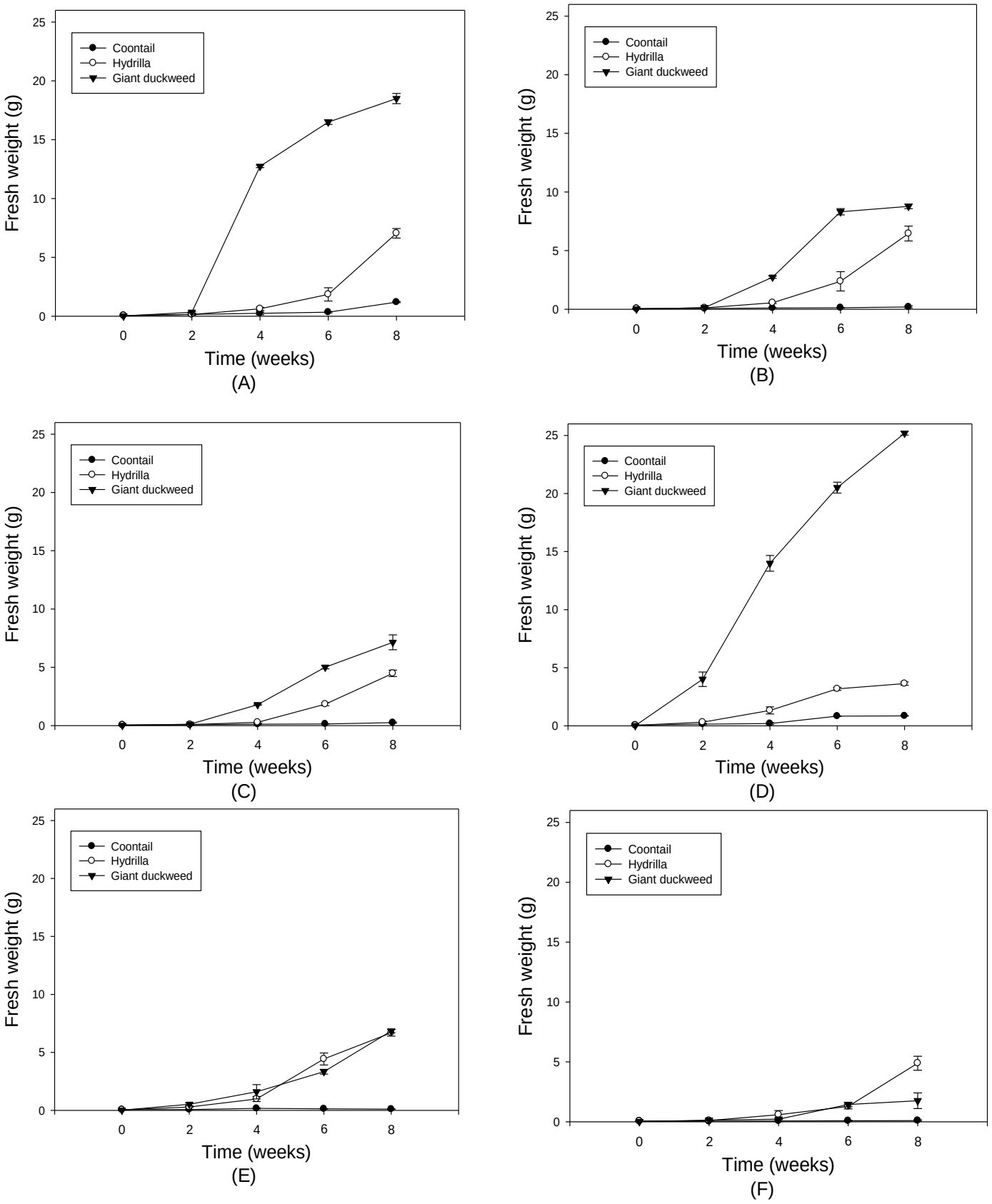

Figure 2. Fresh weight changes during *in vitro* monoculture (A to C) and during *in vitro* polyculture (D to F) of three aquatic plants on basal MS medium supplemented with 0, 0.25 or 0.5 mg/l of paclobutrazol, respectively.

Under polyculture conditions, the fresh weight increases exhibited by hydrilla were similar in the presence or absence of 0.25 mg/l PBZ in MS medium (Figure 2D and E). When the concentration of PBZ was increased to 0.5 mg/l and under polyculture conditions, there were no differences in fresh weights of giant duckweed and hydrilla in the first 6 weeks of culture (Figure 2F). However, by the end of the experiment (8 weeks), hydrilla had a higher fresh weight than giant duckweed (Figure 2F). This was also the only treatment among all the experiments in which the fresh weight of giant duckweed was less than that of hydrilla. Similar to the under monoculture conditions, the fresh weight of coontail did not increase throughout the experiment under polyculture conditions in the presence of 0.25 or 0.5 mg/l PBZ.

From the results obtained, it became clear that both giant duckweed and coontail were sensitive to 0.25 and 0.5 mg/l PBZ but hydrilla was relatively insensitive to these two concentrations of PBZ under polyculture conditions. The patterns in the changes of fresh weights of the three macrophytes under monoculture or polyculture conditions in the absence or presence of PBZ appeared to be different. However, the monoculture and polyculture conditions studied here were probably not ideal to permit direct comparison of the performance of the three macrophytes under monoculture and polyculture conditions as the volume of culture medium in the polyculture experiment was three times that of the monoculture experiment taking into consideration that all three plants were cultured together compared to when each plant was cultured individually, respectively. Nevertheless, it would seem that coontail was most sensitive while hydrilla was relatively insensitive to 0.25 or 5 mg/l PBZ under both monoculture and polyculture conditions. This is consistent with other studies showing that sensitivity to applied PBZ concentrations is dependent on the plant species (Million et al., 2002). Furthermore, the effect of PBZ on giant duckweed might be more severe under polyculture than monoculture conditions. The present results also broadly support the potential use of plant growth retardants such as PBZ and others to aid control and management of aquatic macrophytes (Chand and Lembi, 1994; Fox et al., 1994; van and Vandiver, 1994).

In conclusion, these findings from the present tissue culture studies under highly controllable environmental and aseptic conditions have implications for PBZ application in the control of different aquatic macrophytes being co-present under natural conditions.

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UPCOMING CONFERENCES

2nd Conference of Cereal Biotechnology and Breeding (CBB2), Budapest, Hungary, 5 Nov 2013

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014

ICBEE 2014 : International Conference on Biotechnology and Environment Engineering

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