African Journal of Plant Science

Volume 7 Number 10 October 2013

ISSN 1996-0824

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Vol. 7(10), pp. 445-447, October 2013
DOI: 10.5897/AJPS2013.1054
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Review

Relationship between gibberellins and carbohydrates in vegetable products

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Accepted 16 September, 2013

Gibberellin is a vegetable hormone widely used in agriculture and horticulture. The objective of this review was to show the relationship between gibberellins and carbohydrates in vegetable products. Thus, the study concludes that the involvement of substances may be related with the terminal stages of development of various plants. However, the interactions of carbohydrate content and gibberellin are still discussed.

Keywords: Flowers, hormones, enzymes, postharvest.

INTRODUCTION

Commercially use, the term regulator that is nothing more than substances that reduce the effects of hormones produced by plants (Yamada, 1992). The benefits from the knowledge of the activity of hormones on the various processes of plant development are undeniable. Its discovery has brought great advances in physiology, leading to understanding and controlling cell differentiation, culminating in the emergence of tissue culture isolates *in vitro*, which together with molecular biology, was one of the most important tools for the development agriculture (Taiz and Zeiger, 2004).

In this respect, the objective of this review is to show the relationship of the vegetal hormone, gibberellins, with one of the substances that may be related to senescence in plants, such as carbohydrates.

HORMONES

Hormones regulate source/drain ratios, probably in combined action; where a hormone acts as an event promoter and another as inhibitor of such event. Thus, the plant can control the whole process of absorption, transport and utilization of carbohydrates step by step, making a fine adjustment according to the instant environmental variations, such as contents of CO_2 or H_2O, to maximize the plant production process. Probably none of these processes is controlled by a single hormone but rather by their balance; as example the control of stomatal opening (Mansfield and Mcainsh, 1995). The floral stimulus, for example, is believed to be produced simultaneously on different leaves, through phytochromes, sent and amplified in cascade reactions in two levels, with cooperation between intracellular microfunctions and intercellular macrofunctions and this is in connection with the production and distribution of energy and genetic activation, associated with changes in the balance of phytohormones. Such a response is also dependent on the changes that occurred in the existing

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Abbreviations: CO_2, carbon dioxide; **H_2O**, dihydrogen monoxide; **GA,** gibberellin; **GA_3**, gibberellic acid; **MEP**, metileritritolfosfato.

structures and enzymatic activity that remained in the vegetative growth (Jaegher and Boyer, 1990).

Gibberellins

In the 1950's, gibberellin was characterized as phytohormones. Phytohormones are organic compounds, non-nutrients, produced by plants and in low concentrations (10^{-4} M) they promote, inhibit or modify physiological and morphological processes of vegetables (Davies, 2004). The most important gibberellin is GA_1 and most of the others are precursors of GA_1, except GA_3, GA_5 and GA_6. It has a complex structure, being chemically isoprenoid. It is synthesized by a branch in the route of terpenoids, which are synthesized by the route of mevalonic acid and by the route of metileritritolfosfato (MEP).

The differences between many gibberellin are the number and the localization of the double connections and the hydroxyls group (Taiz and Zeiger, 2004). The gibberellin group covers a large number of compounds, where 1/3 are gibberellin with 20 carbons and the others are gibberellin with 19 carbons, being more active with the GA_1, GA_3, GA_4, GA_7, GA_9 and GA_{20}.

CARBOHYDRATES

The knowledge of phenology and how the reserve carbohydrates content varies during the development of plants have been the basis to developing phenological models of production. These models have been used in the assessment of management techniques in numerous plant species that have been successfully grown around the world (Corsato et al., 2004).

Carbohydrates are a group of compounds including simple sugars and more complex molecules consisting of subunits of simpler sugars, whose main function is to supply energy to the chemical processes of the cell life cycle (Brum et al., 1994; Moalen-Beno et al., 1997). Carbohydrates also present various functions, such as osmotic regulator (Bieleski, 1993) and as a metabolic precursor of other molecules (Sood et al., 2006).

Starch and soluble sugars (sucrose and derivatives) are the main reserve carbohydrates derived from glucose formed during photosynthesis. The first is still in the cell, being synthesized in the chloroplasts of photosynthetic tissues, while the second has high mobility in cells and tissues being discharged into the phloem and cytosol of the cells. Once translocated to non-photosynthetic drains, sucrose is rapidly converted into fructose and glucose (Dennis and Blakeley, 2000). Once in these organs, these sugars can be used in three ways: a) in the breath for the synthesis of energy and other compounds; b) be stored in the vacuole for subsequent use in breathing or c) be converted inside the reserve organs for use in situation of low photosynthetic activity in shoots (Taiz and Zeiger, 2002). By the time that precedes, the start of photoassimilates export by the photosynthetic tissues newly formed in the shoot for every new cycle of the plant is observed; all developing organs are totally dependent on the carbohydrate reserves present in the storage organs. This dependence decreases with the increased amount of assimilates exported by the expanding canopy (Darnell and Birkhold, 1996).

The carbon metabolism in the source and drain, as well as transportation between these tissues is regulated by a "feedback" control by the levels of intermediate and final metabolites. The "feedback" control is performed directly by the metabolite at transcription level of specific enzymes of their metabolism, or mediated by hormones, which control these enzymes. When there is no use of these metabolites their formation decreases (Foyer and Galtier, 1996).

The movement of photoassimilates from the synthesis site, in the source, to the place of use or accumulation in the drain can be regulated at several points. The concentration gradient between the source and drain is generally accepted as the main determinant of the rates of transport and partitioning among organs. Such processes are also controlled in part by hormones that act as messengers among cells, tissues and organs, promoting the activation of specific genes or metabolic processes (Foyer and Galtier, 1996). However, to evaluate the control of source/drain ratios, it is important to first understand where these hormones act and if possible, their place of origin, which are generally in the very drains (HO et al., 1989). According to Kock et al. (1996), the carbohydrate metabolism of plants has a complex signaling mechanism involving sugars and hormones capable of controlling the enzymes involved in the synthesis/degradation of starch and sucrose accumulation.

In the literature were found studies showing the relationship between carbohydrates and gibberellins in vegetable products. In potato, the gibberellins are involved in dormancy and initiation of tuber development, associated with levels of inhibitors of carbohydrate available and environmental factors such as light, temperature and photoperiod (Tamas, 1995). In vines, gibberellins increase the activity of sucrose-phosphate synthase, stimulating the export of sucrose from the leaf, being considered to induce the reproductive development (Brenner and Cheikh, 1995). Spraying with gibberellic acid (GA_3) decreased the diameter of the pseudobulbs of D. *nobile*, probably because this is the main storage organ for water, carbohydrates and mineral nutrients of epiphytic orchids (Zimmermann, 1990). This author also reports that gibberellins act on the metabolism of carbohydrates mainly stored in the pseudobulb, causing decreased pseudobulb diameter due to the hydrolysis of its reserve substances. Furthermore, the accumulation of carbohydrates in the tissue increases the osmotic pressure, making the stored water flow, especially in the

pseudobulb; occur more rapidly in the interior of the cell, promoting its expansion (Cordeiro, 1979; Daykin et al., 1997; Pires, 1998). Martinez-Cortina and Sanz (1991), studying the carbohydrate content in Citrus, found increased levels of carbohydrates in buds and leaves on oranges pears treated with GA_3, and was related to a possible increase in the photosynthetic capacity of leaves or a modification of the model of assimilates distribution in the plant. In the view of these authors, the GA_3 seems to have especially important role as a regulator of hydrolytic enzymes. In an experiment with postharvest of papaya fruits stored under refrigeration, Coneglian (1994) noted that it was more effective when the plant growth regulators auxin, gibberellin and cytokinin were used in combination, since in this way higher levels of total soluble carbohydrates were obtained.

There are few studies that report the changes in carbohydrate contents with gibberellins use in flowers, especially cut flowers. Coorts (1973) reports that glucose is the main constituent of the tissues of rose petals and the accumulation of glucose is due to the fact that, when removed from the plant, the flower acts as a drain where translocation from leaves to the tissues of the petals occur. This transport occurs in the form of sucrose (sugar transport) which form glucose when they reach the flowers (Taiz and Zeiger, 1998). Adachi et al. (2000) noted that high temperatures decreased the concentrations of glucose and fructose in chrysanthemum 'Seiun', but did not contribute to the premature aging of the species. This is due to high temperatures that influenced the complete development of ligules. Vieira et al. (2010) studying the cut chrysanthemum cultivar Faroe' observed decreased total carbohydrate content in the leaves and flowers during the lifetime, especially during senescence. Larcher (2000) mentioned that the distribution of sugars in the plant has been reported in some cultivated species; the leaves closer to the soil supply the root system and those closer to the apex supply the meristems of the shoot and especially the flowers. Vieira et al. (2010) also reported that gibberellin was not effective in altering the carbohydrate content in leaves and chrysanthemum flowers at the concentrations and method of application used. Several studies showed the relationship between these two substances, but also there is need for better discussion.

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Vol. 7(10), pp. 448-467, October 2013
DOI: 10.5897/AJPS2013.1031
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Review

Medicinal orchids and their uses: Tissue culture a potential alternative for conservation

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Accepted 19 August, 2013

Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine. They exhibit incredible range of diversity in size, shape and color of their flowers. Though orchids are grown primarily as ornamentals, many are used as herbal medicines, food, and other have cultural value by different cultures and tribes in different parts of the world. Orchids have been used in many parts of the world in traditional healing system as well as in the treatment of a number of diseases since the ancient time. Though Orchidaceae is regarded as a largest family of plant kingdom, few studies have been done regarding their medicinal properties. Linking of the indigenous knowledge of medicinal orchids to modern research activities provides a new reliable approach, for the discovery of novel drugs much more effectively than with random collection. Many of these orchids face the extreme danger of extinction due to over-exploitation and habitat loss. Plant tissue culture could be one of the most suitable alternative tools to minimize the pressure on natural population of medicinal orchids and their sustainable utilization.

Key words: Medicinal, orchids, propagation, conservation, culture.

INTRODUCTION

Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine (White and Sharma, 2000). They exhibit incredible range of diversity in shape, size and color of their flowers. They are important aesthetically, medicinally and also regarded as ecological indicators (Joshi et al., 2009). Several orchid species are cultivated for their various economic uses especially in floriculture. Orchids are grown primarily as ornamentals and are valued as cut flowers because of their exotic beauty and their long lasting blooming period (Hew et al., 1997). Though orchids are grown primarily as ornamentals, many are used as herbal medicines, food, and other cultural value by many different cultures and tribes in the different parts of world (Khasim and Rao, 1999; Kasulo et al., 2009). Though large population of orchid is still confined in their natural habitat, in many parts of the world their number is decreasing due to their high demand and population pressure. Many orchid species are threatened due to their habitat destruction and indiscriminate collection.

At present, the orchids also figure prominently in the Red Data Book prepared by International Union for Conservation of Nature (IUCN). In fact, the entire family is now included in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where the international trade is strictly controlled and monitored.

Use of orchids in traditional medicine

Orchidaceae is regarded as the largest family of plant kingdom comprising 25,000-35,000 species (Dressler, 1993; Hossain, 2011). Very less study has been done

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regarding their medicinal properties. Limited information on medicinal values of orchids regarding their therapeutic properties in different parts of worlds is available and specifically corresponded to particular regions and community. Compilation of such information is very important to provide the reference for the drug development of many problematic diseases at present.

Scientists have traced orchids as far back as 120 million years ago. The history of orchids might started with their uses in the medicinal purpose. Chinese were the first to cultivate and describe orchids (Jalal et al., 2008). These plants first received recognition in the herbal writings of China and Japan 3,000 to 4,000 years ago, and they were the first to describe orchids for medicinal use (Reinikka, 1995; Bulpitt, 2005). Medicinal orchids belong mainly to genera: *Anoctochilus*, *Bletilla, Calanthe, Coelogyne, Cymbidium, Cypipedium, Dendrobium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nevilia* and *Thunia* (Szlachetko, 2001). Recently, more species belonging to different genera have been reported to have medicinal properties and in future more will be added in the list (Gutiérrez, 2010; Pant et al., 2011). We have listed ninety species of orchids from Nepal with their medicinal uses (Pant and Raskoti, 2013) (Table 1)

Dendrobium nobile, Bletilla striata and *Gastrodia elata* are routinely used in Traditional Chinese Medicine (TCM). Several species of *Anoectochilus* are used in Chinese folk medicines, such as *Anoectochilus formosanus* Hayata, *Anoectochilus koshunensis* Hayata, and *Anoectochilus roxburghii* (Wall.) Lindl. *Anoectochilus roxburghii*, which is distributed in southern China, Japan, Sri Lanka, India, and Nepal (Li and Zou, 1995), is also called "King Medicine" in China (Tseng et al., 2006). Different species of *Dendrobium Sw* are important in Chinese medicine used as Shi-hu (Shi hu: plant living on rocks) since the Han dynasty, 200 BC to 200 AD and are still used as a strengthening medicine and to cure varieties of diseases (Chen et al., 1994). Shi-hu is a term used to describe all *Dendrobium* and some *Flickingeria* species in China.

Different species of *Dendrobium (*Shi-hu) are used in Taiwan, Korea and Japan for various proposes such as a stomache, to treat night sweats, to fortify a person's body, to strengthen the kidneys and to cure impotence and as tonic. The tuber of *Bletilla striata*, which is called Baiji in China, has been used in traditional medicine to treat pneumonorrhagia and pneumono-phthisis. The medicine prepared from these tubers is used to treat tuberculosis, hemoptysis, gastrisis and duodenal ulcers, as well as bleeding, and cracked skin on the feet and hands. Other uses in China, Mongolia, Korea and Japan include the introduction of euphoria, purification of blood, strengthening and consolidation of lungs, as well as the treatment of pus, boils, abscesses, malignant swellings, ulcers and breast cancer (Zhang et al., 2006). *Bulbophyllum kwangtungense* Schlecht (Chinese name "Shi dou-Ian") has long been used in traditional Chinese medicine as a Yin tonic (Yi et al., 2005).

The earliest Middle East report of plant remedies is in a 4000-year-old Sumerian clay tablet which included some orchids (Kong et al., 2003). For flavoring, both Vanilla and Salep are well known and widely used long ago, the former is used as a delicious flavoring and wonderful perfume (Bechtel et al., 1992). Both are used in making ice-cream and beverages (Bulpitt, 2005).

Orchids are also one of the ingredients in ancient Indian systems of medicine called "Ayurveda". Asthavarga an important ingredient in many classical formulations viz., Chavyanprasa is reported to contain 4 species of orchids namely, *Malaxis muscifrea, Malaxis acuminata, Habenaria intermedia, Habenaria edgeworthi* (Singh and Duggal, 2009). *Dendrobium macraei* is another important orchid from Ayurvedic point of view as it is reported to be source of 'Jivanti'. *Cypripedium parviflora* is widely used as aphrodisiac and nervine tonic (Khasim and Rao, 1999). The tubers and pseudobulbs of several orchids like *Orchis latifolia, Orchis mascula, Cymbidium aloifolium, Zeuxine strateumatica,* and some species of *Dendrobium, Eulophia* and *Habenaria* are used as a restorative and in the treatment of various diseases (Puri, 1970).

Dendrobium fimbriatum, Papilionanthe teres, Eria musicucola, Eulophia compestris, Satyrium nepalense, Laparis odorata, Orchis latifolia, Vanda cristata, V. tessalata, V. coerula, V. spathulata, Cymbidium gigantieum, C. aloifolium, C. williomsoni, Dendrobium nobile, D. moschatum, Phaius tancarvilleae are some of the important medicinal plants used by traditional healer in Indian subcontinent (Suresh et al., 2000; Kong et al., 2003; Hossain et al., 2009; Medhi and Chakrabarti, 2009). Other Asian countries such as Indonesia, Malaysia, Taiwan, Singapore, Vietnam, Shri Lanka, Thailand, Myanmar, etc. have been using orchids in traditional medicine since the ancient time till date (Basu et al., 1971; Kumar et al., 2000; Hernández-Romero et al., 2005; Luo et al., 2007).

Similarly, use of orchids in America also has a long history. In Mexico, Vanilla has been used since ancient time to add aroma and flavor cocoa. In America, *Vanilla planifolia* was used as useful herb for the treatment of hysteria, fevers, impotence, rheumatism and to increase the energy of muscular systems since 15th century. *Encyclia citrina*, used by natives on infected wounds was described in the earliest literature. *Laelia autumnal is* for coughs; *Stanhopea hernandezii* for sunstroke; *Arpophyllum spicatum*, *Bletia catenulate* and *Epidendrum pastoris* for dysentery. Different species of *Cypripedium* were used in North America by different ethnic groups for its sedative and antispasmodic properties and to counter insomnia and nervous tension (Wilson, 2007). In North America, species collected for medicinal purposes include *Cypripedium acaule, C. reginae, C. candidum* and *C. parvifolium* (Cribb 1997; Richards, 1998; Duke,

Table 1. Uses of medicinal orchids in Nepal.

S/N	Botanical name	Habitat	Part used	Uses
1	*Acampe papilliosa* (Lindl.) Lindl.	Epiphytic	Root	Used to treat rheumatism (Ref. 1, 3, 5, 6)
2	*Aerides multiflora* Roxb.	Epiphytic	Leaves, Bulbs, Roots	Leaf paste applied to treat cuts and wounds. Plant parts possess antibacterial properties (Ref. 1, 5, 6)
3	*Aerides odoratum* Lour.	Epiphytic	Leaves	Leaf paste is used to treat cuts and wounds. Antibacterial properties (Ref. 1, 5, 6)
4	*Anoectochilus roxburghii* (Wall.) Lindl.	Terrestrial	Whole plant	Consumed to treat tuberculosis (Ref. 5)
5	*Arundina graminifolia* (D.Don) Hochr.	Terrestrial	Root	Root is used to relieve body ache (Ref. 4)
6	*Brachycortis obcordata* (Lindl.) Summerh.	Terrestrial	Root	Used in dysentery. Taken with milk as a tonic, nutritious (Ref. 1, 2, 5, 6)
7	*Bulbophyllum careyanum* (Hook.) Sprengel	Epiphytic	Leaves and pseudobulb	Fresh pulp of pseudo bulb is used in burns, powder of leaves is used to cause abortion and recovery during childbirth (Ref. 6)
8	*Bulbophyllum leopardinum* (Wall.) Lindl.	Epiphytic	Whole plant	Fresh pulp or juice is used in burns (Ref. 6)
9	*Bulbophyllum odoratissimum* (Sm.) Lindl.	Epiphytic	Whole plant	Used to treat tuberculosis and fracture (Ref. 6)
10	*Bulbophyllum umbellatum* Lindl.	Epiphytic	Whole plant	Used to enhance congenity (Ref. 2, 5)
11	*Calanthe plantaginea* Lindl.	Terrestrial	Rhizome	Dry powder with milk is taken as tonic and also as an aphrodisiac (Ref. 6)
12	*Calanthe puberula* Lindl.	Terrestrial	Rhizome	Dry powder with milk is taken as tonic (Ref. 6)
13	*Calanthe sylvatica* (Thou) Lindl.	Terrestrial	Flower	Juice is applied to stop nose bleeding (Ref.1, 6)
14	*Calanthe tricarinata* Lindl.	Terrestrial	Leaf, Pseudobulbs	Leaf paste applied on sores and eczema. Leaves and pseudobulbs are aphrodisiac (Ref. 1, 4)
15	*Cephalanthera longifolia* K. Fritsch		Rhizome	Appetizer, tonic, it heals wound (Ref. 1, 2)
16	*Coelogyne corymbosa* Lindl.	Epiphytic	Pseudobulbs	Juice of pseudobulbs applied in wound, paste applied in forehead to cure headache (Ref. 1, 2, 4, 5, 6)
17	*Coelogyne cristata* Lindl.	Epiphytic	Pseudobulbs	Pseudobulbs are given in constipation as also as an aphrodisiac. Juice of pseudobulbs is applied in wound and boils. Gum from psudobulb are used for sores (Ref. 3, 4, 5, 6)
18	*Coelogyne flaccida* Lindl.	Epiphytic	Pseudobulbs	Paste of pseudobulb is applied to forehead to cure headache and fever, juice is taken for indigestion (Ref. 4, 5, 6)
19	*Coelogyne fuscescens* Lindl.	Epiphytic	Pseudobulbs	Paste and juice for abdominal pain and in burns (Ref. 1, 5, 6)
20	*Coelogyne nitida* (Wall. ex Lindl) D. Don.	Epiphytic	Pseudobulbs	Paste and juice are applied in headache and fever and in burns (Ref. 6)
21	*Coelogyne ovalis* Lindl.	Epiphytic	Pseudobulbs	Aphrodisiac (Ref. 4, 5)
22	*Coelogyne prolifera* Lindl.	Epiphytic	Pseudobulbs	Paste is used to relieve from fever and headache and also applied in burns (Ref. 6), Paste is used for boils and backache (Ref. 4, 5)
23	*Coelogyne stricta* (D. Don) Schltr	Epiphytic	Pseudobulbs	Paste to relieve headache and fever (Ref. 1, 2, 5, 6)
24	*Conchidium muscicola* (Lindl.) Lindl.	Epiphyte	Whole plant	Used in cardiac, respiratory and nervous disorder (Ref. 2, 5)
25	*Crepidium acuminatum* (D. Don) Szlach	Epiphytic	Rhizome, root, psudobulb	Root powder is used for burns (Ref. 6), One of the ingredients of "Astavarga" of Ayurveda. Bulbs are used to treat bronchitis, fever, tuberculosis and weakness. Also given as a tonic (Ref. 1, 2, 5)

Table 1. Cont.

