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IN VITRO CALLUS PRODUCTION IN THE MEDICINAL, RARE AND ENDANGERED SPECIES *ECBALLIUM ELATERIUM* (L.) A. RICHARD

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Ecballium elaterium L. Richard, a rare and endangered species for Romanian Flora, grows in Dobrogea, especially on the coastal area of the Black Sea. It was used from the ancient time in traditional medicine as a resource of bioactive compounds with therapeutic effects for the treatment of sinusitis, nephrites, rheumatism, jaundice, etc. The fruits and the roots contain elaterines (cucurbitacine), compounds derived from tetracyclic triterpenoids with cytotoxic and antitumor properties. The aim of our study was to establish an efficient protocol for production of *Ecballium elaterium* callus for exploitation of the potential of secondary metabolites biosynthesis. The reactivity of some types of tissues and the effect of different plant growth regulators (PGR) were analysed for the efficiency of callus production.

Fragments of somatic tissues from aseptic germinated seedlings (hypocotyl, stem fragment, root and shoot apex) were used as source of explants. The cellular growth rate, cell viability assay based on TTC cytohistochemical method were determined.

The proper explants were roots and the medium for the best cell proliferation was Murashige-Skoog medium added with Dicamba.

Further studies will accomplish the evaluation of antimicrobial effect of produced callus and its secondary metabolites content.

Keywords: *Ecballium elaterium*, callus induction, growth rate, TTC test.

INTRODUCTION

Ecballium elaterium (L.) A. Richard is a rare and endangered species mentioned in different works concerning the conservative status of the Romanian Flora (Oltean *et al*., 1994; Oprea, 2005; Dihoru & Negrean, 2009). It is a perennial, thermophile species, flowering from April to November. As a medicinal remedy, it was used since 2000 years ago. It is also called squirting cucumber, a plant from the *Cucurbitaceae* family, and grows on the Mediterranean Coast and in Romania on the coastal area of the Black Sea in sandy or rocky places, in ruderal places or nearby an anthropic zone.

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This plant has a thick root, bushy growth habit, ramified stems, yellow-green petiolate leaves cordate or triangle shape. Flowers are monoecious. The fruits are green fleshy rounded like melon, but very small (about 4-5 cm) and covered by bristles, having an explosive opening due to the osmotic pressure at maturity scattering seeds and juice. (Figure 1a, b).The spreading of species is unique because the seeds are rejected at 5-10 m and from this point of view it is considered an exceptional didactic plant material (Dihoru & Negrean, 2009).

Figure 1. General view of *Ecballium elaterium* (a); The detaching of stem and eject of seeds and juice (mucilaginous liquid) due to osmotic pressure (b).

The plant contains remedies for the treatment of varicose veins, has antibleeding properties, especially for nasal bleedings, in the treatment of rhinosinusites (Shabbar 2006), of haemorrhoids and in jaundice. It has emetic, anti-inflammatory, purgative and analgesic effects.

The fruit contains a mixture of compounds such as elaterin or cucurbitacine (B, D, E, I, L, R) and also some phenols and glycosylated compounds (Jaradat *et al*., 2012), phytomelin. The cucurbitacines A, E, D, I- tetracyclic triterpenoids have cytotoxic and antitumor properties (Attard and Cushchieri, 2004). Previous studies described the dependency of cucurbitacine content produced from *Ecballium elaterium* fruits, stems and leaves with the active growth and season temperature (Attard and Scicluna-Spiteri, 2003).

The aim of our work is to elaborate an efficient method for the production of high proliferative callus with a high capacity for secondary metabolites synthesis. In the next step, we intend to initiate analyses regarding antimicrobial activity of ethanolic extracts from *Ecballium elaterium*. These studies are justified, *Ecballium elaterium* being an endangered rare species and a medicinal one. Biotechnological methods based on the in *vitro* systems proved to be very useful for its conservation.

MATERIAL AND METHODS

Plant material

The seeds were collected in November 2013 from the coastal area close to Constanta harbour. The mature seeds were surface sterilized with 70° alcohol, then immersed in mercuric chloride 0.1% for 5 minutes, next in Domestos for 4 minutes. They were aseptically germinated on Murashige-Skoog (1962) medium without PGR, by two weeks.

For callus induction, different nutritive media based on MS formula (Murashige & Skoog, 1962) added with 30g/L sucrose, B5 vitamins (Gamborg, 1968), 8g/L agar, pH adjusted at 5.8 and supplemented with different combinations of PGR such as naphthalene acetic acid -NAA, benzyl amino-purine- BAP, Dicamba-3,6 dichlor-2 methoxybenzoic acid, indolyl butyric acid -IBA, 2,4-D phenoxyacetic acid and kinetin- K, were tested (Table 1).

Evaluation of growth rate

Callus induction was made on different experimental variants (M2-M5) characterized by auxin dominance and maintained by repeated subculture on fresh media with the same composition at every 6 weeks. The cells growth rate and viability were evaluated.

For establishing the proper protocol of callus production, about 1.75 g from each experimental variant was repeatedly cultivated on the fresh media M2 to M5 and the relative growth rate was recorded after 2, 4, 6 weeks.

The TTC histochemical staining of callus for the evaluation of cell viability and photonic microscope analysis

For the porpose of testing cell viability the calli cultures were maintained for 6 months and repeatedly cultivated once per month. Histochemical staining with TTC was achieved at 3 weeks after callus transfer on the fresh medium.

TTC staining method (Towil and Mazur, 1975) is based on reduction in mitochondria by the tetrazolium salt accepting electrons from the electron-transport chain or the succinate-dehydrogenases involved in cell respiration and formed formazan, a red compound.

For photonic microscope analyses the fresh samples were prepared by the squash method, staininig in 0.1% TTC solution and visualised at Zeiss microscope connected to a video camera AxioCamICc3 and a computer programme for image Axio Vision Release 4.8.2. The viable cells were stained in red.

Tetrazolium assay

4 samples which represented 2 replicates (of two types of calli cultivated on M2 and M5 media) were analysed.

Each fragment of tissue was incubated in 5mL TTC 0.1% at room temperature at 25°C for 20h. For TTC removal, the tissue was washed with distilled water and then submerged in 5 mL 95% ethanol and incubated in the water bath at 85°C for 30 minutes until total discolouration.

The extract was assessed spectrophotometrically at 485 nm against blank. The formazan contain was expressed in mg/mL callus extract. This method allows to quantitative evaluate the cells viability.

RESULTS AND DISCUSSION

For initiating the experiments the inoculum was represented by explants from plants obtained by aseptic germination of seeds, the rate of *in vitro* germination being about 90% (Figure 2).

Figure 2. *Ecballium elaterium* aseptically germinated seeds on MS medium (1962) without PGR.

Evaluation of *in vitro* response

In vitro reactivity of *Ecballium elaterium* is different concerning the type of inoculum used, the plant growth regulators and their ratio from each cultivation medium (Table 1, Figure 3).

In the M 1 variant (supplemented with NAA and BAP) the response was different as function of the explant type, from the roots fragment a whitish-yellow nonregenerative callus was induced; the culture of apex conducted to regeneration of shoots by direct morphogenesis with callus at the basal end or shoots. The repeated cultivation for a long time conducted to callus necrosis.

The singular presence of auxins 2.4-D and Dicamba (in the M2 and M3 variants) allowed the induction of a brittle, nonregenerative callus from hypocotyl and roots explants. The reactivity of the root fragments was optimal.

The apex and the stem fragments also conducted to shoot development from meristems and formation of basal callus.

The M4 variant added with 2.4-D, IBA and K produced callus from root and hypocotyl fragments. The apex cultivation on M4 variant conducted also to shoots 33 development from pre-existent meristem.

In our previous experiments regarding *in vitro* cultivation of *Ecballium elaterium* (Voichita and Brezeanu, 2005), we observed that the shoot regeneration was stimulated by the presence of BAP, K and IAA or NAA, the rate of regeneration being rather limited. Rooting process was amplified by the singular presence or the combination of IBA and IAA; the nonmorphogenic callus was obtained with a small rate in the presence of BAP and IAA.

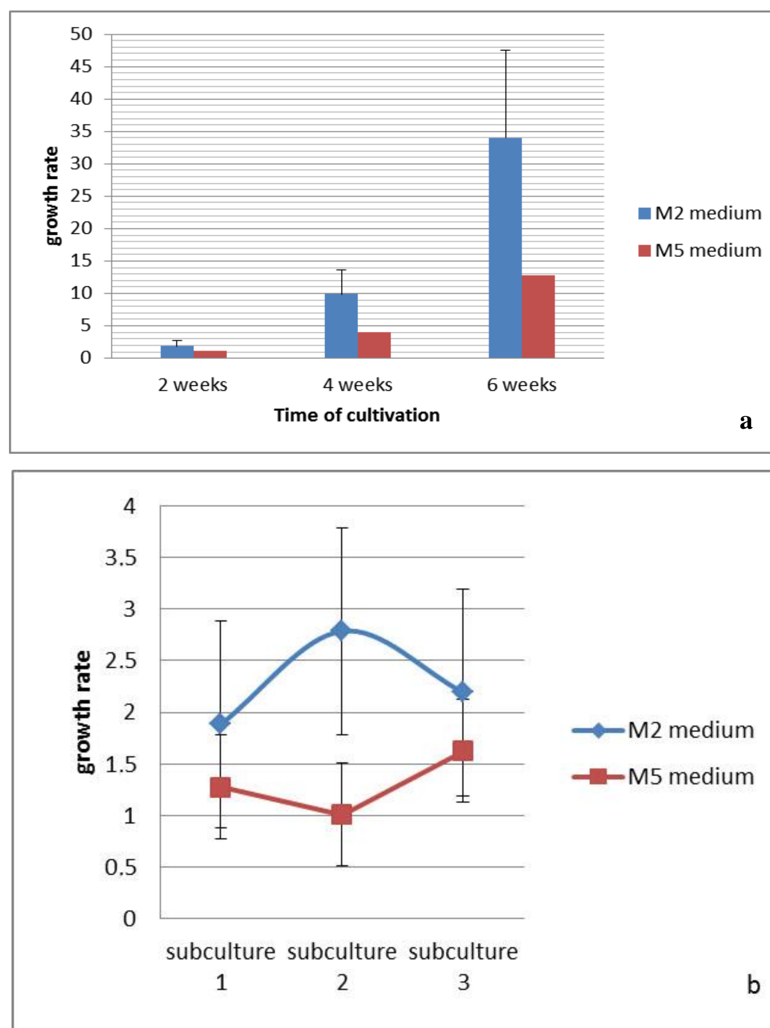

Figure 3. Variation in time of growth rate on M2 and M5 medium (a); Variation of growth rate with number of subculture (b).

Table 1

In vitro reactivity of different types of *explants* cultivated on MS media added with combinations of PGR

Explant	Medium variant	PGR(µM)						*in vitro* response	Reactivity
		Auxin				Cytokinin			
		Dicamba	2.4-D	IBA	NAA	BAP	K		
root	M1	-	-	-	0.54	4.4	-	Whitish-yellow granular, nonregenerative callus	++
hypocotyl								-	-
Apex+ stem fragment								Direct morphogenesis, basal callus	++
root	M2	2.26	-	-	-	-	-	Nonregenerative white friable callus,	+++
hypocotyl								Whitish-green friable, nonregenerative callus	++
Apex+ stem fragment								Shoots, basal callus	++
root	M3	-	2.26	-	-	-	-	White friable nonregenerative callus	+
hypocotyl								Whitish-green friable nonregenerative callus	++
Apex+ stem fragment								Whitish-green friable nonregenerative callus	++
root	M4	-	4.52	4.9	-	-	1	White friable nonregenerativ callus	++
hypocotyl								Whitish-green friable nonregenerativ callus	++
Apex+ stem fragment								Shoots, basal callus	++
root	M5	4.52	-	4.9	-	-	1	White friable nonregenerative callus	+++
hypocotyl								Whitish-green friable nonregenerative callus	++
Apex+ stem fragment								Shoots, basal callus	++

+ weak reactivity
++ moderate reactivity
+++ high reactivity

Dicamba = 3,6 dichloro-2 methoxybenzoic acid 2,4D = dichlorophenoxyacetic acid
NAA = naphthylacetic acid K=kinetin IBA= indolylbutyric acid
BAP= Benzyl aminopurin

Our results suggest that the production of nonregenerative callus with a good rate of proliferation can be efficiently accomplished from root and hypocotyl explants on media culture added with dicamba (M2 and M5). The roots and hypocotyl explants cultivated on M2 variant are recommended for obtaining a large callus mass, useful for cryoconservation and also for establishing a bank tissue.

The proliferative capacity of calli cultivated on M2 and M5 media was estimated through the variation of growth rate assay correlated to the subculture stage (Fig. 3).

The callus cultivated on M2 medium had an increased proliferation; the maximum value was registered in the second subculture and then decreased until the next culture (Fig. 3a). The evolution of the callus cultivated on M5 medium is in reverse correlation with the callus cultivated on M2 medium, the growth rate value decreased between the first and the second culture and then increased.

The proliferative capacity of the callus cultivated on M2 medium is higher comparative with callus cultivated on M5 medium.

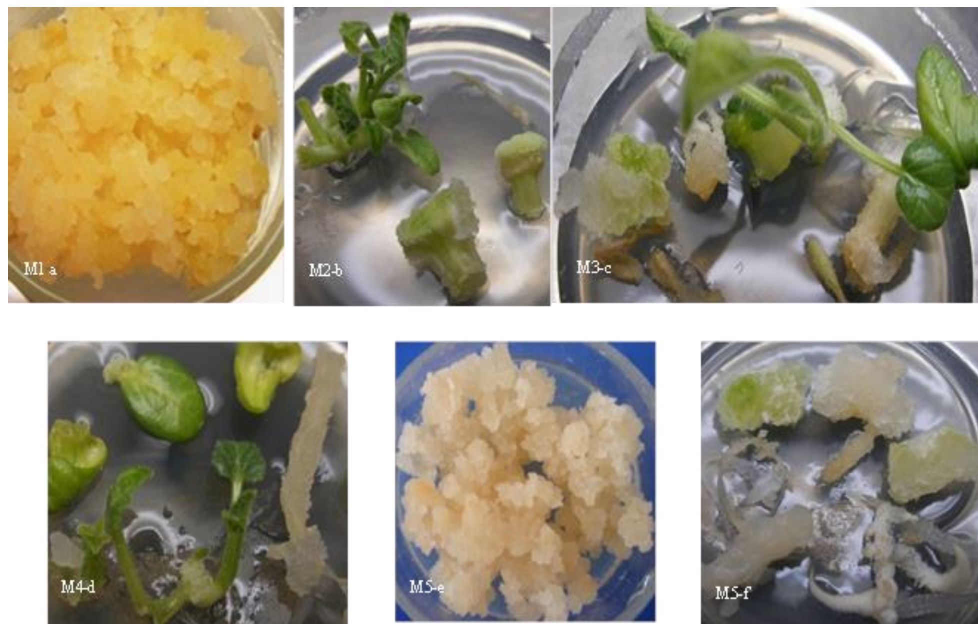

Figure 4. Different *in vitro* cultures on M1-M5 media.

a) Whitish-yellow granular callus derived from roots, cultivated on basal medium MS supplemented with NAA and BAP;

b) Regenerated apex and shoot stem fragments with friable nonregenerative white–green callus on the basal side, cultivated on MS medium supplemented with Dicamba;

c) Friable white-green callus with origin in hypocotyl fragments, cultivated on MS basal medium supplemented with 2.5 D;

d) Shoots regenerated from apex fragments; weak reactivity of cotyledonexplants;

e) Vigorous proliferation of callus differentiated from root inocula;

f) White-green callus derived from hypocotyl and white callus derived from roots fragments.

Toker *et al*., (2003) have found a correlation between *in vitro* reactivity of the explants cultivated on different experimental variants and the callus content in cucurbitacins. Thus, the stem fragments produced callus with a good rate of proliferation and the highest content in cucurbitacin on the medium supplemented with BA and NAA.

Photonic microscopy analysis of calli

The peculiarities of callus produced from root fragments cultivated on MS medium with NAA and BAP

The cells presented different shapes: oval, reniform, elongated, with small vacuoles; spherical, white-yellow or with reduced cytoplasmic content and some starch grains, cells with voluminous vacuoles without content in different stages of senescence (Figure 5). Dhed'a *et al.* (1991) considered that the elongated cells and those with many vacuoles originated from the non meristematic tissues (specialized) and the oval cells with many starch grains had the origin in meristematic cells. We observed also cells in active division. These cells had no capacity to redifferentiate, remaining to undifferentiated callus cell stage.

