MICROPROPAGATION OF *BACOPA MONNIERI*

कषाया मधुरा स्वादुपाका युष्या रसायनी।

स्वर्या स्मृतिप्रदाकुष्ठपाण्डुमेहास्त्रकासजित्।

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(भावप्रकाश)

Introduction

Bacopa monnieri (L.) Pennell is popularly known in India as Brahmi or Jala-brahmi. It is also known as Medhya Rasayana in Ayurveda as it increases mental clarity and has brain-stimulating action (Bhattacharya and Ghosal, 1998). Since the time of Athar-ved (800 BC), the plant has been mentioned in the religious, social and medical treatises of India. The first clear reference of its effect on intellect abilities and memory is present in Charak Samhita written in the 1st century AD, where it is prescribed as a cure for mental retardation (Satyavati et al, 1976).

Synonyms: *Bacopa monniera* (Linn) Wettst., *Herpestis monniera* (Linn) H.B. & K., *Moniera cuneifolia* Michaux, *Gratiola monniera* Linn., *Lysimachia monniera* Linn.

Table-1: Vernacular nomenclature of *Bacopa monnieri* (L.)

Bengali	Brahmi-sak, Jalanimba
English	Thyme-leaved gratiola, Water hyssop
Gujarati	Jalbrahmi
Kannada	Neeru brahmi, Kiru brahmi
Malayalam	Brahmi, Nirbrahmi
Marathi	Neerbrahmi
Sanskrit	Bramhi, Sarasvati, Saumya, Surasreshtha, Suvarcala, Surejya, Jalajabrahmi, Kapotavega, Vaidhatri, Divyateja, Mahausadhi, Jalanimba, Brahakanyaka, Surasa,
Tamil	Priam, Vivitam,
Telgu	Sambrani chettu

Family : Scrophulariaceae

Morphology and Distribution

Bacopa monnieri L. Pennell is an amphibious plant of the tropics where it grows on the banks of rivers and lakes. It is a small prostrate or creeping, glabrous and succulent annual herb, rooting at nodes with ascending branches. Leaves are sessile, obovate-oblong or spatulate, flowers blue or white with purple veins on long pedicels. Fruit is a 2-valved capsule. Seeds are minute and numerous (Sala, 1996). The seeds are ridged, brown to dark brown in colour and 0.2 to 0.3 mm in size. The herb is found in moist or wet places, such as on borders of water channels, wells irrigated fields etc in all parts of India.

Medicinal properties

The plant is astringent, pungent, bitter, cooling, healing, emetic and laxative. The plant is an anti-anxient agent having adaptogenic effect (Joshi, 2000). It is an aphrodisiac and aperient. It also possesses anti-inflammatory, analgesic, antipyretic, epilepsy, insanity, anticancer and antioxidant activities (Satyavati et al, 1976; Jain et al, 1994; Elangovan et al, 1995).

The plant is potent nerve tonic, cardio-tonic and found very effective in cases of anxiety neurosis. Stem and leaves are used as brain tonic, which sharpens dull memory. They are used in catarrhal complaints and in snakebite and a safe cardiac tonic. Leaves are diuretic. It is used in the treatment of asthma, hoarseness, water retention and blood cleaning. Moreover, leaf juice is given to children for relief in bronchitis and diarrhoea. It is useful in vitiated conditions of Kapha and Vata, biliousness, reuralgia, inflammations, insanity, amenia, tumors, ulcer, spenomegaly, ascites, dyspepsia, flatulence, constipation, leucoderma, erysipelas, syphilis,

strangury, elephantiasis, dysmenorrhoea, sterility, fever and general debility (Tripathi et al, 1996; Vohora et al, 1997). It has been demonstrated as anxiolytic, relaxing, bronchodilatory, cognition enhancing, anti-oxidant, anticancer, immuno-modulating and anti-inflammatory effects in pharmacological studies (Bhakuni et al, 1969; Tripathi et al, 1996). The drug is an important ingredient of traditional medicines such as Brahmighritam and Brahmirasayanam. Modern medicines such as Mentat, Memory Plus and Megamind Plus use brahmi as their major component. Mentat has been successfully in children with behavioral problems. With entry of such drugs in the market, there is an increased demand for the raw material, which need to be met by the commercial cultivation and processing of the plant.

Part used: Whole plant, leaves, stem and root

Ayurvedic Properties

Sr. No.	Ayurvedic Properties	Description
1	Rasa (taste)	Tikta (bitter), Kasaya (astringent),
2	Gunna (properties)	Laghu (light, easy to digest), Snigdha (unctuous), Sara (mild laxative).
3	Veerya (potency)	Sita (cooling)
4	Vipaaka (properties after digestion)	Madhura (sweet in the first phase of vipaaka, ie. before the drug reaches the stomach)

(Anonymous, 1999a; Anonymous, 1976)

Active constituents

The medicinal properties of *B. monnieri* have been attributed to the presence of four saponins like Bacosides-A, B, C and D (Rastogi et al, 1994). The bacosides

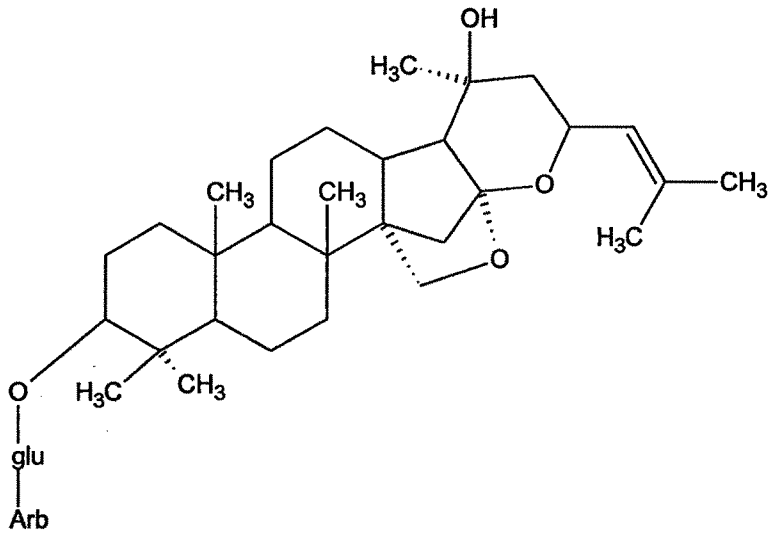


Figure -1 Structure of active compound: Bacoside A_1

known as memory chemicals are the active principles responsible for improving memory-related functions. These constituents are identified as a mixture of dammarane-type triterpenoid saponins designated as Bacosides A and B. Bacoside A (Fig.1), on acid hydrolysis gives three sugars, two of which have been identified as glucose and arabinose. On hydrolysis, Bacoside B also gives glucose and arabinose. These compounds are attributed with the capability to enhance the efficiency of transmission of nerve impulses, thereby strengthening memory and cognition (Singh and Dhawan, 1997). Two new dammarane-type jujubogenin bisdesmosides, bacosaponins E and F of biological interest have been isolated from this herb (Mahato et al, 2000). Other constituents include alkaloids (herpestine and brahmine), flavanoid, glycosides, betulic acid, *d*-mannitol, stigmasterol, β-sitosterol and bacosaponins E and F of biological interest have been isolated from this herb (Mahato et al, 2000). Other constituents include alkaloids (herpestine and brahmine), flavanoid, glycosides, betulic acid, *d*-mannitol, stigmasterol, β-sitosterol and stigmastanol (Joshi, 2000).

Bacopa monnieri has a great market demand due to its high medicinal values. The species propagates mainly by vegetative means. However, it is the most adulterated species in ayurvedic formulation. Its natural regeneration is hampered by death at 2 leaved stage and very specific habitat requirement. The submerged shoots of *B. monnieri* also hardly ramify normally. Further, in a recent study conducted on Indian medicinal plants, *B. monnieri* has been placed second in priority list of most important Indian Medicinal Plants evaluated on the basis of their medicinal importance, commercial value and potential for further research and development (Anonymous, 1997). Therefore, it is necessary to generate efficient protocols for micropropagation. Further, the drug content of the plant is very low (0.2%). Therefore, large amount of the plant material is required for drug extraction (Tejavathi and Shailaja, 1999) and processing.

Materials and Methods

Explant

Healthy plants of *Bacopa monnieri* were collected from Botanical garden of the M. S. University of Baroda. These plants served as the source of explants. These plantlets were well maintained in the medicinal garden of GSFC Science Foundation. The plants were watered twice a day for healthy growth of plants. A day or two before explant collection, the elite plants were sprayed with 1.0 % (w/v) bavistin solution in order to reduce contamination. Shoot tips, axillary bud, internode and leaf were used as explants for experiments.

Establishment of aseptic cultures

The explants (shoot tips, axillary bud, internode and leaf) were initially washed thoroughly under running tap water for 15 minutes to remove soil then treated with (0.2%; v/v) aqueous surfactant Teepol (BDH, India) for 15 minutes followed by repeated rinsing with distill water. Subsequently then explants were pre-treated for 20 minutes with (0.1%; w/v) carbendenzim (BASF, India) and followed by three times rinsing with distill water. Further sterilization was done under aseptic conditions in a Laminar Airflow Chamber (Lab services, India).

A two-step sterilization procedure was followed. Pre-treated explants were surface sterilized with (50%; v/v) ethanol for one minute and followed by (0.05%; w/v) $HgCl_2$ solution for 2 minutes. The remnants of sterilizing agents were washed off by rinsing the explants 5 to 6 times with sterilized double distilled water. The explants were blotted dry for few minutes on a sterile filter paper under aseptic conditions. The explants were cut into appropriate size and placed aseptically onto MS (Murashige and Skoog, 1962) medium supplemented with different concentration of auxins and cytokinins with 3% (w/v) sucrose.