26	*Cymbidium aloifolium* (L.) Sw.	Epiphytic	Rhizome, root, bulbs	Paste is used for bone fracture and dislocated bones. Powder is used as a tonic (Ref. 1, 2, 4, 5, 6), Bulbs is used as demulcent agent (Ref.1,5)
27	*Cymbidium devonianium* Lindl. ex Paxton	Epiphytic	Whole plant	Root paste is applied to treat boils; concentrated decoction is taken in cough and cold (Ref. 4, 5)
28	*Cymbidium elegans* Lindl.	Epiphytic	Leaves, Pseudobulbs, roots	Fresh juice is coagulating, applied in deep wound to stop bleeding (Ref.1, 5, 6)
29	*Cymbidium iridioides* D. Don	Epiphytic	Leaves, Pseudobulbs, roots	Fresh juice is used to stop bleeding. Powder is used as tonic (Ref.1, 5, 6)
30	*Cypripedium cordigerum* D. Don	Terrestrial	Roots	Tonic, edible as a vegetable (Ref.1, 5)
31	*Cypripedium elegans.* Reichenb .f. Nep	Terrestrial	Roots	Nervine tonic in hysteria, spasm, madness, epilepsy and rheumatism (Ref.1, 5)
32	*Cypripedium himalaicum* (Rolfe) Kranzl	Terrestrial	Whole plant	Urine blocks treatment, Stone disease, heart disease, Chest disorder and cough (Ref. 5, 6)
33	*Dactylorhiza hatagirea* (D. Don) Soo	Terrestrial	Tubers	Tonic, wound healing and control bleeding, burns. Also used as a farinaceous food. Used to treat fever and various other body disorders (Ref.1, 2, 3, 4, 5, 6)
34	*Dendrobium amoenum* Wall. Ex Lindl.	Epiphytic	Pseudobulbs	Fresh paste is applied to cure burnt skin and dislocated bones (Ref. 6)
35	*Dendrobium crepidatum Griff.*	Epiphytic	Psudobulbs	Paste is used in fracture and dislocated bone. Ref. 8
36	*Dendrobium densiflorum* Lindl.	Epiphytic	Pseudobulbs	Pulps of the pseudobulbs are used in boils and pimples and other skin eruption (Ref. 4, 5, 6)
37	*Dendrobium eriaeflorum* Griff.	Epiphytic	Pseudobulbs	Paste is used to treat fractured and dislocated bones. Dried powder is used as tonic (Ref. 6)
38	*Dendrobium fimbriatum* Hook.	Epiphytic	Whole plant	Used in Liver upset and nervous debility (Ref.1, 5)
39	*Dendrobium heterocarpum* Wall.ex Lindl.	Epiphytic	Pseudobulb	Paste is used to treat fractured and dislocated bones (Ref. 6)
40	*Dendrobium longicornu* Lindl.	Epiphytic	Whole plant	Plant juice is used to relieve fever; boiled roots are used to feed livestock suffering from cough (Ref. 4, 6)
41	*Dendrobium macaraei* (Lindl.) Seidenf.		Whole plant	Paste is used against snake bite, general stimulant and demulcent (Ref. 6), Used in Asthma, Bronchitis, throat trouble, and fever, aphrodiasic (Ref. 5)
42	*Dendrobium monticola* P.F. Hunt & Summerh.	Epiphytic	Whole plant	Pulps of the pseudobulbs are used in boils and pimples and other skin eruptions (Ref.1, 2, 5)
43	*Dendrobium moschtum* Lindl.	Epiphytic	Pseudobulb	Paste is used to treat fractured and dislocated bones (Ref. 8)
44	*Dendrobium nobile* Lindl.	Epiphytic	Stem	Tonic useful in thirst and dryness of tongue. Given in weakening and fever (Ref. 1, 2, 5)
45	*Dendrobium transparens* Wall. ex Lindl.	Epiphytic	Pseudobulb	Paste is used to treat fractured and dislocated bones (Ref. 6)
46	*Dienia cylindrostycha* Lindl.	Terrestrial	Pseudobulb	Power is used as a tonic (Ref. 6)
47	*Epipactis helleborine* (L.) Crantz.	Terrestrial	Tubers	Used to treat insanity, gouts, headache & stomachache (Ref. 1, 2, 5, 6)
48	*Eria spicata* (D. Don) Hand. Mazz.	Epiphytic	Stem	Paste is taken internally to reduce stomachache and applied externally to reduce, headache (Ref. 1, 5, 6)
49	*Eulophia dabia* (D. Don) Hochr.	Terrestrial	Rhizome	Appetizer, tonic and aphrodisiac. Used in purulent cough and heart trouble. Tubers are given to infants in cough and cold (Ref. 1, 5, 6)
50	*Eulophia nuda* Landl.	Terrestrial	Tubers	Appetizer, useful for tuberculosis glands in neck, tumors and bronchitis (Ref. 5)

Table 1. Cont.

51	*Flickingeria fugax* (Rchb. f.) Seidenf.	Terrestrial	Whole plant	Powder is used as a tonic general debility stimulant (Ref. 6)
52	*Galeris strachaeyi* (Hook. f.) P. F. Hunt	Epiphytic	Tubers	Used as tonic and to cure headache (Ref. 2, 5, 6)
53	*Goodyera repens* (L.) R. Br.	Terrestrial	Tuber	Plant paste externally applied in syphilis, extract is taken as a blood purifier (Ref. 7)
54	*Gymnadenia orchidis* Lindl.	Terrestrial	Roots, Pseudobulbs	Powered pseudobulbs are used to treat cuts and wounds. Also used for liver and urinary disorders and gastric (Ref. 1, 4, 5, 6)
55	*Habenaria commelinifolia* (Roxb.) Wall. ex Lindl.	Terrestrial	Whole plant	Used as Salep in combination of dried tubers of various orchids, and also used as spices (Ref. 5)
56	*Habenaria intermedia* D. Don.	Terrestrial	Tubers	The One of the ingredient of Astavarga of Ayurveda, used as tonic. Tuber paste is used to cure various diseases such as hyperdipsia, fever cough, asthma leprosy skin diseases (Ref. 2, 5, 6)
57	*Habenaria marginata* Colebr.	Terrestrial	Tubers	Thoroughly boiled plant extract taken in flatulence. in wound, tonic (Ref. 7).
58	*Habenaria pectinata* (Sm.) D. Don	Terrestrial	Tubers	Leaf juice applied in snake bites. Tuber used against arthritis (Ref. 6)
59	*Herminium lanceum* (Thunb. ex Sw.) Vuijk	Terrestrial	Whole plant	Extract of plant given in suppressed urination (Ref. 7).
60	*Herminium monorchis* (Linn.) R .Br.	Terrestrial	Roots	Tonic (Ref. 7).
61	*Liparis nervosa* (Thunb) Lindl.	Terrestrial	Tubers	Used to treat stomachache, malignant ulcers (Ref. 2, 5)
62	*Luisia trichorhiza (*Hook.) Bl.	Epiphytic	Tubers	Paste is applied externally to cure muscular pain (Ref. 2, 5, 6)
63	*Luisia zeylanica* Lindl.	Epiphytic	Leaves	Juice is used to treat chronic wounds, boils and burns (Ref. 2, 4, 5, 6)
64	*Malaxis muscifera* (Lindl.) Kuntze	Terrestrial	Swollen stem base	Useful in sterility, seminal weakness, dysentery, fever and general debility as a tonic (Ref. 5, 6)
65	*Neottianthe calcicola* (W.W. Sm.) Soo.	Terrestrial	Rhizome	Tonic (Personal communication)
66	*Nervilia aragoana* Gaudich.	Terrestrial	Whole plant	Used in uropathy, haemoptysis cough asthma, vomiting, diarrhoea & mental instability (Ref. 1, 5)
67	*Oberonia caulescens* Lindl.	Epiphytic	Tubers	Used in liver ailments (Ref. 1, 5)
68	*Otochilus albus* Lindl.	Epiphytic	Whole plant	Powder is used as a tonic (Ref. 6)
69	*Otochilus lancifolius* Griff.	Epiphytic	Pseudobulb	Used to treat fractured and dislocated bones (Ref. 6)
70	*Otochilus porrectus* Lindl.	Epiphytic	Whole plant	Used as tonic & also in the treatment of sinusitis rheumatism (Ref. 3, 5)
71	*Papilionanthe teres* (Roxb.) Schltr.	Epiphytic	Whole plant	Paste is applied to treat dislocated bones (Ref. 4, 5, 6)
72	*Phaius tankervilliae* (Banks) Blume.	Terrestrial	Tubers	Tonic (Personal communication)
73	*Pholidota articulata* Lindl.	Epiphytic	Roots, fruits	Whole plant used as tonic. Root powder is used to treat cancer, juice berries is used to treat skin ulcers and skin eruptions (Ref. 1, 5, 6)
74	*Pholidota articulata* Lindl. var. *grifithii* Hook. f.	Epiphytic	Pseudobulb	Paste is applied to treat dislocated bones (Ref. 4, 5)
75	*Pholidota imbricata* (Roxb.) Lindl.	Epiphytic	Bulbs, Pseudobulb	Juice is applied to relieve naval pain, abdominal pain, and rheumatic pain. Also as a tonic (Ref. 1, 2, 5)
76	*Pholidota pallida* Lindl.	Epiphytic	Roots, Pseudobulb	Juice is applied to relieve naval pain, abdominal pain, and rheumatic pain. Powder is used to induce sleep (Ref. 1, 2, 5, 6)

Table 1. Cont.

77	*Platanthera edgeworthii* (Hook. f. ex Collett) R. K. Gupta.	Terrestrial	Root, Leaves	Powder is used as a blood purifier (Ref. 6)
78	*Platanthera sikkimensis* (Hook. f.) Kraenzlin.	Terrestrial	Bulbs, Pseudobulb	Juice is applied to relieve naval pain, abdominal pain, and rheumatic pain (Ref. 1, 2, 5)
79	*Pleione humilis* (Sm.) D. Don	Epiphytic	Pseudobulb	Dried powder is tonic; Paste is used in cut and wounds (Ref. 4, 5, 6)
80	*Pleione maculata* (Lindl.) Lindl.	Epiphytic	Rhizome	Used for liver and stomach ailments (Ref. 1, 2, 5)
81	*Pleione praecox* (Sm.) D. Don	Epiphytic	Pseudobulb	Dried powder is tonic; paste is used in cut and wounds (Ref. 4, 5, 6)
82	*Rhynchostylis retusa* (L.). Bl.	Epiphytic	Whole plant	Leaves are used to treat rheumatism Root juice is applied to cuts and wounds (Ref. 2, 3, 4, 6)
83	*Satyrium nepalense* D. Don.	Terrestrial	Tubers	As a tonic and also used in diarrhea and malaria, tubers edible, juice is used externally in cut and wounds (Ref. 1, 2, 4, 5, 6)
84	*Smitinandia micrantha* (Lindl.) Holttum	Epiphytic	Whole plant	Root powder as a tonic and stem has antibacterial property (Ref. 1, 5)
85	*Spiranthes sinensis* (Pers.) Ames	Terrestrial	Tuber	Decoction of plant given in intermittent fever, tubers used as tonic. Paste of roots and stem is applied in sores (Ref. 1, 5, 6)
86	*Thunia alba* (Lindl.) Rchb. F.	Epiphytic	Whole plant	Plant paste is applied to treat dislocated bones (Ref. 4, 5, 6)
87	*Trudelia cristata* (Lindl.) Senghas	Epiphytic	Roots & Leaves	Root paste is applied in cuts, wounds, boils and dislocated bones (Ref. 3, 4, 5)
88	*Vanda tessellata* (Roxb.) Rchb. f.	Epiphytic	Roots, leaves	Used in rheumatism and allied disorders, paste of leaves is used for fever (Ref. Subramoniam and Pushpangadan, 2000). Used in rheumatism and allied disorders (Ref. 2, 5).
89	*Vanda testacea* (Lindl.) Rchb.f.	Epiphytic	Leaves	Used as antiviral and anticancer agent. Leaf drops are used for earache (Ref. 1, 2, 5, 6)
90	*Zeuxine strateumatica* (L.) Schltr.	Terrestrial	Roots and tubers	Dry powder is used as tonic (Ref. 6)

References: Vaidhya et al., 2000, (1); Shrestha, 2000, (2); Joshi and Joshi, 2000, (3); Manandhar, 2002, (4); Baral and Kurmi, 2006, (5); Subedi 2011, (6); Joshi et al., 2009 (7). Source: Medicinal orchids of Nepal, Pant and Raskoti, 2013.

2002; Moerman, 1998). Several species of *Goodyera* have been used as herbal remedy by the natives in North America. *Goodyera pubescens*, commonly known as ‘Downy Rattlesnake Orchid’, were used for infallible cure of the bite of a mad dog and to cure scrofula (Moerman, 1986).

The history of use of orchid in Europe is very long and is being used even today in various preparations. Langham, 1579, in his Garden of Health, he reported antipyretic, anti-consumption and anti-diarrhoeal effects of many European terrestrial orchids. The number of orchid species recorded as medicinal throughout Europe such *as Ophrys apifera, O. muscifera, O. fuciflora, O. sphegodes, Orchis simia, O. mascula, Himantoglossum hircinum, Serapias vomeracea, S. lingua, Dactylorhiza majalis*, *D. majalis, foliosa* etc. were used as aphrodisiac and have other healing properties (Turner, 1568). In Europe, some species of *Epipactis* have been used in traditional medicine preparation. The roots of *Epipactis gigantea*, commonly known as ‘Giant Orchid’, have been used in a severe case of illness as a tonic. *Epipactis helleborine* was valued as a remedy for gout in European folklore. Its rhizome is also used as aphrodisiac infusion or decoction (Balzarini et al., 1992). The roots of *E. latifolia* were used in rheumatism. Several species of *Spiranthes* have also been used medicinally in various diseases, for instance *Spiranthes diuretica* is effective as a diuretic in children, *Spiranthes autumnalis*'s roots are used as a strong aphrodisiac as reported by Balzarini.

The early settlers and Australian aborigines in Australia used orchids in the earliest time (Lawler and Slaytor, 1970). Bulbs of many orchids such as *Gastrodia sesamoides, Dendrobium speciosum* and *Caladenia* species were used as emergency food (Bulpitt, 2005). The infusion or decoctions from the leaves of *Dendrobium aurantiacum* were used to cure diabetes (Yang et al., 2005). *Selenipedium chica*, considered as the tallest plants of the orchid family, was used occasionally as a substitute for vanilla.

Pseudobulbs of *Cymbidium madidam* were chewed for dysentery and its seeds were used as an oral contraceptive. In addition, *Cymbidium canaliculatum, Dendrobium teratifolium* and *Dendrobium discolor* were used for treating different ailments such as dysentery, to relieve pain and control ringworm (Lawler and Slaytor, 1970).

In Africa, the Zulus used several orchids for therapeutic purposes. Several species of *Eulophia* were used to prevent miscarriage and cure barrenness. Powdered form of *Eulophia flaccida* were applied to incisions made on the skin to relieve pain. *Eulophia aha,* commonly known as 'Wild cocow', was introduced in South Africa in the early days of the slave trade for its various medicinal uses. The Zulus also used the stems of *Ansellia gigantea* for their aphrodisiac intent. Morris (2003) has described twelve orchids currently used as medicine in Malawi. Nine of these are used for stomach complaints and two for fertility problems. *Cyrtorchis arcuata* and *Eulophia cucullata* are used to treat diabetes or skin infections and *Eulophia cucullata* to prevent epilepsy. An infusion of the leaves and pseudobulbs of *Bulbophyllum maximum* is used to protect against sorcery, and to treat madness. In Zambia and East sub-Saharan Africa, the boiled root tubers of some terrestrial orchid are used to make a food dish (Davenport, 2004). In Africa, an amulet of leaves of *Ansellia africana* infused with a paste made from the pseudobulbs of the same species is said to function as a short term contraceptive (Berliocchi, 2004). Stems infusion or decoction of *Galeola foliate* is used for the treatment of some infections in Morobe, Papua New Guinea (Khan and Omoloso, 2004).

Orchids as a rich source of natural compounds and their pharmacological uses

Researchers have found the various activities of metabolites and extracts of different orchid species in the treatment of various diseases. They have been used variously in different diseases as anti rheumatic, anti-inflammatory, antiviral, anti carcinogenic, anticonvulsive, diuretic, neuroprotective, relaxation, anti-aging, wound healing, hypoglycemic, antitumor and anticancer, antimicrobial, antiviral and many other activities (Ghanaksh and Kaushik, 1999; Shyur et al., 2004; Li et al., 2001; 2006; Shimura et al., 2007; Wang et al., 2006; Prasad and Achari, 1966; Kumar et al., 2000; Zhao et al., 2003; Satish et al., 2003; Watanabe et al., 2007, Won et al., 2006; Lawler and Slaytor, 1970; Balzarini et al., 1992; Nayak et al., 2005; Miyazawa et al., 1999). For drugs derived from orchids, some novel discoveries, both in phytochemical and pharmacological properties, were reported by some researcher. Studies have reported the isolation of wide range of important phytochemicals from different genera of orchids such as alkaloids, flavonoids, stilebnoids, anthocyanins, triterpedoids, orchinol, hircinol, cypripedin, bibenzyl derivatives, phenanthrenes, jibantine, nidemin and loroglossin which are present in leaves, pseudobulb, roots, flowers or in the entire plant (Okamoto et al., 1966; Williams, 1979; Majumder and Sen, 1991; Majumder et al., 1996; Zhao et al., 2003; Yang et al., 2006; Singh and Duggal, 2009).

Thus, from various studies, it is well known that orchids have been used all over the world in traditional healing and treatment system of a number of diseases. Knowledge of different ethnopharmacological studies, linking of the indigenous knowledge of medicinal orchids to modern research activities provides a new reliable approach, which makes the chances of discovery of drugs much more effective than with random collection. In this perspective, orchids which have been used for centuries are the potential resources for many novel drugs. It can be predicted that more genera and species of orchid possesses the possibility of having medicinal properties and in future they can be utilized for the ever demanding life saving drugs (Figures 1a to d).

Threats to orchids

Globally, orchids are the most threatened species among the flowering plants. Due the various reasons like overexploitation, illegal trade and encroachment of land, change in climate, orchids species are threatened rampantly (Shrestha, 2000; Pant et al., 2007). Medicinal orchids are under considerable threat due to habitat destruction, degradation - fragmentation and illegal collection for trade and consumption (Pant et al., 2002). Most of these species has been categorized as critically endangered, rare and listed under appendix II of CITES (IUCN status). There are some species such as *Liparis olivacea*, which have already extinct from the wild (Subedi, 2011). There is a wide gap between the supply and demands of medicinal orchids. Collection and sale of wild orchid from the orchid rich area specially by the rural community is the routine activity, uprooting the whole plant causes the extinction of many species and providing the huge amount of such orchids to the local and international traders (Kala, 2004). Due to such various levels of disturbances, destruction of number of economically important plants in alpine meadows has continued like reduction of *Dactylorhiza hatagirea,* a high valued medicinal orchids of the region from its natural population in the Himalayas which has been categorized as critically endangered listed under appendix I of CITES (Badola and Aitken, 2003; Giri et al., 2008). Due to the recent trend of using traditional medicine in western countries its demand is increasing. Such rapid depletion from the wild requires urgent conservation measures.

CONSERVATION MEASURES

As many valuable orchids are now at the verge of

Figure 1a. Some medicinal orchids of Nepal; *Cymbidium devonianium.*

Figure 1b. *Dendrobium longicornu.*

Figure 1c. *Dendrobium fimbriatum.*

Figure 1d. *Pholidota articulate.*

extinction, so it is high time to conduct effective strategies to conserve them throughout all geographical regions. Conservation of orchids is an important issue that should be seriously considered by both government and private sector of the concern nation in participation with research institutions, non-government organization, community growers as well as through international collaboration. Conservation of medicinal orchids can be addressed by both *in situ* and *ex situ* measures in association with participation of local people.

In situ conservation

In situ conservation, the conservation of species in their natural habitats, is considered the most appropriate way of conserving biodiversity. Habitat protection could be the most important *in situ* conservation strategies for orchids. Because of their small population size and restricted distribution, intensive care and habitat management is highly recommended for their *in situ* conservation. Thus the Protected Areas (PAs) form a central element of any national strategy to conserve biodiversity. However, illegal collections of species from their natural habitat continue even from the PAs in many part of the world due to poor enforcement and regulation of law (Chaudhary et al., 2002; Dixon et al., 2003). Moreover, *in situ* conservation is not always a viable option because of the modification of habitat and migration or absence of the pollinators due to unfavorable modifications environment (Swarts, 2007; Swarts and Dixon, 2009). There is no substitution for conservation of threatened medicinal orchid species in their natural habitat by natural propagation method as their propagation rate is very slow.

Ex-situ conservation

Ex situ conservation is the preservation of components of biological diversity outside their natural habitats. *Ex situ* conservation measures can be complementary to *in situ* methods as they provide an "insurance policy" against extinction. These measures also have a valuable role to play in recovery programmes for endangered species. In this context, *ex situ* conservation is very important aspect of orchid conservation which can include both seed banks and *in vitro* culture plant tissue collections. Therefore there is an urgent need to develop such conservatory for long term conservation and recovery programme specifically for medicinal orchids in the threatened area.

Domestication and participation of community based organization

Propagation techniques for medicinal orchids are yet to be perfected and encouraged to sell the cultivated orchid for the income generations of the people which will promote both *ex situ* and *in situ* conservation. In this regards, participation of community based organization, community forest users' groups (CFUs), private nurseries and orchid enthusiastic is very important. Cultivation of medicinal orchids could be one of the effective ways of income generation of ethnic inhabitants worldwide who are using medicinal orchids for their primary health care and trade to support their livelihood. This will be one of the best alternatives for the more sustainable use of wild orchids (Pant et al., 2007).