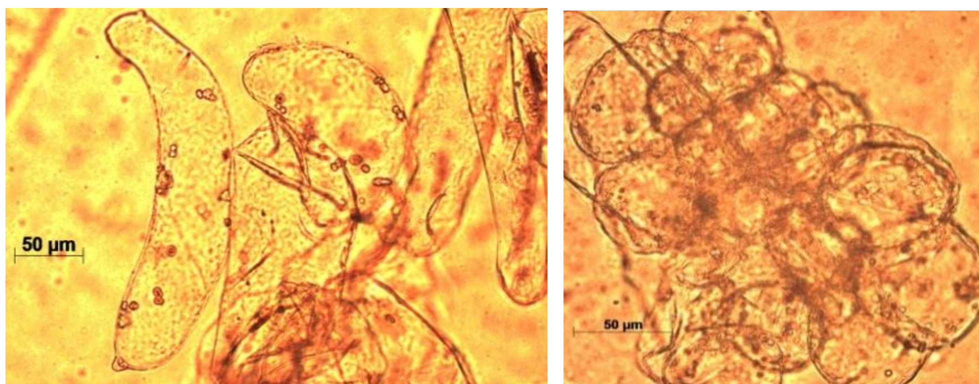

Figure 5. Callus cells produced through culture on M1 medium with origin in root fragments.

Histochemical evaluation of callus tissue viability using TTC staining

We evaluated callus fragments cultured on MS basal medium supplemented with Dicamba (M2) and Dicamba, IBA, K (M5) suitable for the proliferation of callus. The cytological analyses of proliferative callus revealed that the tissue presents general characteristics, with some peculiarities.

Thus, this tissue showed a heterogenic, polymorphic cellular aggregation, formed from nonspecialized parenchymatic cells, with frequently meristematic cells with a continuous growing and division, randomly spread or as aggregation, nodular-like structure but also cells differentiated in tracheary elements. The differentiation of tracheary elements, frequently reported in *in vitro* cell culture is an interesting subject of study because it represents a pattern for cytodifferentiation researches

and, on the other hand, a typical example for plant apoptosis. Through the extension of the duration, especially in' long-term' cultures, these cells were characterized by reduced cytoplasmic content and a growing vacuom which finally extended in all cell, aspect typical for the senescence process.

The photonic microscopy analyses of callus cultivated on M1 medium revealed elongated cells nonspecialized, parenchymatic-like, typical callusal cells and smaller cells, in active divisions, with formazan accumulations (Fig. 6a).

In the case of M5 variant, we also observed the presence of many tracheary elements and many viable cells stained in red with formazan.

The tracheary structures (Fig. 6b) redifferentiated from callusal cells, having a role in the nutrients transport in the callus mass. The auxins 2, 4 D and Dicamba stimulated the dedifferentiation process and also the cell division and the proliferation of the callus mass.

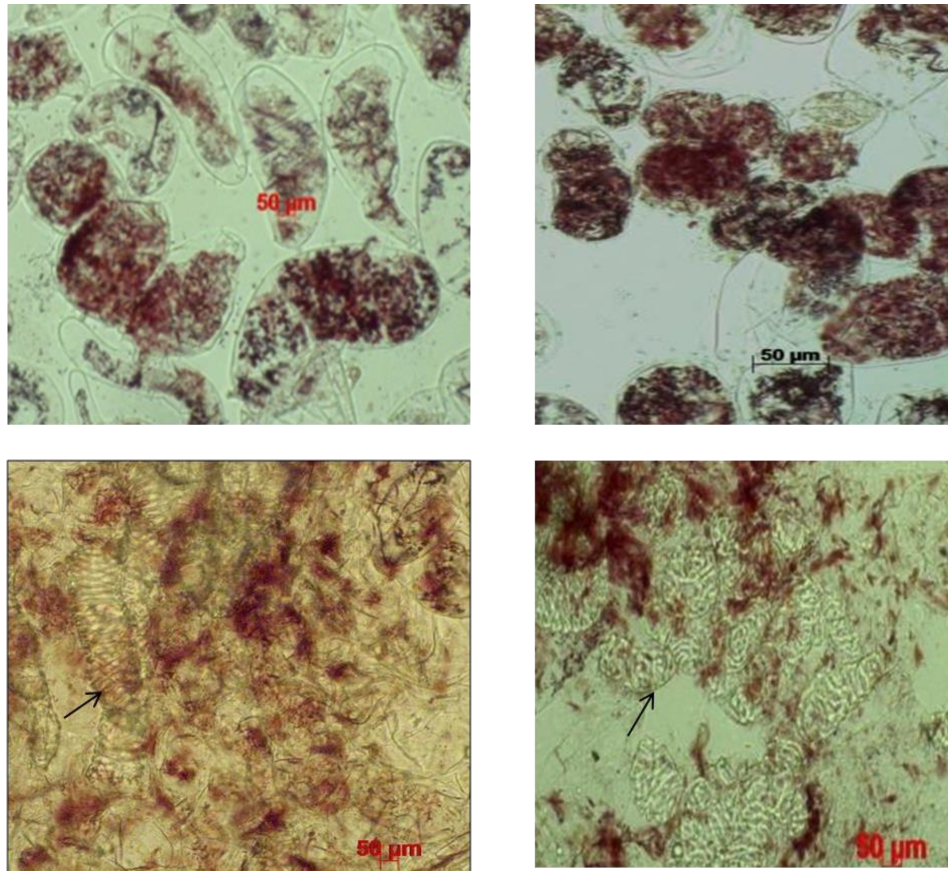

Figure 6. The callus cells cultivated on MS medium supplemented with Dicamba (M2-a) and basal medium MS supplemented with Dicamba, IBA and K (M5-b), histochemical staining with TTC- revealed the presence of tracheary elements aggregates in packages.

Spectrophotometrical assay of formazan extract from viable callus cultivated on MS basal medium with Dicamba (M2) and MS basal medium with Dicamba, IBA and K (M5)

For the evaluation of cell viability, the formazan content was spectrophotometrically determined in the calli.

The results suggest the positive effect on MS medium supplemented with Dicamba, IBA, and K (M5) which ensured the maintenance of a high viability of cells comparatively with M2 medium. This result does not offer information regarding the proliferative capacity of calli cultured on both variant media.

Despite of the high level of formazan measured in the callus cultivated on M5 medium, the capacity of multiplication is in inverse correlation with the viability of the cells. These results might be due to the large heterogeneity of the callused cell mass.

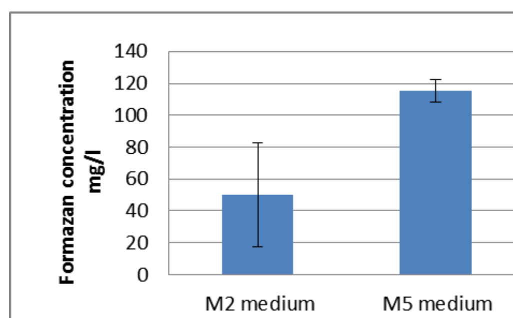

Figure 7. The formazan content in the calli cultured on M2 medium (Murashige–Skoog supplemented with Dicamba) and M5 medium (Murashige–Skoog supplemented with Dicamba, IBA, and K).

The callus produced using this protocol will be further evaluated concerning the antimicrobial potential and the content in secondary metabolites with application in pharmacy.

CONCLUSIONS

Ecballium elaterium was *in vitro* reactive, viable callus with good proliferation rate can be produced for biotechnological purpose.

Root and hypocotyl tissues are the appropriate source of inoculum for the initiation of a proliferative cell culture.

Medium MS added with Dicamba had a stimulatory effect on cell proliferation, being optimal for the production of large mass of friable, non regenerative callus.

The media M2 and M5 are suitable also for micropropagated shoots in conservative purpose and repopulation of endangered areas.

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EVALUATION OF INSECTICIDAL POTENTIAL OF ROOT EXTRACTS OF *RAUVOLFIA TETRAPHYLLA* AGAINST *MUSCA DOMESTICA*

R. THAPAR KAPOOR[1]

Musca domestica L. is a well-known cosmopolitan pest that causes irritation, spoilage of food and acts as a vector for many pathogenic organisms. The control of housefly mainly depends on the application of chemical insecticides, but the use of chemical insecticides is not only detrimental to environment, but its long term utilization also leads to development of resistance among insects. Biological control of housefly by the use of natural plant products is safer than synthetic insecticides on account of their rapid environmental degradation and no toxic impact on human-beings and animals. In the present study, root extracts of *R. tetraphylla* have been screened for their larvicidal activity against *Musca domestica*. The phytochemical analysis of methanolic root extracts of *R. tetraphylla* indicated that it contains several biologically active compounds such as quinones, terpenoids, phenolic compounds, saponins, tannins and flavonoids, etc. The inhibitory impact of the root extracts of *R. tetraphylla* on the *Musca domestica* was in order: 200 ppm > 150 ppm > 100 ppm > 50 ppm > control. The present investigation indicates that methanolic root extracts of *Rauvolfia tetraphylla* can be utilized in the development of bio-insecticides to control the population of *Musca domestica* as these natural products are safer and an eco-friendly alternative to the synthetic pesticides and it seems to be a suitable candidate for commercialization.

Keywords: Insecticidal potential; *Musca domestica*; phytochemicals; *Rauvolfia tetraphylla*.

INTRODUCTION

Musca domestica L., commonly known as housefly, is a well-known cosmopolitan pest that causes irritation, spoilage of food and acts as a vector for many pathogenic organisms such as bacteria, virus, protozoa and metazoan parasites (Barin *et al.* 2010). Housefly feed and breed on decaying matter, human waste and food material and they are known to transmit approximately one hundred human and animal diseases and are responsible for bacterial, protozoan, helmenthic and viral infections (Sasaki *et al.* 2000; Malik *et al.* 2007). The common housefly has no mouth and it has an eating tube through which it vomits a drop of fluid from its stomach and deposits it on its intended food material. This fluid is then sucked up along with the nutrients it has dissolved, leaving behind untold numbers of

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germs. According to the reports of WHO (1991), housefly may also transmit cholera and typhoid diseases. Several viral infections are also transmitted by domestic houseflies include polio, coxsackievirus and enterovirus (Graczyk *et al.* 2001). A wide variety of chemicals have been used to control houseflies such as chlorinated hydrocarbons, organophosphates, carbamates and pyrethroids (Shono *et al.* 2004). Unfortunately, house fly population can rapidly evolve resistance to insecticides, limiting our ability to control them and due to this reason resistance in house flies has become a global problem. The inappropriate application of chemical insecticides has created a number of ecological problems such as resistance in houseflies (Acevedo *et al.* 2009), ecological imbalance, side effects to non-target species and long-lived residues in the environment (Kristensen and Jespersen, 2003). Increased public awareness on the negative impact of conventional insecticides on human - beings and environmental health has increased the need for development of effective alternative strategies for the management of house fly. Several efforts are being made to replace the synthetic pesticides with eco-friendly alternatives (Begum *et al.* 2011) and natural products are well known to have a range of useful biological properties against insect pests (Arthur, 1996). Screening of plant extracts for evaluating their insecticidal efficacy has gained special attention worldwide (Singh and Singh, 1990). Pest control by using plant products is a promising approach (Isman, 2006; Bakkali *et al.* 2008) as the plant extracts are usually pest specific, easily biodegradable, eco-friendly and risk free natural products (Bowers, 1992). There are several reports that plant-derived products are highly effective against insecticide-resistant insect pests (Ahn *et al.* 1997).

Many plants have been reported about their potential for insecticidal action on larvae and on the adults of houseflies via crude extracts or active compounds present in different parts of the plants (Schmutterer, 1990). Ahmed *et al*. (1981) reported incomplete emergence, deformity and death of the imago from maggots fed with the seeds of *Peganum harmala*. The leaves of *Acalypha indica, Carica papaya* and *Santalum album* have been used as insecticidal agents for the control of mosquito (Sccoy and Smith, 1983). The seed as well as foliar extracts of several plants have been reported to have toxic and potent growth reducing activity to insects (Champagne, 1993). Brousseau *et al*. (1999) reported the insecticidal activities of *G. simplifolia* extracts and insecticidal activities of *Z. xantholoides* on storage insect pests have been reported by Owusu *et al*. (2007). Zebitz (1984) reported the anti-ecdysteroid activity of neem seed kernel extracts on *Aedes aegypti*, resulting in growth inhibition and prolonged developmental period. Eucalyptol has been documented to be very toxic to male housefly at LD50 of 118 µg/fly concentrations (Sukontason *et al.* 2004). Jesikha (2012) reported the larvicidal efficacy of *Aloe vera* extract against *Musca domestica*. He also observed that fruit extract of *Capsicum annuum* was quite effective as larvicide for providing a better and excellent alternate for the control of *M. domestica.* Shalaby *et al*. (1998) evaluated the efficacy of orange oil against the larvae and adults of *Musca domestica* and *Culex pipiens* and he also reported reduction in the weight of emerged pupae from

treated larvae as well as harmful effect on the fecundity of adult female. The peel oil of *C. sinensis* was also found to have potent insecticidal effects on larvae of *Culex pipiens quinquenfasciatus* (Yang *et al*. 2005). Sukontason *et al*. (2004) reported deformation in the inter-segmental spines in the *Eucalyptus* treated housefly larvae. Linalool, which is the component of *C. sinensis* showed 87% inhibition in egg hatching in treated *Musca domestica* (Rice and Coats, 1994). Amer and Mehlhorn (2006) also reported the activeness of essential oil against housefly. However, no report is available in literature to indicate that *R. tetraphylla* root extracts possess insecticidal activity against housefly. Keeping these facts in view, the present study was undertaken to investigate the larvicidal property of methanolic root extracts of *R. tetraphylla* and to assess the chemical nature of the active components present in the root extracts along with their effect on biochemical components of housefly. Hence this study provides first-hand information on the use of methanolic root extracts of *R. tetraphylla* as a novel biological agent for the control of *Musca domestica* population as these natural products are eco-friendly, cost effective, better in efficacy and seem to be suitable candidate for commercialization.

MATERIALS AND METHODS

R. tetraphylla belongs to the Apocynaceae family and it is commonly known as Devil pepper or Be still tree. *R.* genus is mainly known for its phytochemical reserpine, which is widely used as an antihypertensive drug. Roots of *R. tetraphylla* have been employed from centuries for relief of various nervous disorders like anxiety, schizophrenia, insomnia and epilepsy. Root extracts of *R. tetraphylla* are also used in the treatment of diarrhoea, dysentery, cholera and fever.

Collection of plant material

The experiment was conducted in the Plant Physiology Laboratory, Amity Institute of Biotechnology, Amity University, Noida, India. The fresh and healthy plants of *R. tetraphylla* were collected at the vegetative stage from the Gautam Buddh Nagar district of Uttar Pradesh. Fresh roots of *R. tetraphylla* were removed, washed gently with tap water only for few seconds to avoid leaching losses of water soluble components, followed by quick rinsing in distilled water and drying with clean absorbent paper.

Preparation of dry root powder of *R. tetraphylla*

The roots of *R. tetraphylla* were cut into small pieces with sterilized knife and kept in single layer on plastic tray under the shade for 8-10 days. After air drying under the shade, roots were powdered mechanically by using commercial electrical stainless steel blender and dry root powder was stored in sterilized air tight container at room temperature in dark for further analysis.

Phytochemical screening

For the analysis of phytochemical components present in the roots of *R. tetraphylla,* the root extracts were prepared in methanol.

Preparation of methanolic root extracts

10 g of dried root powder of *R. tetraphylla* was taken in a beaker and 100 ml of methanol was added. The mixture was kept on rotary shaker for 24 hours at 190-220 rpm. After 24 hours, mixture was filtered and pellet was discarded and supernatant was collected and evaporated to $1/4^{th}$ of its original volume. After the preparation of the methanolic root extracts from *R. tetraphylla*, the root extracts were stored at 4ºC in refrigerator when these were not in use (Vaghasiya, 2011). After the preparation of methanolic extracts from the roots of *R. tetraphylla*, different phytochemicals were analyzed by using the standard procedures described by Harborne (1973) and Trease and Evans (1989).