For the multiplication, shoots generated from different explants were excised and sub-cultured as *in vitro* explants to MS medium supplemented with various concentrations of BA /KN and IAA. Cultures were incubated with at 25 ± 1°C under a 14-hr photoperiod and 50 $\mu Em^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes (Phillips, India).

Various explants (apical tip, axillary node, leaf and internode portion) were used for the study. Size of explants used for the *in vitro* study was (1cm) for all the explants. Different cytokinins (BA / KN) and auxins (IAA / IBA) alone or in

combination were studied during the course of the *in vitro* studies of the plants. The efficacy of the PGR's used was assessed by counting the number of shoots per explant during proliferation of shoots.

To check the effect of physical nature of the medium, *in vitro* derived shoots of were transferred to the shoot proliferation medium in both liquid (devoid of agar) and solid medium. The response was observed in terms of increase in shoot number and length of shoots.

Various numbers of nodes (1^{st} - 8^{th}) were inoculated to check the bud proliferation on the optimal media. The efficacy was assessed by counting the number of shoots formation on the nodes.

The experiment for optimal medium and volume required for carrying out the subcultures for commercialization was also undertaken. The subcultures were carried out on large scale.

Root induction of regenerated plantlets

In vitro well-developed regenerated shoots (3-5cm) of *Bacopa* were isolated and subjected to induction of roots on MS medium supplemented with different concentrations of auxins (IAA /IBA) alone. The effectiveness of the PGR's was assessed by counting number of roots formation on *in vitro* shoots. Subsequently, they were transferred to the greenhouse and then to field for acclimatization.

Acclimatization of Plantlets

Well-rooted plantlets of *B. monnieri,* were removed from the culture vessels and gently washed to remove agar. The regenerated plantlets (4-6cm) were transferred

to small sized plastic bags containing various potting mixtures in different ratios. Small plantlets (2-3cm) were transferred to portray with different potting mixtures. They were initially kept under greenhouse for 2-3 weeks. Initially, high humidity was maintained with water spray at regular intervals (Jasrai et al, 1999). Later the hardened plants were transferred to medicinal garden of GSFC Science Foundation. Tissue culture raised as well as vegetative grown plants were compared for their morphology and growth parameters.

Phytochemical analysis

Phytochemical evaluation was done by High performance liquid chromatography of shoots for six-month-old micropropagated plants, market samples and field-grown plants. All the three samples were air-dried and crushed to fine powder form. The mobile phase consisting of methanol and water was degassed for 15 minutes before analysis.

Drug extracts and standard solution: Optimal extraction procedure was established during preliminary studies, varying the degree of fitness of powdered drug and the concentration of methanol in water between 80% and 100% as well as changing the drug and the solvent ratio. Optimal extraction was achieved by heating one gm of fine powdered drug with 20ml methanol on a hot water bath under reflux for 5 hours at 110°C. The extract was cooled and transferred to separating funnel. Further, it was extracted with chloroform 3 times using 30ml of solvent for each extraction. All the chloroform layers were collected and 0.5 ml of (0.01 %; v/v) ammonia solution was added and washed with water until neutral. The combined chloroform layers were collected through sodium sulfate into a beaker. The

chloroform was evaporated on hot water-bath, residue was dissolved in methanol, and volume was made upto 100ml.

HPLC analysis: Separation and determination of Bacoside was performed with HPLC column (250 mm x 4.6mm) column that contained ODS (18) packing (Sigma- Aldrich Hypersil ODS 5mm). Injection volume was 5µl. The solvent flow rate was 0.5ml /min. The column eluent was monitored by UV detector at 240 nm.

Results and Discussions

The two-step sterilization procedure of the explants (apical tip, axillary node, leaf and internode) with ethanol (50%; v/v) for one minute and (0.05%; w/v) $HgCl_2$ for 2 minutes was found best for generating contamination free *in vitro* explants. The explant establishment rate was found 95%. Similar sterilization procedures have also been reported by other workers in *Dolichos lablab* by Saunder Raj et al, (1991) and in *Tylophora indica* by Sharma and Chandel, (1992).

Earlier reports available on *Bacopa monnieri* demonstrated plant regeneration through various explants in medium containing very high concentrations of cytokinin. (Shrivastava and Rajni, 1999;Tiwari et al, 2000; Tiwari et al, 2004; Mohapatra and Rath, 2005). *Scrophularia yoshimurae* (Scrophulariaceae family) micropropagation has also been reported through various explants: shoot-tip, leaf-base, stem-node and stem-internode explants (Sagare, 2001). The nodal segment (Plate-2.1A) implanted on MS medium supplemented with BA (1.1μM) showed initiation of shoot bud after 9th day of inoculation (Plate-2.1B). Several workers have reported induction of shoot with cytokinins in the growth medium in *Vitis* sps. (Stamp et al, 1990; Clog et al, 1990). Ali et al, (1996) have also reported *in vitro* regeneration from nodal explants in *Bacopa monnieri.* Within two weeks of incubation, multiple shoot formation (3-4 shoots) was observed on the explant. Tiwari et al, (1998) reported shoot regeneration after 4 weeks from nodal explant on MS medium supplemented with 22.2μM BA. Mohapatra and Rath (2005) had reported less shoot regeneration on MS medium supplemented with 44.4μM BA through leaf and nodal explants. Further, increase in the levels BA resulted in a decrease in the extent of shoot regeneration. This is in agreement with earlier results of Shrivastava and Rajni, (1999) in same species.

Synergistic effect of auxin and cytokinin for large multiple shoot formation has been reported in many plant species. The same results have been observed for the present plant. The explant when cultured on MS medium with addition of IAA (0.2μM) with BA (1.1μM) resulted in the formation of large multiple shoot formation (7-8 shoots) from the nodal explant (Plate-2.1C, D). Similarly, multiple shoots of *Scrophularia yoshimurae* has been reported on Murashige and Skoog (MS) medium supplemented with 4.44 μM BA and 1.07 μM NAA (Sagare, 2001). Shoot regeneration potential of IAA has also been reported (Tejavathi and Shailaja, 1999) in *Bacopa monneria* with stem and flower bud as an explant. The combination of BA and IAA for multiple shoot formation has been also reported in *Catha edulis* cultures by Elhag, (1991). Proliferation of shoot buds was observed in the both liquid and solid optimal medium respectively. Liquid medium showed uniform response and higher biomass with 8 shoots from the nodal explant compared to the solid medium with 6 shoots (Table-2). Similar responses have been reported in apple and pear (Singha, 1982). The propagation ratio of *Cynara scolymus* was higher in liquid than in solid medium (Debergh et al., 1981) as well as that the use of a liquid medium gave more vigorous seedlings in *Epidendrum radicalis* (Pateli et al, 2003). Furthermore, the costs for preparing a liquid medium are lower than an agar-solidified one, since agar is one of the most expensive components of the culture medium.

Increase in shoot length was also found faster in liquid medium, 5-6 cm within 15-18 days than solid medium (20-25 days) (Plate-2.2A, B). This might be due to better uptake of nutrients as large surface of the explant is in contact with the medium leading to increased growth and multiplication. Further liquid medium helps in maintaining O_2:CO_2 balance (Biondi and Thorpe, 1981).

Plate-2.1

A. Establishment of nodal explant of *B. monnieri* on the medium

B. Bud break on 9th day of inoculation

C & D. Multiple shoot formation from nodal explant on MS medium supplemented (0.2μM) IAA with BA (1.1μM)

Photoplate - 2.1

Table-2: Effect of different combinations of BA and IAA in MS medium on shoot formation through nodal explants of *B. monnieri* and % survival rate in the field

Concentrations (µM)		Number of shoots/explant Mean ± SE	Response (%)	Shoot length (cm) Mean ± SE	Field Survival (%)
BA	IAA	**Agar based solid medium**			
0.0	0.0	3.5 ± 0.26	75	3.1± 0.06	80
0.5	0.2	3.2 ± 2.43	84	2.1 ± 0.29	91
1.1	0.2	6.9 ± 1.15	100	5.4 ± 0.14	100
2.2	0.2	4.1± 1.20	95	4.1 ± 0.21	98
4.4	0.2	3.1± 0.26	95	3.2± 1.3	98
Liquid medium (stationary)					
0.0	0.0	1.1± 0.3	76	2.1± 0.6	
0.5	0.2	1.1 ± 0.23	91	3.2 ± 0.32	100
1.1	0.2	7.8 ± 1.13	100	5.6 ± 1.19	100
2.2	0.2	3.9 ± 0.12	100	3.5 ± 0.12	100
4.4	0.2	3.4 ± 0.43	97	2.1 ± 0.35	100

The slow growth and fewer shoots on agar solidified medium might be a result partly due to its specific habitat needs, a lower diffusion rate of molecules through the medium, an undefined agar-borne inhibitors and may be even due to reduced availability of water to tissues growing on agar solidified medium (Stoltz, 1971; Kohlenbach and Wernicke, 1978; Stevenson and Haris, 1980). Earlier, suitability of liquid medium for this plant has been reported (Shrivastava and Rajni, 1999; Tiwari et al, 2000). There are several reports on shoot proliferation in liquid medium but the major disadvantage is the vitrification of shoots when grown in liquid medium (Ziv et

Plate-2.2

A. Multiple shoot formation on semi solid medium

B. Multiple shoot formation on liquid medium

C. Performance of the nodal explants with reference to their position on *in vitro* developed shoots (Ranging from shoot tip to 7^{th} node)

Photoplate - 2.2

al, 1983). The problem was slowed in *Bacopa* by transferring the cultures to the fresh medium after three weeks.

Performance of the nodal explants with reference to their position on *in vitro* developed shoots was evaluated in optimal liquid medium. It was observed that upper nodes (2-3) were slow in growth, while lower nodes (4-7) were found to be more promising (Plate-2.2C) and resulted in better multiplication of shoots (8 shoots). Shoot tip explants were also used however proliferation response was found poor with single shoot formation (Table-3).