Propagation of orchids

Orchids are propagated either sexually or asexually. Propagation of orchids through sexual means is a very slow process as their seeds lack endosperm and need fungal stimulant for germination in nature. Since most of the commercial orchids are highly heterozygous they are not raised through seeds and are propagated through vegetative means to get true-to-type plants. Conventional methods such as cuttings division of shoots are applied for the vegetative propagation of orchids. Orchids are highly heterozygous and their vegetative propagation through division of clumps of rhizomes, bulbs or by the rooting of off shoots also takes long time and difficult to obtain desired number of orchids. This difficulty in natural population drives the many orchids including medicinal orchids to be threatened and some are reached to extinction. It is therefore important to take initiative for their mass propagation and reestablish them in nature.

Propagation through seeds: Symbiotic seed germination

The physiology of its seed germination has made the family Orchidaceae most interesting as their seeds are unique and adaptive in several respects. The small, dust like seeds of the orchids produced in each capsule, are highly fragile, nearly microscopic in size and are produced in very large numbers (Mitra, 1971). As many as 1,300 to 4,00,000 seeds per capsules are produced(Figures 2 and 3). Their color may be white, cream, pale green, reddish orange or dark brown and have very diverse shapes. Orchid seeds are characterized by lack of storage tissues required for seed germination and seedling development.

Orchid requires a combination of multiple factor for their continued reproduction in nature. In nature, association with a specific fungal partner, the orchid mycorrhiza is a pre-requisite for orchid seed germination (Mitra, 1986). Most of the mycorrhizal fungi of orchids fall into a non-sporing group known as Rhizoctonia, the major species

Figure 2. Immature capsules of orchids in their natural habitat.

being *Rhizoctonia repens, Rhizoctonia mucoroides* and *Rhizoctonia languinosa*. Orchid seeds cannot utilize their own reserve or do so very slowly, they also cannot hydrolyse large molecules like starch or cellulose. As a result, asymbiotic germination in the absence of sugar proceeds only to the early protocorm stage, after which they wait for external supply of simple sugars through the help of mycorrhizal fungus. The fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid which is called symbiotic germination (Arditti et al., 1992). Symbiotic associations between orchids and mycorrhizal fungi are a competitive struggle. The fungi always try to invade the cytoplasm of orchid cells to obtain nutritional compounds. On the other hand, the orchid cells restrict the growth of the infecting hyphae and obtain nutrition by digesting them. It is assumed that antifungal compounds are involved in the restriction of fungal growth inside the orchid (Shimura et al., 2007). The rate of seed germination in nature is very poor, that is, 2-5% (Rao, 1977; Vij, 2002). Even in the symbiotic germination, the seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population.

Plant tissue culture, a breakthrough for the orchid propagation

Plant tissue culture technique has been accepted as a potential alternative method for mass scale propagation and conservation of rare, threatened and endangered orchids. Invention of *in vitro* propagation technique has saved the many naturally growing orchids and their collection from the wild has reduced. Increasing popularity of orchids for cut flower and medicinal purpose has added new dimension to *in vitro* propagation technique through which a significant number of identical clones can be raised from a single protocorm or shoot tip explants (Arditti and Ernst, 1993; Deb and Pongener, 2012). Thus methods for rapid multiplication of orchids are essential to meet the commercial demand. Various works on *in vitro* culture include propagation from seed or

Figure 3. LS of orchid capsule showing immature seeds.

different explants such as shoot tips, stems, rhizomes protocorms, etc. More information on *in vitro* culture of orchids is described under separate heading below.

Asymbiotic seed germination in synthetic nutrient media

The propagation and cultivation of orchid was revolutionized after the discovery of Knudson (1922), media. The asymbiotic method for orchid seed germination developed by Knudson (1884-1958) was the first procedure for *in vitro* propagation of any plant in pure culture (Yam et al., 2009). Lewis Knudson in 1916, while working on the influence of the carbohydrates on green plants, also started experimenting with the germination of orchid seeds on the basis of the analysis of orchid salep which contained starch, protein, sugars and minerals. He formulated a medium and successfully germinated seeds of *Cattleya, Laelia, Epidendrum* and concluded that fungus was not necessary for orchid seed germination. Development of asymbiotic germination methods of orchid seeds took place following the formulation of Knudson B and C medium (Knudson, 1922, 1946). After his demonstration of the possibility of by-passing the fungal requirement for germination of orchid seeds during *in vitro* culture, non-symbiotic seed germination has been accepted as an important tool for propagating orchids (Ernst, 1982). This method was a major methodological, biological and technological advance which contributed to modern biotechnology (Johnson et al., 2007).

Different media used for Orchid culture

Successes of plant tissue culture primarily depend on the formulation of nutrient media. With the advent of Knudson medium, a large number of modified media have been standardized and became available in market even with pre-adjusted pH. The most common media used for orchid culture are Knudson C medium (Knudson, 1922, 1925, 1927, 1946); MS medium (Murashige and Skoog, 1962); Vacin and Went medium (Vacin and Went, 1949); and many others (Arditti, 1968; Ernst, 1974; Mitra, 1986; Jonn, 1988). Different media range from simple three-salt solution to complex containing 20 or more salts of macro

Figure 4. Germinating seeds of *Cymbidium aloifolium* in MS medium after 21 weeks of culture.

and micro elements (Chang and Chang 2000a). Some media are designed for specific genera, while others for a broad spectrum. Plant growth regulators such as auxins and cytokinins are added in the media to enhance the seed germination in some orchids (Pant and Gurung, 2005; Stewart and Kane, 2006; Deb and Temjensangba, 2006; Johnson et al., 2007; Hossain, 2008; Pradhan and Pant, 2009; Pant et al., 2011). A large number of complex additives like coconut water, banana pulp, peptone, tomato juice, salep, honey and beef extract have been used in different media to show their effect on orchid seed germination (Mitra, 1971; Vij, 1993; Hua and Zhiguo, 1998; Mohammad et al., 2009). The germination rate of seeds, protocorm formation and complete development of seedlings depend on the genotype, maturity of seeds and culture condition.

Importance of seed culture in orchids

In vitro germination of seeds is an important aspect in the orchid multiplication and conservation program since the dust like tiny seeds have the capability of developing into complete seedlings without any fungal aid, Arditti (1967). After the successful pioneer work of Knudson, commercial propagation of orchids advanced remarkably. The hybridizing potential found in orchids, both at the inter-specific and inter-generic level, has been utilized by amateur and commercial orchid growers to produce thousands of artificial hybrids. The desire to observe the resulting hybrids quickly leads to research and advances in *in vitro* culture techniques which have led to many innovations. Production of artificial seeds and propagation through them is another potential area of orchid conservation and propagation (Rederbangh et al. 1993; Fujii et al., 1989; Datta et al., 1999) (Figures 4, 5 and 6 a,b).

Propagation of orchids using different explants

Micropropagation, monoculture of desirable clones under uniform conditions and stopping the use of plants collected from the wild may solve problem of loss of orchid gene pool and help to conserve the gene bank of medicinal orchids. *In vitro* propagation using seed culture is less desirable in many cases especially for horticultural uses due to the long juvenile period before flowering (Decruse et al., 2003). As orchids are outbreeders, their propagation using seeds leads to the production of heterozygous plants. To obtain similar clones from the superior mother plants, regeneration from various vegetative parts of mature plants are essential. After the development of protocol for *in vitro* micropropagation by

Figure 5. Development of protocorms from germinating seeds of *Cymbidium iridioides.*

Figure 6a. *In vitro* propagation of *Cymbidium aloifolium* through artificial seeds.

Figure 6b. *In vitro* propagation of *Cymbidium aloifolium* through artificial seeds.

Morel (1960) who cultured shoot tips for the production of large number of virus free *Cymbidiums*, commercial orchids were predominantly produced by tissue culture and this technique is routinely used worldwide for mass scale production of orchids by the orchid growers (Wimber, 1963). During the last 50 years, tissue culture techniques using different explants have been extensively exploited, not only for the rapid and large-scale propagation of orchids but also for their *ex situ* conservation. Different protocols have been developed for the large-scale propagation of a number of orchid species and hybrids through *in vitro* culture of various parts including shoot tips, flower stalk, nodes, buds, stems, root tips and rhizome segments (Vij, 1993; Nayak

Figure 7. *In vitro* propagation of *Dendrobium densiflorum* from root culture.

et al., 1998; Kanjilal et al., 1999; Chang and Chang, 2000b; Chen et al., 2003b; Chugh et al., 2009; Pant and Shrestha, 2011; Deb and Pongener, 2012; Paudel and Pant, 2012). Mass propagation of medicinal orchids using *in vitro* culture technique has been reported by some workers (Sharma and Chandel, 1996; Liu and Zhang, 1998); Nalawade et al., 2003; Shiau et al., 2005; Basker and Bai 2006; Sharma et al., 2007; Pant et al., 2008; Hossain et al., 2009, 2012; Kaur and Bhutani, 2009, 2010; Nongdam and Chongtham, 2011; Pant and Thapa, 2012; Pradhan et al., 2013). There are very few reports on reintroduction of *in vitro* propagated species of medicinal orchids to natural habitat (Stewart and Kane, 2006; Aggarwal and Zettler, 2010; Lesar, 2012.) or their cultivation, which are always collected from the wild for trade. To substitute for the habitat protection and species recovery, it is very important to reintroduce over exploited species (Figures 7, 8 and 9). At the mean time, there are ample opportunity and possibility of production of desired phytochemicals in culture (Pant, 2008; Mazumder et al. 2010).

CONCLUSION

Extensive research is still necessary to be able to fullyrecommend the orchid species for their medicinal uses. Due to their small population size and restricted distribution, intensive care and habitat management is highly recommended. Very little effort has been made to cultivate the medicinal orchids for commercial scale. The species which has reached the threatened category because of the human activities can survive only with human support. Plant tissue culture could be one of the most suitable alternative tools to minimize the pressure on natural population of medicinal orchids and their sustainable utilization.

ACKNOWLEDGEMENTS

The author gratefully acknowledges Ms Shreeti Pradhan, Ms Tripti Regmi, Mr. Babulal TIruwa and Mr. Bishnu Joshi for their cooperation in the research and Dr. Basant

Figure 8. *In vitro* grown plantlets of *Phaius tancarvilleae* form shoot tip culture.

Figure 9. Acclimatization of *in vitro* grown plantlets of *Cymbidium aloifolium.*

Pant for the photography of some medicinal orchids. All the tissue culture photographs are used from our research work. The author gratefully acknowledges all the team members involved in orchids research project.

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academicJournals

Vol. 7(10), pp. 468-475, October 2013
DOI: 10.5897/AJPS2012.0716
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Review

Sesbania sesban (L.) Merrill: Potential uses of an underutilized multipurpose tree in Ethiopia

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Accepted 29 July, 2013

Sesbania sesban **(L.) Merrill is the most productive multipurpose tree widely distributed in tropics and subtropics; and usually planted by smallholder farmers mostly for its fodder and soil improvement values. The objective of the present study was to review the various aspects (with emphasis on its importance) of *S. sesban* and generate comprehensive technical information for scholars who wish to study the plant in detail. Different parts of *S. sesban* is reputed for various purposes such as weed control, phytoremediation, anti-inflammation and antioxidant effect, abortion and antifertility agent, antimicrobial activity, firewood source, livestock feed and pasture improvement, green manure, mosquito repellant, live support and *Schistosoma* control. Having these and other multiple uses, *S. sesban* can contribute to sustainable livelihoods by improving household food, nutrition and health security.**

Key words: *Sesbania sesban*, phytoremediation, anti-inflammation, antioxidant, livelihood.

INTRODUCTION

Ethiopia is an agriculture based country where the majority of the population engage in subsistence level crop and livestock production (Nigussie, 2012), that is, mixed crop-livestock farming systems. Soil nutrient depletion and poor nutritive forage are the major causes of low crop and animal productivity in smallholders' farms in Ethiopia, especially in highland areas. As small-scale farmers cannot afford to use chemicals (Wakjira et al., 2011) and improved feeds in their agricultural production system, they resort to the use of natural ways of replenishing soil fertility and feeding livestock through agroforestry.

It is since 1970s that different exotic multipurpose fodder trees like *Sesbania sesban* got promoted by different organizations in Ethiopia to alleviate feed shortages (Mekoya et al., 2009b), maintain soil fertility and prevent land degradation.

In the highland part of Ethiopia, *S. sesban* an N-fixing and deep rooting shrub with good-quality foliage is one of the most promising species for short-duration cover cropping (Desaeger and Rao, 2001) and serve as protein supplement to poor quality roughages or as substitute for commercial protein supplements (Mekoya et al., 2009). Apart from this, its capacity to control soil erosion and hence restore and maintain soil fertility makes it a useful component of traditional agroforestry (Degefu et al., 2011). The authentic product or by-product served by *S. sesban* to human beings is not limited to this, it includes medicinal role to cure diseases and relieve physical sufferings. Therefore, promoting *S. sesban* in Ethiopia, where more than 80% of the people are dependent on plants for their health service (Wondimu et al., 2007), could seem right. Keeping this in mind, the aim of the present work was to blend existing information on the various attributes and potential uses of *S. sesban* for its application in health, agriculture and other sectors.

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Figure 1. *S. sesban* trees, leaves, flowers and pods.

BOTANICAL DESCRIPTION

The species, *S. sesban* (L.) Merrill belongs to the sub-family Papilionoideae, family Leguminosae or Fabaceae (Dinendra and Azad-ud-doula, 2001; Pandhare et al., 2011; Gupta et al., 2011) under the tribe Robinieae (Forni-Martins and Guerra, 1999) with 2n = 12 chromosome (Gillett, 1963; Heering, 1995) and n = 6, 7, 8, 12 and 14 haploid chromosome (Heering, 1995).

The genus *Sesbania* Scop. (*Leguminosae*) contains about 50 species, which are widely distributed in the tropics and subtropics. The majority of *Sesbania* species are annuals, and some are relatively short-lived woody perennials. The greatest species diversity occurs in Africa (distributed widely in northern, eastern, southern and central Africa) with 33 species described (Gillett, 1963; Degefu et al., 2011; Yang et al., 2003; Vadivel et al., 2012; Gupta et al., 2011). Five varieties of *S. sesban* are recognised botanically (Mani et al., 2011; Gutteridge, 1993) viz., *S. sesban* var. *sesban, S. sesban* var. *bicolor, S. sesban* var. *nubica, S. sesban* var. *zambesiaca* and *S. sesban* subsp. *punctata.* The first three varieties are all similar and have been noted for their vigorous growth and high yields, while the rest are less known varieties (Gutteridge, 1993).

S. sesban is a narrow-crowned, deep-rooting single or multiple stem shrub or short-lived tree, which may grow up to 8 m (Hang et al., 2011; Mani et al., 2011) and up to 20 cm stem diameter. The plant is fast growing (Makatiani and Odee, 2007) and it grows 4.5 to 6.0 m high in one year (Siddiqi et al., 1985); and normally flowers and produces ripe pods within the first year after planting (Heering, 1995). If the trees planted are widely spaced they usually develop many side branches. The many branches give the tree a shrubby appearance (Orwa et al., 2009). Leaves are paripinnate, long (compound 12 to 18 cm long) and narrow; leaflets in many pairs (made up of 6 to 27 pairs of leaflets) (Mani et al., 2011), rounded or oblong, usually asymmetric at the base, often glaucous and stipules are minute or absent (Figure 1).

S. sesban tree has up to 20 flowers which are yellow with purple or brown streaks on the corolla. Flowers are attractive, yellow (Pandhare et al., 2011; Mani et al., 2011), red, purplish, variegated or streaked, seldom white, large or small on slender pedicels, solitary or paired in short axillary racemes, usually unpleasantly scented; all petals are long clawed, standard orbicular or obovate. Pods are pale yellow and linear (Pandhare et al., 2011) or slightly curved with 10 to 20 cm long and 5 mm wide containing up to 50 seeds. Seeds are oblong or sub-quadrate, brown or dark green mottled with black (Orwa et al., 2009; Pravin Gomase et al., 2012; Mythili and Ravindhran, 2012).

Origin

The exact origin of *S. sesban* is unclear, but it is widely distributed and cultivated throughout tropical Africa and Asia. It has also been introduced in tropical America (Mani et al., 2011; Heering et al., 1996; Wiegand et al., 1995). It is an exotic plant to Ethiopia (Mekoya et al., 2009a; Orwa et al., 2009) and is originally from east Africa.

Local names

S. sesban is known by different vernacular names such as rivierboontjie (Afrikaans); girangire (Amharic); sesaban (Arabic); jainti or jayant (Bengali); Egyptian sesban (Dande et al., 2010), common sesban, Egyptian rattle pod, frother, river bean, sesban or sesbania (English); dien-dien (Vietnamese); umsokosoko (Zulu); janti, jayanti or puri (Indonesian); Añil francés, tamarindillo (Spanish); and mubimba or muzimbandeya (Luganda) (Orwa et al., 2009; Pravin Gomase et al., 2012).

Nutritional profile

The seeds of *S. sesban* are reported to contain 30 to 40% crude protein (Hossain and Becker, 2001), 5 to 6% of crude lipid and 2.7 to 3.3% of ash (Hossain and Becker, 2002). Debela et al. (2011) reported that the

crude protein contents of *S. sesban* fractions varied from 194 g/kg dry matter in twigs to 297 g/kg dry matter in leaves. In addition, Akkasaeng et al. (1989) found that the *in vitro* dry matter digestibility of *S. sesban* was 75%.

Phytochemical properties

S. sesban seed contains various antinutritional factors such as tannins, saponins and trypsin inhibitors. These antinutritional factors are the major problems when the seed is used as animal feed (Hossain and Becker, 2001). Phytochemical investigations in the seeds led to the isolation of oleanolic acid, stigmastane-5.24(28)-diene-3β-O-β-D-galactopyranoside and galactomannan (Das et al., 2011). The extracts had a high content of phenols, flavonoids and anthocyanins (Kathiresh et al., 2012).

The pods and leaves contain campesterol and beta-sitosterol. Flowers contain cyanidin and delphinidin glucosides. Pollen and pollen tubes contain alpha-ketoglutaric, oxaloacetic and pyruvic acids (Pandhare et al., 2011). From the root extracts of *S. sesban,* Das et al. (2011) isolated Oleanolic acid 3-β-D-glucuronide and found that it has potential spermicidal activity. Among the glucuronide derivatives of oleanolic acid, saponin was responsible for the molluscicidal activity of the plant (Vadivel et al., 2012).

On their phytochemical screening study, Mythili and Ravindhran (2012) found that the stem and root extracts of *S. sesban* contain alkaloids, carbohydrates, proteins, phytosterol, phenol, flavonoids, fixed oil and gum. Moreover, the leaf extract showed the presence of all these except phenol and fixed oil.

Ecological adaptation

S. sesban can be found in areas with a semi-arid to subhumid climate (Heering, 1995; Degefu et al., 2011), with a rainfall between 500and 2000 mm per year (Heering, 1995; Orwa et al., 2009) and temperature of 18 to 23°C (Orwa et al., 2009). In the regions with low precipitation however, they occur primarily on poorly drained soils which are subjected to periodic water-logging or flooding. Because of its good tolerance to low temperatures (Heering, 1995), it can be grown at an altitude of 100 to 2300 m (Orwa et al., 2009). It has moderate shade tolerance as well, and it is adapted to a wide variety of soil types, ranging from loose sandy soils to heavy clays. Furthermore, it has an excellent tolerance to waterlogging and flooding (Heering, 1995) as well as saline, acidic, alkaline soils (Orwa et al., 2009) and soils laden with heavy metals (Gupta et al., 2011).

Pests and diseases

S. sesban is attacked by nematodes, insects, fungi and viruses. *Mesoplatys ochroptera,* the leaf-eating beetle, has been reported as a serious pest of *S. sesban* in Ethiopia, Kenya, Malawi, Mozambique, Tanzania, Zambia and Uganda; while the other leaf eating beetle, *Exosoma* and *Ootheca* spp., has been reported so far only from Malawi and Zambia (Sileshi and Hailu, 2006). *M. ochroptera* can reduce forage yield or completely defoliate leading to mortality, if controlling measures were not taken during establishment (mostly 2 months after planting) (FAO, 2007; Orwa et al., 2009). Insects such as *Anoplocnemis curvipes*, *Exosoma* sp., *Formicomus* sp., *Hilda patruelis* and *Medythia quaterna*, have also been reported to attack *S. sesban* in southern Africa (Sileshi et al., 2000). *Alcidodes buho* is a weevil that damages the plant. The larvae of *Azygophelps scalaris* attack the plant boring the stems. The bacterium, *Xanthomonas sesbaniae* affects the stems and foliage. The seeds are often destroyed by a number of bruchid and other beetles (FAO, 2007; Orwa et al., 2009).

USES OF *Sesbania sesban* (L.) Merrill

S. sesban is a multipurpose tree with different parts of the plant (bark, root, seed, leaf and stem) used for various purposes:

Green manure

S. sesban is a fast growing nitrogen-fixing leguminous tree species which has the capacity of rapid decomposition when incorporated into soil serving as a green manure (Patra et al., 2006) in alley cropping (Heering, 1995) which could bring about substantial increment in crop available nitrogen and soil organic carbon (Table 1).

Forage source

S. sesban tree has a high level of foliage nitrogen and is an excellent supplement to protein-poor roughage (Sabra et al., 2010; Manaye et al., 2009; Orwa et al., 2009). The leaves and tender branches of this tree have high levels of protein (with 20 to 25% crude protein), and easily digestible when consumed by ruminants (Pravin Gomase et al., 2012). It has a long history of use as a source of cut-and-carry forage (Naik et al., 2011). In Ethiopia, feeding *Sesbania* leaves and young twigs have become increasingly important as a protein rich supplement to a basal diet of either grass or poor quality forage for ruminants (Tessema and Baars, 2004). For example, Manaye et al. (2009) reported that the sheep fed with diet containing 300 g/kg *S. sesban* foliage showed 103 g/day average daily body weight gain.

Table 1. Uses of *Sesbania sesban* (L.) Merrill.