Test for tannin

Approximately 0.5 g of the dried root powder of *R. tetraphylla* was boiled in 20 ml of distilled water and then filtered. A few drops of 0.1% ferric chloride solution were added in the filtrate. A brownish green or blue black colour of the test solution indicated the presence of tannin in a given sample.

Test for saponin

2 g of the powdered root sample was boiled with 20 ml of distilled water in a water bath and filtered. The 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The foamy leather formation showed the presence of saponin in the test solution.

Test for quinone

To 0.5 ml of root extract of *R. tetraphylla*, 5 drops of 40% 1N NaOH was added. Blue green or red colour indicated the presence of quinone in the test sample.

Borntrager's test for anthraquinone

Two ml of the root extract of *R. tetraphylla* was mixed with 10% of 5 ml ammonia. Appearance of pink red or violet colour at the lower phase indicated the presence of anthraquinone in the roots of *R. tetraphylla.*

Keller - Killani test for cardiac glycosides

Few drops of glacial acetic acid and 2-3 drops of ferric chloride were added to 2 ml of root extract along with 1 ml of concentrated sulfuric acid. Appearance of brown ring at the interface confirmed the presence of cardiac glycosides in the test sample.

Test for phenol

Dry root powder of *R. tetraphylla* (500 mg) was dissolved in 5 ml of distilled water. To this, few drops of 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds in the test sample.

Test for flavonoids

One gram of the root powder of *R. tetraphylla* was boiled with 10 ml of distilled water for 5 minutes and filtered. Few drops of 20% NaOH solution were added to 1 ml of cool filtrate. Appearance of yellow colour which on addition of acid changed to colourless solution depicted the presence of flavonoids.

Test for terpenoids

The root extract of *R. tetraphylla* (5 ml) was mixed with 2 ml of chloroform and 3 ml of concentrated H_2SO_4 which was added from the sides of the test tubes. A reddish-brown colour of test solution showed the presence of terpenoids in the root extracts.

Test for steroids

Two ml of acetic anhydride were added in 0.5 ml of root extract of *R. tetraphylla.* They were mixed properly and 2 ml of concentrated H_2SO_4 were also added from the sides of the test tube. The change in colour of test solution from violet to blue green indicated the presence of steroids in the root extracts.

Rearing technique of housefly colony

Adult house flies were collected from the garbage site of Amity University, Noida through a sweep net method and reared in the cylindrical box covered with a muslin cloth in the laboratory at 28±2°C, 65±5% relative humidity and photoperiod 12:12 (L:D). The plywood floor was fixed at the base of the box. The mixture of wheat flour and milk was prepared at a ratio of 1:3 and 40 g of this mixture was placed on a Petri dish with wet cotton and food material was changed after the 24 hours interval. The cotton swab served as the site for oviposition. The eggs were transferred to plastic jar on the medium containing finely crushed 30 g groundnut, 1 g yeast mixed with 20 ml of distilled water. The eggs were allowed to develop in this medium up to the larval stage.

Bioassay tests

The different concentrations such as 50 ppm, 100 ppm, 150 ppm and 200 ppm were prepared by dissolving root residue of *R. tetraphylla* in methanol for further study.

Repellency assay

In this assay, thirty adult houseflies were released in a box containing five conical flasks. Four flasks contained different concentrations of methanolic root extracts of *R. tetraphylla* such as 50 ppm, 100 ppm, 150 ppm and 200 ppm mixed with 5 ml of milk, while the fifth conical flask contained methanol and 5 ml of milk to serve as control. Funnels were introduced in each flask to avoid the escape of the houseflies. The total number of houseflies trapped in these flasks was counted after 24 hours. The results were expressed in terms of percentage attraction/repulsion. The percentage repellency (R%) was calculated by the following formula (Campbell, 1983);

$$R\% = [100(C-T)/C]$$

where, C= number of houseflies trapped in the control flask
T= number of houseflies trapped in the treated flask.

Larvicidal bioassay

Thirty housefly larvae were used for the larvicidal bioassay by the dipping method. Different concentrations of root extracts of *R. tetraphylla* such as 50 ppm, 100 ppm, 150 ppm and 200 ppm were kept in a separate bowl, while one bowl with methanol was used to serve as control. The larvae were dipped carefully in different concentrations of root extracts of *R. tetraphylla* for 60 seconds, whereas those for the control were dipped in the bowl containing the methanol only and transferred back in the rearing medium. Mortality of these larvae followed by the exposure in different concentrations of root extracts of *R. tetraphylla* was monitored after 24 hours by touching each larva by a soft, number zero paint brush and those not responding to the touch were considered as dead larvae.

Biochemical Analysis

Preparation of homogenate

After 48 h exposure of methanolic root extracts of *R. tetraphylla,* housefly larvae were homogenized with the mortar and pestle in 50 mM Tris-HCl buffer (pH 7.5) and homogenate was centrifuged at 4°C for 10 minutes at 15,000 g in a refrigerated centrifuge. The supernatant of different treatments was either used fresh or kept frozen at -20°C until further use for determining the different biochemical components of housefly.

Total protein content estimation

Quantitative estimation of protein content was done by following the method of Lowry *et al*. (1951). Stock solution of the following reagents was prepared:

Reagents:

A. Alkaline sodium carbonate solution (0.2% Na_2CO_3 in 0.1 N NaOH).

B. Copper sulphate - sodium potassium tartarate solution (0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartarate).

C. Alkaline copper reagent: Mixed 50 ml of reagent A and 1 ml of reagent B.

D. Folin - Ciocalteu reagent (Folin - Ciocalteu reagent with equal volume of distilled water just before use).

E. 1 N NaOH.

10 mg tissue extracts of housefly were homogenized with 1 ml of 1N NaOH and kept for 5 minutes at 100°C into the boiling water bath. Added 5 ml of alkaline copper reagent to it and allowed the mixture to stand at room temperature for 10 minutes. Added 0.5 ml of Folin - Ciocalteu reagent immediately and mixed the contents properly in the test tube. The absorbance of the solution was measured at 650 nm after 30 minutes. The amount of protein was calculated with reference to standard curve of lysozyme.

Estimation of nucleic acids

Nucleic acids (DNA and RNA) were extracted by using the method described by Schneider (1957) and calf thymus DNA was used as a standard. Total RNA was estimated by the orcinol method using yeast RNA as a standard.

Statistical analysis

All the values in the tables are given as mean ± standard error of mean (sem) of three independent experiments.

RESULTS

Screening of the phytochemicals present in the root extracts of *R. tetraphylla*

In the present study, the methanolic root extracts of *R. tetraphylla* were analysed against the housefly. The phytochemical analysis of the methanolic root extracts of *R. tetraphylla* revealed the presence of different phytochemicals such as tannin, quinone, phenol, saponin, flavonoid, steroids and terpenoids in the roots of *R. tetraphylla* (Table 1). These phytochemicals may be responsible for the insecticidal nature of the root extracts of *R. tetraphylla*.

Repellency percentage of *Musca domestica*

The treatment of larvae of *M. domestica* with different concentrations of the root extracts of *R. tetraphylla* such as 50 ppm, 100 ppm, 150 ppm and 200 ppm exhibited significant mortality in the larvae of housefly in a dose dependent manner. The lower percentage of mortality was observed after shorter duration (24 h) than at longer duration (48 h). The results presented in (Tables 2 and 3)

exhibit the toxicity of root extracts of *R. tetraphylla* against *M. domestica* larvae. The repellency percentage of *M. domestica* and mortality percentage of larvae of *M. domestica* with the different concentration of root extracts of *R. tetraphylla* was in the order: 200 ppm > 150 ppm > 100 ppm > 50 ppm. The maximum repellency percentage 93.75% was observed in *M. domestica* with 200 ppm concentration of root extracts of *R. tetraphylla.* In the larvicidal assay, all the larvae were found dead with the treatment of 200 ppm root extracts of *R. tetraphylla.*

Table 1

Identification of phytochemicals present in the methanolic root extract of *R. tetraphylla*

Phytochemicals	Status of phytochemicals in root extracts of *R. tetraphylla*
Tannin	+
Saponin	+
Quinone	+++
Anthraquinone	+++
Glycosides	++
Phenol	++
Flavonoid	+
Terpenoid	++
Steroid	++

where: + = moderately present; ++ = highly present; +++ = very highly present.

Table 2

Effect of methanolic root extract of *R. tetraphylla* on the repellency percentage (%) of *Musca domestica* L

Treatment	Repellency percentage (R %)
Control	0.00 ± 0.00
50 ppm	62.5 ± 0.05
100 ppm	75 ± 0.18
150 ppm	81.25 ± 0.63
200 ppm	93.75 ± 0.29

Values are mean of three replicates ± sem

Table 3

Effect of methanolic root extract of *R. tetraphylla on* the mortality percentage (%) of larvae of *Musca domestica* L

Treatment	Number of alive larvae		Mortality (%)	
	24 h	48 h	24 h	48 h
Control	30 ± 0.04	30 ± 0.02	0 ± 0.00	0 ± 0.00
50 ppm	28 ± 0.13	25 ± 0.04	6.67 ± 0.09	16.67 ± 0.15
100 ppm	22 ± 0.14	19 ± 0.12	26.67 ± 0.17	36.67 ± 0.28
150 ppm	14 ± 0.03	11 ± 0.06	53.33 ± 0.34	63.33 ± 0.45
200 ppm	5 ± 0.02	0 ± 0.00	83.33 ± 0.96	100 ± 1.28

Values are mean of three replicates ± sem

Protein content in the larvae of *M. domestica*

The protein content in the larvae of *M. domestica* was the highest in control 427.24 mg/g and it was reduced in different treatments and the lowest amount of protein content 189.69 mg/g was observed with the 200 ppm concentration of root extracts of *R. tetraphylla* (Table 4). In this way maximum inhibition (55.6%) in protein content of larvae of *M. domestica* was observed with the treatment of 200 ppm concentration of root extracts of *R. tetraphylla.*

Table 4

Effect of methanolic root extract of *R. tetraphylla* on the protein content (mg/g) of larvae of *Musca domestica* L

Treatment	Protein content (mg/g)
Control	427.24 ± 0.95
50 ppm	369.57 ± 0.75 (13.49)
100 ppm	311.16 ± 0.64 (27.17)
150 ppm	297.28 ± 0.42 (30.42)
200 ppm	189.69 ± 0.25 (55.60)

Values are mean of three replicates ± sem. Figures in parentheses indicate percent inhibition over control.

Nucleic acid content in the larvae of *M. domestica*

The content of nucleic acids (DNA and RNA) was significantly reduced in larvae of *M. domestica* with different concentrations of root extracts of *R. tetraphylla.* The highest DNA content 0.364 mg/g was observed in the larvae of *M. domestica* in control and it was reduced in different treatments and the lowest amount 0.129 mg/g was observed with the 200 ppm concentration of root extracts of *R. tetraphylla.* Similarly, the highest RNA content 0.245 mg/g was observed in larvae of *M. domestica* in control and it was reduced in different treatments and the lowest amount 0.132 mg/g was observed with the 200 ppm concentration of root extracts of *R. tetraphylla* (Table 5). Maximum inhibition (64.56% and 46.12%) in DNA and RNA contents in the larvae of *M. domestica* was observed with 200 ppm concentration of root extracts of *R. tetraphylla.*

Table 5

Effect of methanolic root extract of *R. tetraphylla* on the DNA and RNA contents (mg/g) of larvae of *Musca domestica* L

Treatment	DNA content (mg/g)	RNA content (mg/g)
Control	0.364 ± 0.06	0.245 ± 0.03
50 ppm	0.315 ± 0.05(13.46)	0.209 ± 0.04 (14.69)
100 ppm	0.246 ± 0.03(32.42)	0.184 ± 0.02 (24.89)
150 ppm	0.218 ± 0.02 (40.11)	0.173 ± 0.03 (29.39)
200 ppm	0.129 ± 0.01 (64.56)	0.132 ± 0.04 (46.12)

Values are mean of three replicates ± sem. Figures in parentheses indicate percent inhibition over control.

DISCUSSION

In the present study, the methanolic root extracts of *R. tetraphylla* were quite effective against the larvae of housefly. The larvicidal property of root extracts of *R. tetraphylla* adversely affected the housefly larvae in a dose dependent manner. The high susceptibility of houseflies to the root extracts may be attributed to the absence of hard sclerotized thoracic cuticle which is characteristic of skeletal tissue of Coleoptera (Talukder and Howse, 1994). This may indicate possible contact neurotoxic action of the active constituents present in the root extracts of *R. tetraphylla*. It has been observed that insects contain a very less amount of carbohydrates and due to the presence of the less amount of carbohydrates, proteins are used by the insects to meet their energy demand. The protein and nucleic acid contents play an important role in regulating the different vital activities of the cells. The protein content of the insects is the main source of nitrogenous metabolism and proteins are also involved in the architecture of the cell in the insects. In the present investigation, decrease in the protein content in the larvae indicates the high protein hydrolytic activity due to increase in protease activity (Begum *et al.* 2010). The results of the present study clearly indicate that methanolic root extracts of *R. tetraphylla* is potent inhibitor of DNA synthesis and inhibiton in DNA synthesis might reduce the RNA content as well as protein synthesis via adverse effect on protein synthesis machinery. The decrease in protein and nucleic acid contents in the larvae of *M. domestica* with the methanolic root extracts may be due to the presence of active ingredients present in the root extracts of *R. tetraphylla* which causes interference in the normal metabolism of insects. The results obtained from the present study clearly indicate that methanolic root extracts of *R. tetraphylla* were quite effective as bioinsecticide for providing excellent alternate for the control of housefly, but further investigations are required for its large scale production.

CONCLUSION

The present study clearly indicates that methanolic root extracts of *R. tetraphylla* have quite effective repellency and larvicidal potential against *Musca domestica.* The findings of the present investigation show that methanolic root extracts of *R. tetraphylla* can find a place in the development of eco-friendly bioinsecticides for the housefly management programme. The aqueous formulation prepared from the roots of *R. tetraphylla* may be used to control housefly eggs and larvae in cattle and poultry manure or as component of washing liquids for cleaning the floors to repel the houseflies. Further investigation on the active constituents present in the *R. tetraphylla* may open new avenues in the development of housefly control strategy.

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GENETIC VARIABILITY FOR GROWTH, EARLINESS AND YIELD ATTRIBUTES IN OKRA (*Abelmoschus esculentus* (L.) Moench)

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Okra (*Abelmoschus esculentus* L. Moench) is a nutrient-rich and medicinally valuable pod vegetable commonly grown in a wide range of agro-ecosystems in different habitats across India. Forty five single crosses were derived from crossing 10 germplasm lines of okra (IC282248, IC27826-A, IC29119-B, IC31398-A, IC45732, IC89819, IC89976, IC90107, IC99716 and IC111443) in a half-diallel fashion during summer, 2009. 45 F_1s and their 10 parents were evaluated in a randomized block design with three replications at the Vegetable Research Station, Rajendranagar, India during early (June-September), mid (July-October) and late (August-November) rainy (*kharif*) season, 2009 to determine their genetic variability, heritability and genetic advance from thirteen yield related characters. The pooled analysis of variance revealed significance ($P<0.01$) of mean squares due to genotypes for all thirteen agronomic characters namely plant height (cm), number of branches per plant, internodal length (cm), days to 50% flowering, first flowering node, first fruiting node, fruit length (cm), fruit width (cm), fruit weight (g), total number of fruits per plant, number of marketable fruits per plant, total yield per plant (g) and marketable yield per plant (g) indicating an appreciable amount of variability among the genotypes. High magnitude of genotypic coefficient of variation (>20%) for plant height, number of branches per plant, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant indicated high degree of genetic variability offering great scope for selection of these characters. The characters plant height, internodal length, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant having high heritability (>60%) along with high expected genetic advance (>20%) suggest that a very significant improvement is possible through selection for all these characters.

Keywords: Genetic advance, genetic variability, heritability in broad sense, selection parameters.