At the base of node, group of nodules surrounding the stem was observed which later developed into shoots (Plate-2.3A). The internode portion (1cm) was excised and cultured on the optimal solid medium. Thakur and Ganpathy (1978) used internode sections less than 1 mm which necrosed within 1 week on basal medium. In present study a large number of shoot (28) were regenerated within a period of two and half weeks (Plate-2.3B, C, D). This is in agreement with results observed by Tiwari et al (1998) with (23) shoots on higher concentration of BA (4.4 µM) after 4 weeks. In contrast, Shrotri and Mukundan (2004) observed fewer shoots (8) from internode explants on high concentration of BA (4.44 µM) and IAA (5.71 µM). While Mathur and Kumar (1998) recorded 15 shoots from internode explants after a long incubation period (six weeks) but without growth hormone. Thakur and Ganpathy (1978) had reported shoot regeneration from the distal ends of 0.1-1.2 cm long internode segments and reported longer internodes being more conducive to regeneration for *B. monniera.*

Plate-2.3

A. Formation of nodules at the base of the node

B. Induction of multiple shoot formation from the nodules

C & D. Multiple shoot formation

Photoplate - 2.3

Table-3: Effects of different number of nodal explants and apical tip on shoot proliferation within 15 days of inoculation in liquid medium

Explant		No. of shoots/ node Mean ± SE	Mean shoot length (cm) Mean ± SE
Axillary node	2nd	3.6 ± 0.12	2.8 ± 0.21
	3rd	3.8 ± 1.15	2.8 ± 0.12
	4th	4.3 ± 0.01	2.8 ± 0.31
	5th	5.9 ± 0.10	4.0 ± 0.25
	6th	6.8 ± 1.13	4.1 ± 0.01
	7th	6.2 ± 0.10	4.1 ± 0.11
	8th	5.9 ± 0.21	4.7 ± 0.03
Apical tip		1.1 ± 0.11	3.9 ± 0.21

Leaf explant was also tried and found the most responsive explant, producing the greatest number of shoot buds. The explant was excised and cultured on solid medium. Within 4-5 days, the leaf explants were established on the medium. Initiation of bud sprouts was observed on the dorsal side, near the proximal cut end of leaf (Plate-2.4A) on MS basal medium which is similar to the findings of Thakur and Ganpathy (1978). On BA and IAA supplemented media, shoot buds regenerated near the proximal cut ends of leaves and later the whole explant surface became covered with buds. Shoot bud proliferation was observed initially from the base (Plate-2.4B) and then extended all over the surface (Plate-2.4C).

Large number of shoot buds was also observed on *in vitro* leaf explants on liquid and agar based MS media with BA (1.1μM) and IAA (0.2μM) without the intervention of callus (Plate-2.4C). Shoot buds developed on leaf did not correlated to the somatic embryos, as different stages of embryos were not formed. This result is in

agreement with earlier results (Mathur and Kumar, 1998). A maximum of 110 shoots was observed (Plate-2.4E) within three weeks of incubation in first sub-cycle (Table-4).

Table-4: Effect of different combinations of BA and IAA on direct organogenesis from leaf explants and % survival in the field

Concentrations (µM)		Number of shoots/explant Mean ± SE	Shoot length (cm) Mean ± SE	Response (%)	Field Survival (%)
BA	IAA				
0.0	0.0	1.2 ± 0.45	0.5 ± 1.4	55	64
0.5	0.1	5.4 ± 0.69	2.1 ± 0.22	67	69
0.5	0.2	25 ± 1.32	2.2 ± 0.61	74	81
1.1	0.2	110 ± 2.31	3.2 ± 0.25	100	98
2.2	0.2	35 ± 0.12	2.9 ± 0.24	86	92

Research is said to be more successful if it is cost effective. The number of shoots per subculture and media quantity per subculture was standardized from commercial point of view. The number of shoots at each subculture generated from leaf, axillary node and internode were noted. Within a period of 3 subcultures, leaf, node and internode explants were able to generate 12100, 49, 784 shoots respectively (Plate-2.5A). While in the case of *Scrophularia yoshimurae*, stem-node and shoot-tip explants showed the highest response (100%) followed by stem-internode (74.4%) and leaf-base (7.7%) explants (Sagare, 2001). The system demonstrated a continuous supply of shoot uptil 9 cycles without any decline in the number of shoots in each subculture (Fig.2). While Tiwari et al, 2006 reported consistency of shoots per leaf

Plate –2.4

A. Initiation of shoot bud from leaf explant

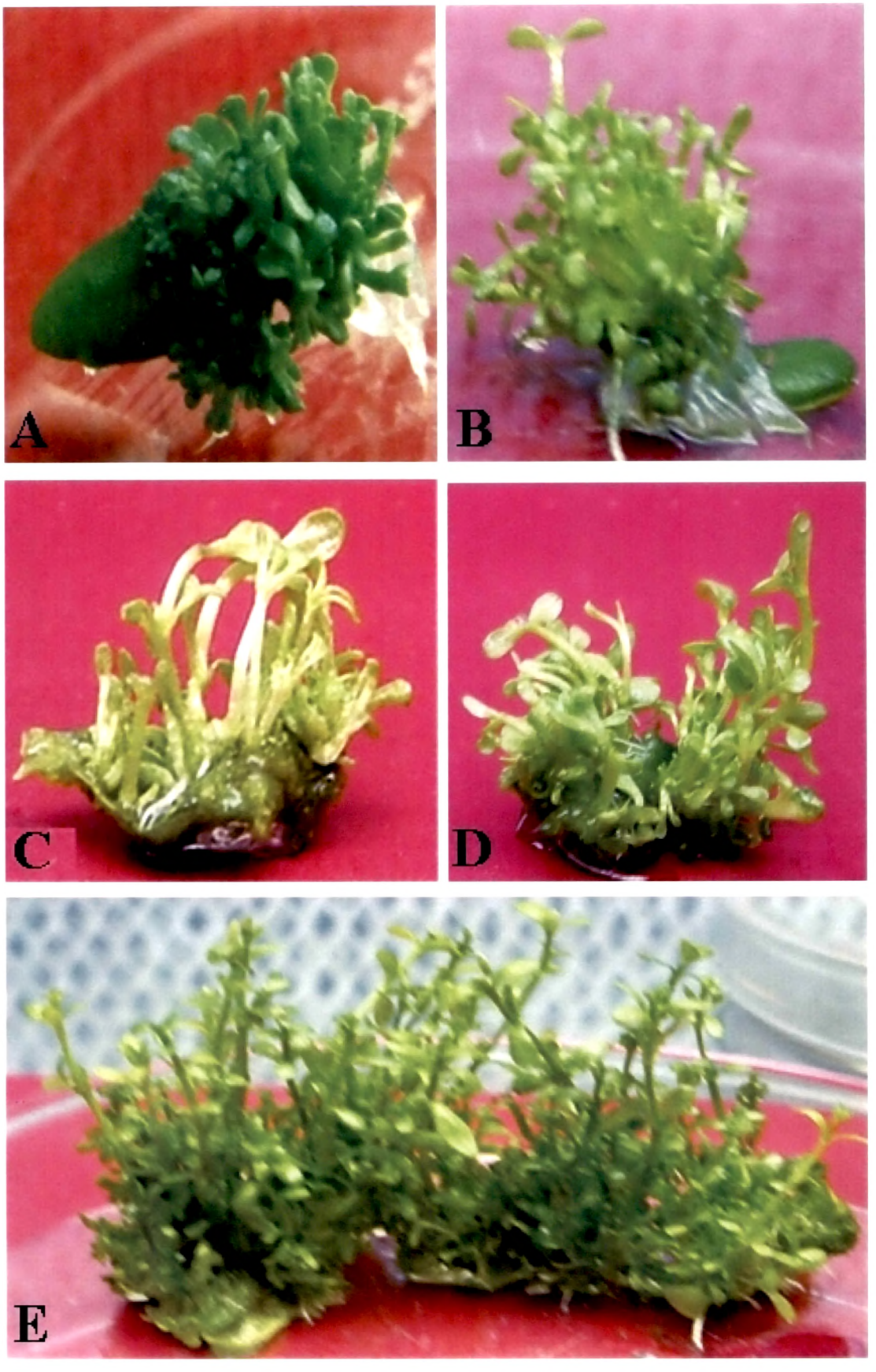
B. Shoot bud proliferation from the base

C. Extended all over the leaf

D. Large number of shoot formation on MS medium supplemented with BA (1.1μM) and IAA (0.2μM)

E. Maximum number of shoot formation from leaf explant

Photoplate - 2.4



explant upto first 3 subculture only. Subcultures were performed frequently (3 weeks) as delayed subcultures (more than four weeks) in liquid medium caused vitrification of shoots which correlates with results observed in carnation plantlets (Ziv et al, 1983). Further 20 ml of basal media for leaf and 40 ml liquid media for nodal and internode explants with 10 explants was found to be optimal for each subculture.