Use	Type of use	Parts used	References
Agricultural uses	Reproduction and milk production enhancement	Leave and young twigs	Sabra et al., 2010; Mekoya et al., 2009a; Mekoya et al., 2009b
	Green manure	Whole tree	Patra et al., 2006; Heering, 1995
	Forage source	Leaves and young twigs	Sabra et al., 2010; Manaye et al., 2009; Orwa et al., 2009; Pravin Gomase et al., 2012; Naik et al., 2011; Tessema and Baars, 2004; Manaye et al.; 2009
	Nitrogen fixation	Whole tree	Shaheen et al., 2004; Degefu et al., 2011
	Controlling *Striga asiatica*	Leaves	Gomba and Kachigunda, 2005; Matata et al., 2011; Sikwese et al., 2003
	Live support	Tree	Kathiresh, 2011; Orwa et al., 2009; Naik et al., 2011; Orwa et al., 2009; Sarkar and Prodhan, 2001
	Weed control	Leaves	Tamado and Milberg, 2000
Medicinal uses	Anti-inflammatory effect	Leaves	Nirmal et al., 2012; Shaikh Sajid et al, 2012
	Traditional medicine	Leave and root	Dande et al., 2010; Pravin Gomase et al., 2012; Pravin Gomase et al., 2012; Orwa et al., 2009; Vadivel et al., 2012; Orwa et al., 2009
	Anti-diabetic role	Leaves and seeds	Anil Boddupalli et al., 2012; Pandhare et al., 2011; Ramdas et al., 2010; Ramdas et al., 2012; Vadivel et al. (2012)
	Antioxidant effect	Flower	Kathiresh et al., 2011; Mani et al., 2011
	Antimicrobial activity	Flower	Kathiresh et al., 2012; Mythili and Ravindhran, 2012; Vadivel et al., 2012; Alagesaboopathi, 2012
	Antifertility agent	Seed	Tirkey, 2006; Singh, 1990; Saravanan et al., 2012; Das et al., 2011
	Ethnoveterinary use	Leaves	Vadivel et al., 2012; Orwa et al., 2009; Wondimu et al., 2007; Harun-or-Rashid et al., 2010
Other uses	Bioenergy source	Stem and thick branches	Heering, 1995; Orwa et al., 2009; Naik et al., 2011; Pravin Gonase et al., 2012; Sileshi et al., 2000; Orwa et al., 2009; Pravin Gonase et al., 2012
	Stimulant effect	Bark	Naik et al., 2011; Pravin Gomase et al., 2012
	Schistosoma control	Wood	Nirmal et al., 2012; Mahmoud et al., 2011; Hasheesh et al., 2011
	Fiber source	Bark	Orwa et al., 2009
	Decorative food ingredient	Flower	Kathiresh et al., 2011; Orwa et al., 2009; Pravin Gomase et al., 2012
	Polluted water and soil treatment	Whole tree	Indieka and Odee, 2005; Dan et al., 2011; Dan and Brix, 2009; Indieka and Odee, 2005; Dan et al., 2011; Dan and Brix, 2009; Yang et al., 2003; Gupta et al., 2011.

Anti-inflammatory effect

S. sesban leaf is reported to be used in the treatment of inflammatory rheumatic conditions (Nirmal et al., 2012; Shaikh et al., 2012).

Reproductive and milk production enhancement

Supplementation of ration with *S. sesban* is reported to improve the reproductive performance of sheep; and its inclusion up to 30% of the ration improved feed intake, growth rate, onset of puberty and sexual development (Sabra et al., 2010; Mekoya et al., 2009a). Moreover, ewes supplemented with *S. sesban* at 30% of the ration showed a 13% increase in milk production over ewes supplemented with concentrates (Mekoya et al., 2009b).

Nitrogen fixation

In symbiosis with *Rhizobium* (nitrogen fixing bacteria), *S. sesban* can fix up to 542 kg N ha^{-1} (Shaheen et al., 2004). Even, Degefu et al. (2011) reported nitrogen fixation level of 500 to 600 kg N/ha/year and is particularly promoted for soil fertility replenishment through ‘improved fallow’ agroforestry practice.

Bioenergy source

The stem and thick branches of *S. sesban* is popular for firewood and charcoal production because it produces a relatively smokeless, quick kindling and hot burning woody biomass in a short time (Heering, 1995; Orwa et al., 2009; Naik et al., 2011; Pravin Gonase et al., 2012).

S. sesban has been found to yield 10 to 20 t ha^{-1} of fuelwood when planted as pure fallows, and up to 2 t ha^{-1} in relay intercropping (Sileshi et al., 2000). The calorific yield for a 3-years-old tree is approximately 4350 kcal/kg (Orwa et al., 2009). Because of its rapid growth, the plant also has a potential for pulpwood production (Pravin et al., 2012).

Ethnomedicine

The cataplasm prepared from leaves of *S. sesban* facilitates discharge of boils and abscesses and absorption of inflammatory rheumatic swellings (Dande et al., 2010; Pravin et al., 2012). In addition, juice of fresh leaves is credited with anthelmintic properties (Pravin et al., 2012; Orwa et al., 2009). The fresh root of *S. sesban* is also said to be an excellent remedy for scorpion stings (Vadivel et al., 2012; Orwa et al., 2009).

Anti-diabetic role

Different research works (Boddupalli et al., 2012; Pandhare et al., 2011; Ramdas et al., 2010, 2012) reported that the aqueous leave extract of *S. sesban* have anti-diabetic effect which could be associated with the presence of flavonoids. More specifically, to manage type II diabetes (Vadivel et al., 2012) recommend the use of *S. sesban* seeds as a natural source of dietary antioxidants.

Controlling *Striga asiatica*

Studies have reported that exposing *S. asiatica* to the leaf crude extracts obtained from *S. sesban* reduced the germination of the parasite by 50 to 100%. This property of *S. sesban* makes the plant with contributory effect on the control of *Striga asiatica* as a parasite of economic importance (Gomba and Kachigunda, 2005). Application of *S. sesban* manure to the soil is also reported to reduce the *Striga* infestation or incidence by 88% (Matata et al., 2011). Furthermore, other research works reported various mechanisms through which *S. sesban* control *Striga* in an intercropping system like trapping or catching crops to the parasite in a rotation, shading the *Striga* from receiving enough sunshine to proliferate (Sikwese et al., 2003) and stimulating suicidal germination of *Striga* seeds (Matata et al., 2011).

Stimulant effect

The crude drug extract obtained from the bark of *S. sesban* have been examined and found to have a potential central nervous system stimulant effect that can be explored for therapeutic advantage as an alternative treatment in medical conditions associated with dizziness and sedative (Naik et al., 2011; Pravin et al., 2012).

Schistosoma control

Shade, dried and coarsely powdered wood of *S. sesban* is reported to have potent antinociceptive activity (Nirmal et al., 2012). The dry powder of the plant *S. sesban* exhibited an acceptable toxic effect against the snails which can make the plant good candidate for interrupting and minimizing the transmission of *Schistosoma mansoni* (Mahmoud et al., 2011; Hasheesh et al., 2011).

Fiber source

The bark of *S. sesban* can be used for making ropes and fishnet (Orwa et al., 2009).

Antioxidant effect

The flower petals of *S. sesban* may be valuable natural antioxidant sources that protect the cells against the effect of free radicals by scavenging them and retard the progress of many chronic and degenerative diseases such as cardiovascular diseases and cancer (Kathiresh et al., 2011). This activity is attributed to the presence of saponins and flavonoids (Mani et al., 2011) which make the plant potentially applicable in both pharma and food industry (Kathiresh et al., 2011).

Ethnoveterinary use

One of the most promising uses of *S. sesban* is as mosquito repellant. Washing the bodies of animals with its water extract can serve as protection against mosquito bites (Vadivel et al., 2012). Orwa et al. (2009) reported the use of decoctions of leaves of this plant as a drench for cattle to repel tsetse fly from cattle by the Hausa people of Ghana. *S. sesban* in Southeastern Ethiopia, is also reported for its veterinary use in crushed and homogenized water form (Wondimu et al., 2007). Moreover, the leave of this plant has been used by Bangladesh farmers to treat retention of urine (Harun-or-Rashid et al., 2010).

Decorative food ingredient

Flowers of *S. sesban* are known to be added to stews and omelets in some areas, perhaps mainly as a decorative or festive ingredient in foods (Kathiresh et al., 2011; Orwa et al., 2009; Pravin et al., 2012).

Antifertility agent

Inserting *S. sesban* seed paste (15 g) placed in cotton swap into vagina causes abortion in females (human beings). In addition, taking fresh root decoction twice a day for 3 to 4 days following menstrual phase serve as an antifertility agent (Tirkey, 2006). Therefore, *S. sesban* seed powder hinder the ovarian normal function, change the uterine structure and prevent implantation, thus, control the fertility of female (Singh, 1990; Saravanan et al., 2012). With this respect, Das et al. (2011) reported that the Kandha tribe of India uses the root extracts as contraceptive.

Antimicrobial activity

Studies also witnessed that extracts from *S. sesban* flower petals serve as antimicrobial activity (Kathiresh et al., 2012; Mythili and Ravindhran, 2012). In traditional medicine system of Nigeria, the plant is pounded in milk and taken as an internal remedy for Guinea worm (Vadivel et al., 2012). Furthermore, Alagesaboopathi (2012) reported that decoction of the leaf is mixed with hot milk and given once a day for seven days for treatment of diarrhea, itches and skin diseases.

Live support

S. sesban can be used as a live support for black pepper, grapes, cucurbits and betel vine. In addition, its spreading canopies can serve as a shade tree for coffee, tea, cocoa and turmeric (Kathiresh, 2011; Orwa et al., 2009; Naik et al., 2011). It has also been used as a windbreak for bananas, citrus and coffee (Orwa et al., 2009) and as fencing materials (Sarkar and Prodhan, 2001).

Polluted water and soil treatment

The ability of *S. sesban* to grow at different ammonium concentrations soil culture has been studied by different workers (Indieka and Odee, 2005; Dan et al., 2011; Dan and Brix, 2009), and it was shown that its seedlings can tolerate ammonium concentrations up to 800 mg/L. This high tolerance suggests that this plant has a potential for use in treatment systems of waste or polluted water (Indieka and Odee, 2005; Dan et al., 2011; Dan and Brix, 2009) and removal of heavy metals from soil, that is, phytoremediation of sites contaminated with heavy metals (Yang et al., 2003; Gupta et al., 2011).

Weed control

Leaf aqueous extract and dry residue of *S. sesban* could serve in the inhibition of germination and seedling growth of parthenium, which is currently considered as the most serious weed in Ethiopia in both arable and grazing lands as it caused severe crop losses (Tamado and Milberg, 2000).

CONCLUSION

S. sesban (L.) Merrill is an extremely versatile plant which has significant contribution in attaining rural household food security in Ethiopia through its contribution to production, service and ethnomedicinal functions (for example, weed control, phytoremediation, anti-inflammation and antioxidant effect, abortion and antifertility agent, antimicrobial activity, firewood source, livestock feed and pasture improvement, green manure, mosquito repellant, live support and *Schistosoma* control) to the subsistence crop-livestock mixed farming system. It is a species of wide-ranging soil and climatic adaptations. Currently, the use of the plant as animal feed and manure has been reported in Ethiopia by many researches and its further use need to be explored for its better utilization in the present mixed farming system.

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Vol. 7(10), pp. 476-481, October 2013
DOI: 10.5897/AJPS2013.1058
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Full Length Research Paper

Antifungal activity of aerial parts as well as *in vitro* raised calli of the medicinal plant, *Balanites aegyptiaca* Del.

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Accepted 18 September, 2013

The antifungal potential of an endangered medicinal plant, *Balanites aegyptiaca* Del. and its *in vitro* raised calli was evaluated by agar well diffusion method. The alcoholic extract of fruits of parent plant as well as its callus showed good antifungal activity against various pathogenic and opportunistic fungi. Minimum inhibitory concentrations (MIC) of the extracts were determined by broth microdilution method. The MIC of alcoholic fruit extract of parent plant against tested fungi ranged from 3.05 to 24.0 µg/ml, whereas, the MIC of extract of *in vitro* raised callus ranged from 1.53 to 12.0 µg/ml. The present study shows that extracts of *B. aegyptiaca* contain good antifungal activity which could be used in the treatment of fungal infections showing resistance to currently used antifungal agents. As its calli also gave good results, *in vitro* cultivation of the explants may be used to obtain novel antifungal compounds even at places where it does not grow naturally and thus can be used in the treatment of various opportunistic and life threatening fungal infections especially in immunocompromised patients.

Key words: *Balanites aegyptiaca*, *in vitro* raised callus, antifungal activity, agar well diffusion, minimum inhibitory concentration.

INTRODUCTION

Fungi cause opportunistic infections in patients who are immunocompromised, either by an underlying disease process or immunosuppressive agents. Candidiasis has become a major public health problem as an opportunistic infection of HIV/AIDS and considered as a leading fungal infection in immune-suppressed population (Al Ashaal et al., 2010). Patients with cancer often develop serious fungal infections, especially during periods of cancer treatment. Antibiotic usage for the prevention and treatment of infections in these high-risk patients leads to selection pressures resulting in the emergence and spread of resistant organisms (Panghal et al., 2011).

Nosocomial fungal infections have gained more importance especially in association with or as a consequence of the extraordinary progress in the management of seriously ill patients during the past few decades. However,

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despite this increase, there has been comparatively little progress in elucidating treatment of such nosocomial and opportunistic fungal infections. The treatment of these infections in the immunocompromised patients is a challenging task. Although many antifungal agents have been developed so far but only a few are clinically effective and safe to use. Also, resistance to antifungal agents has been reported in AIDS patients who suffer from recurrent azole-resistant oropharyngeal or oesophageal candidiasis (Chander, 2002).

This situation has forced the researchers to search for new antimicrobial substance from various sources including medicinal plants (Scazzocchio et al., 2001; Erdogrul, 2002; Bandow et al., 2003; Parekh and Chanda, 2008). Antimicrobials of plant origin have proved effective in the treatment of several infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Samy and Ignacimuthu, 2000).

But unfortunately, many plant species, possessing medicinally important compounds are disappearing at an alarming rate due to the destruction of their natural habitats (Vanila et al., 2008). Through *in vitro* cultivation, it would be possible to preserve and conserve these important endangered plant species and also *in vitro* cultivation of explants may be used to obtain phytotherapeutic compounds, especially, at places where the parent plants cannot be grown because of the adverse atmospheric conditions (Shahid et al., 2009b).

Balanites aegyptiaca Del. (Zygophyllaceae or Simaroubaceae), known as 'desert date' in English, 'Hingoli' in Hindi and 'Angavriksha' in Sanskrit (Iwu, 1993; Gaur et al., 2008), is a savanna tree that grows in Sahel-Savanna regions and drier parts of middle-belt zones of Nigeria, Ghana, Ivory Coast and India. In India, it is particularly found in Rajasthan, Gujarat, Madhya Pradesh and Deccan. The tree produces date-like fruits between March and October (Chothani and Vaghasiya, 2011).

Various parts of the *Balanites* tree have been used for folk medicines in many regions of Africa and Asia (Hall and Walker, 1991; Mohamed et al., 2000, 2002). Various literatures have revealed antifeedant, antidiabetic, molluscicide, anthelminthic and contraceptive activities in various *Balanites* extracts (Liu and Nakanishi, 1982; Kamel et al., 1991; Ibrahim, 1992; Rao et al., 1997). The bark, unripe fruits, and leaves of this plant are reported to have anthelminthic, antifertility, purgative and antidysenteric properties (Chopra et al., 1956a, b; Kirtikar and Basu, 1996). The root has been indicated for the treatment of malaria, herpes zoster and venereal diseases (Irvine, 1961; Ayensu, 1978). It is traditionally employed in treatment of jaundice, yellow fever, syphilis, diarrhea, epilepsy, cough, wound healing and even for snake bites (Inngerdingen et al., 2004; Maregesi et al., 2008; Chothani and Vaghasiya, 2011; Abdallah et al., 2012).

Although, *B. aegyptiaca* is a versatile medicinal plant its use is restricted in few localities of Indian sub continents and parts of Africa. The present study was carried out to evaluate the antifungal potential of medicinal plant *B. aegyptiaca* and its *in vitro* raised callus by testing their activity against an exhaustive range of fungal isolates, including both standard as well as clinical strains.

MATERIALS AND METHODS

Collection of plant materials

The fruit pulp of 15 years old plant of *B. aegyptiaca* was obtained from Tissue Culture Laboratory, Department of Botany, Gujarat University, Ahmedabad (Gujarat) and its *in vitro* cultivation was done in Botanical Garden, Department of Botany, Aligarh Muslim University, Aligarh.

Sterilization of the collected explants

The young explant was washed under running tap water for 30 min to remove any adherent particles and then washed thoroughly in sterile double-distilled water (DDW). Then these explants were kept in 1% (w/v) Bavistin (Carbendazim Powder, BASF India Limited) for 25 min, followed by thorough washing with 5% (v/v) Teepol (Qualigens Fine Chemicals, India), for 15 min by continuous shaking method (Shahid et al., 2007, 2009b).

The treated explant was washed in sterilized DDW 3-4 times under an aseptic condition, to remove the chemical inhibitors. It was then treated with 70% (v/v) ethanol for 30 - 40 s followed by a rapid washing with sterile DDW and then surface sterilized by emersion in a freshly prepared aqueous solution of 0.1% (w/v) $HgCl_2$ (Qualigens Fine Chemicals, India) (Bhojwani and Razdan, 1996), for 4 min under laminar flow. Finally, the explants were washed 5-6 times with sterile DDW with intervals of 5 min to remove all traces of sterilants (mercury ions).

Inoculation of sterilized explants on MS medium

Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) containing 2.5 µM of 2,4-dichlorophenoxy acetic acid was prepared in culture tubes (25 × 150 mm, Borosil). The pH of the medium was adjusted at 5.8 ± 0.2. 1% agar (HiMedia Lab. Ltd., India) was added to the medium and it was autoclaved at 15 lb pressure per square inch, 121°C temperature for 15 min. The sterilized explant was then inoculated aseptically and incubated at 25 ± 2°C with relative humidity of 55 ± 5% and exposed for 16 h photocycle of 2,500 Lux intensity (Shahid et al., 2009b). 5 weeks old callus was used for evaluation of the antimicrobial effect.

Preparation of plant extracts

The alcoholic extracts of the plant were tested for antifungal activity. The extracts were prepared according to the method of Singh and Singh (2000) with some modifications (Shahid et al., 2007, 2009a, b). To prepare alcoholic extracts, fresh fruit (15 g) from parent plant was surface sterilized in 70% ethyl alcohol for 1 min and then washed 3 times with sterilized double distilled water (DDW). The calli of the explants were aseptically removed from the culture tubes and all the plant materials, including calli, were grounded with a sterilized pestle and mortar in 150 ml of 95% ethanol and centrifuged at 5000 rpm for 15 min. The resultant supernatant was filtered and taken as the alcoholic extracts which were immediately used for experimentation.

Fungi tested

The clinical fungal strains included in our study were *Candida albicans*, *Candida krusei*, *Candida parapsilosis*, *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium* spp. and *Fusarium* spp. isolated in the Department of Microbiology, Jawaharlal Nehru Medical College and Hospital, Aligarh Muslim University, Aligarh, India. The fungal control strains tested were *C. parapsilosis* (ATCC 22019), *C. krusei* (ATCC 6258) and *A. fumigatus* (ATCC 204305), obtained from New Drug Discovery Research, Ranbaxy, Gurgaon, India. The fungi were grown at 25°C and maintained on Sabouraud's Dextrose agar (SDA) slants.

Antifungal susceptibility testing

Antifungal testing was performed on SDA plates using agar well diffusion method (Vanden-Berghe and Vlietinck, 1991; Akinpelu, 2001), with some modifications (Shahid et al., 2007). An inoculum size of 2×10^6 yeast cells or fungal spores was used for inoculating the susceptibility plates. These plates were lawn cultured with fungal suspensions with the help of sterile swabs. Wells of 5 mm diameter were made in each plate using a sterile borer. Plant extracts in a volume of 20 µl were poured in the wells using a micropipette. 20 µl of 95% ethanol was used to serve as negative control, whereas, antifungal agent voriconazole (500 µg/20 µl) was used as positive control. The plates were kept upright for 5-10 min until the solution diffused into the medium. SDA plates were then incubated aerobically at 25°C in a biological oxygen demand (BOD) incubator for 2-5 days and the zone of inhibition was measured and recorded. All experiments were performed in triplicate.

Determination of minimum inhibitory concentrations (MIC)

MIC was determined by broth micro-dilution method. It was performed according to Clinical and Laboratory Standards Institute (CLSI), formerly NCCLS (1997) for yeasts and NCCLS (2002) for filamentous fungi, with minor modifications (Shahid et al., 2007). Doubling dilutions of the extract was prepared using RPMI-1640 (HiMedia, India) broth supplemented with 0.3 g/L L-glutamine (HiMedia, India), 0.165 mol/L of 3-[N-morpholino] propanesulfonic acid (MOPS) buffer (HiMedia, India) and 0.01% of dimethyl sulphoxide (DMSO) (Qualigens Fine Chemicals, India). Extracts were dissolved in DMSO, and further diluted 1:50 in RPMI-1640 medium, and each resulting solution was used for a doubling dilution series. Microtitre plates were prepared containing 100 µl of undiluted extracts in the first well, followed by doubling dilutions of extracts from second well. The standardized inoculum of each fungal species was added to the respective dilution wells including the first well. The final concentrations of the extracts ranged from 25×10^3 to 48×10^{-3} µg/ml. For each test there was a sterility control well containing alcoholic extract in RPMI-1640 broth plus DMSO and a growth control well containing fungal suspension without alcoholic extract. The microtitre plates were incubated at 35°C for 48 h with their upper surface covered by sterile sealers. The lowest concentration that did not show any visible growth was considered the MIC of that extract for the tested fungal species. All the MIC experimentations were performed in duplicate.