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INTRODUCTION

Okra (*Abelmoschus esculentus* (L.) Moench) is an important vegetable of the family Malvaceae. It is one of the oldest cultivated crops and presently grown in many countries and is widely distributed from Africa to Asia, southern Europe and America (Oyenuga, 1969; Hamon, 1991; Ariyo, 1993). It is a multifarious and multipurpose crop valued for its tender and delicious pods (Chinatu and Okocha, 2006). It is a nutrient-rich and medicinally valuable tropical vegetable with huge socio-economic and industrial potential (Reddy *et al.*, 2013). Okra is commonly cultivated in a wide range of agro-ecosystems in different habitats across India. The economic and ecological sustainability of okra in these agro-ecosystems is under question because of the low-yielding potential, suboptimal pod quality and susceptibility to biotic and biotic stresses especially the yellow vein mosaic virus (YVMV) of the traditionally cultivated open-pollinated varieties (OPVs) and local varieties (LVs) or indigenous landraces (Kumar and Reddy, 2015). Being an often cross-pollinated crop, good amount of variability exists for various growth, earliness and yield attributes (Reddy *et al.*, 2012). In okra, yield improvement in the past was based on selection in locally adapted populations of local varieties (Reddy *et al.*, 2012). Through the genetic breeding, using hybridization, improved varieties of okra can be obtained with a better adaptation to certain environments, more productive, resistant and/or tolerant to some specific insects or diseases and, in general, plants with desirable agronomic characters to be cultivated commercially, in particular (Reddy *et al.*, 2011).

The ultimate goal of any okra breeding program is the maximization of pod yield. Improvement of complex characters such as yield may be accomplished through the component approach of breeding. Using yield components as a quantification and selection criterion is superior to using yield alone. Plant breeders commonly select for yield components which indirectly increase yield. The magnitude of the coefficient of variation, heritability and repeatability of traits are important guides to selecting polygenic yield determining traits (Ortiz and Ng, 2000). Yield improvement has been achieved through directional selection for yield contributing traits (Akbar and Kamran, 2006). Pod yield improvement through breeding for yield components would be the most effective if their variability, heritability and genetic advances are understood.

The assessment of the variation in the yield determining quantitative traits of crop is the primary aspect in the breeding for yield. The improvement in any crop is proportional to the magnitude of its genetic variability present in the population. Genetic variability for agronomic traits is the key component of breeding programs for broadening the gene pool of crops. The success of a breeding program depends on the variability of the initial material (Fick and Miller, 1997). In order to apply an optimum breeding strategy for targeted quantitative traits, a genetic variability analysis of those traits needs to be performed (Has, 1999; Nistor *et al.*, 2005). The

genotypic coefficient of variation together with heritability estimate would give the best picture of the amount of advance to be expected from selection. The amount of genetic advance under selection depends mainly on the amount of genetic variability. The phenotype of an individual is affected both by genotype and environment. Most agronomically important characters are inherited quantitatively and are known to be affected by environmental factors (Baghizadeh *et al.*, 2007). Selection based on the phenotype would be difficult for such difficult traits. In breeding programs, it is often difficult to manipulate such traits, since several inter-componential characters indirectly control those (Hittalmani *et al.*, 2003).

Heritability (h^2) of a trait is important in determining its response to selection. Genetic improvement of plants for quantitative traits requires reliable estimates of heritability in order to plan an efficient breeding program. Heritability, a measure of the phenotypic variance attributable to genetic causes, has predictive function in breeding crops. Heritability represents the ratio between genetic and all factors (including non-genetic ones) that influence the variability (Fick and Miller, 1997; Bernardo, 2002). It provides an estimate of genetic advance, a breeder can expect from selection applied to a population under certain environments. The higher the heritability estimates the simpler are the selection procedures (Khan *et al.*, 2008). The higher ratio of the genetic component in phenotypic expression of a certain trait, the higher is the heritability, and selection for these traits can be performed in earlier generations in field trials set up at a smaller number of locations, years, and replications. Heritability accompanied with an estimation of genetic gain is more useful than heritability alone in accurate prediction of the selection effects (Johnson *et al.*, 1955).

It is known that phenotypic expression of quantitative traits is highly influenced by environmental fluctuations (Allard and Bradshaw, 1964). The progress in breeding for yield and its contributing characters of any crop is polygenically controlled, environmentally influenced and determined by the nature and magnitude of their genetic variability (Wright, 1935; Fisher, 1981). Different okra genotypes differ significantly for most of the traits like plant height, number of branches per plant, internodal length, days to 50% flowering, first flowering and fruiting nodes, pod length, width and weight, total number of pods per plant, number of marketable fruits per plant which ultimately determine total and marketable pod yields of the cultivar, and also respond differently to the varying environments with respect to their agronomic characteristics and ultimately yield components (Dash and Misra, 1995; Gondane and Bhattia, 1995; Shridhar and Dhar, 1995). Several researchers have observed low to high variability, heritability (in broad sense), genetic advance and genetic advance as percent of mean for these growth, earliness and yield attributes in okra (Jindal *et al.*, 2010; Pradip *et al.*, 2010; Prakash and Pitchaimuthu, 2010; Ramanjinappa *et al.*, 2011; Nwangburuka *et al.*, 2012; Reddy *et al.*, 2012; Duggi *et al.*, 2013; Ibrahim *et al.*, 2013; Barche *et al.*, 2014).

In view of the above, it is necessary to study the genetic architecture of okra genotypes in relation to the environment for which they have to be developed. Knowledge and the nature and magnitude of variation existing in available breeding materials are requisite to choose characters for effective selection of desirable genotypes to undertake planned breeding program. This study, therefore, aimed at revealing the variability, the broad sense heritability and genetic advances of some quantitative traits of okra.

MATERIALS AND METHODS

Ten elite and nearly homozygous lines of okra, namely IC282248, IC27826-A, IC29119-B, IC31398-A, IC45732, IC89819, IC89976, IC90107, IC99716 and IC111443) were crossed in n(n-1)/2 possible combinations during summer 2009 to generate the breeding material. The resulting 45 one way crosses along with their ten counterpart parental lines were evaluated in a randomized block design with three replications at the Vegetable Research Station, Rajendranagar, Hyderabad, Andhra Pradesh, India during early (June - September), mid (July - October) and late (August- November) rainy (*kharif*) season, 2009. Each entry was grown in a double-row plot of 3.0 m length and 1.2 m width. The rows were spaced 60 cm apart, while plants within rows were spaced 30 cm apart. All the recommended package of practices were followed to get complete expression of traits under study. The observations were recorded on five randomly selected plants from each genotype in each replication for plant height (cm), number of branches per plant, internodal length (cm), fruit length (cm), fruit width (cm) and fruit weight (g) and on whole plot basis for days to 50% flowering, first flowering node, first fruiting node, total number of fruits per plant, number of marketable fruits per plant, total yield per plant (g) and marketable yield per plant (g). Analysis of variance computed as devised by Panse and Sukhatme (1985). Phenotypic and genotypic components of variance were estimated as per the formulae suggested by Lush (1940). Estimates of phenotypic and genotypic coefficients of variation (PCV and GCV) were calculated as per the standard formulae (Burton, 1952). The PCV and GCV values were classified as low (<10%), moderate (10-20%) and high (>20%) as suggested by Sivasubramanian and Menon (1973). The broad sense heritability was estimated for all the characters as the ratio of genotypic variance to total or phenotypic variance (Lush, 1940). The heritability values were classified as low (<30%), moderate (30-60%) and high (>60%) as suggested by Johnson *et al.*, (1955). The expected genetic gain or advance under selection for each character was estimated by following the method suggested by Johnson *et al.* (1955). The estimates of genetic advance and genetic advance as percent of mean were classified as low (<10%), moderate (10-20%) and high (>20%) as suggested by Johnson *et al.* (1955).

RESULTS

From the analysis of variance, it is evident that the mean squares due to genotypes were highly significant (Table 1) for all the traits among the 55 genotypes under study. The mean and range values for thirteen characters are presented in Table 2. The ranges of mean values revealed sufficient variation for all the traits under study. In the parental lines under study, maximum range of variability (Table 2) was observed for total yield per plant (209.56-303.82 g) followed by marketable yield per plant (170.92-252.21 g) and plant height (129.52-158.78 cm). In the cross combinations under study, maximum range of variability (Table 2) was observed for total yield per plant (202.46-345.28 g) followed by marketable yield per plant (171.28-272.46 g) and plant height (125.58-166.14 cm).

Table 1

Analysis of variance for yield and yield attributes in okra

Character	Mean sum of squares		
	Replications (2)	**Treatments (54)**	**Error (108)**
Plant height (cm)	929.3071**	4896.0087**	172.6755
Number of branches per plant	0.4922*	0.6708**	0.12993
Internodal length (cm)	2.1070*	3.5201**	0.5358
Days to 50% flowering	0.2566	2.8237**	0.7322
First flowering node	0.0844**	0.3956**	0.0135
First fruiting node	0.0844**	0.3956**	0.0135
Fruit length (cm)	0.1975**	3.0441**	0.0413
Fruit width (cm)	0.0002	0.0158**	0.0002
Fruit weight (g)	0.6029**	4.0109**	0.1076
Total number of fruits per plant	14.1464**	75.0229**	2.1609
Number of marketable fruits per plant	8.4065**	53.4139**	1.6398
Total yield per plant (g)	2626.5020**	19626.3896**	526.2435
Marketable yield per plant (g)	1624.2230**	13772.8295**	397.1559

*, ** Significant at 1and 5% levels, respectively; Values in parenthesis denote degrees of freedom

Table 2

Mean values and ranges for yield and yield attributes in okra

Character	Mean ± S.Em*	Range	
		Parents	**Hybrids**
Plant height (cm)	147.0791±7.5637	129.52-158.78	125.58-166.14
Number of branches per plant	2.0554±0.2070	1.47-3.00	1.18-3.36
Internodal length (cm)	6.2009±0.4213	4.86-7.19	4.91-7.32
Days to 50% flowering	38.5899±0.4925	37.33-39.94	37.33-39.89
First flowering node	4.6861±0.0670	4.01-5.13	3.87-5.33
First fruiting node	4.6861±0.0670	4.01-5.13	3.87-5.33
Fruit length (cm)	14.2180±0.1170	11.41-15.54	12.16-16.12
Fruit width (cm)	1.8909±0.0077	1.69-2.07	1.70-2.01

Table 2 (continued)

Fruit weight (g)	15.1470±0.1888	12.67-17.21	13.70-17.70
Total number of fruits per plant	17.7282±0.8461	15.13-18.67	13.18-21.00
Number of marketable fruits per plant	14.9398±0.7371	12.66-15.74	12.07-17.92
Total yield per plant (g)	269.3250±13.2042	209.56-303.82	202.46-345.28
Marketable yield per plant (g)	226.8530±11.4710	170.92-252.21	171.28-272.46

* Significant at 1% level

For all the characters under study, phenotypic variances were higher than the corresponding genotypic variances (Table 3). The phenotypic variance was highest for total yield per plant (6892.96) followed by marketable yield per plant (4855.71) and plant height (1747.12). Similarly, the genotypic variance was also highest for total yield per plant (6366.72) followed by marketable yield per plant (4458.56) and plant height (1574.44). The phenotypic variance was lowest for fruit width (0.01) followed by first flowering and fruiting node (0.14) and number of branches per plant (0.31). Similarly, the genotypic variance was lowest for fruit width (0.01) followed by first flowering and fruiting node (0.13) and number of branches per plant (0.18).

Table 3

Genetic parameters for yield and yield attributes in okra

Character	**Variance**		**Coefficient of variation**		h^2	**GA***	**GAM***
	Genotypic	**Phenotypic**	**Genotypic**	**Phenotypic**			
Plant height (cm)	1574.44	1747.12	26.98	28.42	90.10	77.60	52.76
Number of branches per plant	0.18	0.31	20.67	27.08	58.30	0.67	32.51
Internodal length (cm)	1.00	1.53	16.08	19.95	65.00	1.66	26.71
Days to 50% flowering	0.70	1.43	2.16	3.10	48.80	1.20	3.11
First flowering node	0.13	0.14	7.62	8.01	90.40	0.70	14.92
First fruiting node	0.13	0.14	7.62	8.01	90.40	0.70	14.92
Fruit length (cm)	1.00	1.04	7.04	7.18	96.00	2.02	14.21
Fruit width (cm)	0.01	0.01	3.81	3.88	96.70	0.15	7.72
Fruit weight (g)	1.30	1.41	7.53	7.84	92.40	2.26	14.91
Total number of fruits per plant	24.29	26.45	27.80	29.01	91.80	9.73	54.88
Number of marketable fruits per plant	17.26	18.90	27.81	29.10	91.30	8.18	54.74
Total yield per plant (g)	6366.72	6892.96	29.63	30.83	92.40	157.97	58.66
Marketable yield per plant (g)	4458.56	4855.71	29.43	30.72	91.80	131.81	58.10

* Significant at 5% level; h^2 = Heritability in broad sense; GA = Genetic advance; GAM = Genetic advance as percent of mean

The estimates of PCV (Table 3) were highest for total yield per plant (30.83%), followed by marketable yield per plant (30.72%) and number of marketable fruits per plant (29.10%), while lowest for days to 50% flowering (3.10%) followed by fruit width (3.88%) and fruit length (7.18%). The estimates of GCV (Table 4) were highest for total yield per plant (29.63%), followed by marketable yield per plant (29.43%) and number of marketable fruits per plant (27.81%), while lowest for days to 50% flowering (2.16%) followed by fruit width (3.81%) and fruit length (7.04%).

The estimates of PCV (Table 3) were of high magnitude (>20%) for plant height (28.42%), number of branches per plant (27.08%), total number of fruits per plant (29.01%), number of marketable fruits per plant (29.10%), total yield per plant (30.83%) and marketable yield per plant (30.72%), of moderate magnitude (10 to 20%) for internodal length (19.95%), and of low magnitude (<10%) for days to 50% flowering (3.10%), first flowering node (8.01%), first fruiting node (8.01%), fruit length (7.18%), fruit width (3.88%) and fruit weight (7.84%).

The estimates of GCV (Table 3) were of high magnitude (>20%) for plant height (26.98%), number of branches per plant (20.67%), total number of fruits per plant (27.80%), number of marketable fruits per plant (27.81%), total yield per plant (29.63%) and marketable yield per plant (29.43%), of moderate magnitude (10 to 20%) for internodal length (16.08%), and of low magnitude (<10%) for days to 50% flowering (2.16%), first flowering node (7.62%), first fruiting node (7.62%), fruit length (7.04%), fruit width (3.81%) and fruit weight (7.53%).

In general, the magnitude of phenotypic coefficients of variation (PCV) was higher than the corresponding genotypic coefficients of variation (GCV) for all the thirteen characters under study (Table 3). However, the magnitudinal differences between the estimates of GCV and PCV were highest for number of branches per plant (6.41) followed by internodal length (3.87).

The estimates of heritability (Table 3) were of high magnitude (>60%) for plant height (90.10%), internodal length (65.00%), first flowering node (90.40%), first fruiting node (90.40%), fruit length (96.00%), fruit width (96.70%), fruit weight (92.40%), total number of fruits per plant (91.80%), number of marketable fruits per plant (91.30%), total yield per plant (92.40%), marketable yield per plant (91.80%) and of moderate magnitude (30-60%) for number of branches per plant (58.30%) and days to 50% flowering (48.80%).

The estimates of genetic advance as percent of mean (Table 3) were of high magnitude (>20%) for plant height (52.76%), number of branches per plant (32.51%), internodal length (26.71%), total number of fruits per plant (54.88%), number of marketable fruits per plant (54.74%), total yield per plant (58.66%), marketable yield per plant (58.10%), of moderate magnitude (10 to 20%) for first flowering node (14.92%), first fruiting node (14.92%), fruit length (14.21%), fruit weight (14.91%), and of low magnitude (<10%) for days to 50% flowering (3.11%) and fruit width (7.72%).

DISCUSSION

The success of a breeding program for the genetic improvement of quantitative characters depends on the magnitude of genetic variability available in the germplasm and the extent to which the desirable characters are heritable. The determination of genetic variability and partitioning it into heritable and non-heritable components using the genetic parameters, namely phenotypic and genotypic coefficients of variation, heritability and genetic advance is necessary to have an insight into genetic nature of yield and its components on which selection can be effectively carried out. Character like yield is complex in inheritance and is improved through its component traits. High yield can be achieved by selection of those yield contributing characters that have high heritability coupled with high genetic advance. Therefore, the components of variance and heritable components with genetic parameters such as genotypic coefficient of variation, heritability estimates and genetic advance as per cent of mean are important tools to plan a suitable breeding strategy. Hence, for the improvement of okra, detailed investigation on genetic architecture of pod yield and its attributes should be the main concern.