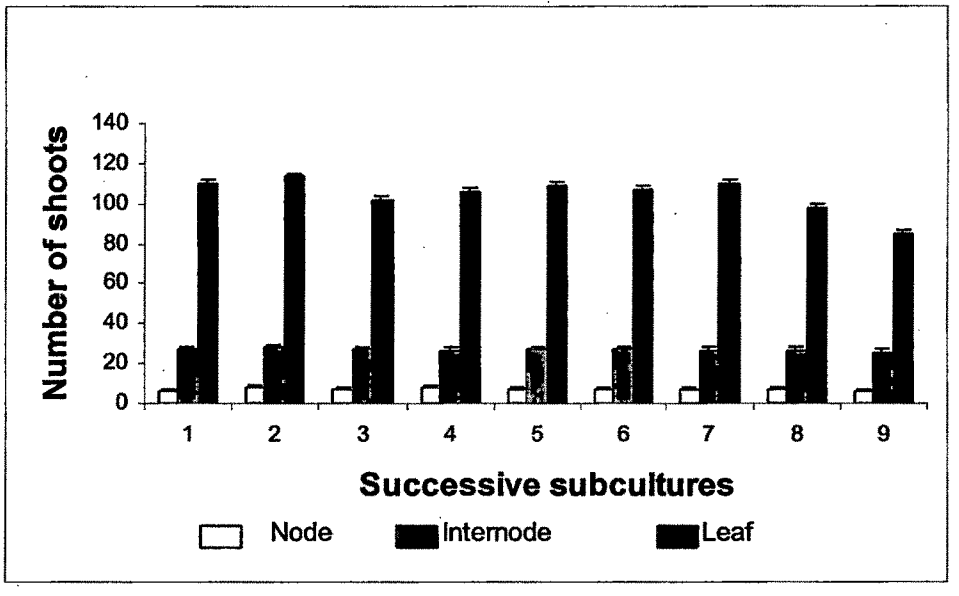


Figure-2 Rate of shoot formation from different explants during successive subcultures

The source of the leaf explant as well as gelling agents in the medium were found to influence shoot induction and eventual shoot growth in *Bacopa monniera* (Shrivastavaand Rajani, 1999). Interestingly in *Adhatoda beddomei* split halves of shoots were used for subculture; and resulted in regeneration of 23–27 shoots from each of the two halves, which was far more than that (15–17 shoots) obtained from culture of the intact nodes (Sudha and Seeni, 1994). Ajithkumar and Seeni (1998) reported that by repeated subculturing of node and leaf explants of *Aegle marmelos* through five cycles enabled continuous production of healthy callus-free shoots

without any sign of decline. In some species, viz., *Vitex negundo* (Sahoo and Chand, 1998), *Ocimum americanum* and *O. sanctum* (Pattnaik and Chand, 1996) frequency of shoot proliferation and growth were greatly influenced by the month of the year during which the explants were collected.

Root induction

Well-grown shoots (3-4cm) were isolated and transferred to the basal and the induction medium for root induction. Root induction was found to better in the optimal medium as compared to the basal medium. Although multiple shoot formation was accompanied with the formation of roots in the same medium, basal medium and auxins like (IAA and IBA) were also tested for *in vitro* rhizogenensis. *In vitro* grown plantlets with 3-4 nodes were found to respond well when inoculated on MS medium supplemented with various auxins. Root initiation was observed within a week of transfer of *in vitro* shoots onto auxin supplemented medium. Among the two auxins tested, IAA proved to be very effective in inducing profuse roots at the base of the regenerated plantlets (Plate-2.5B, C). Similar results have been reported by Niranjan and Sudrshan, (2000) in *Bacopa* cultures. Faria and Illg, (1995) reported 100% rooting in *Zinger spectabile* when the basal medium was supplemented with 5 μM IAA. It is reported that in several species, especially herbaceous, are able to root in the presence of reduced levels of auxin or even in culture medium without this growth regulator (Cuzzuol et al., 1996). The maximum numbers of roots obtained are 25 at a concentration of 0.2 μM IAA, followed by 12 roots at 4.9 μM IBA. Effectiveness of IAA compared to other auxins on rooting has been reported in carnation by Malczweka et al, (1979). Although IAA is considered to be weaker auxin

Plate-2.5

A. Formation of shoot during different subculture

B & C. Root formation on MS medium supplemented with 0.2 µM IAA

Photoplate - 2.5

than NAA (Hu and Wang, 1983). While on the basal medium the root formation was restricted to 3-4 roots. At higher levels of IBA, root growth was poor and root formed was short.

The induction, proliferation and rooting could be achieved on a single one-step medium with low concentrations of cytokinin and auxin from various explants with large and rapid multiplication at a cost-effective level.

Acclimatization of plantlets

Commercial success of tissue culture technique depends on the survival rate of the *in vitro* grown plants and their establishment in field. Thus, acclimatization is crucial step to ensure maximum survival of *in vitro* grown plantlets. Here plants have to adapt to new environmental conditions such as low relative humidity, higher intensity and fluctuations in temperature and stress (Preece and Sutter, 1991).

The shoots (5-6 cm) with roots of size (3-4 cm) were washed with water and planted to portray containing sand, soil and farmyard manure (1:1:1) under shade for hardening. All the plants regenerated from different explants were hardened directly in the net-house skipping the greenhouse stage. Initially, high humidity was maintained by five sprays of water at regular interval (5-6 hrs). The plantlets so hardened for 2 weeks in net-house (Plate-2.6A, B) were subsequently transferred to the open beds (Plate-2.6C) with 100% and 98% survival rate for node/internode and leaf based explant respectively. No morphological variation of any nature was observed among the *in vitro* raised plants when compared with the mother stock.

Plate-2.6

A. The plantlet (2-3 cm) transferred to plastic small pot for hardening in agri-net house

B. The hardened plantlet (5-6 cm) in the pot

C. Field transferred *in vitro* grown plantlets showing vigorous growth

Photoplate - 2.6

Phytochemical analysis

For HPLC, different solvent strengths of mobile phase were used, a mixture of methanol and water in proportion of (80:20) was found to be suitable for the separation of bacoside A in *Bacopa monnieri* plant extract. A matching profile of representative chromatograms of *in vitro* generated plants, market sample and field-grown plants of *Bacopa monnieri* was observed (Fig.-3).

In the present investigation positive response was observed with all the explants which were tested viz. leaf, stem tip, nodes and internodes in contrast to the above mentioned work of various researchers wherein they obtained success with one or two type of explants only.

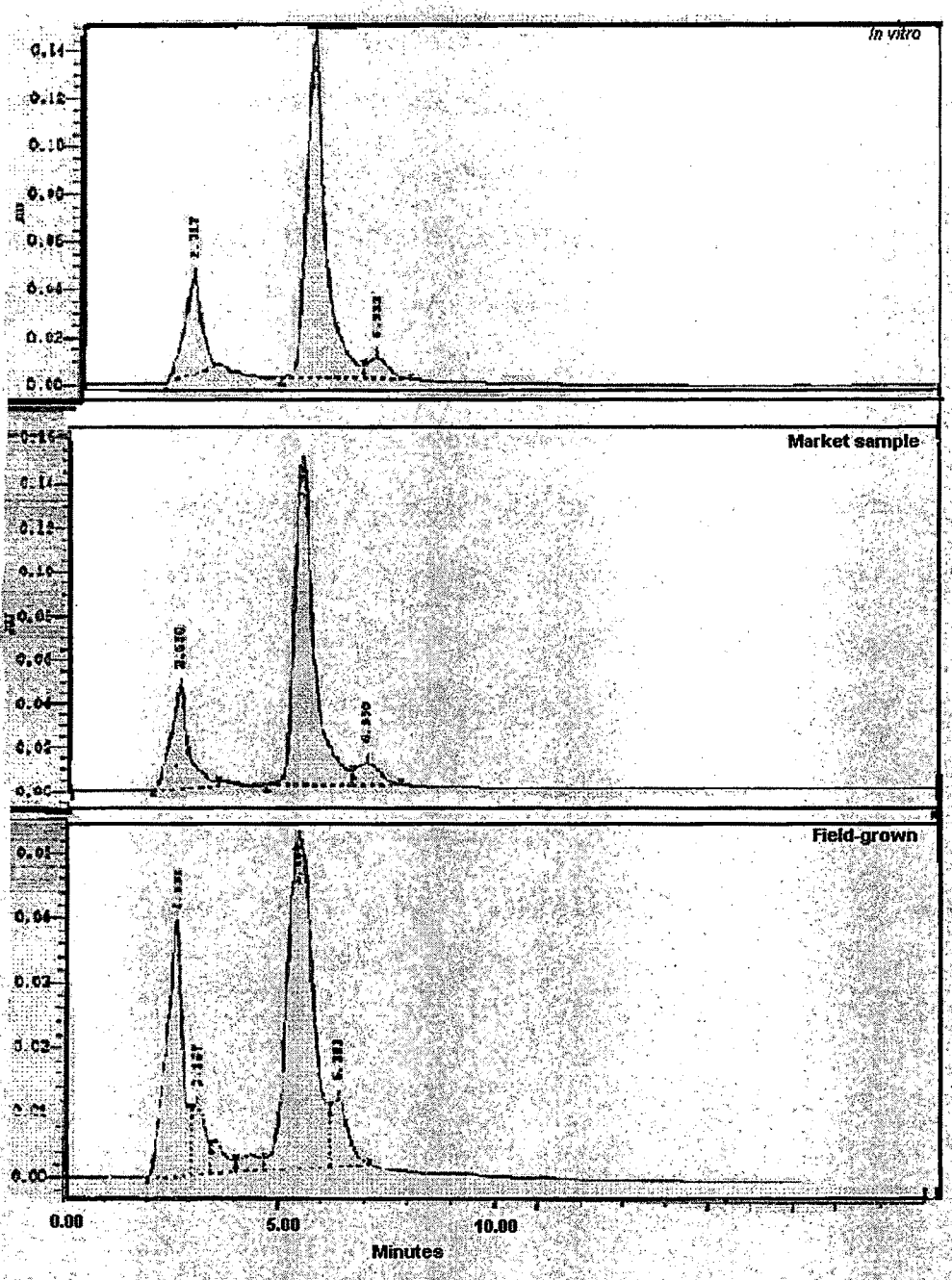


Figure-3 separation of bacoside A present in the methanol extracts of Bacopa monnieri by HPLC using mobile phase MeOH-Water (80:20) of in vitro generated plants, market sample and field-grown plants