Statistical analysis

All the experiments of antimicrobial susceptibility testing were performed in triplicate. The results were expressed as the mean ± standard error (SE). Data were analyzed statistically by one way analysis of variance (ANOVA) followed by Tukey's multiple analysis test using SPSS Software, Chicago, III, version 10. P values were calculated by one-sample T-test and $P < 0.05$ was taken as statistically significant.

RESULTS AND DISCUSSION

Antifungal activity of alcoholic extracts of parent plant as well as its *in vitro* raised callus against the tested fungal species is shown in Table 1. Negative control (ethanol) showed the zone of inhibition in the range of 0.00 to 8.67 ± 0.33 mm. Positive controls (voriconazole) showed the zone of inhibition in the range of 9.33 ± 0.33 to 13.00 ±0.58 mm. The extracts derived from both parent plants as well as callus gave good antifungal activity (Table 1). The alcoholic fruit extract of parent plant showed significant antifungal activity ($P < 0.05$) against *C. albicans* ($P = 0.003$), *C. parapsilosis* ($P=0.012$) and *Penicillium* spp. ($P = 0.017$), with MIC ranging from 3.05 to 24.0 µg/ml (Figure 1).

Various studies have been under taken previously by different researchers to analyze the antifungal potential of *B. aegyptiaca* (Runyoro et al., 2006; Maregesi et al., 2008; Al Ashaal, 2010; Panghal et al., 2011). They showed significant antifungal activity of this plant against *C. albicans*, which supports our present research findings.

Since we tested an exhaustive range of fungal isolates, both standard and clinical strains as compared to previously studies, we were able to detect a wide spectrum of antifungal activity of this plant extract. In the present study, it was also found that alcoholic fruit extract of *B. aegyptiaca* also showed good antifungal activity against *C. parapsilosis* and *Penicillium* spp., which were not tested in earlier studies. Another study done by Abdallah et al. (2012) showed significant antifungal activity of this plant against *A. niger* and *Fusarium* species. These findings are in contrast with our study. This could be due to different concentrations of extracts used in their study as well as variation in active metabolites present in plant extracts derived from different places.

As seen in Table 1, the alcoholic extract of *in vitro* cultivated callus also showed significant antifungal activity against *C. albicans*, *C. parapsilosis* and *Penicillium* spp. which were comparable to the parent plant extract. In addition, it also showed good antifungal activity against *C. krusei*. The MIC of *in vitro* raised alcoholic callus extract ranged from 1.53 to 12.0 µg/ml (Figure 2). To the best of our knowledge, this is the first study analyzing the antifungal potential of *in vitro* raised calli of this plant; therefore, our findings could not be compared.

Conclusion

Extracts of *B. aegyptiaca* have remarkable antifungal potential against clinical and standard strains and thus could be used to derive antifungal agents especially against *C. albicans*, *C. parapsilosis* and *Penicillium* species. Hence, it could be used in the treatment of various opportunistic and life threatening fungal infections especially

Table 1. Antifungal activity of alcoholic extracts of parent plant of *B. aegyptiaca* and its *in vitro* raised callus against pathogenic and opportunistic fungi.

Fungi tested	Zone of inhibition (mm) ± SE			
	Alcoholic fruit extract of parent plant$^{\Delta}$	Alcoholic extract of *in vitro* raised callus$^{\Delta}$	Ethanol† (negative control)	Voriconazole$^{£}$ (positive control)
Candida albicans	15.33±0.67[a]	13.67±0.33[a]	8.33±0.33[b]	12.33±0.33[b]
Candida krusei	0.00±0.00[d]	12.67±0.33[bc]	7.67±0.33[c]	11.67±0.33[c]
Candida parapsilosis	14.67±0.33[ab]	13.00±0.58[b]	8.67±0.33[a]	12.67±0.33[ab]
Aspergillus fumigatus	0.00±0.00[d]	0.00±0.00[d]	7.67±0.33[c]	10.33±0.33[de]
Aspergillus flavus	0.00±0.00[d]	0.00±0.00[d]	7.33±0.33[d]	9.67±0.33[e]
Aspergillus niger	0.00±0.00[d]	0.00±0.00[d]	0.00±0.00[e]	9.33±0.33[f]
Penicillium spp.	13.00±0.58[c]	12.33±0.33[c]	8.33±0.33[b]	11.67±0.33[c]
Fusarium spp.	0.00±0.00[d]	0.00±0.00[d]	0.00±0.00[e]	9.33±0.33[f]
C. parapsilosis (ATCC 22019)	14.33±0.33[b]	13.33±0.33[ab]	8.67±0.33[a]	13.00±0.58[a]
C. krusei (ATCC 6258)	0.00±0.00[d]	13.00±0.58[b]	8.33±0.33[b]	12.33±0.33[b]
A. fumigatus (ATCC 204305)	0.00±0.00[d]	0.00±0.00[d]	7.67±0.33[c]	10.67±0.33[d]

† = 20 µl of 95% ethanol was used as negative control. ∆ = concentration of extracts used in the test, that is, 2 mg/20 µl. £ = concentration of voriconazole used in test, that is, 500 µg/20 µl. Diameter of zone of inhibition is a mean of triplicates ± SE (mm). Differences were assessed statistically using one way ANOVA followed by Tukey's test. P<0.05 was considered as significant. The mean represented by same letter is not significantly different within the column.

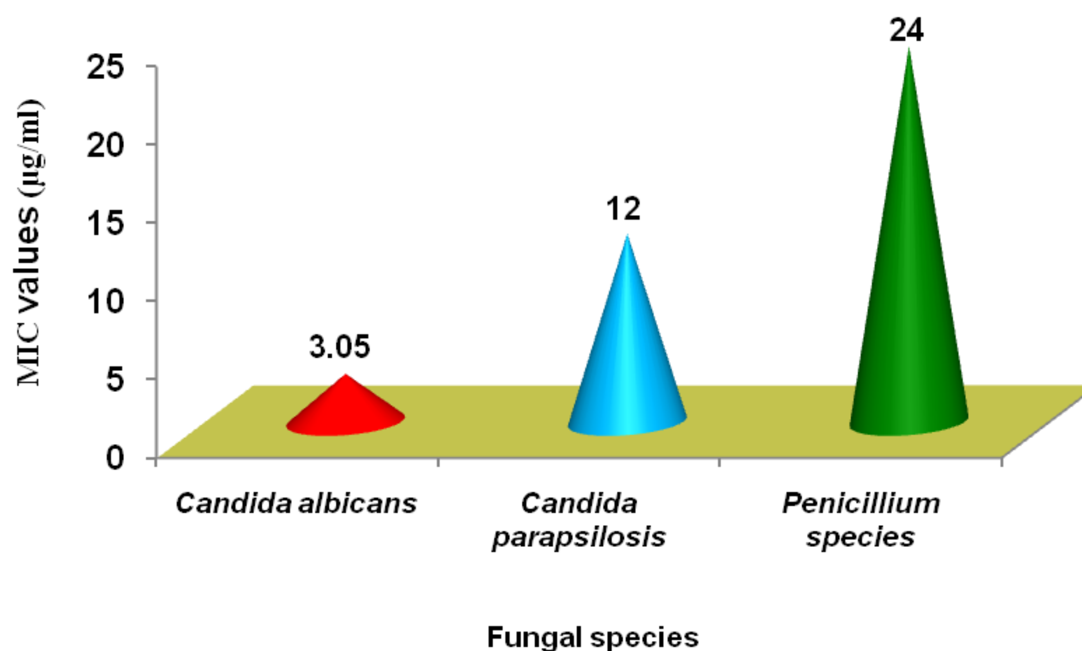

Figure 1. MIC determination of alcoholic fruit extract of *B. aegyptiaca* against tested pathogenic and opportunistic fungi.

in diabetics, cancer patients and immunocompromised patients. As the extracts from *in vitro* raised callus showed significant and even better antifungal potential as compared to parent plant, *in vitro* cultivation of this plant may be used to obtain phytotherapeutic compounds, even at places where it does not grow naturally because of adverse atmospheric conditions. In the future, bioactive compounds responsible for antifungal activity could further be enhanced by nutritional and hormonal manipulations of the cultivation medium, as depicted in our study.

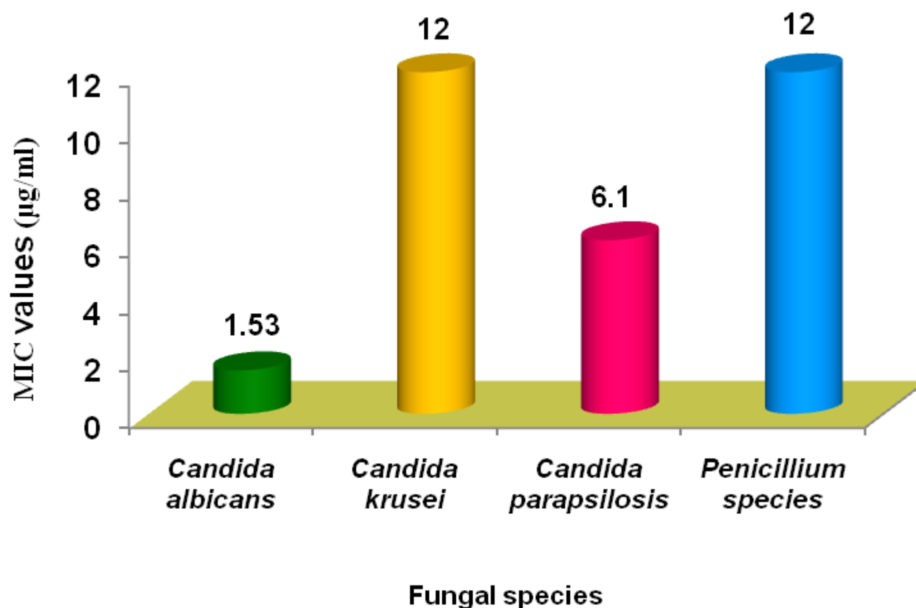

Figure 2. MIC determination of alcoholic extract of *in vitro* raised callus of *B.aegyptiaca* against tested pathogenic and opportunistic fungi.

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Vol. 7(10), pp. 482-487, October 2013
DOI: 10.5897/AJPS2013.1092
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Full Length Research Paper

Characterization of dekoko *(Pisum sativum var. abyssinicum)* accessions by qualitative traits in the highlands of Southern Tigray, Ethiopia

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Accepted 16 September, 2013

Cultivated *Pisum* is dominated with *Pisum sativum* subgroup, but *P. sativum var. abyssinicum* (Dekoko) is a unique subgroup developed and cultivated in Ethiopia. The objectives of the study were the characterization of the accessions using three qualitative traits and understanding the genetic diversity of the crop. Twenty-four (24) accession/local collections of Dekoko collected from South Tigray and North Wello by Alamata Agricultural Research Centre were planted in three replications of the Randomized Complete Block Design (RCBD) at Mekhan farmers' Training Centre in Endamekhoni in 2010. Characterization of the accessions by three qualitative traits viz., flower color, seed size and seed shape had revealed the existence of high genetic diversity of the population (H'=0.84, 0.95, 0.98, respectively, with mean H'=0.92) implying that the crop can be improved through breeding. The distribution of the various categories of the three qualitative traits (flower color, seed shape and seed size) was independent of region and altitude. Clustering of the accessions by qualitative traits produced seven distinct clusters which did not classify the accessions according to regions or Weredas, but according to three altitude classes.

Key words: Characterization, Dekoko, *Pisum sativum* var. *abyssinicum*, accession/collection.

INTRODUCTION

The origin of field pea is controversial. Ethiopia is undoubtedly the centre of diversity for this crop since wild and primitive forms are known to exist in the high elevations of the country. Ethiopia is one of the major Vavilovian centers of diversity for several grain legume crops including lupine, field pea and wild ancestors of cow pea Ali et al. (2003).

Cultivated *Pisum* is dominated by *P. sativum*, but *P. sativum* species *abyssinicum* (or simply *P. abyssinicum*) is a unique species independently developed and cultivated in Ethiopia. The existing germplasm in the country shows tolerance/resistance to disease (IBC, 2007; Sentayehu, 2009; Jing et al., 2010). *P. sativum* is widespread across the Middle East and has affinity with the wild *P. elatius* while *P. abyssinicum* is restricted to highland regions of Ethiopia (South Tigray and North Wello) and Southern Yemen and shows a greater affinity to *P. fulvum* (Yemane and Skjelvåg, 2002; Jing et al., 2010). However, *P. fulvum* is found around the eastern edge (Syria, Lebanon, Israel, Palestine and Jordan) and not common in Ethiopia (Maxted and Ambrose, 2001).

P. sativum abyssinicum is locally known as Dekoko (minute seeded) in Tigrigna and Yagere Ater (pea of my country) or Tinishu Ater (the smallest pea) in Amharic.

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Dekoko is capable of producing seed yield of up to 1.95 t/ha under phosphorus fertilization and is known for its high market price (more than double of the price of faba bean and field pea) and for its food preference Yemane and Skjelvåg (2002). Farmers and consumers call it as the "Dero-Wot of the poor" (chicken stew of the poor) probably to express its high nutritional value. Most often, the dry seeds of Dekoko are decorticated and split ('split peas') before boiling. Sometimes they are boiled without decortications and consumed as soup (personal observation). In Ethiopia, the annual consumption per person of field pea including Dekoko seeds is estimated at 6-7 kg (Messiaen et al., 2006; Sentayehu, 2009).

The genetic diversity of a species is the outcome of cumulative mutation, recombination and selection on individuals by the environment and selection by man for traits desirable for cultivation or consumption (Ali et al., 2007). The largest collection of *P. sativum* germplasm in Africa is located at the Institute of Biodiversity Conservation, Addis Ababa, Ethiopia, with over 1600 accessions (Messiaen et al., 2006).

A large genetic diversity has been found in *P. sativum* collections from both Africa (Ethiopia) and Asia. High to medium field pea genetic diversity in Ethiopia was observed in collections from Shoa, Gojam, Gondar, Wello, and Tigray while low to trace genetic diversity was observed in collections from Arsi, Gamo-gofa, Wellega, Illubabur and Kafa (Ali et al., 2003).

Morphological characterization or description of germplasm is the first step in the description and classification of the germplasm (Smith and Smith, 1989). An understanding of morphological characters facilitates the identification, selection of desirable traits, designing new populations, in transferring their desirable genes into widely grown food legumes through biotechnological means, resistance to biotic and abiotic stresses that are known to individual accessions that increase the importance of the germplasm (Santall et al., 2001; Tar'an et al., 2005; Jorge, 2006).

In Ethiopia, more than 15 cultivars of field pea, with better yield potential, seed size, seed color and disease resistance than the farmers' varieties, have been released for different agro-ecological conditions (MoARD, 2008). Some of these varieties were obtained from local collections while others were obtained through hybridization of landraces with introduced germplasm. Land races are the genetic wealth that a crop acquires over many years of its existence and have considerable breeding values as they contain valuable adaptive genes to different circumstances (Messiaen et al., 2006; Ali et al., 2003).

Even though Dekoko (*P. sativum var. abyssinicum*) is important both for the local farmers and consumers, the existing germplasm was not characterized morphologically and/or at genetic level; neither was there any improvement work on this crop so far. The current study was, therefore, conducted with the objectives of characterizing Dekoko accessions collected from northern Ethiopia (South Tigray and North Wello regions) using qualitative traits and understanding the genetic diversity of the crop.

MATERIALS AND METHODS

Accessions evaluated

The research was conducted in Southern zone of Tigray regional state, Wereda Endamekhoni at tabia Mekhan farmers' training center which is one of the mandate areas of Alamata Agricultural Research Center (AlARC). It is located at a latitude of 120 78'N and longitude of 390 53'E Endamekhoni (BoANR, 2002).

Twenty four (24) accessions collected from two regions; South-Tigray and North-Wello were tested at Mekhan farmers training center (FTC) at an elevation of 2100 m above sea level. The accessions were collected in 2008 by AlARC from weredas: Alamata, Ofla, Endamekhoni, Alaje, and Hintalo-Wejerat in South Tigray, and Kobo, Guba-lafto, Srinka and Habru in North-Wello regions. Table 1 explains the sources and altitude of the accessions with their major agro-ecological condition.

Experimental design and trial management

The trial was conducted using Randomized Complete Block Design with three replications and the plot size for each accession was 1.5 m^2 with inter- and intra-row spacing of 25 and 5 cm, respectively. Accessions were sown in six rows each 1 m long. Phosphorus and Nitrogen fertilizers with normal recommendation rates to other pulse crops (46 kg P_2O_5 and 18 kg N ha-1, that is 100 Kg DAP (Di-Ammonium Phosphate ha-1) and seed rate of 150 kg ha-1 were applied.

Analysis of qualitative data

Frequency distribution of the various categories of qualitative traits was studied. Deviation of the frequency distribution from the theoretical distribution was tested by χ^2. Proc Freq of SAS SAS (1994) was used for the χ^2 test.

The Shannon- Weaver diversity index

The Shannon-Weaver diversity index (H') was computed using the phenotypic frequencies to assess the overall phenotypic diversity for each character by the classification variable. The Shannon-Weaver diversity index as described by Hutchenson (1970) was used to calculate phenotypic diversity for j^{th} trait with n sub classes:

$$h_j = \sum_{i=1}^{n} pi \ln pi$$

Where, pi is the relative frequency in the ith category of the jth trait. To keep Shannon-Weaver diversity index between 0 and 1, the formula suggested by Hennink and Zeven (1991) was used:

$$H' = \frac{-\sum Pi \ln Pi}{\ln n}$$

Where, pi is the relative frequency in the ith category of the jth trait. *H'* of 0 indicates that the trait is monomorphic, that is all individuals belong to one and the same category (clan), where as H' of 1 indicates maximum diversity i.e., individuals are equally dispersed among the *n* class.

Table 1. Accessions of Dekoko Included in the Study and Their Sources with their altitude.

Accession name	Source of accessions			
	Region	Wereda	Major agro-ecology	Altitude of wereda (masl)
T-001/08 Of	Tigray	Ofla	High land	2457
T-002/08 Of	Tigray	Ofla	High land	2457
T-003/08 Of	Tigray	Ofla	High land	2457
TK-004/08 Al	Tigray	Alamata	Low land	1178-3148
TK-005/08 Al	Tigray	Alamata	Low land	1178-3148
TK-006/08 Al	Tigray	Alamata	Low land	1178-3148
TK-008/08 Al	Tigray	Alamata	Low land	1178-3148
T-023/08 Mw	Tigray	Endamekhoni	High land	2100
T-022/08 E/A	Tigray	Emba-Alaje	High land	2116
T-024/08 E/A	Tigray	Emba-Alaje	High land	2116
T-021/08 H/W	Tigray	Hintalo-Wejerat	Mid –altitude	1400-3050
T-007/08 Ko	Amhara	Kobo	Low land	1100-3000
T-009/08 Ko	Amhara	Kobo	Low land	1100-3000
T-010/08 Ko	Amhara	Kobo	Low land	1100-3000
T-017/08 Ko	Amhara	Kobo	Low land	1100-3000
T-018/08 Ko	Amhara	Kobo	Low land	1100-3000
T-019/08 Ko	Amhara	Kobo	Low land	1100-3000
T-020/08 Ko	Amhara	Kobo	Low land	1100-3000
T-012/08 G/L	Amhara	Guba-lafto	High land	2061
TA-013/08 Sr	Amhara	Srinka	Mid –altitude	1868
TA-014/08 Sr	Amhara	Srinka	Mid –altitude	1868
TA-015/08 Sr	Amhara	Srinka	Mid –altitude	1868
T-011/08 Hb	Amhara	Habru	Low land	700-1900
T-016/08 Hb	Amhara	Habru	Low land	700-1900

RESULTS

Qualitative traits

Cluster analysis using qualitative traits

Dekoko accessions were clustered into seven distinct groups based on three qualitative traits. Dendrograms summarizing the genetic similarity among 24 Dekoko accessions based on their qualitative traits is given in Figure1. The characters used for clustering are flower color, seed size and seed shape. The seven clusters (I–VII) are also depicted on the Dendrograms. The number of accessions in each cluster varied from 7 in cluster II to 1 in cluster V, VI and VII (Table 2).