In the present investigation, the significance of mean squares due to genotypes for all the characters under study indicated the presence of an appreciable amount of variability among the genotypes. All the thirteen characters under study exhibited high variability as evident from the major components of variability such as phenotypic and genotypic coefficients of variation, heritability and genetic advance. Hence, there is lot of scope for improvement of these characters in okra by selection.

The range of mean values could present a rough estimate about the variation in magnitude of variability present among genotypes. From the mean and range values, which are the simple measures of variability, it is also evident that there was a wide range of variability for all the traits under study. The characters showing high range of variation have more scope for improvement. All the thirteen characters under study exhibited high variability as evident from the ranges of mean values. However, the characters total yield per plant, marketable yield per plant and plant height having wide range of variation in mean values indicated the presence of high variability for these characters and thus offering greater scope for selecting desirable genotypes. These findings are in consonance with the findings of earlier workers (Dhankar and Dhankar, 2002; Singh *et al.*, 2006; Mohapatra *et al.*, 2007) in okra.

The genotypic variance was very low for fruit width indicating that major part of the total variation was not heritable. High genotypic variance as observed for total yield per plant, marketable yield per plant and plant height indicated greater stability of the genotypes under different environmental conditions. Therefore, genotypes with such characters are likely to exhibit uniform performance over locations and seasons. Such a high genotypic variance was also reported by Vijay and Manohar (1990) for total yield per plant in okra.

The estimates of phenotypic variability cannot differentiate between the effects of genotype and environment. Hence, the study of genetic variability is effective in partitioning out the real genetic differences. The estimates of GCV and PCV are of greater use in determining the variability present in the material. In general, the magnitude of PCV was higher than the corresponding GCV for all the thirteen characters under study, indicating that the apparent variation was not only due to genotype, but also to the favorable influence of environment and selection for these traits sometimes may be misleading. This environmental effect could be due to heterogeneity in soil fertility status and other unpredictable factors. Similar projections and findings have been made by Vijay and Manohar (1990), Chandra *et al.* (1996), Jindal *et al.* (2010), Pradip *et al*. (2010) and Reddy *et al*. (2012). However, there was a close correspondence between the estimates of phenotypic and genotypic coefficients of variation for majority of the characters under study indicating the fact that the environment influence is very low. These findings are in line with those of Duggi *et al*. (2013) who also reported that the PCV and GCV were very close for most of the characters. In contrast, the high magnitudinal differences between the estimates of GCV and PCV for number of branches per plant and internodal length revealed that these traits were influenced by the environmental effects to a large extent and the greater role of environment in the expression of these traits. This also implies that one should not rely on mean phenotypic values for direct selection of these traits. Gandhi *et al.* (2001) also reported high magnitudinal differences between GCV and PCV for number of branches per plant in okra.

In the present investigation, the genotypes were found to possess a high to low phenotypic and genotypic variation as revealed by phenotypic and genotypic coefficients of variation. The characters like plant height, number of branches per plant, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant, having high genotypic coefficients of variation possesses better potential for further gain and improvement through selection. The higher the genotypic coefficient of variation, the more are the chances of improvement in those characters. High magnitude (>20%) of genotypic and phenotypic coefficients of variation were also reported by Prakash and Pitchaimuthu (2010) and Ramanjinappa *et al.* (2011) for plant height, Jaiprakashnarayan *et al.* (2006), Mehta *et al.* (2006), Singh *et al.* (2006), Jindal *et al.* (2010), Ramanjinappa *et al.* (2011) and Duggi *et al*. (2013) for number of branches per plant, Dhall *et al.* (2003), Duggi *et al*. (2013) and Barche *et al*. (2014) for total number of fruits per plant, Gandhi *et al.* (2001), Bendale *et al.* (2003), Mehta *et al.* (2006) and Singh *et al.* (2006) for total yield per plant and Dhall *et al.* (2003) for marketable yield per plant. Low magnitude (<10%) of genotypic and phenotypic coefficients of variation was also reported by Gandhi *et al.* (2001), Jaiprakashnarayan *et al.* (2006), Mehta *et al.* (2006), Singh *et al.* (2006), Dakahe *et al.* (2007), Mohapatra *et al.* (2007), Ramanjinappa *et al.* (2011) and Prakash and Pitchaimuthu (2010) for

days to 50% flowering, Jindal *et al.* (2010) for first flowering node, Jindal *et al.* (2010) and Ramanjinappa *et al.* (2011) for fruit length, Singh *et al.* (2006), Mohapatra *et al.* (2007), Pradip *et al.* (2010) and Jindal *et al.* (2010) for fruit width and Ramanjinappa *et al.* (2011) for fruit weight in okra. Moderate magnitude (10-20%) of genotypic and phenotypic coefficients of variation was also reported by Bendale *et al.* (2003), Pradip *et al.* (2010) and Ramanjinappa *et al.* (2011).

It is very difficult to judge whether observed variability is highly heritable or not. Heritability plays a vital role in deciding the suitability and strategy for selection of a particular character. Moreover, knowledge of heritability is essential for selection based improvement, as it indicates the extent of transmissibility of a character into future generations (Sabesan *et al.*, 2009). Heritability in broad sense is the ratio of genotypic variance to total variance in non-segregating population (Hanson *et al.*, 1956). The estimates of heritability were of high magnitude (>60%) for plant height, internodal length, first flowering node, first fruiting node, fruit length, fruit width, fruit weight, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant indicating that though the characters are least influenced by the environmental effects, the selection for the improvement of such characters may not be useful, because broad sense heritability is based on total genetic variance which includes both fixable (additive) and non-fixable (dominance and epistatic) variances. High magnitude (>60%) of heritability estimates were also reported by Bendale *et al.* (2003), Jaiprakashnarayan *et al.* (2006), Singh *et al.* (2006), Mohapatra *et al.* (2007) and Ramanjinappa *et al.* (2011) for all the three growth attributes (plant height, number of branches and internodal length), Singh *et al.* (2006), Mohapatra *et al.* (2007) and Prakash and Pitchaimuthu (2010) for first flowering node, Jaiprakashnarayan *et al.* (2006) and Prakash and Pitchaimuthu (2010) for first fruiting node, Singh *et al.* (2006), Mohapatra *et al.* (2007) and Ramanjinappa *et al.* (2011) for all fruit traits (fruit length, width and weight) and total number of fruits per plant, Mehta *et al.* (2006), Singh *et al.* (2006), Mohapatra *et al.* (2007), Prakash and Pitchaimuthu (2010) and Ramanjinappa *et al.* (2011) for total yield per plant. The estimates of heritability were of moderate magnitude (30-60%) for number of branches per plant and days to 50% flowering indicating that these characters are moderately influenced by environmental effects and genetic improvement through selection will be moderately difficult due to masking effects of the environment on the genotypic effects.

Knowledge of extent of improvement possible through selection is useful in designing breeding program. Genetic advance under selection is the improvement in the mean genotypic value of the selected families over the base population. The genetic advance shows the improvement that can be made in a particular character by applying certain amount of selection intensity. The genetic advance to be expected depends up on the selection differential, the genotypic coefficient of variation and the square root of the heritability ratio (Johnson *et al.*, 1955). The genotypic coefficient of variation × selection differential estimates the maximum effectiveness of

selection and heritability indicates how closely the goal can be achieved. However, by increasing the diversity of genotypes of okra, the expected genetic advance can still be increased. The estimates of genetic advance as percent of mean were of high magnitude (>20%) for plant height, number of branches per plant, internodal length, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant indicating that these characters are governed by additive genes and selection will be rewarding for improvement of such traits. This suggests that such characters can be improved by direct selection. Therefore, such characters having high heritability coupled with high genetic advance would be effective in crop improvement program through selection methods. High magnitude (>20%) of genetic advance as percent of mean was also reported by Singh *et al.* (2006), Mohapatra *et al.* (2007), Jaiprakashnarayan *et al.* (2006), Jindal *et al.* (2010) and Ramanjinappa *et al.* (2011) for all growth attributes, Singh *et al.* (2006), Dakahe *et al.* (2007), Mohapatra *et al.* (2007), Pradip *et al.* (2010), Prakash and Pitchaimuthu (2010) and Ramanjinappa *et al.* (2011) for total number of fruits per plant, Mehta *et al.* (2006), Singh *et al.* (2006), Dakahe *et al.* (2007), Mohapatra *et al.* (2007), Pradip *et al.* (2010), Prakash and Pitchaimuthu (2010) and Ramanjinappa *et al.* (2011) for total yield per plant and Dhall *et al.* (2003) and Jindal *et al.* (2010) for marketable yield per plant. The estimates of genetic advance as percent of mean were of moderate magnitude (10-20%) for first flowering node, first fruiting node, fruit length and fruit weight. Moderate magnitude of genetic advance as percent of mean was also reported by Pradip *et al.* (2010) and Ramanjinappa *et al.* (2011) for fruit length, Pradip *et al.* (2010) and Ramanjinappa *et al.* (2011) for fruit weight. Low magnitude (<10%) of genetic advance as percent of mean was also reported by Ramanjinappa *et al.* (2011) for days to 50% flowering in okra.

Estimates of heritability along with genetic advance are more useful in predicting the value of selection than heritability alone (Johnson *et al.*, 1955). Panse (1957) concluded that a character with high heritability in association with high genetic advance as percent of mean is an indication of expression of additive gene action. Characters without such combination appear generally because of non-additive gene action (Liang and Walter, 1968). Therefore, it may be stated that among the characters under study with high estimates of heritability (>60%) coupled with high genetic advance as percent of mean (>20%) were plant height, internodal length, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant, which are most likely to be operated by additive genes and selection may be effective. These findings are in line with those of Reddy *et al*. (2012) for plant height, internodal length, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant, Duggi *et al*. (2013) for plant height and marketable yield per plant and Barche *et al*. (2014) for plant height, number of fruits per plant and marketable yield per plant.

CONCLUSIONS

From the results obtained it was concluded that genetic variation existed among the genotypes in all characters. On the whole, there was high magnitude of genetic variability and high degree of transmission of majority of the growth, earliness and yield associated traits under study. Characters like plant height, internodal length, total number of fruits per plant, number of marketable fruits per plant, total yield per plant and marketable yield per plant showed high genotypic coefficient of variation, heritability and genetic advance, indicating that selection of these characters will show a positive response in improvement.

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CHOROLOGY OF *MYCARTHOPYRENIA* KEISSL. GENUS IN ROMANIA

IOANA VICOL[1]

The investigation on the spatial distribution of *Mycarthopyrenia pyrenastrella* Keissl. and *Mycarthopyrenia stenospora* Keissl. was performed in sites from which these were firstly cited (Retezat Mountains and Cerna Valley) and also within a lot of localities from Romania, but the two lichen species have not been identified. The two lichen species have not been found in the studied localities during 2009-2014. According to field observations, the majority of the investigated sites are strongly anthropogenic modified and probably therefore the two lichen species were not identified in the studied area.

Keywords: chorology, *Mycarthopyrenia pyrenastrella*, *Mycarthopyrenia stenospora*, Valeriască Valley (Retezat Mountains), Cerna Valley at Băile Herculane, Romania.

INTRODUCTION

The main causes of absence or dimitigation of the epiphytic lichen diversity are the forest fragmentation (Caruso *et al.*, 2011), atmospheric pollution (Kapusta *et al.*, 2004), and forest management practices (Nascimbene *et al.*, 2010).

At European level, the lichen species tabulated within *Mycarthopyrenia* genus have been cited as follows: (1) *Mycarthopyrenia pyrenastrella* identified in Swedish within Skåne and Torne Lappmark provinces (Santesson, 1993), Norway in Aust – Agder and Finnmark provinces (Santesson, 1993), Ukraine in the Carpathian Mountains (Transcarpathian or Zakarpat'ye, mountainous part), Ukraine Plains (forest-steppe zone) and Crimea in mountains (Kondratyuk *et al*., 1996) and Transcarpathian region (Zakarpatska), Chornohora Mountains, Rahiv District, Goverlu and Petros peaks (Окснер, 1956); (2) *Mycarthopyrenia stenospora* found in Småland – Ångermanland and Jämtland provinces from Sweden (Santesson, 1993) and in Vestfold and Troms provinces from Norway (Santesson, 1993). Nordin *et al.* (2004) have found *Mycarthopyrenia stenospora* in Sweden but they have not provided any informations as regards localities.

A reevaluation of the spatial distribution of *Myarthopyrenia* genus on the Romania territory represents the aim of this study. The main objective is based on the mapping of *Mycarthopyrenia pyrenastrella* and *Mycarthopyrenia stenospora* including them within a database that integrates lichen chorology from Romania.

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MATERIAL AND METHODS

The field activities were performed during 2009-2014 in the Retezat Mountains, Cerna Valley that have been mentioned in literature and as an own initiative within forest sites from Suceava (Codrul Secular Slătioara Forest Reserve), Neamţ (Ceahlău National Park), Bacău (Nemira Mountains, forested area near Slănic Moldova locality), Vaslui (forested area near Igești and Mălușteni villages), Buzău (Ruginoasa Forest), Prahova (Bucegi National Park), Călărași (Goștilele and Călăreţilor forests), Ilfov (Afumaţi, Vlădiceasca, Snagov and Biglaru forests), Giurgiu (Căscioarelor and Crevedia forests), Mehedinţi (Porţile de Fier Natural Park), Gorj (Parâng Mountains) Brașov (Pojorta Valley and Brezcioarei Valley from the Făgăraș Mountains), Sibiu (Podu-Olt and Tălmaciu forests), Alba (Șureanu Massif), Arad (Zărandului Mountains between Socilor Valley and Gurahonţ), Hunedoara (Retezat National Park), Cluj (Turda Gorges and forested area near to Tarniţa Dam) and Bihor (Apuseni Natural Park, Crișului Repede Valley) counties. The nomenclature and spatial distribution of investigated lichen species is according to Ciurchea (2004) and www.mycobanc.org. UTM codes (Lehrer and Lehrer, 1990) were used to construct the map of the two species chorology using Corolog Software (Ștefănuţ *et al.*, 2009).

RESULTS AND DISCUSSION

Within field activities during 2009-2014, the two lichen species from *Mycarthopyrenia* genus have not been identified. According to field observations, the main cause of this fact might be anthropogenic activities such as forest fragmentation, expanding settlements and tourism.

From the taxonomic point of view both *Mycarthopyrenia pyrenastrella* Keissl. syn. *Arthopyrenia pyrenastrella* Vain., *Arthopyrenia punctiformis* var. *pyrenastrella* Migula and *Mycarthopyrenia stenospora* Keissl. syn. *Arthopyrenia stenospora* Körb. are tabulated within Ascomycota Class, Pyrenocarpeae Series, Dothideales Order, Arthopyreniaceae Family (Ciurchea 2004). Beside the two species mentioned above, the genus *Mycarthopyrenia* is represented by the other species which have not been cited for Romania lichen flora, such as: *M. aeruginella* (Nyl.) Keissl. (1937), *M. cembricola* (Anzi) Keissl. (1937), *M. desistens* (Nyl.) Keissl. (1937), *M. grisea* (Schleich ex Schaer.) Keissl. (1937), *M. ilicicola* (Nyl.) Keissl. (1937), *M. intersistens* (Nyl.) Keissl. (1937), *M. juglandis* (A. Massal.) Keissl. (1937), *M. lapponina* (Anzi) Keissl. (1937), *M. pinicola* (Hepp) Keissl. (1937), *M. subcerasi* (Vain.) Keissl. (1937) revised by Keissler Karl (1937). The same author has described *M. sorbi* Keissl. as new species (Keissler, 1921). With the exception of *M. sorbi*, all the other lichen species are published by Zahlbruckner (1938-1940) in *Catalogus Lichenum Universalis*.

The morpho-anatomy features of *Mycarthopyrenia pyrenastrella* Keissl. are the following: perithecia are grouped (2-4-7) in a common integument, ascospores are 1-septate, colourless, with a strongly median constriction, with unequal locules and different apexes. As regards *Mycarthopyrenia stenospora*, its thallus is non-lichenized, perithecia 0.2-0.3 mm diameter and it has an endophloedal development of the hyphae (Ciurchea, 2004).