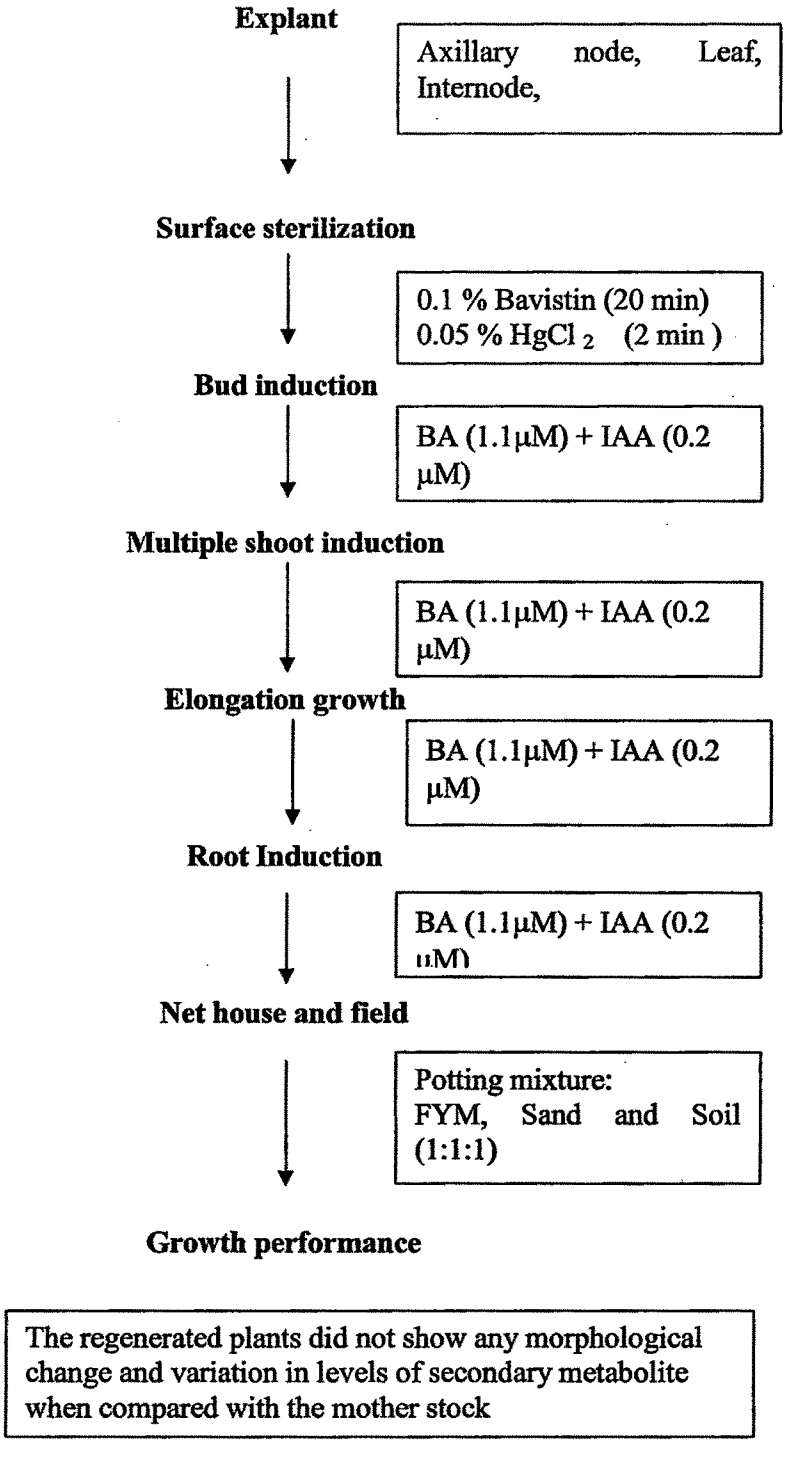


Figure-4 Schematic representation of *in vitro* multiplication of *Bacopa monnieri* L Pennell

MICROPROPAGATION OF *CENTELLA ASIATICA*

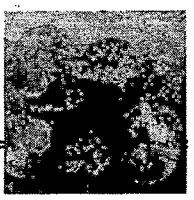
रक्तपित्तरहान्याहु हृधानी सुलघुनि च।

कुष्ठमेहज्वरश्वासकासारूचिहरानि च।

कषाया तु हिता पित्ते स्वादुपाकरसा हिमा।

लघ्वी मण्डूकपर्णी तु .....।।

(सुश्रुतसंहिता)



Introduction

Centella asiatica (L.) is an ethnomedical plant widely used in the health food and cosmetic industries. In India, it is usually described under the name of Mandukparni or Indian Pennywort in the Ayurvedic system of medicine that resembles the claw of a frog (Sudha et al, 2002). The genus comprises of 33 species inhabiting tropical and subtropical regions.

Synonym: *Hydrocotyle asiatica* (L.)

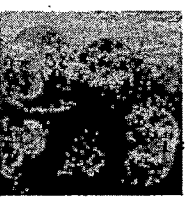
Table-1: Vernacular nomenclature of *Centella asiatica* (L.)

Bengali	Thankuni
English name	Indian pennywort
Gujarati	Motibrahmi
Kannada	Urage, Vondelga
Malayalam	Muttil, Kutakan
Marathi	Karinga, Karivana
Oodia	Thalkudi
Sanskrit	Mandukaparni, Manduki, Brahamanduki
Tamil	Vallarai
Telgu	Brahmi, Saraswataku

Family: Apiaceae

Morphology and Distribution

Centella asiatica is a prostrate, stoloniferous perennial herbaceous creeper. Stems are glabrous, long, prostrate, filiform often reddish and with long internode and rooting at the nodes. Leaves are long, petiolated, several from rootstock and 1-3 from each node of stem. They are orbicular, reniform, and broader than length, 1.3–3.3 inch diameter, glabrous on both sides and with numerous slender nerves from a deeply



cordate base. Fruit is 8 mm long, ovoid, hard with a thick pericarp (Sala, 1996). It grows on moist, sandy or clay soils, forming a dense green carpet. In India, it is distributed from Himalayas to south at altitude upto 2000 ft above sea level.

Medicinal properties

Mandukparni enjoys considerable reputation in Indian System of Medicine as diuretic, febrifuge, alterative, nervine, rejuvenative and tonic (Kirtikar and Basu, 2001). The whole plant possesses antibacterial, anti-inflammatory, antifebrite, antifillarial, antileprotic, antifeedant, antistress, antituberculosis activities and galactagogic properties (Chakraborty et al, 1996; Srivastava et al, 1997).

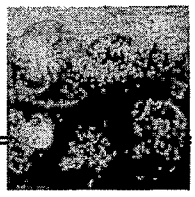
Part used: Whole plant and leaves

Ayurvedic properties:

Sr. No.	Ayurvedic Properties	Description
1	Rasa (taste)	Tikta (bitter), Kasaya (astringent),
2	Gunna (properties)	Laghu (light, easy to digest), Sara (mild laxative).
3	Veerya (potency)	Sita (cooling)
4	Vipaaka (properties after digestion)	Madhura (sweet in the first phase of vipaaka, ie. before the drug reaches the stomach)

(Anonymous, 1999a; Anonymous, 1976)

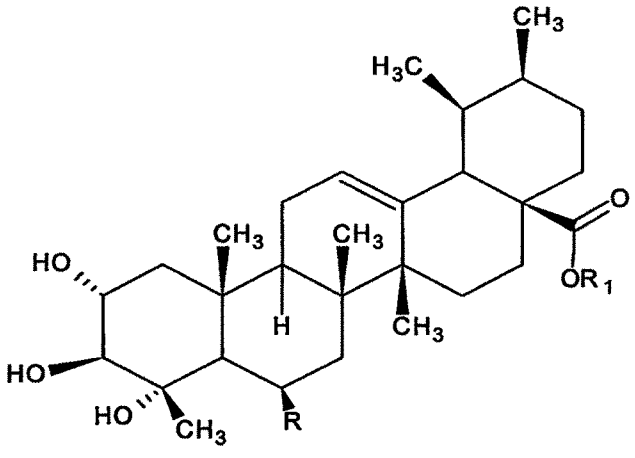
Various uses are claimed for the plant, the more common uses are as a wound healing agent and a major constituent of brain tonics. Its extracts and active metabolites are reported to have many medicinal and therapeutic properties, ranging from enhanced memory and longevity to the treatment of leprosy. It is also used in the treatment of asthma, bronchitis, dropsy, elephantiasis, fever, measles, epistaxis,



diarrhoea, dysentry, constipation, leucorrhea, jaundice, furunculosis and dysmenorrhoea. It is also used in the preparation of various products for diseases associated with skin, nervous systems and the blood. Leaves are taken as tonic and for improving memory. Juice of leaves is useful in catarrh and other eye troubles.

Active constituents

The major active metabolites are asiatic acid -A, asiaticoside -B, madecassic acid -C and madecassoside -D (Fig-1).

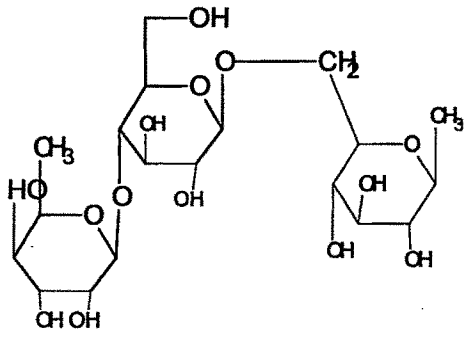


A) R=H R1= H

C) R=OH R1=H

B) R=H R1=

D) R=OH R1=



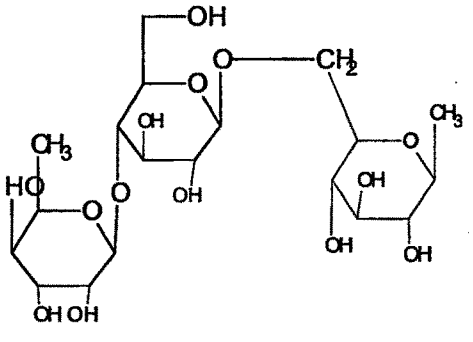
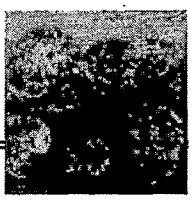


Figure-1: Structures of Asiatic acid (A), Asiaticoside (B), Madecassic acid (C) and Madecassoside (D)

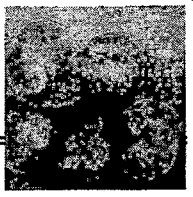


These are pentacyclic triterpenes and belong to the β-amyrin ursolic acid group (Kakkar, 1988). The plant also contains ascorbic acid (13.8 mg per 100gms). Other constituents are Brahmoside, Brahminoside, Thiankuniside, isothaniside, isobrahmic acid, quercetin, betulic acid, hydrocotyline and polyacetylenes (Rao and Sheshadri, 1969).