DISCUSSION

The seven clusters correspond to 7 of the 12 possible combinations of the 3 seed colors, 2 seed shapes and 2 seed sizes. The first cluster contains 6 accessions with red flowers and wrinkled large seeds. Accessions from five Weredas (Ofla, Alamata, Hintalo-Wejerat, Kobo and Srinka) are grouped into this cluster. A similar trend is observed in other clusters. Accessions from different weredas of the two regions are grouped into the same cluster and accessions from a single wereda are scattered among different clusters. For example accessions collected from Alamata wereda of Tigray are found in clusters 1, 2, 5 and 6 and accessions of Kobo wereda are distributed in clusters 1, 2 and 3. There were no accessions with the following combinations of the three qualitative traits: accessions with pink flowers and smooth large seeds, with red flowers and wrinkled small seeds, with red flowers and smooth large seeds, with white flowers and wrinkled small seeds and those with white flowers and smooth large seeds. Whether farmers have deliberately selected against these genotypes or whether this happened by mere chance or other factors needs further investigation. The only two accessions with white flowers (accession 7 from Alamata and accession 23 from Habru) formed solitary clusters VI and VII with only one member. There is some tendency of the accessions to be grouped according to altitude. Although cluster I contains accessions from all three altitudes, in Cluster II, four of the seven accessions are from high lands. Cluster III is composed of two accessions from lowlands and one from highland while cluster IV contains accessions from mid- and low-lands. Comparison of the seven clusters by quantitative data showed that accessions in cluster I and

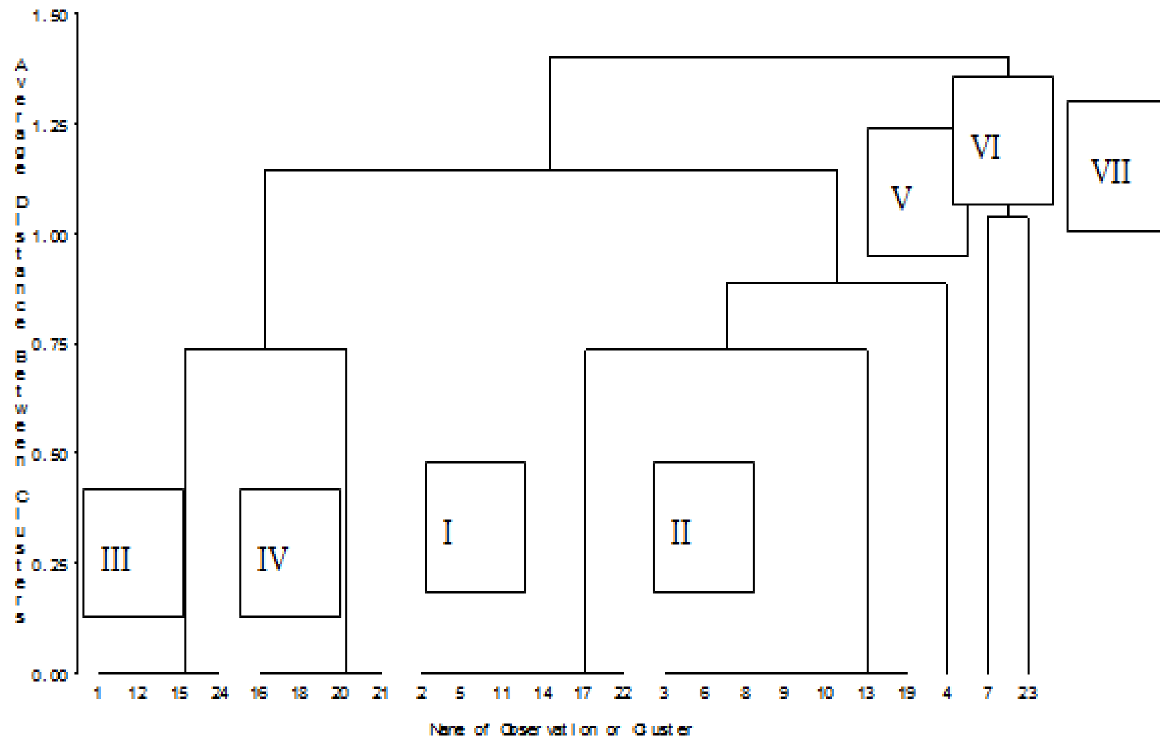

Figure 1. Dendrograms showing the distribution of 24 Dekoko accessions into 7 clusters.

Table 2. Classification of 24 Dekoko accessions into clusters by leaf color, seed shape &size

Cluster	S/N	Traits	Weredas (sources of accessions)
I	2, 5, 11, 14, 17,22	Red flower, Wrinkled and Large Seed	Ofla, Alamata, Hintalo-Wejerat, Kobo, Srinka
II	3, 6, 8, 9, 10, 13,19	Pink flower, Wrinkled and Large Seed	Ofla, Alamata, Endamekhoni, E/Alaje, Kobo, Guba-lafto
III	1, 12, 15, 24	Red Flower, Smooth and Small Seed	Ofla, Kobo, Habru
IV	16, 18, 20, 21	Pink Flower, Smooth and Small Seed	Kobo, Srinka
V	4	Pink flower, Wrinkled and Small seed	Alamata
VI	7	White flower, Wrinkled and Large seed	Alamata
VII	23	White flower, Smooth and Small Seed	Habru

VI emerged later (10.1 and 10.7 days, respectively, while the third latest emerging cluster was cluster 4 (9.4 days). Clusters V and VII emerged faster (8.7 days). Cluster VI was late in flowering (45.3 days), while clusters II and V were the earliest flowering (41 and 42 days, respectively). Clusters VII and II had the highest number of seeds per plant (5.3 and 5.1 seeds, respectively), while cluster VI had the lowest number of seeds per plant (4.1). Clusters V, II and I had high protein content (37.0, 30.4 and 30.1%, respectively), while clusters IV, VII and VI had low protein content (25.4, 26.3 and 27.3%, respectively).

Frequency distribution based on qualitative traits

Flower color varied from accession to accession with the dominant color being pink (50%) followed by red (41.67 %). White color was with least percentage (8.33%) which was expressed only in few accessions. Seed shape was dominantly wrinkled (62.5%) while seeds with smooth surface were 37.5%. Similarly, seed size was dominated by larger-sized seeds (58.33%) while smaller seeds constituted 41.67%. These two characteristics of Dekoko may, therefore, be used to characterize seeds into Kik ('split pea') and Shiro ('ground pea') type. Accordingly, at least 58% of the accessions are Kik ('split peas') type. Summary on the qualitative traits is given in Table 3.

The distribution of the various categories of the three qualitative traits (flower color, seed shape and seed size) was independent of region and altitude except that in Tigray region more large-seeded accessions than small-seeded ones (81.8 vs. 18.2%) are grown while in Amhara region (North Wello) more small seeded varieties are grown

Table 3. Frequency Distribution and Shanon-Weaver Diversity Index (H') of the Qualitative Traits of Dekoko Grown at Mekhan, Endamekhoni, in 2010.

Qualitative trait	Index and description adopted	Frequency (%)	H'
Flower color	Pink	50.00	0.84
	Red	41.67	
	White	8.33	
Seed shape	Smooth	37.5	0.95
	Wrinkled	62.5	
Seed size	Large	58.33	0.98
	Small	41.67	
Overall mean			0.92

grown (38.5 vs. 61.5%) (P = 0.03). More wrinkled seeded Dekoko varieties than smooth-seeded ones are grown in Tigray (90.1 vs. 9.1%), while in Amhara this ratio was 38.5 vs. 61.5% (P = 0.01). There was a strong association between seed size and seed shape (p < 0.001); all except one accession with big seeds had wrinkled seed surface, i.e., and all accessions with small seeds had seeds with smooth surface. These traits might be linked tightly; that is big seed size might be tightly linked with wrinkled seed shape while small seed size might be tightly linked with smooth seed surface. We have found out that Accessions from high lands had the highest protein content (31.3 vs. 25.58% and 24.33% of the mid altitudes and low lands, respectively). Seed yield was highest (0.70 t/ha) from collections of the lowlands followed by accessions from mid-altitudes (0.68 t/ha) and 0.66 t/ha for the high land accessions. This is reasonable that all the accessions were grown in more suitable environments (high lands) and as a result, the accessions collected from low lands might have expressed their potential for seed yield at the expense of protein. White flowered accessions flowered late (44.2 days) and accessions with pink flowers were the earliest to flower (42 days), while red-flowered accessions were intermediate in flowering (43days). Seed size had no effect on many of the quantitative traits except that 29 accessions with smaller seeds produced the highest leaf area index (2.14) as compared to 2.04 of accessions with bigger seeds.

The Shannon-Weaver Diversity Index (H')

In this study, Shannon-weaver diversity index was considered to measure the diversity of Dekoko accessions based on the frequency distributions of three qualitative traits. The result of 'H' values for the phenotypic characters showed high levels of diversity among 24 Dekoko accessions that ranged from 0.83 in flower color to 0.98 in seed size (Table 3). Furthermore, the overall mean of 'H' value of 0.92 confirms the existence of high level of phenotypic diversity among Dekoko accessions. Low 'H' value indicates low level of diversity and uneven distribution of accessions Hennink and Zevan (1991) while high "H" indicates high level of diversity with even distribution of accessions in the various categories. Except in flower color where the white color is rare, but the red and pink are almost equally abundant, farmers did not discriminate among genotypes according to seed size and seed shape, distribution of small and big and smooth and wrinkled seeds being even.

ACKNOWLEDGEMENTS

The authors duly thank the Ethiopian Institute of Agricultural Research and Tigray Agricultural Research Institute for covering the research fund through the Rural Capacity Building project.

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Vol. 7(10), pp. 488-491, October 2013
DOI: 10.5897/AJPS2013.1078
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Full Length Research Paper

Direct regeneration of the medicinal herb *Cucumis anguria* L from shoot tip explants

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Accepted 16 September, 2013

A protocol was developed by *in vitro* regeneration by shoot induction of *Cucumis anguria*, a plant used as medicine and vegetable belonging to the family Cucurbitaceae. High frequencies of multiple shoot regeneration were achieved from nodal explants on MS fortified with 2 mg/l 6-benzyl amino purine (BAP) and 0.5 mg/l indole acetic acid (IAA). Five to ten shoots per explant were obtained. The elongated shoots were sub cultured for rooting on MS supplemented with 2 mg/l napthalin acetic acid (NAA). The *in vitro* raised plantlets were acclimated in green house and successfully transplanted to natural condition with 75% survival.

Key words: *In vitro* regeneration, *Cucumis anguria*, shoot tip explants, MS medium.

INTRODUCTION

Tissue culture techniques are being increasingly explanted for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened with extinction. Greater demands of these plants especially for the purpose of food and medicine are the causes of their rapid depletion from primary habitats. Plant tissue culture is a tool which ensures mass production of plants for their improvement.

Cucumis anguria, a climber belongs to the family Cucurbitaceae. It is distributed in Manapparai and Dindigul area. In Tamil, the plant is called as *Vizavellari* and its fruits are traditionally used for reducing the body heat and stomach pain. The fruits are rich in carbohydrates. This plant is dioecious in nature, limiting pollination and fertilization. Hence, the present study was undertaken to develop a suitable micropropagation technique to contract the natural in their population. There has been progress in tissue culture studies in many Cucurbitaceae members such as *Momordica dioca* (Shiragave and Chavan, 2001), *Coccinia indica* (Venkateswaralu, 2001), *Cetrullus vulgaris* (Dong and Ja, 1991), *Cucumis melo* (Mackay et al., 1989), *Cucumis sativus* (Cade et al., 1987) and *Mormordica charantia* (Mala Agarwal and Reka Kamal, 2004). The present investigation elucidates *in vitro* multiple shoot regeneration through nodal explants of *C. anguria* for better exploitation and also preservation of this valuable germplasm. The objective of the research was to find out the suitable protocol for tissue culture.

MATERIALS AND METHODS

C. anguria (L) seeds were collected from IAP Farms (P) Ltd., Nilakkottai, Dindigul District, Tamil Nadu. The collected seeds were planted at College Garden. The nodal segments were excised from the garden grown plants and washed thoroughly in running tap water for 10-15 min, 1% teepol solution for 5 min and washed five times with distilled water followed by sterile distilled water under aseptic condition. For surface sterilization, explants were sensed by 0.1% $HgCl_2$ solution for 3-5 min and rinsed with sterile distilled

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Table 1. Production of plantlets from shoot tip explants through direct organogenesis.

Hormone concentration (mg/l)		Number of explants cultured	Number of explants Responded	Percentage of Response	Number of Shoot/ Explants mean ± SD
BAP	IAA				
0.5	0.5	20	8	40	3.50 ± 0.53
1.0	0.5	20	12	60	4.00 ± 0.85
1.5	0.5	20	15	75	4.40 ± 0.99
2.0	0.5	20	17	85	5.71 ± 0.77
2.5	0.5	20	11	55	4.45 ± 0.69

water five times to remove the traces of $HgCl_2$.

Nodal segments were further trimmed to remove excess tissues. The explants were cultured on MS medium with different concentration of plant growth hormones along with 3% sucrose and 0.8% of agar. The pH of the medium was adjusted to 5.7 using 0.1 N NaOH or 0.1 N HCl before autoclaving and adding agar. About 10 ml of the medium were dispensed in each culture tube and sealed with non-absorbent cotton plugs prior to autoclaving at 121°C for 20 min. All cultures were maintained at 16 h photoperiod with 3000 Lux light intensity at 25 ± 2° C.

Results were observed at regular intervals and tabulated for each treatment; 20 replicates were used and all experiments were conducted thrice.

RESULTS AND DISCUSSION

Direct organogenesis is the development of an organ from the explants itself without intermediate tissue culture development. Production of plantlets by direct organogenesis either from shoot apex where apical meristem is present or from the nodal explants where lateral meristem is present is called micropropagation.

Studies were carried out to produce plantlets from shoot tip explants through direct organogenesis without intervention of callus a significant development, and it was observed that multiple shoot buds originated from Shoot tip explants, when MS was supplemented with various concentrations of 6-benzyl amino purine (BAP) (1.0-10 mg/l) along with indole acetic acid (IAA) (0.5 mg/l). The nodal explants showed slight swelling prior to the emergence of short buds development from the pre-existing material 20 days after inoculation; initially two to five shoot buds per explant emerged 35 days after inoculation and gradually the number of shoot buds per explant increased upto eight to ten (Table 1) on MS fortified with 2.0 mg/l BAP along with combination of 0.5 mg/l IAA. Similar results were observed with BA at a concentration of 2.5 mg/l.

It can be concluded that 1.0 mg/l BAP and 0.5 mg/l IAA are suitable phytohormones for suitable phytohormones for shoot proliferation and shoot elongation from shoot explants of *C. anguria.* This is accordance with the results as reported earlier (Anand and Jeyachandran, 2004; Kulkarni et al., 2002; Yokoya and Handro, 2002; John Britto et al., 2001).

Following multiple shoot elongation, the healthy shoots (4-5 cm long) were transferred on MS supplemented with different concentrations of NAA. Shoot elongation was simultaneously observed along with root induction in 2.0 mg/l NAA (Table 2). Anitha and Pulliaiah (2002) in *Decalipis hamiltoni* demonstrated similar results.

After 35 days, well developed shoots and shoots were observed. Subsequently, cultures were removed from agar medium washed thoroughly and placed in pots containing a mixture of sterilized vermiculate and sterilized soil (1.1). *In vitro* raised plants were acclimated in greenhouse and successfully transplanted into the field with 60% survival.

In the present investigation, high frequency of multiple shoot induction was achieved in *C. anguria* through shoot explants with BAP 2.0 mg/l and combination of IAA (0.5 mg/l). Further, an increase or decrease of this hormone level showed negative trend in multiple shoot formation. NAA (2 mg/l) was found to be an ideal growth regulator for root induction as well as shoot elongation.

Multiple shoots from shoot tip explants on MS medium fortified with BAP (1.0 mg l^{-1}) in combination with abscisic acid (2.0 mg l^{-1}) showed maximum percentage of response (74.4%) with 24.6 shoots per explants. Varisai Mohamed et al. (1999) demonstrated high frequency of shoot multiplication from shoot tip explants of *Macrotyloma uniflorum* on MS medium supplemented with BAP (1.5 mg l^{-1}) and GA_3 (0.5 mg l^{-1}). The above study contradicted the results reported in *Cucurbita pepo* (Pink and Walkey, 1984) where multiple shoot induction from shoot tip explants occurred on modified MS medium with IBA (1.7 µM), KIN (0.5 µM) and GA_3 (0.3 µm). Plate 1 and Figure 1.

In *C. anguria* for shoot-tip culture, BAP (2.0 mg/l) with IAA (0.5 mg/l) was a suitable hormone concentration. When concentration increased or decreased the rate of shoot initiation and shoot proliferation also decreased.

ACKNOWLEDGEMENT

The authors are grateful to the management of Jamal

Table 2. Rooting response of excised shoots of *Cucumis anguria.*

NAA (mg/l)	Response (%)	Root length / shoot (cm)
0.5	-	-
1.0	24	1.6 ± 0.20
1.5	52	1.9 ± 0.08
2.0	64	2.7 ± 0.08
2.5	51	1.8 ± 0.06

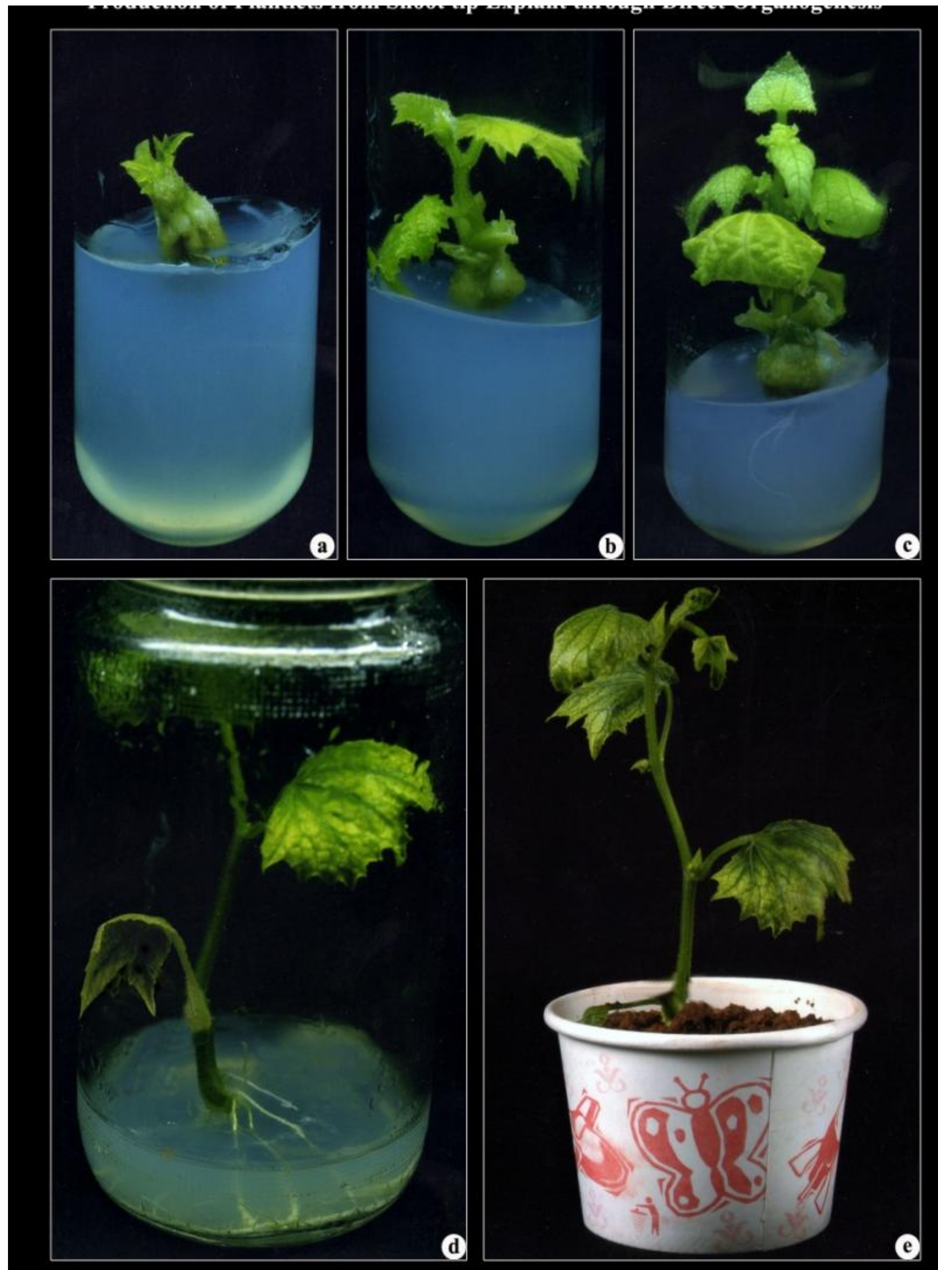

Plate 1. Production of plantlets from shoot tip explants through direct organogenesis A and b, Shoot initiation from shoot tip explants on MS + BAP (2.0 mg/l) + IAA (0.5 mg/l); c, shoot elongation on MS+BAP (2.0 mg/l) + IAA (0.5 mg/l); d, rooting on MS with 1.5 mg/l of NAA; e, hardened plantlet.

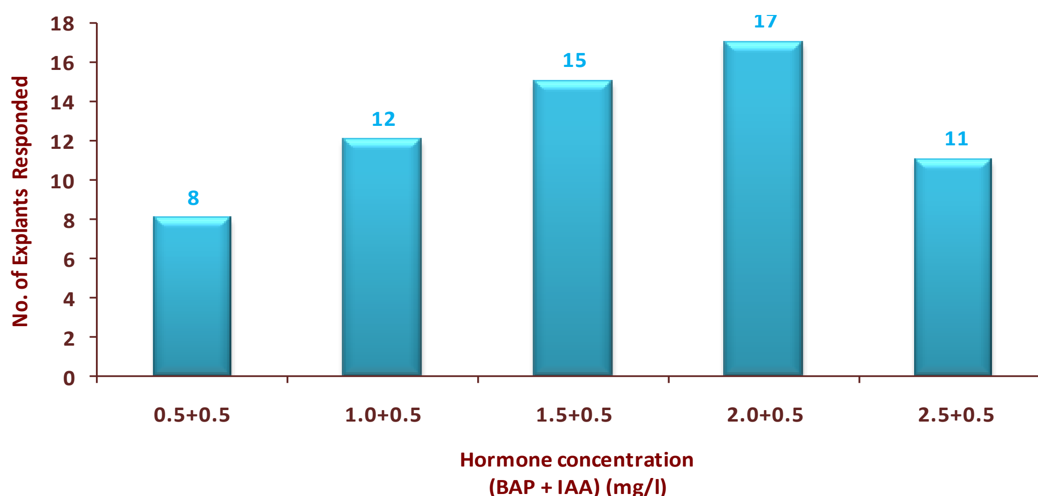

Figure 1. Production of plantlets from shoot tip explants through direct organogenesis.

Mohamed College (Autonomous), Tiruchirappalli, Tamil Nadu-620020, for providing necessary laboratory and other facilities to perform all the experiments.