The two studied lichen species are growing on corticolous substrata represented by deciduous trees, especially with a smooth rhytidome (Santesson, 1993)

According to bibliographic sources (Hazslinszky, 1884; Szatala, 1932; Ciurchea, 2004), the chorology of studied lichen species is mentioned below:

Mycarthopyrenia pyrenastrella

Hunedoara County: Retezat Mountains, Valeriască Valley (FR42/43) cited for the first time by Szatala (1932) and afterwards by Ciurchea (2004), but without checking the presence of this species in the cited localities (Fig. 1).

Mycarthopyrenia stenospora

Caraş-Severin County: Cerna Valley at Băile Herculane (FQ17) the initial datum as respects the presence of this species in Romania was mentioned by Hazslinszky (1884) and then by Ciurchea (2004), but without any checking on its chorology (Fig. 2).

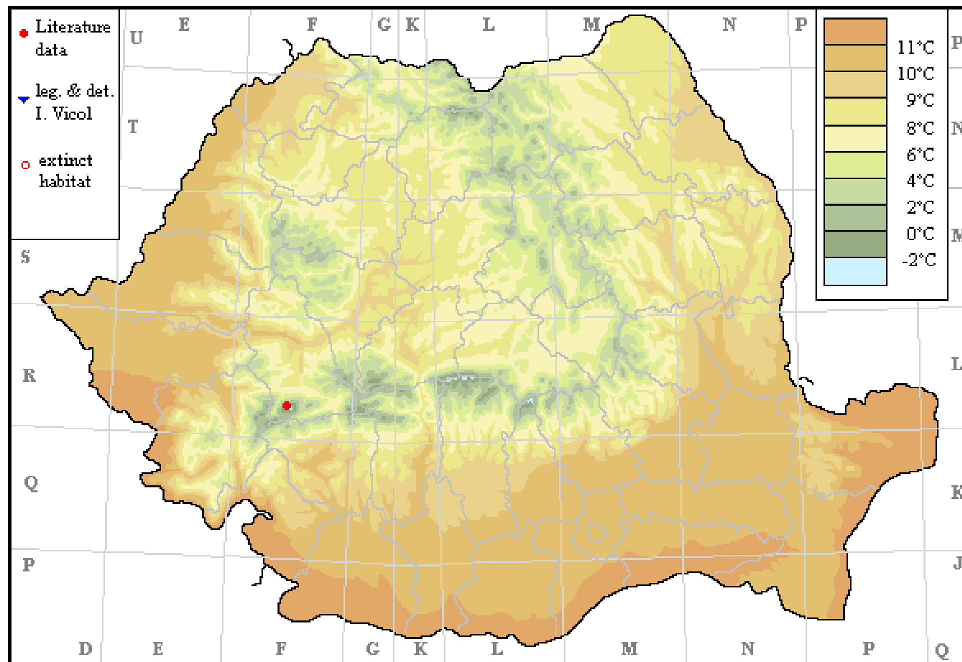

Figure 1. The chorology of *Mycarthopyrenia pyrenastrella* in Romania.

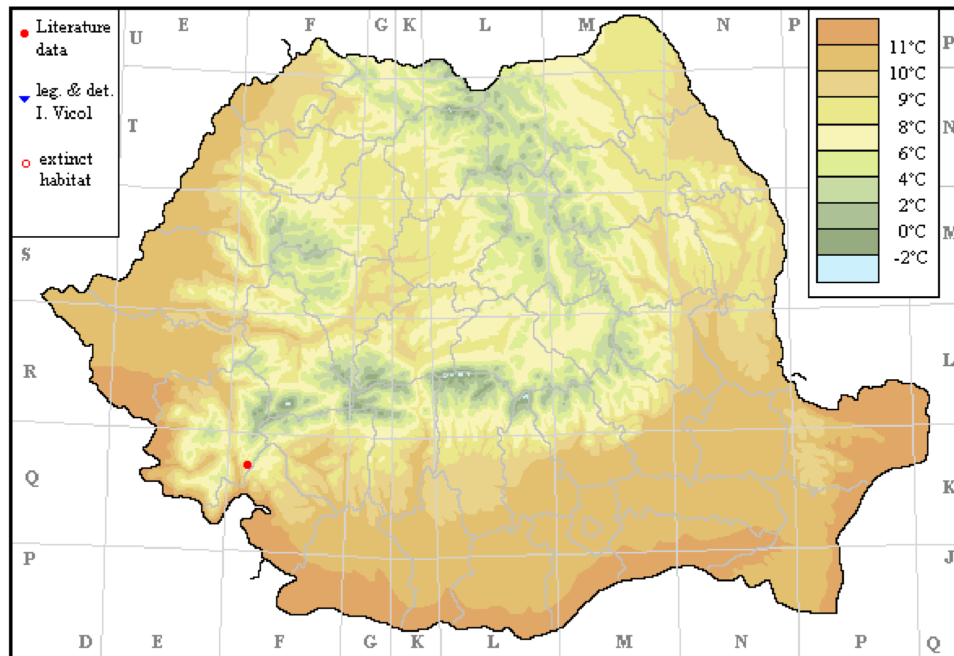

Figure 2. The chorology of *Mycarthopyrenia stenospora* in Romania.

CONCLUSIONS

Within studied area *Mycarthopyrenia pyrenastrella* and *Mycarthopyrenia stenospora* have not been identified. The anthropogenic impacts might be a major cause of the absence of lichen species taken into account from investigated sites. This is a challenge based on reinvestigation of sites, especially from the two lichen species have been cited.

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THE GENETIC VARIABILITY OF *ALTERNARIA PORRI* IN BANGLADESH

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Twenty-seven (27) isolates of *Alternaria porri* were isolated from diseased samples collected from different onion growing regions of Bangladesh and characterized for molecular variability of *Alternaria porri*. In molecular characterization no band was found by using seven (7) RAPD primers and band found from three isolates (DSSA, DSTR 02 and RBHR 02) by using two ITS primers ITS1F and ITS4 in polymerase chain reaction (PCR). Band producing isolates DSSA and RBHR 02 were explored for sequencing. The sequenced designated sample A. DSSA found 75% similarities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima* partial sequence (KM513592.1) and 74% similarities with *Alternaria mali* partial sequence (JF817299.1). Another sample A.RBHR 02 found 86% similarities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% similarities with *Alternaria tenuissima* HLJ-KS-BH-TY-A (KF996744.1). The results demonstrated the existence of considerable variation in molecular characters by DNA sequencing of *Alternaria porri* isolates prevalent in the environment of Bangladesh.

Key words: ITS, molecular, PCR, phylogenetic tree, RAPD.

INTRODUCTION

Onion (*Allium cepa*) is an important spices crop commercially grown in many countries of the world. Out of 15 important vegetables and spice crops listed by FAO, onion stands second in terms of annual world production (Ali, 2008). It ranks first in production among the spices crops cultivated in Bangladesh (BBS, 2008). It is also used as the condiments for flavoring a number of foods and medicines (Hassan *et al*., 2006). The production of onion is nearly 1.159 million metric tons from 335000 acres of land. In Bangladesh requirement of onion per year is around 1.95 million metric tons per year (BBS, 2012). The production of onion in Bangladesh is 8.95 t ha^{-1} (AIS, 2011) which is very lower in comparison of other onion producing countries. Onions are attacked by ten diseases in Bangladesh caused by various pathogens (Ahmed and Hossain, 1985). Purple

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blotch of onion is noted as a major disease throughout the world including Bangladesh (Islam *et al*., 2001). The fungus can cause a reduction in yield ranged from 30 to 50 % (Pascua *et al*., 1997). Many research works have been conducted on the management of purple blotch of onion (Ashrafuzzaman and Ahmed, 1976; Rahman *et al.,* 1988; Rahman, 1990). Rovral 50 WP (0.2%), Dithane M-45 80 WP (0.2) and some other options are suggested as foliar spraying against the disease. But nowadays the fungicides are not working properly against the disease. This might be due to the genetic variability or introduction of new races of the pathogen. Understanding of pathogen population structures and mechanisms by which variation arises within a population is of paramount importance for devising a successful disease management strategy. Continuous monitoring requires of the development of pathogen variability for the breeding programme aimed at developing resistant genotypes to the given set of pathogenic races (Sartorato, 2002). By molecular characterization variation in pathogen populations generally can be detected. DNA markers have become a powerful tool to study the molecular genetics of a variety of organisms. The internal transcribed spacer (ITS) region and random amplified polymorphic DNA (RAPD) perform quick assessment of genetic variability, and has been used to study inter- and intra-specific variability amongst the isolates of several fungal species. The present study was therefore undertaken to determine the genetic variability of the isolates of *Alternaria porri* through molecular characterization.

MATERIAL AND METHODS

Collection of diseased leaf samples

Diseased leaf samples having typical symptoms (Fig. 1) were collected from 9 districts of Bangladesh, namely Dhaka, Mymensingh, Rajshahi, Gazipur, Comilla, Jamalpur, Manikgonj, Jessore, and Faridpur (Table 1). The diseased leaves were cut from the plants grown in the field and put into a brown paper envelope. Then the brown paper envelopes of each collection were taken to the laboratory, Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka for isolation.

Isolation and identification of the pathogen

The pathogen was isolated by tissue culture technique method (Mohsin *et al.* 2016). The disease leaves were cut into pieces (4 mm diameter) and surface sterilization with $HgCl_2$ (1:1000) for 30 seconds. Then the cut pieces were washed in sterile water thrice and dried in keeping untreated blotting paper then placed on to acidified PDA in petri dish. The plates containing leaf pieces were incubated at 25^0C temperature and near ultraviolet for seven days. When the fungus grew well and sporulated then the pathogen slide was prepared and examined under stereomicroscope (Model: Motic, SMZ-168) and compound microscope (Model:

Omana, OMTM-85) for identification of the pathogen with the help of relevant literature (CMI Description Vol. No. 338). Pathogenic conidia were muriform, tapering beak and brown in color (Fig. 2). After identification of *A. porri* it was purified for further study in PDA and preserved in refrigerator at 4±0.5^{0}C for further use.

Figure 1. Collected leaf samples showing typical symptom of purple blotch of onion.

Table 1

Sample collected area of purple blotch of onion caused by *Alternaria porri*

Sl. No	Isolates	Sample collected region			
		District/ Region	**Upazila/ Thana**	**Union/ Organization**	**Village/ Place**
1	DSSA	Dhaka (SAU, Savar)	Sher-e-Bangla Nagar	SAU	Agronomy Field
2	DSTR 01		Savar	Tetuljhora	Rajfulbaria
3	DSTR 02				
4	MMBH	Mymensingh (BAU, Trishal)	Mymensingh Sadar	BAU	Horticulture Field
5	MTBB 01		Trishal	Balipara	Balipara
6	MTBB 02				
7	RBHR 01	Rajshahi (Taherpur)	Bagmara	Hamirkutssa	Ramrama
8	RBHR 02				
9	RBHR 03				
10	GJBS	Gazipur (BARI, Gazipur Sadar)	Joydebpur	BARI	Spices Field
11	GGBB 01		Gazipur Sadar	Baria	Bandan
12	GGBB 02				
13	CCKH 01	Comilla (Chandina)	Chandina	Keronkhali	Harong
14	CCKH 02				
15	CCKH 03				
16	JJLL 01	Jamalpur (Nandina)	Jamalpur Sadar	Lakhirchor	Lakhirchor
17	JJLL 02				
18	JJLL 03				

Table 1 (continued)

19	MSMM 01	Manikganj (Shibalaya)	Shibalaya	Mahadebpur	Mahadebpur
20	MSMM 02				
21	MSMM 03				
22	JMSA 01	Jessore (Monirumpur)	Monirumpur	Shempur	Aminpur
23	JMSA 02				
24	JMSA 03				
25	FFKU 01	Faridpur (Faridpur Sadar)	Faridpur Sadar	Kanaipur	Ulukanda
26	FFKU 02				
27	FFKU 03				

SAU = Sher-e- Bangla Agricultural University
BAU = Bangladesh Agricultural University
BARI = Bangladesh Agricultural Research Institute

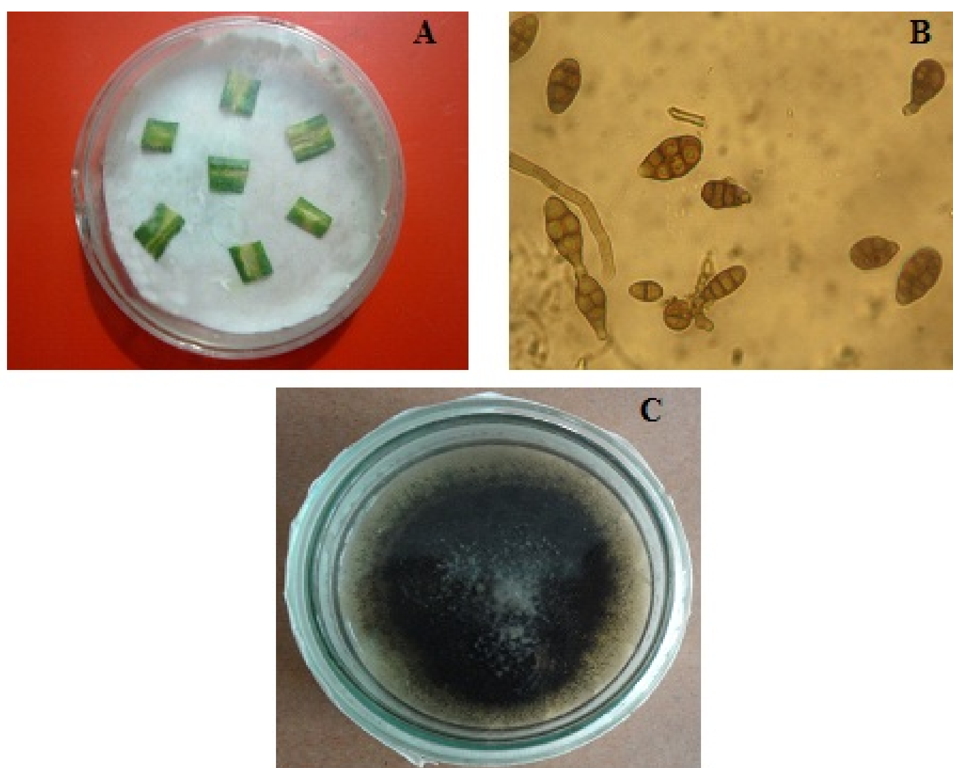

Figure 2. Isolation, identification and pure culture of *Alternaria porri*, (A) Isolaion by blotter method (B) *Alternaria porri* conidia under compound microscope (400x) (C) Pure culture in PDA media.

Designation of collected isolates

The collected isolates were designated following Aminuzzaman *et al*. (2010) based on its locations and sources. For example, an isolate designated by JMSA 01 represents that this isolate was collected from district- Jessore (J), upazilla-

Monirumpur (M), union- Shempur (S), village- Aminpur (A) and 01 denotes collection number.

Collection of mycelium and genomic DNA isolation

To extract genomic DNA, mycelium of *Alternaria porri* was used of 15 days old pure culture of twenty seven (27) isolates. Total genomic DNA of each isolate was extracted using CTAB method (Murray and Thompson, 1980). DNA was stored at -80°C till further use.

RAPD analysis using polymerase chain reaction (PCR)

Seven (7) decamer primers were used for RAPD amplification; which were OPA-01(CAGGCCCTTC), OPA-02(TGCCGAGCTG), OPA-03(ATGCAGCCAC), OPA-04(AATCGGGCTG), OPA-13(CAGCACCCAC), OPB-04(GGACTGGAGT) and OPB-18(CCACAGCAGT). The PCR was carried out in 0.2 ml PCR tube with 25µl reaction volume containing 3.00µl Template DNA 50 ng/µl, 2.50µl Taq Buffer A 10X, 2.50µl dNTPs, 1.00µl Primer, 0.05µl Taq DNA Polymerase and 15.95µl MilliQ H_2O. Reaction mixture was vortexed and centrifuged in a microfuge. Amplifications were performed using thermal cycler (Biometra, UNO II) programmed for initial denaturation at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 30°C for 1 min, 72°C for 3 min and a final extension at 72°C for 5 min using fastest ramp time between transitions.

ITS region identification

Two ITS primer was used for ITS region identification; which were ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC). The PCR was carried out in 0.2 ml PCR tube with 25µl reaction volume containing 3.00µl Template DNA 50 ng/µl, 2.50µl Taq Buffer A 10X, 2.00µl dNTPs, 2.50µl $MgCl_2$, 0.1µl Forward Primer, 0.1µl Reverse Primer, 0.02µl Taq DNA Polymerase and 14.60µl MilliQ H_2O. Reaction mixture was vortexed and centrifuged in a microfuge. Amplifications were performed using thermal cycler (Biometra, UNO II) programmed for initial denaturation at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 49°C for 1 min, 72°C for 3 min and a final extension at 72°C for 10 min using fastest ramp time between transitions.