***In vitro* approaches**

Patra et al, (1998) reported regeneration of *Centella* from stem callus on MS medium supplemented with different combinations of auxins and cytokinins. Earlier *in vitro* propagation of Mandukparni was achieved through leaf explants (Banerjee et al, 1999) with intervention of callus on medium containing BA and NAA. While (Tiwari et al, 2000) and Shashikala et al, (2005) reported shoots regeneration from nodal explants using higher concentration of BA and NAA.

Due to high therapeutic and healing properties of *C. asiatica* almost all the Ayurvedic companies use this extensively in their formulations. Further, the requirement has sharply risen due to its popularity owing to large-scale unrestricted exploitation. As per an estimate an annual requirement of *C asiatica* was around 12700 tonnes of dry biomass valued at Rs.1.5 billion (Ahmad, 1993). The Ayurvedic companies in Gujarat State consume around 40,000 kg alone annually (Singh and Parabia, 2003). To meet such an ever-increasing demand by the Indian pharmaceutical industry there is an urgent need to undertake extensive cultivation of Mandukparni. Moreover, there is large-scale destructive and unrestricted exploitation of this natural resource. Further, there is a limited cultivation and very insufficient attempts for its replenishment. The wild stock of this medicinally important plant



species has been markedly depleted and was thus listed as threatened species by the International Union for Conservation of Nature and National Resources-IUCN (Pandey et al, 1993; Tiwari et al, 2000). In view of the above facts, plant tissue culture techniques can play an important role in the clonal propagation of elite clones. This can also complement efforts for germplasm conservation of *Centella asiatica.*

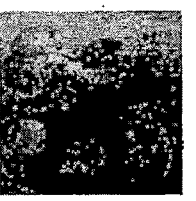
Materials and Methods

Plant material

Healthy plants of *Centella asiatica* (L.) were collected from the Botanical Garden of the Maharaja Sayajirao University of Baroda. These plantlets were well maintained in the Medicinal Garden of GSFC Science Foundation and served as the source of explants. The plants were watered twice a day for healthy growth. A day or two before the explant collection, the plants were sprayed with 1.0 % (w/v) bavistin solution in order to reduce contamination. Nodal explants were used as explant for experiment on micropropagation.

Sterilization

The explants were washed thoroughly under running tap water (15 min) to remove soil particles then treated (15 min) with 0.2% (v/v) aqueous surfactant Teepol (BDH, India) followed by repeated rinsing with distilled water. Subsequently, explants were pre-treated (20 min) with 0.1% (w/v) fungicide-Bavistin (Carbendenzim; BASF, India) and followed by three rinses with distilled water. Further, surface sterilization was done under aseptic conditions in a Laminar Airflow Chamber (Lab Services, India).



A two-step sterilization procedure was followed. Pre-treated explants were surface sterilized with 50% (v/v) ethanol (45 sec) and followed by 1.2% (w/v) sodium hypochlorite (7 min) or by 0.05 % (w/v) $HgCl_2$ (3min) remnants of sterilizing agents were washed off by rinsing the explants 5 to 6 times with sterilized double distilled water. The explants were dipped in the solution of antibiotics for (10min) and then blotted dry for few minutes on a sterile filter paper under aseptic conditions.

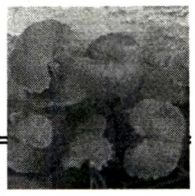
Culture medium and conditions

The sterilized explants were trimmed into appropriate size (0.5 cm) and placed aseptically onto MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of auxins and cytokinins with 3% (w/v) sucrose for bud break. Cultures were incubated at 25 ± 1°C under a 16-hr photoperiod (50 $\mu Em^{-2}s^{-1}$ irradiance) provided by cool white fluorescent tubes (Philips, India).

The single node explants excised from the *in vitro* raised cultures was further subcultured on MS media containing growth hormones. The subculturing was done at every 3 weeks interval. Various concentrations of BA/KN and IAA were used to study the rate of multiplication.

Induction of roots and acclimatization

In vitro well-developed shoots (3-4 cm) were isolated and cultured on MS medium supplemented with different concentrations of auxins for induction of roots. Subsequently, they were transferred to the greenhouse for hardening and then to the field. Well-rooted shoots were removed from the culture vessels, washed gently with water to remove agar. The regenerated plantlets (4-5 cm) were transferred to plastic

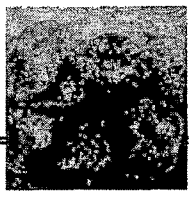


bags (250 gms) containing various potting mixtures. Small plantlets (2-3cm) were transferred to portray with different potting mixtures. The plantlets were initially kept under greenhouse conditions for 2-3 weeks. Later, the hardened plants were transferred to the Medicinal Garden of the GSFC Science Foundation.

Tissue culture raised as well as seed grown plants were compared for their morphology and growth parameters after 20 days of their transplantation.

Phytochemical analysis

Centella asiatica is characterized by the major ester saponins - asiaticoside and madecassoside, a mixture of asiaticoside A and B. Thin layer chromatography was carried out for asiaticoside. Market sample, mother plant and tissue culture raised plant samples were subjected to phytochemical analysis. Solvent system used was chloroform-glacial acetic acid-methanol-water (60:32:12:8). The detection of saponin was carried out by anisaldehyde-sulphuric acid reagent (Wanger et al, 1984).

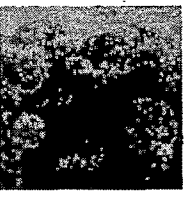


Results and Discussions

Sterilization of nodal explants for the initiation of cultures proved rather difficult due to heavy bacterial contamination and sensitivity to the mercuric chloride. In place of $HgCl_2$, 1.2 % Na hypochlorite (7 min) was used and found optimal for the establishment of explants. The nodal explants were pretreated in the solution containing streptomycin, rifampicin and ampillicin (10 min), which caused apparently all cultures to be contamination free.

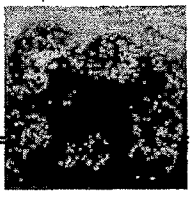
Various combinations of cytokinins (BA/KN) and auxin (IAA) were tried for induction of bud sprouting. Explants cultured in the MS medium without any growth hormones did not showed any response. Within a week's time the explants senescence. This showed the requirement of growth regulators in the medium. While emergence of shoot in the nodal explant cultured in basal medium supplemented with, PGR's is observed in many sps. *Woodfordia fruticosa* (Krishnan and Seeni, 1994), *Ocimum sanctum* (Pattnaik and Chand, 1996) and *Tanacetum parthenium* (Stojakowska and Kiesiel, 1997) is in contrast. There are various reports on cytokinin induced shoot initiation *in vitro* (Sen and Sharma, 1991; Purohit et al, 1994; Jain and Nessler, 1996; Patil and Jayanthi, 1997). Further the role of BA in bud breaking has been recorded for many medicinal plants such as *Bacopa monnieri* (Chaplot et al, 2005), *Hedeoma muliflorum* (Aldofina et al, 1997), *Tridax procumbens* (Sahoo and Chand, 1998), *Wedelia calendula* (Emmanuel et al, 2000), *Plumbago zeylanica* (Chaplot et al, 2006), and many more.

Although BA and KN are equally effective in axillary bud proliferation, BA has been considered to be more active (Rahman and Blake, 1988; Misra, 1996). BA has been reported for bud induction and shoot differentiation in *Bixa orellana*



(Ramamurthy et al, 1999). The medium containing alone cytokinins (BA or KN) showed delay in bud sprouts (3-4 weeks). However, on addition of auxin (IAA) in the medium containing cytokinin gave significant results in the case of *Centella asiatica.* It was observed that when nodal segments were inoculated on MS medium supplemented with BA and IAA, sprouting occurred within 2 weeks of inoculation (Plate-3.1A) whereas on MS medium containing KN and IAA sprouting occurred after 3 weeks. This is in agreement with results obtained in *Bixa orellana* of early bud break with presence of BA and IAA compared to KN and IAA in the medium (George and Jasrai, 2002). Thus, BA elicited faster response as compared to KN when used in combination with IAA for bud outgrowth. In general, KN has been reported to be less effective than BA (Murashige, 1974). BA and IAA have been also reported for induction of bud and shoot differentiation from nodal explants in *Gentiana lutea* (Feijoo and Iglesias, 1998) and *Hemidesmus indicus* (Sharma and Yelne, 1995). In the present study, it was observed that MS medium containing 22.2 μM BA and 2.88 μM IAA gave the fastest response (2 weeks) for bud sprouting (Plate-3.1B). Further leaf and internodal explants however, failed to induce bud break when inoculated in any culture media. This is in agreement with results of Shrotri and Mukundan, (2004) in the same species. Hence, nodal explants were considered to be the best explant to regenerate multiple shoots in *Centella.*

The *in vitro* raised nodal explants proved more beneficial for shoot proliferations.The micropropagated plants showed greater capacity for morphogenesis than the cuttings excised from the field grown plants (Jha and Sen, 1992). Since MS medium supplemented with BA and IAA was giving good response, different concentrations of BA and IAA were studied for the multiple shoot formation. Earlier,

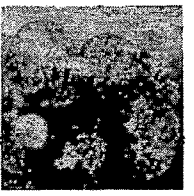


Tiwari et al, (2000) have reported axillary shoot formation on medium containing higher concentration of 22.2 μM BA and 2.68 μM NAA with maximum frequency 91% and optimum number (5.3) of shoots within a long period of 6 weeks. While Shashikala et al, (2005) reported multiple shoot formation (6) on higher concentration of 44.4 μM BA and 2.68 μM NAA within 2 weeks. Nath and Buragohain (2003) also reported multiple shoot (3.38) formation on MS medium supplemented with 88.8 μM BA and NAA (0.1 mg/l). Successful regeneration of *Centella asiatica* has also been reported by Patra et al, (1998) using stem and leaf explant in the medium containing BA or KN in combination with NAA.