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Vol. 7(10), pp. 492-503, October 2013
DOI: 10.5897/AJPS2013.1051
ISSN 1996-0824 ©2013 Academic Journals
http://www.academicjournals.org/AJPS

African Journal of Plant Science

Full Length Research Paper

Lactuca sativa mediated chitinase activity and resistance in pearl millet against *Sclerospora graminicola*

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Accepted 29 August, 2013

Pearl millet seeds were treated with latex extracts (L1- 50 µl, L2- 100 µl and L3- 200 µl) of *Asclepias curassavica, Calotropis gigantea*, *Croton* sp., *Eucalyptus alba*, *Morus alba*, *Nerium odoratum, Lactuca sativa* and *Tridax procumbens* were applied on pearl millet seeds for 3 and 6 h to evaluate their role in growth promotion and downy mildew resistance. Latex extracts of all tested plants improved vegetative parameters when compared with the control. *L. sativa* (L2) at 3 h showed 38 and 18% increase in seed germination and vigor, respectively, over the control. Plant height, shoot fresh and dry weight, number of tillers and leaf surface area also increased upon latex treatment. Green house studies indicated that *L. sativa* required four days after challenge inoculation to build up maximum resistance and offered 54% protection against downy mildew. Resistance induction was evident with increased activities of phenylalanine ammonia lyase (PAL) (four fold), peroxidase (POX) (three fold) and chitinase (three fold) in *L. sativa* (L2) treated seedlings challenged with the pathogen as compared to control seedlings.

Key words: Downy mildew, induced resistance, *Lactuca sativa,* pearl millet, plant extracts, *Sclerospora graminicola.*

INTRODUCTION

The knowledge on biochemical basis of resistance has become a major area of investigation to understand the possible defense mechanisms during pathogen infection. The disease resistance in plants is acquired by conventional fungicides and during recent years, eco-friendly approaches using biotic agents have been carried out in an attempt to build up durable resistance in host plants. Among the new biological approaches, induction of plant's own defense system is considered as one of the most promising alternative strategy for crop protection (Heil and Bostock, 2002; Walters et al., 2005; Anderson et al., 2006; Walters and Fountaine, 2009). Numerous studies have shown the appearance of defense responses against several important plant diseases (Abo-Elyousr and El-Hendawy, 2008).

Various studies have focused on developing eco-

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friendly, long lasting and effective biocontrol methods such as use of plant products (Latha et al., 2009; Modafar et al., 2012) and biocontrol agents for the management of plant diseases (Alizadeh et al., 2012; Chowdappa et al., 2013). Microbial biocontrol agents (Naher et al., 2012; Senthilraja et al., 2013) and plant extracts (Amadioha, 2003; Bowers and Locke, 2004; Nisha et al., 2012; Pal et al., 2013; Sangeetha et al., 2013) have been found efficient against a wide range of pathogens. Numerous plant extracts have been explored as natural resistance inducers like *Azadirachta indica* against *Alternaria* leaf spot (Guleria and Kumar, 2006), *Datura metel* against *Rhizoctonia solani*, *Xanthomonas oryzae* and *Alternaria solani* (Kagale et al., 2004; Latha et al., 2009).

Latex is a stable dispersion of naturally occurring polymer of micro particles in an aqueous medium. It is also rich in enzymes like proteases, glucosidases, chitinases and lipases. It has been proved to be a source of natural fungicides (Barkai-Golan, 2001) which is regarded as safe and effective against various diseases of banana, papaya and other fruits. Latex extracted from several plants has shown a strong antifungal activity against *Botrytis cinerea*, *Fusarium* sp. and *Trichoderma* sp. (Barkai-Golan, 2001) and evidences concerned with likely participation of laticifer proteins (LP) in the plant defense mechanisms (Agrawal and Konno, 2009) are available. Though widespread studies have been done on isolation and characterization of different proteins in latex, restricted information exists on the use of latex as elicitor in inducing resistance against phytopathogens.

Based on the above reports we hypothesized that latex extract derived from latex plants might contain certain metabolites which can effectively manage pearl millet downy mildew disease in an eco-friendly manner. Pearl millet (*Pennisetum glaucum* [L.] R. Br) is one of the important crop (D'Andrea et al., 2001) cultivated in about 25 million ha (Naylor et al., 2004) in semi-arid tropical zone of the world. India alone accounts for 11.2 million ha with a total annual production of 7 million tonnes (Khairwal et al., 2007). The crop is a staple cereal of 90 million people and also used as fodder (Singh and Shetty, 1990). Downy mildew is caused by *Sclerospora graminicola* resulting in high economic losses which can be attributed to disease development at early stages, poor tillering and ear-head malformation (Singh and Shetty, 1990). The disease was known in most of the pearl millet-growing regions, but remained sporadic until the high-yielding hybrids with susceptible parental lines were introduced (Singh, 1995). A number of new resistant varieties and hybrids developed have turned into highly susceptible owing to breakup of host resistance and continuous change in pathogen's nature (Chaudhry et al., 2001). This has drawn the attention of pearl millet growers (Ball, 1983) in search of new line of resistance factors against this devastating disease.

The growing demand on isolation of bioactive molecules from different parts of the plant, paved way for the use of latex and its utility in inducing disease resistance in plants. However, limited information on the role of the latex in induction of resistance in plants is available. An attempt has been made in the present study to evaluate some latex-producing plants for their ability to induce systemic resistance in pearl millet against downy mildew disease. Further study is required to determine the nature of the factors controlling the induced resistance.

MATERIALS AND METHODS

Collection of seed samples

Seeds of pearl millet cultivar cv. 7042S highly susceptible to downy mildew pathogen were obtained from All India Co-ordinated Pearl Millet Improvement Project (AICPMIP), Agricultural Research Station, Mandore, Rajasthan, India and were used throughout the study.

Pathogen and inoculum preparation

The downy mildew pathogen *S. graminicola* maintained on its susceptible host 7042S under greenhouse conditions was used as a source of inoculum throughout the study. The leaves of pearl millet infected with downy mildew disease were collected in the evening hours. The collected leaf samples were washed under running tap water to remove the remnants of sporulation and dust particles, they were blot dried, cut into 1-2 cm pieces and placed on Petri dishes lined with moist blotters and incubated over night. Next day, fresh sporangia were harvested into sterile distilled water (SDW) and the concentration of sporangia was adjusted to 4 x 10^4 zoospores/ml in SDW using haemocytometer which served as inoculum for all the experiments (Safeeulla, 1976).

Plant material and latex collection

Latex yielding plants viz., *Asclepias curassavica*, *Calotropis gigantea* (Asclepiadaceae), *Croton* sp. (Euphorbiaceae), *Eucalyptus alba* (Myrtaceae), *Morus alba* (Moraceae), *Nerium odoratum* (Apocynaceae), *Lactuca sativa* and *Tridax procumbens* (Asteraceae) were collected in and around Mysore District. The plant materials were washed under running tap water, blot dried and small downward v-shaped incisions were made at the base of the stem to collect the latex extracts (droplets). 50, 100 and 200 µl latex extracts (L1, L2 and L3) of each plant were immediately mixed with 10 ml of methanol containing 1% phosphoric acid (Sessa et al., 2000). The latex extracts (L1, L2 and L3) of each plant were evaporated to dryness and suspended in 10 ml sterile distilled water (SDW) which served as inducer for seed treatment.

Seed treatment

Pearl millet seeds of 7042S cultivar were surface sterilized with 0.02% mercuric chloride for 5 min and thoroughly washed for 2-3 times in SDW. After surface sterilization, the seeds were soaked in inducers (L1, L2 and L3) prepared as mentioned above and then incubated at 28 ± 2°C in an incubator rotary shaker at 100 rpm (rotation per minute) for 3 and 6 h, respectively. After incubation,

the seeds were air-dried aseptically. Seeds soaked in SDW served as control.

Effect of latex extracts on seed germination and seedling vigor

Germination test was carried out by paper towel method (ISTA, 2003). The latex extracts treated (L1, L2 and L3) seeds were placed on moist germination sheets equidistantly and another presoaked paper towel was placed on the first one in order to hold the seeds in position, rolled and wrapped with polythene to prevent drying and incubated for seven days at 25 ± 2°C. After seven days of incubation, seed germination and seedling vigor were analyzed (Abdul Baki and Anderson, 1973). The experiment consisted of four replicates of 100 seeds in all the treatments and repeated thrice.

$$\text{Percent germination} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds planted}} \times 100$$

Vigor Index = (Mean root length + Mean shoot length) × Germination (%)

Effect of latex extracts on growth parameters of pearl millet under greenhouse conditions

Based on percent germination results, the L2 treatments of all the latex extracts for 3 h time duration was continued for further studies. Evaluation of growth promotion under greenhouse conditions was carried out in pearl millet cv. 7042S seeds treated with L2 treatments for 3 h time duration. After treatment, the seeds were blot-dried and sown in earthen pots (9 x 12 cm) filled with sand, soil and manure in the ratio of 1:2:1. The experiment consisted of four replicates per treatment with 10 pots in each replicate (10 seeds/ pot) and repeated thrice. The pots were maintained at 25 ± 2°C with 95% RH (relative humidity) and watered regularly. Seeds treated with SDW served as control. 30 days after sowing, seedling height, fresh and dry weight of shoot, leaf surface area and number of basal tiller per plant were measured and recorded accordingly.

Effect of latex extracts on pearl millet-downy mildew disease under greenhouse conditions

The pearl millet seeds primed with latex extract and distilled water treatments for 3 h time duration were sown in earthen pots filled with autoclaved soil (1:2:1 ratio of sand, soil and manure). Leaf-whorls of two-day old seedlings were inoculated with a suspension of 4 x 10^4 zoospores/ml of *S. graminicola* by whorl inoculation method (Singh and Gopinath, 1985). Each treatment consisted of four replicates of 10 pots per replication with 10 seedlings per pot of 14 inch diameter. The pots were arranged in a randomized complete block design (RBD), maintained under greenhouse conditions (90 to 95% RH, 20 to 27°C). Plants were observed daily and were rated diseased when they showed any one of the typical symptoms of downy mildew, that is, sporulation, chlorosis, stunted growth or malformation of the earheads. At the end of 60 days, disease incidence was recorded as the percentage of plants showing symptoms of downy mildew disease. The experiment was repeated thrice with four replicates of 100 plants each. Downy mildew disease protection was calculated using the formula:

$$\text{Downy mildew disease protection} = \frac{C-T}{C} \times 100$$

Where, C, is percent downy mildew disease incidence in control; T- percent downy mildew disease incidence in treated plants.

Spatio-temporal time gap studies

Spatio-temporal time gap studies were carried out in order to understand the nature of disease protection offered by seed treatment and maintaining spatio-temporal separation between inducer treatments and the pathogen inoculation (Amruthesh et al., 2005). The susceptible pearl millet seeds (7042S) treated with latex extract of *L. sativa* (L2) along with control seeds were sown in autoclaved potting medium as mentioned above and arranged in RBD. Two-day-old seedlings were challenge inoculated with zoospore suspension of *S. graminicola* (4 x 10^4 zoospore/ml) by whorl inoculation method with a time gap of 1, 2, 3, 4, 5 and 6 days of emergence in different set of plants. Plants were maintained under greenhouse conditions as mentioned above and were observed for typical symptoms of downy mildew, that is, sporulation, chlorosis, stunted growth or malformation of the earheads at every 15-day time intervals and were rated for disease when they showed any one of the typical downy mildew symptoms. At the end of 60 days, disease incidence was recorded as the percentage of plants showing symptoms of DM disease. The experiment was repeated thrice with four replicates of 100 plants each.

Biochemical studies

Sampling of seedlings

Effect of seed treatment on the activity of defense enzymes like phenylalanine ammonia-lyase, peroxidase and chitinase activity during host-pathogen interaction in pearl millet was carried out. Susceptible pearl millet seeds treated with L2 (*L. sativa*) treatments for 3 h time duration were placed on petri plates lined with moistened blotter discs. Two day old seedlings were harvested at different time intervals (0, 2, 4, 6, 8, 12, 18, 24, 48 and 72 h) after challenge inoculation with *S. graminicola* (4 x 10^4 zoospres/ml) and were stored at -30°C until used for further studies. SDW treated seedlings served as control.

Estimation of phenylalanine ammonia lyase (PAL) activity

Two-day-old pearl millet seedlings (1 g) of all the samples were homogenized in 1 ml of ice cold 25 mM Tris buffer, pH 8.8, containing 32 mM of 2-mercaptoethanol in a prechilled pestle and mortar. The extract was centrifuged at 10,000 rpm for 25 min at 4°C and the supernatant was used as enzyme source. Reaction mixture containing 0.5 ml of enzyme extract was incubated with 1 ml of 25 mM Tris-HCl buffer, pH 8.8 and 1.5 ml of 10 mM L-phenylalanine in the same buffer for 2 h at 40°C. The activity was stopped using 5 N HCl (Geetha et al., 2005). PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm. Enzyme activity was expressed as µmol of trans-cinnamic acid min/mg protein/h.

Estimation of peroxidase (POX) activity

Two-day-old pearl millet seedlings (1 g) of all the samples were macerated with 0.2 M sodium phosphate buffer (pH 6.5) in a prechilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C to get the supernatant. Peroxidase (POX) activity was determined following the method of

Table 1. Effect of seed treatment of latex extract on pearl millet for 3 h on seed germination and seedling vigor.

Plant extract	Plants extracts at different modes					
	Percent germination			Seedling vigor		
	L 1	L 2	L 3	L 1	L 2	L 3
Asclepias curassavica	81±2.41[bc*]	88±1.82[ab]	80±2.27[c]	1188±20.14[c]	1254±30.38[de]	1170±29.07[c]
Calotropis gigantea	88±1.08[ab]	93±0.91[a]	88±1.58[ab]	1352±20.35[ab]	1418±26.28[ab]	1304±25.41[ab]
Croton sp.	87±2.41[abc]	93±1.29[a]	87±0.91[ab]	1320±37.02[ab]	1394±23.84[abc]	1296±35.69[ab]
Eucalyptus alba	83±1.73[abc]	88±1.35[ab]	82±0.9[1bc]	1186±42.47[c]	1272±12.51[de]	1154±23.54[c]
Lactuca sativa	92±1.87[a]	95±1.35[a]	90±2.12[a]	1416±15.11[a]	1482±29.87[a]	1384±30.35[a]
Morus alba	81±2.73[bc]	85±2.34[b]	80±0.91[c]	1190±27.95[c]	1208±39.86[e]	1174±27.67[c]
Nerium odoratum	84±2.41[abc]	90±1.82[ab]	83±0.57[bc]	1245±35.80[bc]	1298±25.22c[de]	1240±26.54[bc]
Tridax procumbens	86±1.29[abc]	91±1.58[ab]	84±0.91[abc]	1282±24.50[bc]	1340±31.48[bcd]	1228±23.64[bc]
Control	78±1.29[c]	78±1.29[c]	78±1.29[c]	916 ± 20.12[d]	916 ± 20.12[f]	916 ± 20.12[d]

*Mean from four repeated experiments with four replicates of 100 plants per treatment in each experiment. *Values are means ± SD of four replicates.

Hammerschmidth et al. (1982). The reaction mixture of 3 ml consisted of 0.25% (v/v) guaiacol in 10 mM potassium phosphate buffer (pH 6.9) containing 10 mM hydrogen peroxide. 5 µl of crude extract was added to initiate the reaction, which was followed calorimetrically at 470 nm (Hitachi 2000). PO activity was expressed as the increase in absorbance at 470 nm/mg protein/min.

Estimation of chitinase

One gram of seedlings of each sample were macerated using 0.05 M sodium acetate buffer, pH 5.2 (1 ml/g fresh weight) and acid washed glass beads at 4°C in prechilled mortar and pestle. The homogenate were centrifuged at 10,000 rpm for 30 min at 4°C (Himac Centrifuge, HITACHI) and the supernatant was used as crude extract. Chitinase was assayed following the method of Isaac and Gokhale (1982) with N-acetyl glucosamine (Sigma) as standard. Colloidal chitin in 0.05 M sodium acetate buffer (pH 5.2), purified from chitin (Sigma-Aldrich, USA) was used as a substrate as suggested by Skujins et al. (1965). Monomers of N-acetyl glucosamine released after incubation were measured spectrophotometrically at 585 nm using dimethyl amino benzaldehyde reagent (Reissig et al., 1955). The enzyme activity was expressed in terms of nmol/min/mg protein.

Protein estimation

Protein content in the crude extracts was estimated by dye binding method (Bradford, 1976) using bovine serum albumin (Sigma) as a standard.

Statistical analysis

Data from four replicates were analyzed for each experiment and subjected to arcsine transformation and analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by magnitude of F values ($P \leq 0.05$). Treatment means were separated by Tukey's HSD test.

RESULTS

Effect of inducers on seed germination and seedling vigor

The latex extract(s) as inducer treatment to the pearl millet susceptible seeds significantly enhanced the seed germination and vigor in general. However, the percent germination and vigor differed greatly with treatments and time duration of treatments.

The seeds treated for 3 h with L2 treatment showed higher seed germination and vigor followed by other treatments viz., L1 and L3 when compared with all the treatments at 6 h (Tables 1 and 2). Among the L2 treatment for 3 h, *L. sativa* showed highest seed germination (95%) and vigor (1482) followed by *C. gigantea* with 93% germination and 1418 vigor. The germination and vigor of pearl millet seeds in response to latex extracts of other plants were higher when compared with the control which showed 78% germination and 916 vigor.

Effect of latex extracts on growth parameters of pearl millet under greenhouse conditions

The latex extract (L2) treatment on seeds for 3 h were further tested for their efficiency on growth promotion by evaluating different growth parameters like plant height, shoot fresh weight, shoot dry weight, leaf surface area and number of basal tillers per plant.

The data on vegetative parameters revealed a significant increase upon inducer treatment. Among the different plants tested, L2 of *L. sativa* treated seeds recorded highest readings in all the growth parameters with 39.2 cm height, 14.5 g shoot fresh weight, 4.5 g shoot dry weight, 39.6 cm^2 leaf surface area and 4.5 number of basal tillers per plant followed by *C. gigantea, Croton* sp., *T. procumbens, N. odoratum, E. alba, A. curassavica* and *M. alba.*

The distilled water treated seeds showed 26.4 cm height, 8.2 g shoot fresh weight, 2.8 g shoot dry weight, 30.6 cms^2 leaf surface area and 3.2 number of basal tillers per plant (Table 3).

Table 2. Effect of seed treatment of latex extract on pearl millet for 6 h on seed germination and seedling vigor.

Plant Extracts	Plants extracts at different modes					
	Percent germination			Seedling vigor		
	L 1	L 2	L 3	L 1	L 2	L 3
Asclepias curassavica	79±0.91^{bc*}	79±1.82^{d}	74±1.58^{bc}	980±10.97^{b}	1024 ± 30.08^{cde}	844±26.04^{c}
Calotropis gigantea	85±1.08^{a}	87±1.08^{ab}	82±1.29^{a}	1178±36.22^{a}	1214±41.90^{ab}	1124±21.89^{ab}
Croton sp.	82±1.47^{ab}	85±0.91^{abc}	80±1.82^{ab}	1148±34.14^{a}	1190±22.66^{ab}	1106±36.34^{ab}
Eucalyptus alba	80±0.91^{abc}	81±0.91^{cd}	76±0.91^{abc}	986±16.45^{b}	1078±61.22^{bcd}	872±13.19^{c}
Lactuca sativa	85±0.91^{a}	89±0.91^{a}	82±0.91^{a}	1205±12.29^{a}	1253±26.47^{a}	1185±30.70^{a}
Morus alba	75±0.91^{c}	78±1.29^{d}	72±1.29^{c}	886±10.43^{b}	976±28.87^{de}	820±11.83^{c}
Nerium odoratum	81±1.29^{ab}	82±1.29^{bcd}	79±2.04^{abc}	1116±32.17^{a}	1154±35.97^{abc}	1023±29.14^{b}
Tridax procumbens	82±1.58^{ab}	83±1.29^{bcd}	79±2.41^{abc}	1148±20.89^{a}	1162±43.17^{abc}	1040±25.88^{b}
Control	78±1.29^{bc}	78±1.29^{d}	78±1.29^{abc}	916±20.12^{b}	916±20.12^{e}	916±20.12^{c}

*Mean from four repeated experiments with four replicates of 100 plants per treatment in each experiment. *Values are means ± SD of four replicates.

Table 3. Effect of latex extract treatment on plant height, shoot fresh weight, shoot dry weight, leaf area and number of basal tillers under green house conditions after 30 DAS.

Plant extracts	Height (cm)*	Shoot fresh weight/plant (g)*	Shoot dry weight/ plant (g)*	Leaf surface area (cm^2)*	No. of basal tillers/plant*
Asclepias curassavica	27.9±0.26^{g}	9.6±0.09^{g}	3.2±0.09^{g}	32.8±0.34^{e}	3.4±0.09^{d}
Calotropis gigantea	35.6±0.14^{b}	13.6±0.09^{b}	4.2±0.09^{b}	38.5±0.23^{b}	4.1±0.12^{ab}
Croton sp.	32.4±0.12^{c}	12.6±0.09^{c}	3.9±0.09^{c}	37.3±0.09^{c}	4.1±0.07^{ab}
Eucalyptus alba	29.1±0.13^{f}	10.8±0.05^{f}	3.3±0.09^{f}	34.8±0.34^{d}	3.4±0.09^{d}
Lactuca sativa	39.2±0.20^{a}	14.5±0.09^{a}	4.5±0.09^{a}	39.6±0.20^{a}	4.5±0.15^{a}
Morus alba	26.5±0.12^{h}	8.9±0.09^{h}	3.1±0.09^{h}	34.8±0.21^{d}	3.2±0.09^{d}
Nerium odoratum	30.5±0.09^{e}	11.6±0.09^{e}	3.5±0.05^{e}	36.5±0.18^{c}	3.6±0.12^{cd}
Tridax procumbens	31.7±0.13^{d}	12.2±0.12^{d}	3.7±0.10^{d}	37.4±0.23^{c}	3.9±0.09^{bc}
Control	26.4±0.09^{h}	8.2±0.12^{i}	2.9±0.09^{i}	30.8±0.43^{f}	3.2±0.13^{d}

*Mean of three repeated experiments with four replicates of 100 plants per treatment in each experiment. Means within columns sharing the same letters are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

Effect of latex extracts on pearl millet downy mildew disease under greenhouse conditions

The potential of latex extracts in managing the pearl millet downy mildew disease was assessed under green house conditions by treating the pearl millet susceptible seeds with L2 treatments for 3 h. Significant ($P \leq 0.05$) disease protection was observed in seedlings raised from latex extract treatments (Table 4). Among the latex extracts, *L. sativa* offered maximum (54%) disease protection against downy mildew disease followed by 43 and 42% by *C. gigantea* and *Croton* sp. respectively. The least disease protection of 8% was found with *M. alba* (Table 4). The control plants showed 100% disease incidence.