Gel electrophoresis

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder containing ethidium bromide and 100 ml 1×TAE buffer. Agarose gel electrophoresis was conducted in 1× TAE buffer at 50 Volts and 100 mA for 1.5 hours. One molecular weight marker 1kb DNA ladder was electrophoresed alongside the RAPD and ITS reactions. DNA

bands were observed on UV-transilluminator and photographed by a Gel Documentation system.

Purification and sequencing of PCR product

PCR amplified products were purified by alcohol precipitation and sequenced through automated sequencer in Centre for Advanced Research in Sciences (CARS), University of Dhaka, Dhaka - 1000.

Data analysis

The sequences obtained through sequencing were analysed through BLAST search in order to confirm that it was from *Alternaria porri* genome. Molecular variability sequences were analyzed using the software MEGA version 6.0. Database searches with *Alternaria* sp. sequences were carried out by NCBI-BLAST program (http://blast.ncbi.nlm.nih.gov).

RESULTS

Extraction of genomic DNA

Twenty-four (24) genomic DNA were selected among twenty seven (27) isolates after extraction by CTAB extraction method and this was confirmed by gel running of genomic DNA. Three isolates DSTR 01, FFKU 01 and FFKU 02 were discarded because of low concentration of genomic DNA (Fig. 3).

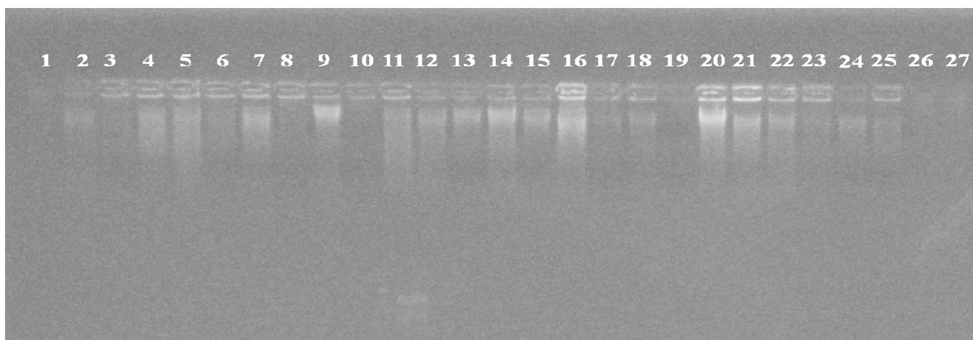

Figure 3. Gel electrophoresis of the genomic DNA of *Alternaria porri* isolates .

RAPD analysis

RAPD analysis was done by seven decamer primers to find out the variation among the isolates. These primers were selected for fingerprinting of twenty-four (24) isolates of *Alternaria porri*. But there was no band found by using these primers.

ITS region identification

For ITS region identification, two ITS primers ITS1F and ITS4 were used as forward primer and reverse primers respectively. By using these primers the ITS regions were identified from three (3) isolates among twenty four (24) and those isolates were DSSA, DSTR 02 and RBHR 02 (Fig. 4).

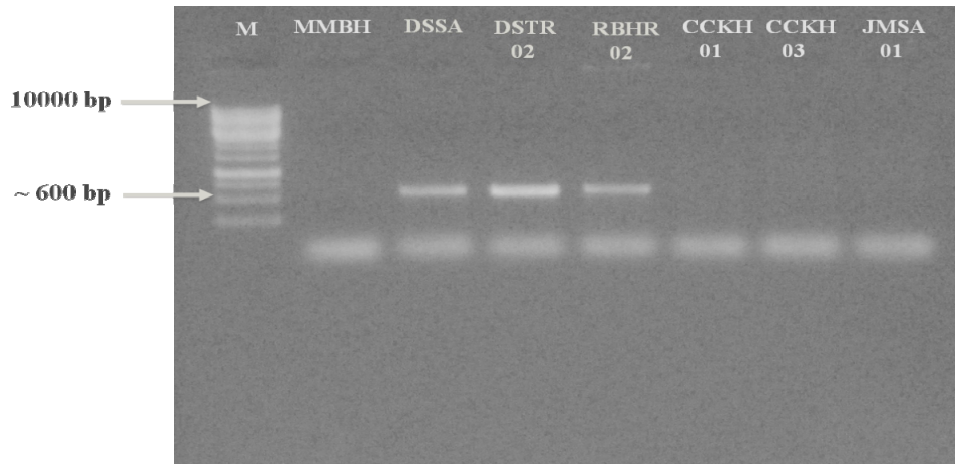

Figure 4. Gel electrophoresis of the PCR product performed by ITS1F and ITS4 primers and showing ~600 bp amplification.

PCR products purification and sequencing

Purification of PCR products was done by alcohol precipitation. Two (2) isolates DSSA and RBHR 02 were purified among three (3) and isolate DSTR 02 was missed during purification. In order to confirm at the gene sequence level, PCR amplified bands were subjected to automated sequencing. For this purpose, a 600 bp band from two samples was used for sequencing. The sequenced samples were designated as Ap-DSSA and Ap-RBHR 02, respectively.

Analysis of the sequence results

Partial sequences of two samples were analyzed through NCBI-BLAST program database search system. Obtained results from the BLAST database showed that one sample A.DSSA has 75% nucleotide (nt) identities with *Alternaria porri* genomic DNA (LN482533.1), *Alternaria alternata* partial sequence (KF269242.1), *Alternaria tenuissima* partial sequence (KM513592.1), and 74% nucleotide (nt) identities with *Alternaria mali* partial sequence (JF817299.1) fungus gene (Table 2). Another sample A.RBHR 02 has 86% nucleotide (nt) identities with *Alternaria* sp. UFMGCB 4425 fungus complete genome (KJ404206.1) and 81% nucleotide (nt) identities with *Alternaria tenuissima* HLJ-KS-BH-TY-A (KF996744.1) fungus

gene reported from this geographic area as well as with others from worldwide (Table 3).

Table 2

Percent identities of nucleotide of A.DSSA isolate with selected *Alternaria* fungus reported worldwide

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Identity
LN482533.1	*Alternaria porri* genomic DNA	167	167	80%	1e-37	75%
KF269242.1	*Alternaria alternata* partial sequence	170	170	80%	1e-38	75%
KM513592.1	*Alternaria tenuissima* partial sequence	167	167	80%	1e-37	75%
JF817299.1	*Alternaria mali* partial sequence	170	170	86%	1e-38	74%

Table 3

Percent identities of nucleotide of A.RBHR 02 isolate with selected *Alternaria* fungus reported worldwide

Accession	Description	Max score	Total score	Query coverage (%)	E-value	Identity
KJ404206.1	*Alternaria* sp. partial sequence	69.4	69.4	24%	2e-08	86%
KF996744.1	*Alternaria tenuissima* partial sequence	147	147	72%	1e-31	81%

Phylogenetic analysis

The phylogenetic analysis done by using MEGA 6.0 software revealed that the A.DSSA isolate forms a cluster by separate clade. This analysis revealed that this isolate is the major ancestor of all the strains (Fig. 5). Another isolate A.RBHR 02 also forms a cluster by separate clade. This analysis also revealed that this isolate has close relationship with KF996744.1 (Fig. 6).

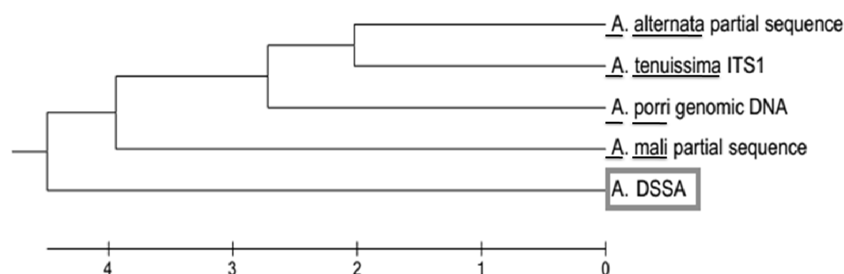

Figure 5. The phylogenetic tree for A.DSSA isolate showing ancestral relationship with other reported *Alternaia* isolates.

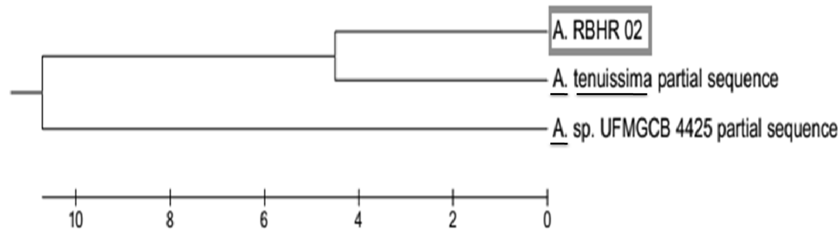

Figure 6. The phylogenetic tree for A.RBHR 02 isolate showing ancestral relationship with other reported *Alternaria* isolates.

DISCUSSION

Our study was aimed to critically determine the genetic variability of the isolates of *A. porri* collected from different locality and possible morphological classification and to evaluate the relationship between host/geographic association and phylogenetic lineage. Due to the economic importance of small-spored *Alternaria* species, especially within the alternate species-group, a predictive association of species names with biology is needed to allow rapid identification. As a genus, *Alternaria* encompasses considerable morphological diversity and there have been a number of attempts to organize taxa into subgeneric groupings based on shared morphological characters. Several workers have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. (Singh *et al*., 2003; Slavov *et al*., 2004; Tetarwal *et al*., 2008). Mohsin *et al.* (2016) reported growth rate of *Alternaria porri* isolates 2.43 to 3.95 mm/day, amount of conidia 7.72×10^3 to 47.02×10^3/ mm^2, sporulation time 3.33 to 11.00 days and average conidial size ranged from 28.31 to 19.38 × 8.147 to 6.740 µm. Also reported leaf infection rate by *Alternaria porri* isolates 2.77 to 7.55 mm/day. It appears that the variation amongst the isolates may be inherent since isolates were collected from different/ distant sites. Phenotypic characters are influenced by environmental conditions so they may be responsible for such a diversity (Sofi *et al*., 2013). Moreover, the isolates in these sites may have acclimatized for many years which may be responsible for this diversity. Many of the phenotypic markers are controlled by many genes most of which have additive effect. Molecular relationships amongst *Alternaria* species based on nuclear ribosomal DNA and hostspecific toxins (Kusaba and Tsuge, 1995) or with other related fungi have been analyzed (Pryor and Gilbertson, 2000; Chou and Wu, 2002). Variation in nuclear ribosomal DNA sequences among *Alternaria* species pathogenic to crucifers has been reported from one isolate each of *A. brassicae*, *A. brassicicola*, *A. raphani* and *A. alternata* (Jasalavich *et al*., 1995).

Two primers ITS1F and ITS4 were used to identify ITS region. After purification a 600 bp band from two samples was used for sequencing. The isolate A.DSSA showed 75% nt similarities with *Alternaria porri*, *Alternaria alternata*, *Alternaria tenuissima* and 74% nt similarities with *Alternaria mali*. Another isolate A.RBHR showed 86% nt similarities with *Alternaria* sp. and 81% nt similarities with *Alternaria tenuissima*. The results are in agreement with McKay *et al*. (1999) who reported 99.6% level of homology in the ITS regions of *Alternaria alternata* and *Alternaria lini*. Similarly, Kusaba and Tsuge (1995) also observed that the close relationship between *Alternaria dauci* and *Alternaria bataticola*, is shown by the high level of homology between the two species. Sharma *et al*. (2013) reported isolates were characterized by using internal transcribed spacer region where all the isolates were found 56% similar to each other and 99% similar to the *A. brassicae* isolates. Pusz (2009) reported low genetic variability among the isolates of *Alternaria alternata* isolated from *Amaranthus*. Kumer *et al*. (2008) also found no effect of origin of isolates; rather two isolates of *Alternaria solani* from two different locations were closer to each other. BLAST analysis of the internal transcribed spacer region of *Alternaria porri* isolates in this study showed high similarity among the isolates with other *Alternaria* of the NCBI database. The phylogenetic analysis done by using MEGA 6.0 software revealed that the isolates form a cluster by separate clade.

In our study we have also performed RAPD analysis using seven decamer primers which were selected for fingerprinting of twenty-four isolates of *Alternaria porri*. However, there was no band found by using these primers. Therefore, further investigation is necessary. Our results suggest that all stable characters, both molecular and morphological, should be considered in developing a more predictive classification system. It also suggests that in such cases breeding efforts should concentrate on quantitative resistance or on the development of cultivar mixtures that can be used in combination with other control strategies (McDonald and Linde, 2002).

CONCLUSIONS

Sequencing DNA of fungal isolates showed substantial identities with *Alternaria porri* after being analyzed through NCBI-BLAST program database. The isolates form cluster by separate clade by phylogenetic analysis and indicate that they have variation from others. Further detailed variation and diversity can be studied from the full genome sequence analysis. This could help us to reveal the true nature of this fungus from Bangladesh and the proper sustainable management for purple blotch of onion disease can be implemented.

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COMPARATIVE STUDY OF ESSENTIAL OIL CHEMICAL CONSTITUENTS OF *CALOTROPIS PROCERA* LEAVES COLLECTED FROM DIFFERENT NATURAL LOCALITIES

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Medicinal plants are the major sources of traditional medicine worldwide. *Calotropis procera* is a plant with many medicinal properties and other economic values with the following features: a perennial shrub, salt and drought resistant, growing in tropical and subtropical environment. The plant parts contain many biologically active molecules which made it a good candidate for herbal medicine. In this project, chemical constituents of essential oil of *C. procera* plants, collected from some natural localities of South of Kerman province, have been investigated using GC-MS chromatography. Essential oil prepared using Clevenger and steam distillation techniques. 31 compounds were totally identified by GC-MS analysis. Different types of chemical compounds with vary functional groups were found. Decane derivatives were the predominant chemical compounds in all localities. The highest and the least number of constituents among the localities were respectively found in leaves of Chah dadkhoda and Shamsabad, respectively. Samples from Jiroft, Chah dadkhoda and Kahnooj had the highest to the lowest percentage of chemical compounds content, respectively. Based on the results, decane and its derivate are predominant in leaf essential oil of calotropis from all localities in Kerman province. Chah dadkhoda locality showed the highest percentage of constituents in essential oil of calotropis leaves.

Keywords: *Calotropis procera*, Essential Oil, GC-MS analysis.

INTRODUCTION

Many plants accumulate organic substances and bioactive molecules which can be used in herbal drug medicine (Aliyu *et al*., 2015). *Calotropis procera,* a flowering shrub of family Asclepiadaceae, is one of the important numbers of traditional herbal medicine in many countries. This shrub is distributed in tropical and subtropical area of the world (Suresh Kumar *et al*., 2013, Aliyu *et al*., 2015) and also throughout south of Iran (Oloumi, 2014). *Calotropis procera* has been reported to possess numerous medicinal properties (Alencar *et al*., 2004). The

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ethnobotanical study on *C. procera* revealed anti-inflammatory (Alencar *et al*., 2004) antioxidant (Kumar *et al*., 2013), antibacterial activity (Ali *et al*., 2014), anticancer (Ibrahim *et al*., 2014), cytotoxic properties (Choedon *et al*., 2006), analgesic activity, schizontocidal activity, antifertility activity, etc. (Sharma & Sharma, 1999) of the plant parts. The plant is also known for its toxic properties that include dermatitis, iridocyclites, and acts like a poison and produces lethal effects (Muzammal, 2014, Mohamed *et al*., 2015, Patil & Makwana, 2015, Iqbal *et al*., 2005).

The *Calotropis procera* plant has many medicinal properties due to the presence of secondary metabolites, and numerous phytochemicals (Begum *et al*., 2013, Sheth, 2011, Moronkola *et al*., 2011, Tsala *et al*., 2015). Medicinal importance of these plants is due to some chemical substances with physiological effects on the cell (Radhakrishnan *et al*., 2013, Singhi *et al*., 2015, Joshi *et al*., 2015). It has been shown that plant parts such as leaves, flowers and roots contained a high amount of ash, protein and carbohydrates (Kalita & Saikia, 2004) with vary quantities of alkaloids, leaves contained calotropin (Kanojiya & Madhusudanan, 2012) and calotropegenin (Khairnar *et al*., 2012, Shehab *et al*., 2015).