The present study exemplifies a positive modification of shoot induction efficacy by with lower concentrations of an auxin in combination of cytokinin (Plate-3.1C) (Table-2). The induction of multiple shoots in MS medium fortified with 17.7 μM BA and 2.88 μM IAA exhibited maximum number of multiple shoots (Plate-3.1D).

Table-2: Efficacy of MS medium fortified with different combinations of growth regulators on proliferation of shoots of *Centella asiatica* (L.)

Growth regulators (μM)		Response (%) (Mean ± SE)	Shoots/node (Mean ± SE)
BA	**IAA**		
0.0	0.0	0	0
2.2	2.88	85 ± 0.01	1.0 ± 0.03
4.4	2.88	82 ± 0.01	1.3 ± 0.19
8.9	2.88	86 ± 0.03	2.1 ± 0.02
13.3	2.88	89 ± 0.11	4.1 ± 0.18
17.7	**2.88**	**94 ± 0.21**	**8.6 ± 1.02**
17.7	3.45	86 ± 0.09	6.6 ± 0.05
22.2	2.88	79 ± 0.13	4.5 ± 0.12
22.2	3.45	76 ± 0.15	3.2 ± 0.19



It was observed that the multiple shoots response decreased in the MS medium supplemented with greater concentration of 3.45 μM IAA. This indicates that higher concentration of IAA did not elicit good response. Similar results have been reported for *Bougainvillea* where the shoot apices cultured initially on MS medium containing IAA and BA (Sharma et al, 1981).

A comparison of means for responding frequency and number of shoots/node revealed that the maximum frequency 94% and maximum number of shoots (8.6) within 3 weeks (Plate-3.1E).

Elongation growth

Shoot elongation growth failed in higher concentration of BA and IAA. The generated multiple shoots (2-3 cm) were transferred to MS medium supplemented with lower concentration of 4.4 μM BA and IAA 2.88 μM for elongation growth. Earlier Anand et al (1999) had also observed the similar results for an important species *Syzygium travancoricum*. Maximum shoot elongation (6.8 cm) was observed on lower concentration of growth hormones 4.4 μM BA and 2.88 μM IAA within 3 weeks of transfer (Plate-3.1F) (Table-3).

A significant effect was also observed on number of nodes/shoot on elongation medium. An optimal number of nodes/shoots (10.5) was observed on MS medium supplemented with 4.4 μM BA and 2.88 μM IAA (Plate-3.2A). Earlier Tiwari et al, (2000) reported half the number of nodes/shoot in the same plant on higher concentration of BA and IAA.

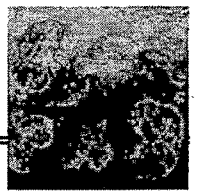


Plate: 3.1

A. Initiation of bud sprouts

B. Bud sprouts on MS medium containing BA (22.2 μM) and IAA (2.88 μM) within 2 weeks

C. Induction of multiple shoot formation

D. Induction of multiple shoot formation on medium containing BA (17.2 μM) and IAA (2.88 μM)

E. Multiple shoot formation

F. Shoot elongation

Photoplate - 3.1

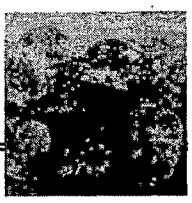


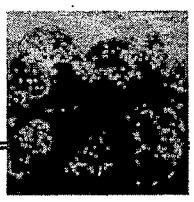
Table-3: Effect of different combinations of cytokinin and auxin in MS medium for shoot elongation growth. Data scored after 3 weeks

Growth regulators (μM)		Shoot length (cm) (Mean ± SE)
BA	IAA	
0.0	0.0	0.62 ± 1.02
1.1	1.14	1.6 ± 1.02
1.1	2.88	2.6 ± 0.05
2.2	1.14	2.5 ± 0.19
2.2	2.88	2.2 ± 0.19
4.4	1.14	4.1 ± 0.19
4.4	**2.88**	**6.8 ± 1.02**
6.7	2.88	5.0 ± 0.05

The subculturing of nodal segments harvested from the *in vitro* derived axenic shoots on the multiplication medium enabled continuous production of healthy shoots with similar frequency (Plate-3.2B, C).

Root induction

There was no rooting on half or full strength MS medium without auxins, but when it was supplemented with auxins (IAA and IBA) moderate to profuse rooting occurred in 80-100% of shoots. When full strength MS medium was supplemented with 2.88 μM IAA profuse rooting was observed on the shoots. Half strength medium gave poor response in number (6.2) and length of roots (4.5 cm) in comparison with full strength medium giving (12.1 roots) and length (14.3 cm) (Plate-3.2D). While other reports on root induction in Mandukparni was noted in MS medium containing higher concentration of 5.16 μM IAA (Shashikala et al, 2005; Grazia et al, 2000;



Bhattacharya and Bhattacharya, 2001). In contrast, Shrotri and Mukundan, (2004) reported poor rhizogenesis on addition of IAA in the medium and stimulatory response on higher concentration of 4.9 µM IBA. Banerjee et al, (1999) also found the stimulatory effect of IBA on rooting in *Centella asiatica*. Media supplemented with 2.88 µM IAA could produce twice the number of rootlets compare to the half strength MS media (Fig.-2). Along with plant growth substances, the quantity of carbohydrates source that is applied exogenously plays a pronounced role in maintaining the growth and metabolic events of the cells and tissues (Maretzki et al, 1974). It was further enhanced when the concentration of sucrose was lowered to 2%.

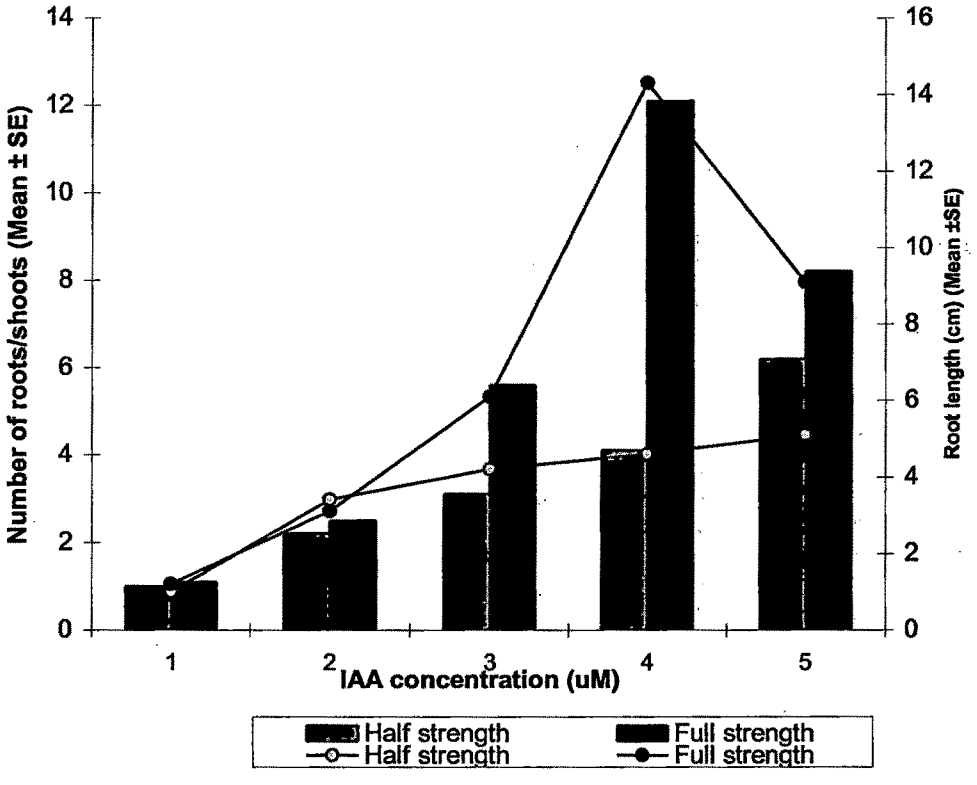
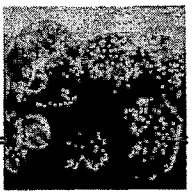


Figure – 2 Effect of half / full strength medium on root induction

Root induction of micro shoots in solanaceae family has been reported on MS medium with IAA or IBA in *Lycopersicon esculantum* (Dwivedi et al, 1990; Camillo



et al, 1991) in *Tylophora indica* (Sharma and Chandel, 1992). The beneficial effects of IBA rather than IAA for inducing *in vitro* rooting are documented in *Plumbago zeylanica* and also in many other plants (Rout and Das, 1993; Raghuvanshi and Srivastava, 1995). But in contrast, amongst the two auxins used to induce rooting in *Centella asiatica*, IAA was most effective, giving maximum number of roots (Plate-3.2D) while with IBA, roots were short and thin. These results are in accordance with that of Joshi and Thengane, (1996) on *Azadirachta indica* and Islam and Karim, (1994) on *Aegle marmelos*. Higher concentration of IAA caused delayed root initiation. This is in the view of George, (1996) who stated that when concentration of auxins, becomes supra optimal, callus formation is promoted and roots if formed, have an abnormal appearance. There was a significant effect on the frequency of cultures showing root regeneration, number of roots/shoot and mean root length. Maximum frequency (98%), number of roots/shoot (12.1) and mean root length (14.3cm) on shoots cultured on full-strength MS media with 2 % sucrose within 3 weeks was observed. This is in the agreement with results obtained earlier (Patra et al, 1998). Madhura and Mukundan (2005) observed (9.33) roots/shoot on medium containing 5.37 µM NAA in *Centella asiatica*. In fact, comparatively, low response for roots/shoot (8.5) and mean root length (6.3) on IBA medium have been reported earlier (Tiwari et al, 2000). Thus amongst the two auxins used to induce rooting, IAA was most effective, giving maximum number of roots.