Spatio-temporal time-gap studies

Spatio-temporal time gap studies were carried out in order to study the systemic nature of resistance offered by susceptible pearl millet seeds upon treatment with latex extract of *L. sativa* followed by pathogen inoculation at different time intervals. Since L2 treatment of *L. sativa* showed higher disease protection under greenhouse conditions, it was further used for time gap studies. The spatio-temporal time gap studies showed varied degrees of downy mildew disease protection depending upon the time interval between the inducer treatment and challenge inoculation. The downy mildew disease protection on the first day was 28%, which subsequently increased to a maximum of 56% on the fourth day after inoculation

Table 4. Effect of latex extract treatment (L2) on percent downy mildew disease protection.

Plant extracts	Percent downy mildew disease protection
Asclepias curassavica	12.9^{g}
Calotropis gigantea	43.2^{b}
Croton sp.	41.5^{c}
Eucalyptus alba	16.6^{f}
Lactuca sativa	54.6^{a}
Morus alba	8.5^{h}
Nerium odoratum	32.7^{e}
Tridax procumbens	39.7^{d}

Mean of four repeated experiments with four replicates of 100 plants per treatment in each experiment. Means within columns sharing the same letters are not significantly different according to Tukey's HSD test at $P \leq 0.05$.

and it sustained upto 6-day-time gap period (Figure 1). The results showed that, disease resistance was noticed as early as 24 h and maximum disease resistance developed when a time gap of 4 days was given between latex treatment and pathogen inoculation and was maintained throughout the cropping period.

Enzyme assay

PAL

Two-day-old seedlings raised from latex extract (L2- *L. sativa*) treated susceptible seeds when subjected to estimation of PAL activity at regular time intervals after post inoculation with the pathogen showed a sequential increase in activity at time points tested when compared with that of control. The maximum activity of 378.29 was

achieved as early as 4 hpi in latex extract treated seedlings which decreased thereafter maintaining higher activity than the control at all time points tested. The uninoculated latex extract-treated seedlings also showed same pattern of enzyme activity as that of latex extract treated inoculated seedlings with maximum activity of 253.46 at 4 hpi when compared with susceptible uninoculated (92.94) and inoculated control (79.21). The enzyme activity of uninoculated control varied at all time points which upon inoculation with the pathogen showed decreased activity (Figure 2).

POX

A significant difference in POX activity was observed between the treated and control seedlings and also between the different time intervals. POX enzyme activities increased in susceptible seedlings after latex extract (L2- *L. sativa*) treatment when compared with control plants. Latex extract treatment induced the POX activity in both inoculated and uninoculated susceptible seedlings as early as 2 hpi. The rate of increase was more pronounced in the treated seedlings after inoculation with the pathogen which reached maximum at 8 hpi with 61.06 units when compared with treated uninoculated (43.98). The induced activity of POX however either decreased thereafter or more or less remained constant at all other time points tested. Susceptible control showed lower activity when compared with latex extract treated which further decreased or remained constant on challenge inoculation (Figure 3).

Chitinase

The chitinase activity in all the samples tested showed sequential increase up to 24 h time interval. The chitinase activity estimated in latex extract (L2- *L. sativa*) treated pearl millet seedlings at different time intervals prior to inoculation revealed high activity at 24 hpi (5.21) and low at 0 hpi (2.33). An increase in chitinase activity over control was recorded in susceptible treated seedlings immediately after pathogen exposure. The percentage of increase in enzyme activity was maximum in induced resistant inoculated at 24 hpi (6.32). The activity in the untreated susceptible seedlings after inoculation showed decreased activity when compared with uninoculated control (Figure 4). Though all the samples showed high activity at 24 hpi, the activity declined thereafter.

DISCUSSION

In the present study, latex extracts of seven different plants were tested for their ability to induce resistance against pearl millet-downy mildew disease. The effort revealed the efficacy of latex extracts at low concentration (L2- 100 µl) in escalation of plant growth and inhibition of downy mildew severity in pearl millet when compared with L1, L3 and control. However, the seeds treated with latex extract of *L. sativa* (L2) for 3 h showed maximum seed germination and seedling vigor when compared with 6 h treatment and all other treatments. Our results are in agreement with that of Shivakumar et al. (2009) and Chandhrashekara et al. (2010) who have showed that seed germination and seedling vigor accelerated in pearl millet seeds treated with plant extracts of *Datura* and *Viscum*. Experiments on vegetative growth parameters like plant height, shoot fresh and dry weight, leaf area and tillering capacity by latex extracts showed better results as compared to the untreated control (Table 3). Comparable raise in vegetative parameters in various crops and also in pearl millet using plant extracts (Shivakumar et al., 2009; Chandhrashekara et al., 2010) and various other indu-

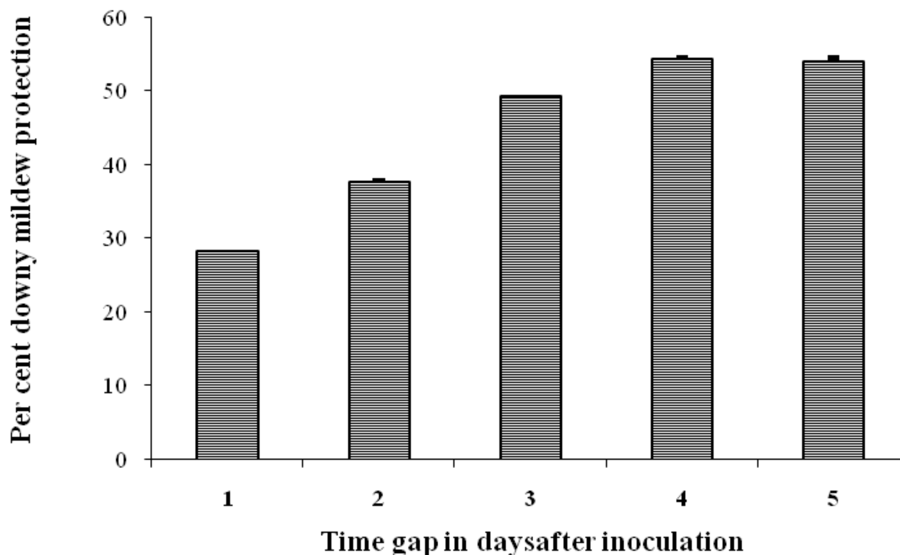

Figure 1. Nature of resistance by *L. sativa* (L2) treatment with spatio-temporal separation of the inducer and pathogen inoculation. The bars represent standard error.

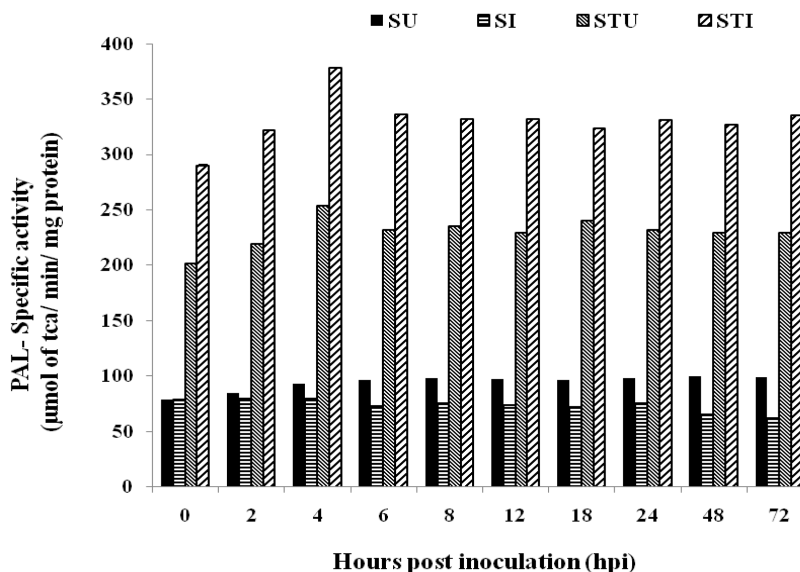

Figure 2. Temporal pattern of accumulation of the phenylalanine ammonia lyase (PAL) enzyme in pearl millet seedlings upon seed treatment with latex extract (L2- *L. sativa*). Bars represent standard errors. SU- susceptible uninoculated; SI- susceptible inoculated; STU- susceptible treated uninoculated; STI- susceptible treated inoculated.

cers has been reported (Amruthesh et al., 2005; Pushpalatha et al., 2007; Sudisha et al., 2011).

Green house studies on temporal and spatial separation of the pathogen and inducer action showed that at least four days were vital to put up maximum resistance against *S. graminicola.* Leaf extracts of spinach and rhubarb induced systemic resistance in cucumber to anthracnose disease caused by *Colletotrichum lagenarium* (Doubrava et al., 1988). Parallel studies on pearl millet under green house conditions have been proven to create resistance towards downy mildew pathogen after treatment with unsaturated fatty acids (Amruthesh et al., 2005), plant extracts (Chandhrashekara et al., 2010; Shiavkumar et al., 2009), raw cow milk and

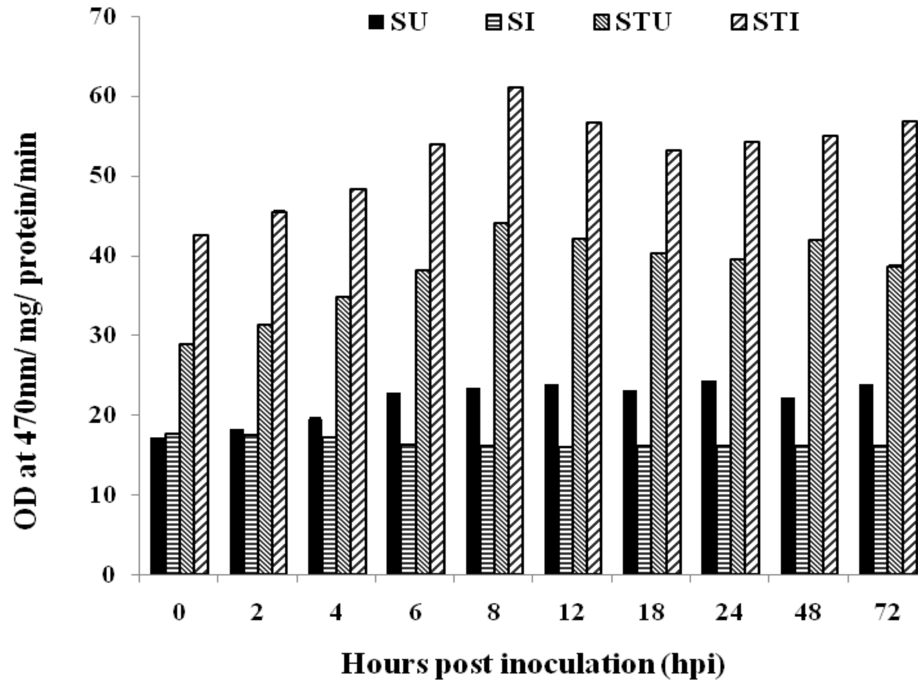

Figure 3. Temporal pattern of accumulation of the peroxidases (POX) enzyme in pearl millet seedlings upon seed treatment with latex extract (L2- *L. sativa*). Bars represent standard errors. SU- susceptible uninoculated; SI- susceptible inoculated; STU- susceptible treated uninoculated; STI- susceptible treated inoculated.

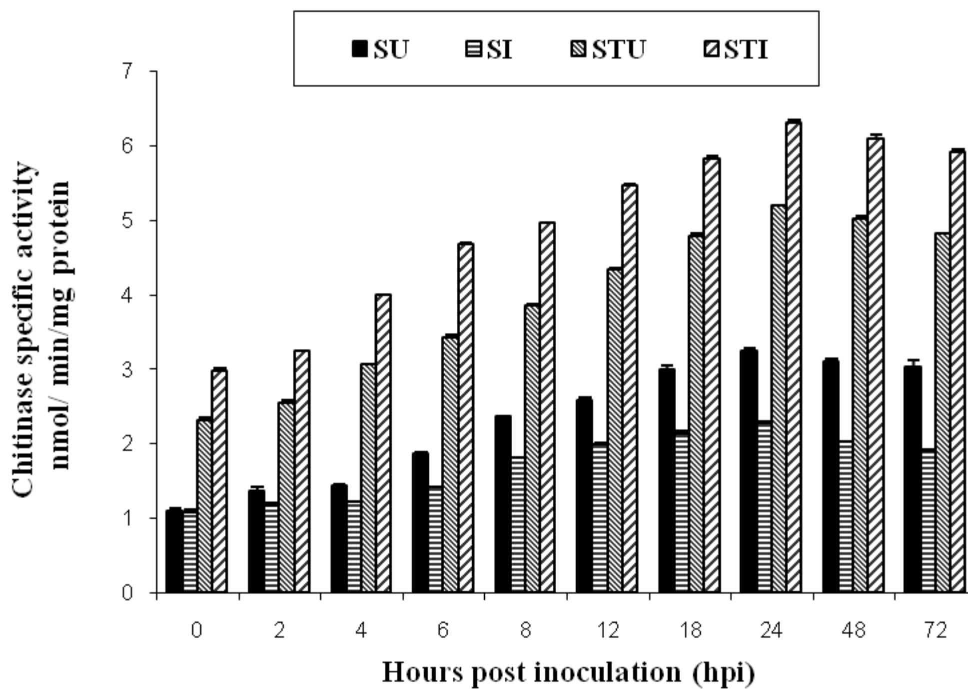

Figure 4. Temporal pattern of accumulation of the chitinase enzyme in pearl millet seedlings upon seed treatment with latex extract (L2- *L. sativa*). Bars represent standard errors. SU- susceptible uninoculated; SI- susceptible inoculated; STU- susceptible treated uninoculated; STI- susceptible treated inoculated.

amino acids (Sudisha et al., 2011). Our results are also consistent with those obtained by other investigators where natural plant products have suppressed plant pathogens leading to resistance towards diseases and consequently, increasing growth parameters and seed yield (Nakatani, 1994; Mohamed et al., 2006; Mohamed and El-Hadidy, 2008). Baraka et al. (2006) reported that extracts of rosemary leaves and *Citrullus colocynthis* fruits, leucarna seeds and alfalfa roots reduced significantly lupine root infection by *F. oxysporum*, *Fusarium solani*, *Rhizoctonia solani* and *Macrophomina phaseolina*. Active principles present in plant products may either act on the pathogen directly (Baraka et al., 2006) or induce resistance in host plants resulting in reduction of disease development (Narwal et al., 2000; Paul and Sharma, 2002; Kagale et al., 2004).

The changes in the activity of defense related enzymes like chitinase, PAL and POX in reaction to pathogen invasion have been illustrated in various plant-pathogen systems (van Loon and van Strien, 1999). The time course analyses of chitinase, PAL and POX activities were made in two-day-old seedlings of non-inoculated and inoculated pearl millet seedlings after inclusion with latex extract of *L. sativa* (L2) for 3 h at different time intervals starting from 0 to 72 hpi.

PAL, a key enzyme of the phenylpropanoid biosynthesis pathway is involved in the biosynthesis of plant defense-related secondary metabolites including salicylic acid, phytoalexins and lignin-based polymers (La Camera et al., 2004), leading to the production of physical and chemical barriers against pathogen infections. In the present study, PAL activity was induced in *L. sativa* (L2) treated seedlings as early as 2 hpi and receded after reaching a peak at 4 hpi. The enzyme activity remained higher at all time points tested when compared with the control and uninoculated seedlings. Similar outcome of increased PAL activity has been recorded against downy mildew pathogen in pearl millet treatment with oligosaccharide of *Trichoderma* spp. (Nandini et al., 2013) and other pathogens in rice on treatment with aqueous extract of *Datura metel* leaves (Kagale et al., 2004), ethanol extracts of *Cymbopogan citrus* and *Ocimum sanctum* (Pal et al., 2013), carrot with sea weed (Jayaraj et al., 2008) and tomato with zimmu leaves (Latha et al., 2009) and ulvans (oligosaccharide) of green algae (*Ulvan lactuca*) (Modafar et al., 2012). Corresponding initial raise and subsequent decline in PAL activity are presented in other incompatible host-pathogen interactions (Borner and Grisebach, 1982; Ralton et al., 1988). An incompatibility thus generated subsequent to latex extract inclusion in susceptible pearl millet seeds suggests that in latex extract induced defense of pearl millet, PAL might be involved in triggering phenylpropanoid pathway resulting in discharge of toxic phytoalexins at the site of *S. graminicola* penetration (Geetha et al., 2005).

Numerous studies have revealed the involvement of greater POX activity with induced resistance in plants against fungal, bacterial and viral pathogens (Hammerschmidt et al., 1982; Reuveni et al., 1990; Hassan and Buchenauer, 2007). POX are recognized to contribute plant defense mechanisms, by catalyzing oxidative polymerization of simple phenols to lignin, synthesis of antimicrobial oxidized phenols, oxidative cross-linking of plant cell walls and also generation of active oxygen species (Lamb and Dixon, 1997; Mehdy, 1994). In the present investigation, POX activity increased in latex extract treated seeds which further increased upon pathogen infection as compared to the control. The figures specify that the level of POX activity remained high at 8 hpi after which the enzyme activity gradually dropped in both infected and uninfected samples. Increase in POX activity is reported in chilli treated with cerebrosides of *F. oxysporum* (Naveen et al., 2013), *Brassica carinata* treated with BABA (β-aminobutyric acid) (Cahavan et al., 2013) against *Colletotrichum capsici* and *Alternaria brassicae*. Similary, there are several other reports on increased POX activity in response to pathogen infection and elicitor treatment as studied by Jayaraj et al. (2008) (*Ascophyllum nodosum* extract: carrot against *A. radicina* and *Botrytis cinerea*), Latha et al. (2009) (Zimmu extract: tomato against *A. solani*), Cawood et al. (2010) (*Agapanthus africans* extract, wheat against *Puccinia triticina*), Nisha et al. (2012) (*Vitex nigundo* extract: rice against *X. oryzae*), Pal et al. (2013) (*C. citrus* and *O. sanctum* extract: rice against *R. solani*) and Senthilraja et al. (2013) (*Pseudomonas fluorescens* bioformulation: groundnut against *sclerotium rolfii*). According to our results, POX can be suggested as typical marker of ISR-mediated defense reaction in pearl millet plants.

Chitinases are present constitutively at a low level in some plants, which is induced by wounding, infection with pathogens (Majeau et al., 1990; Robey et al., 1990) or by abiotic elicitors (Boller et al., 1983) and during plant developmental processes like embryogenesis or fruit ripening. Expression of higher levels of hydrolases like chitinases has been proved to provide enhanced resistance to fungal pathogens (Kasprzewaka, 2003). Chitinase induction after pathogen attack provides protection directly by degrading fungal cell wall components and indirectly by releasing some elicitors from the decaying fungal cell wall that excite other plant defense mechanisms like phytoalexin accumulation in the host (Edreva, 2004). An increased level of chitinase activity was evident on seed treatment with latex extract of *L. sativa* as compared to the control. The activity was induced immediately after challenge inoculation and reached highest at 24 hpi and decreased thereafter. Our results are comparable with those obtained in many other crops on treatment with *Ocimum* extracts (Colpas et al., 2009), zimmu extracts (Latha et al., 2009; Sangeetha et

al., 2013) and *Agapanthus africans* extracts (Cawood et al., 2010), plant growth promoting fungi- *Trichoderma harzianum* (Naher et al., 2012) and plant growth promoting bacteria like *P. fluorescens* (Senthiraja et al., 2013). Bioactive compounds of plants act as elicitors and induce resistance in host plants following reduced disease development (Vidhyashekaran et al., 1992). Plants that are characterized by reduced chitinase activity are significantly more vulnerable to attack by unspecific fungi under natural conditions (Heil et al., 1999) and plants apparently tend to reduce the activity of these enzymes when they are not required due to the presence of other defense mechanisms (Heil et al., 1999, 2000). We therefore assume that activities of these three enzyme classes give an ecologically relevant measure of a plant's overall resistance to pathogens.

Additional investigations are requisite to instigate the bioactive constituents of *L. sativa* involved in bringing about resistance in pearl millet against downy mildew. It is recommended that latex extract induced activity of PAL and PO enzymes in pearl millet, which might have resulted in increased biosynthesis and metabolism of phenols which might have protected pearl millet. In conclusion, the application of latex extracts *L. sativa* can possibly lead to certain defense responses in pearl millet, through elicitation of defense responses induced by its components and its concentration. Further studies are essential to determine the components and concentration for application/seed treatment. The potential of latex extract as elicitors of resistance in plants could be a surplus for other chemical control of diseases in future that cannot be discarded.

ACKNOWLEDGEMENTS

The authors are grateful to the University Grants Commission (UGC) and Council for Scientific and Industrial Research (CSIR) for financial assistance and also Indian Council of Agricultural Research (ICAR)- All India Co-ordinated Pearl Millet Improvement Project (AICPMIP), Government of India, Mysore Center for Facilities and Institution of Excellence (IOE) Project Authorities, University of Mysore, Mysore, India.

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