There are many reports on the phytochemical properties and antibacterial effects of this plant collected from different localities such as India (Ranjit *et al*., 2012, Alam & Ali, 2009, Tiwari *et al*., 2015, Shrivastava *et al*., 2013, Gajare *et al*., 2012, Patel *et al*., 2014, Tsala *et al*., 2015), Nigeria (Mainasara *et al*., 2012, Kawo *et al*., 2010, Okiei *et al*., 2009, Kareem *et al*., 2003, Muraina *et al*., 2010) and Pakistan (Khanzada *et al*., 2008, Hussain *et al*., 2011). The major phytochemicals such as alkaloids, carbohydrates, glycosides, phenolic compounds/tannins, proteins and amino acids, flavonoids, saponins, sterols, acid compounds, resins has been screened in different parts of *C. procera* plant like latex, leaf, flower, bud and root of *Calotropis* (Suresh Kumar *et al*., 2013, Seiber *et al*., 1982, Mainasara *et al*., 2012, Kumar & Sehgal, 2007). Different parts (flowers, leaves and roots) of *C. procera* were analyzed for the metabolites concentrations. The results showed the presence of nutritive chemicals and secondary metabolites in almost all parts of the plant which has made this plant as acceptable forage and effective medicine for human as well as for the livestock (Khattak *et al*., 2015).

In Iran *C. procera* is found in Khouzestan, Lorestan Andimeshk, between Shush and Dehloran, Dezful and Fars, and it is also widespread in the south of Kerman, near the Persian Gulf and Bandar Abbas, Chah Bahar, Iran Shahr, Nikshahr (Zaeifi, 1990, Mozaffarian, 1991). Although there are some reports showing application of different parts of *C. procera* in traditional medicine of Iran (Sadeghi *et al*., 2014), there is very little information about the phytochemical constituents of calotropis in this country. Due to the increasing trend in the use of herbal medicines worldwide, phytochemicals of *C. procera* gathered from different natural localities of Kerman province with the high range of the plant distribution has been studied in this investigation.

MATERIAL AND METHODS

Plant materials

Fresh leaves of *Calotropis procera* were collected from the natural habitats of Kerman province (latitude, longitude) including Orzueeyeh (28.4582494, 56.3628409), Jiroft (28.6751124, 57.7371569), Chah dadkhoda (27.2836629 58.2619308), Shamsabad (30.3500225, 56.7798278) and Kahnooj (28.0250371, 57.745955) during June-July, 2014. The plant material was identified using standard keys and descriptions. The leaves were stored at 4ºC until use.

GC/MS analysis

The essential oil of the leaves was used for GC-MS (Gas chromatography–mass spectrometry) analysis. To isolate the essential oil, air dried leaves of the plants were ground to a fine powder. Then, the plant material was hydro-distilled in a glass Clevenger's apparatus during approximately 3 hours. The essential oil was analyzed using gas chromatography/mass spectroscopy.

The gas chromatography analysis was performed using GC Agilent Technologies 7890A fitted with HP-5 capillary column (30.0 m×250 µm× 250 µm) using helium as a carrier gas. The column temperature was initially 50ºC for 10 min and temperature was increased at 4ºC/min up to 300ºC at 30 min. The flow rate of helium gas was 1 ml/min.

Mass spectroscopy was carried out using MS Agilent Technologies 5775 CA. The ionization mass spectroscopic analysis was done with 70 eV. Identification of components was based on comparison of their mass spectra with dose of Wiley library and comparison of their retention indices with literatures. Phytochemical compounds were represented based on the peak area (which represents the percentage of that compound).

Statistical analysis

Data from antibacterial studies were analyzed using the analysis of variances (ANOVA) followed by Duncan test (SAS v. 9.1). Each data was the mean of three replicates and means were considered as significant differences at $p \leq 5\%$ level.

RESULTS AND DISCUSSION

The constituent rich essential oil of *C. procera* leaf was subjected to Gas Chromatography-Mass spectrometry (GC-MS) analysis. Phytochemicals of leave essential oil from *Calotropis procera* of five natural populations of Kerman province are given in Tables 1 to 5.

Table 1

Chemical constituents of leaf essential oil of *C. procera* using GC-MC analysis in Orzueeyeh

	Peak number	Identified compounds	R.Time (min)	RI	Area%	Qual
2.	1.	Decane	13.960	1000	11.36	96
4.	3.	Menthon (trans)	21.534	1153	0.61	97
6.	5.	Iso menthone	21.979	1164	0.38	90
8.	7.	Dodecane	23.536	1200	2.97	97
10.	9.	Pulegone	24.995	1239	1.33	96
12.	11.	Tetradecane	30.658	1400	0.92	97
14.	13.	Geranyl acetone	32.388	1454	1.07	95
16.	15.	Germacrene D	33.222	1481	0.74	96
18.	17.	Beta-Ionone	33.396	1486	3.26	96
20.	19.	Hexadecane	36.759	1600	1.06	96
22.	21.	Octadecane	42.248	1800	0.82	97
24.	23.	2-Pentadecanone, 6,10,14-trimethyl	43.437	1846	3.28	98
26.	25.	1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl)ester (phthalic acid)	44.034	1869	1.36	90
28.	27.	Nonadecane	44.819	1899	0.43	81
30.	29.	5,9,13-Pentadecatrien-2-one, 6,10,14- trimethyl	45.327	1919	1.15	81
32.	31.	Hexadecanoic acid	46.522	1967	4.67	98
		Total			35.41	

Table 2

Chemical constituents of leaf essential oil of *C. procera* using GC-MC analysis in Jiroft

Peak number	Identified compounds	R.Time (min)	RI	Area%	Qual
1.	Decane	13.960	1000	21.07	96
2.	Undecane	19.234	1100	0.45	90
3.	Nonanal	19.491	1105	3.65	91
4.	Dodecane	23.535	1200	10.54	97
5.	Naphthalene, decahydro-1,5-dimethyl	24.432	1224	0.71	94
6.	Tridecane	27.274	1300	0.83	92
7.	Beta-Damascenone	30.157	1385	1.49	96
8.	Tetradecane	30.658	1400	3.84	98
9.	Geranyl acetone	32.381	1454	8.89	95
10.	Beta-Ionone	33.409	1487	9.28	98
11.	Hexadecane	36.766	1648	2.34	97
12.	Octadecane	42.248	1800	1.01	90
13.	1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl)ester (phthalic acid)	44.062	1870	0.94	83
14.	5,9,13-Pentadecatrien-2-one, 6,10,14- trimethyl	45.347	1920	2.66	93
	Total			67.7	

Table 3

Chemical constituents of leaf essential oil of *C. procera* using GC-MC analysis in Chah dadkhoda

Peak number	Identified compounds	R.Time (min)	RI	Area%	Qual
1.	Alpha-pinene	9.298	930	0.43	90
2.	Nonane, 5-methyl	11.375	961	0.74	86
3.	Decane	13.960	1000	25.04	97
4.	Nonanal	19.484	1105	3.02	87
5.	Dodecane	23.535	1200	6.16	96
6.	Damascenone A	30.164	1285	0.69	90
7.	Tetradecane	30.658	1400	1.76	97
8.	Caryophyllene	31.248	1418	3.91	98
9.	Geranyl acetate	32.402	1455	2.41	83
10.	Beta-Ionone	33.409	1487	3.53	98
11.	Caryophyllene oxide	36.293	1640	7.16	87
12.	Hexadecane	36.766	1648	0.93	93
13.	Heptadecane	39.573	1700	0.58	81
14.	Octacosane	42.248	1800	0.63	80
15.	2-Pentadecanone, 6,10,14-trimethyl	43.436	1846	4.66	90
16.	1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (phthalic acid)	44.048	1869	0.79	90
17.	Hexadecanoic acid	46.508	1966	2.74	97
18.	10-Methylnonadecane	47.334	1999	0.46	80
	Total			66.27	

Table 4

Chemical constituents of leaf essential oil of *C. procera* using GC-MC analysis in Shamsabad

Peak number	Identified compounds	R.Time (min)	RI	Area%	Qual
1.	Nonane, 5-methyl	11.403	961	1.68	90
2.	Nonane, 3-methyl	12.133	972	2.57	83
3.	Decane	13.974	1000	56.58	97
4.	Dodecane	23.535	1200	14.19	96
5.	Tetradecane	30.679	1400	3.25	94
6.	Decane, 2-methyl	36.793	1649	1.77	86
	Total			80.04	

Table 5

Chemical constituents of leaf essential oil of *C. procera* using GC-MC analysis in Kahnooj

Peak number	Identified compounds	R.Time (min)	RI	Area%	Qual
1.	Decane	13.932	1000	24.42	96
2.	Dodecane	23.528	1200	5.34	94
3.	Tetradecane	30.672	1400	1.43	87
4.	Beta-Ionone	33.416	1487	2.40	95
5.	2-Pentadecanone, 6,10,14-trimethyl	43.436	1846	2.56	91
6.	1, 2-Benzenedicarboxylic acid, bis (2-methylpropyl)ester	44.055	1870	1.74	83
7.	Hexadecanoic acid	46.522	1967	2.99	90
	Total			40.88	

By comparison of retention indices and the mass spectra of GC component with those of standards and with reported data, 31 compounds were totally identified in Kerman natural localities of calotropis. In the GC-MS analysis of *C. procera* leaves, the most and the least numbers of constituents were respectively found in leaves of Chah dadkhoda and Shamsabad.

The number of chemical contents in essential oils were 16, 14, 18, 6, 7 constituents in Orzueeyeh, Jiroft, Chah dadkhoda, Shamsabad and Kahnooj, respectively. Several groups of chemical compounds including alkanes (decane, dodecane, hexadecane, octadecane, nonadecane, tridecane, heptadecane, octacosane, undecane, nonane), alkenes (Alpha- pinene), ketone (menthone, beta-Ionon, (beta)-damascenone), ester (Benzene dicarboxylic acid, bis (2-methylpropyl) ester or phthalic acid) were observed in the essential oil (Table 5). Our results showed the existence of terpenods such as menthone, pulegone, geranyl acetate, sesquiterpene germacrene (having antimicrobial and insecticidal properties) was only found in Orzueeyeh sample and (iso) caryophyllene, an essential oil of the plant. Presence of terpenoids was previously reported by Tiwari *et al*., in *C. procera* leaf extract (Tiwari *et al*., 2014, Saratha & Subramanian, 2012). The results also lead to the identification of numerous compounds from the GC fractions of the ethanolic extract of *C. procera* with various functional groups such as hydrocarbon (decane derivaties, decahydro-1,5-dimethyl-naphthalene), carboxylic acid (1,2-Benzene dicarboxylic acid, bis (2-methylpropyl, methyl nonanoate), ester (Phthalic acid)) and methyl (2-Pentadecanone, 6,10,14-trimethyl, Methylnonadecane, Nonane, 5(or 3)-methyl).

1,2 -Benzene dicarboxylic acid, bis (2-methylpropyl)ester and Beta-Ionone in appreciable concentration was present in almost all oil populations except for Shams Abad. Similar to our results, Verma *et al*. (2013) found the presence of various types of compounds in *C. procera* leaves using GC-MS analysis of extracts,. They reported 26 compounds in the GC-MS analyses in the ethanolic extract and 17 compounds in chloroform extract of *C. procera*. In another species, Dhivya and Manimegalai (2013) reported the presence of 14 major compounds in ethanolic extract of *C. gigantae*. Similar to our results, they found the presence of hydrocarbon, methyl, hydroxyl, carbonyl, carboxylic acid functional groups in the extract. They observed 6,10,14-trimethyl 2-Pentadecanone as an effective repellent against Anopheles species in the essential oil of *C. procera.* Authors stated that presence of various bioactive compounds justifies the use of the flower of *C. gigantea* for various ailments by traditional practitioners. This compound was found in appreciable concentrations in leaves essential oil of *C. procera* collected from Orzueeyeh, Chahdadkhoda and Kahnooj.

Decane, dodecane and tetradecane were common compounds in all regions. Decane derivatives were the predominated compounds in all regions and decane had the highest percentage of peak area in all localities. Calotropis leaves of Shamsabad had the highest decane (56.58%) and dodecane (14.19%) content in their essential oil. Although based on the results of Okiei *et al*., (2009), hexadecanal was present in all fractions of *C. procera*, this compound being as not

observed in plants from any localities, in this study. Okiei *et al*., (2009) identified phytol, tetramethyl hexadecene-1-ol isomers and trimethyl pentadecanone as major constituents of hydro-distillates of *Calotropis procera* by GC-MS analysis (Okiei *et al*., 2009).

Menthon (trans), Iso menthone, germacrene D, and nonadecane were exclusively found in samples from Orzueeyeh. Jiroft sample was unique in terms of the hexadecane, octadecane, tridecane, naphthalene, decahydro-1,5-dimethyl-, and Beta-Damascenone. Alpha- pinene, damascenone A, caryophyllene, caryophyllene oxide, heptadecane, octacosane, and 10-methylnonadecane existed only in Chah dadkhoda samples. Nonane, 5-methyl, nonane, 3-methyl, and Decane, 2-methyl were exclusive compounds of shamsabad (Table 6).

Table 6

Phytocomponents identified in the leaf ethanolic extract of *Calotropis procera* by GC-MS analysis in Kerman natural localities

No.	**Name of the compound**	**Orzueeyeh**	**Jiroft**	**Chah dadkhoda**	**Shamsabad**	**Kahnooj**
		Area (%)				
1.	Decane	11.36	21.07	25.04	56.58	24.42
2.	Menthon (trans)	0.61	—	—	—	—
3.	Iso menthone	0.38	—	—	—	—
4.	Dodecane	2.97	10.54	6.16	14.19	5.34
5.	Pulegone	1.33	—	—	—	—
6.	Tetradecane	0.92	3.84	1.76	3.25	1.43
7.	Geranyl acetone	1.07	8.89	2.41	—	—
8.	Germacrene D	0.74	—	—	—	—
9.	Beta-Ionone	3.26	9.28	3.53	—	2.40
10.	Hexadecane	1.06	2.34	0.93	—	—
11.	Octadecane	0.82	1.01	0.63	—	—
12.	2-Pentadecanone, 6,10,14-trimethyl	3.28	—	4.66	—	2.56
13.	1,2-Benzene dicarboxylic acid, bis (2-methylpropyl) ester (Phthalic acid)	1.36	0.94	0.79	—	1.74
14.	Nonadecane	0.43	—	—	—	—
15.	5,9,13-Pentadecatrien-2-one, 6,10,14- trimethyl	1.15	2.66	—	—	—
16.	Hexadecanoic acid	4.67	—	2.74	—	2.99
17.	Nonane, 5-methyl	—	—	0.74	1.68	—
18.	Nonane, 3-methyl	—	—	—	2.57	—
19.	Decane, 2-methyl	—	—	—	1.77	—
20.	Undecane	—	0.45	—	—	—
21.	Nonanal	—	3.65	3.02	—	—
22.	Naphthalene, decahydro-1,5-dimethyl	—	0.71	—	—	—
23.	Tridecane	—	0.83	—	—	—
24.	Beta-Damascenone	—	1.49	—	—	—

Table 6 (continued)

25.	Alpha- pinene	—	—	0.43	—	—
26.	Damascenone A	—	—	0.69	—	—
27.	Caryophyllene	—	—	3.91	—	—
28.	Caryophyllene oxide	—	—	7.16	—	—
29.	Heptadecane	—	—	0.58	—	—
30.	Octacosane	—	—	0.63	—	—
31.	10-Methylnonadecane	—	—	0.46	—	—
Total		35.41	67.7	66.27	80.04	40.88

CONCLUSION

GC-MS analysis showed the existence of various compounds with variable chemical structures in *Calotropis procera* leaf essential oil collected from five natural localities of Kerman. Totally, 31 chemical constituents were found in *C. procera* leaves collected from natural localities of Kerman province. The highest (n=18) and the lowest (n=6) number of chemicals were found in Chah dadkhoda and Shamsabad, respectively. Alkane hydrocarbons like decane, dodecane and tetradecane were found in all populations. Decane derivatives were predominant in all regions. The highest content of decane was found in Shams Abas.

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