Acclimatization of *in vitro* plantlets

The ultimate commercial success of *in vitro* propagation lies in the successful establishment of plantlets in the soil. The plants have to adapt to new environmental conditions such as low relative humidity, higher light intensity and fluctuations in

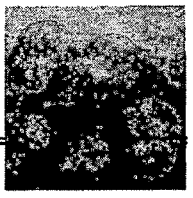


Plate: 3.2

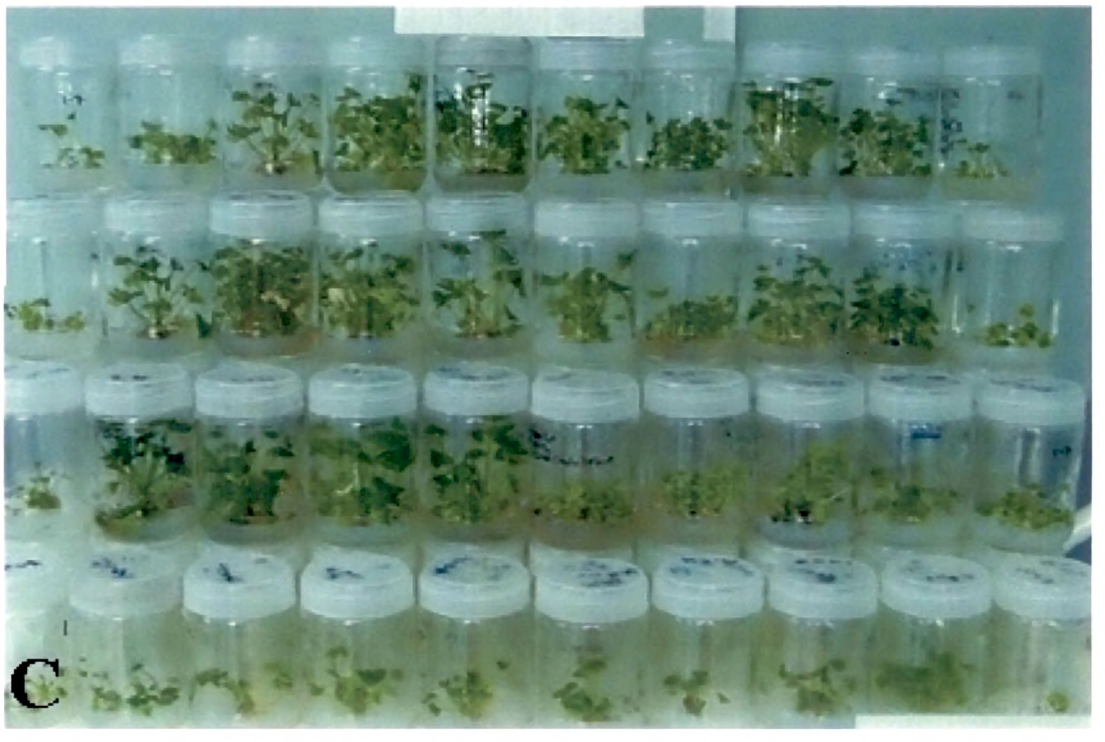
A. Formation of number of nodes per shoot

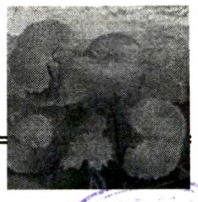
B. Subculture of regenerated shoots

C. Formation of regenerated shoots during (4-5) subcultures

D. Root induction on full strength medium supplemented with IAA (2.88 μM)

Photoplate - 3.2





temperature and stress (Preece and Sutter, 1991). In greenhouse the regenerated small plantlets (2-3 cm) to small plastic pots and big plantlets (4-5 cm) were washed with water and transferred to plastic bags containing different sterile potting mixtures (Plate-3.3A, B). Shrotri and Mukundan, (2004) irrigated the rooted shoots with Hoagland and Arnon's nutrient medium for better acclimatization but no such requirement was needed as the plantlets were readily acclimatized in greenhouse.

The experiments were carried out using various potting mixtures for *Centella* plantlets hardening. The mixture containing sand-soil-FYM (1:1:1) supported the highest survival of plantlets (100%), while establishment in other potting mixtures like sand-soil was 55%, vermiculite-soil was 65%, vermiculite-soil-sand was 71%, vermiculite-soil-FYM was 82% and sand-soil-cocopeat was 65% (Table-5).

Table-5: Acclimatization and survival of regenerated plantlets of *C. asiatica* in Greenhouse and Open field

Potting Mixture	**Proportion**	**Survival (%) (Mean ± SE)**	
		Greenhouse	**Field**
Sand + Soil	1:1	55.2 ± 0.01	60.21 ± 0.10
Vermiculite + Soil	1:1	65.2 ± 0.03	67.21 ± 0.10
Vermiculite + Soil + Sand	1:1:1	71.6 ± 0.14	80.05 ± 0.21
Vermiculite + Soil + Sand + FYM	1:1:1:1	82.1± 1.04	75.21 ± 0.10
Sand + Soil + FYM	**1:1:1**	**100**	**99.16 ± 0.01**
Cocopeat + Sand + Soil	1:1:1	65.6 ± 0.0 1	42.23 ± 0.16

Survival rate was found to be 100% in greenhouse (Plate-3.3C). High humidity (80%) and temperature (25 ± 1°C) was maintained by frequent water sprays. Shrotri and Mukundan, (2004) and Sivakumar et al, (2006) have also reported

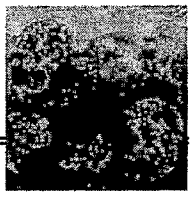


Plate: 3.3

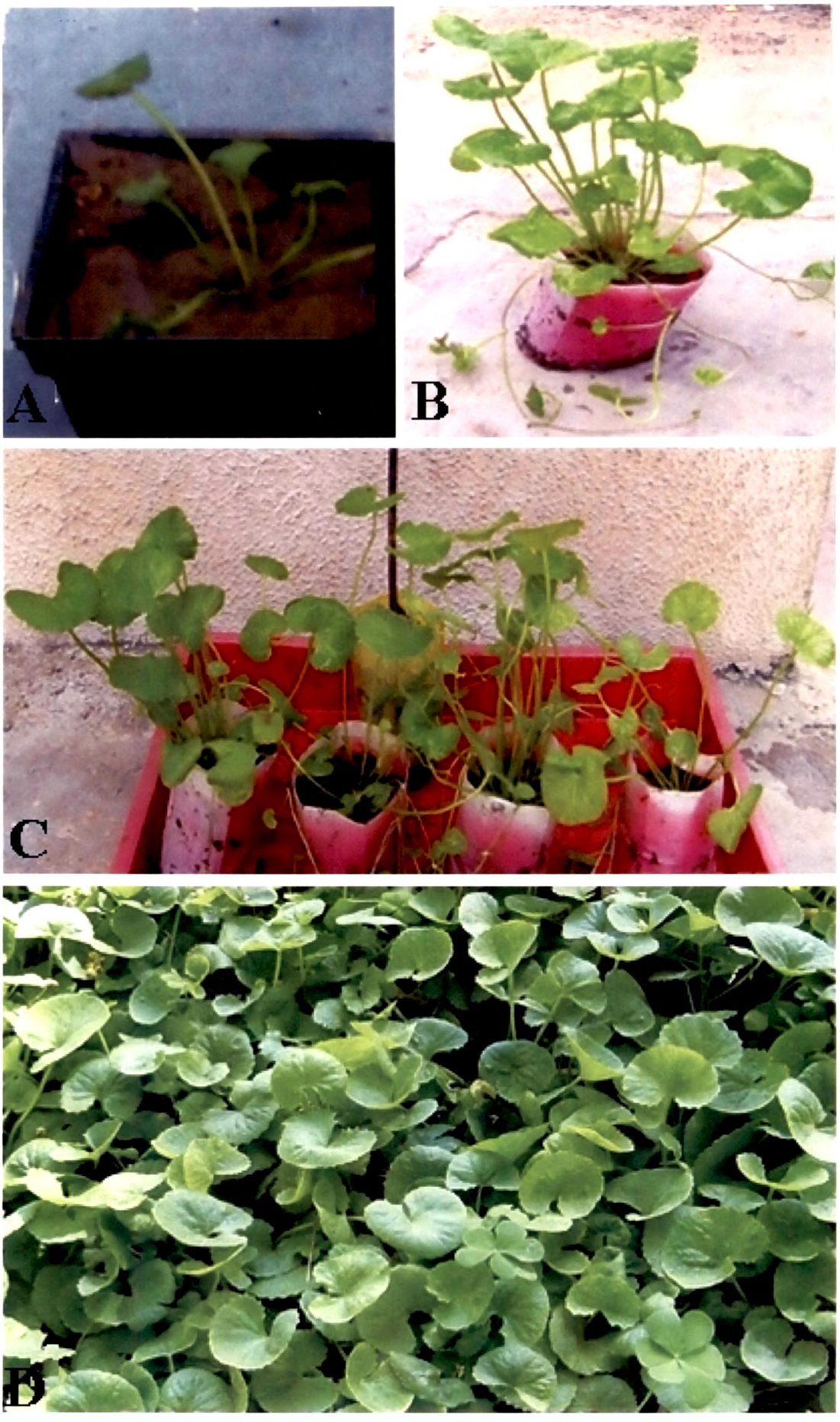
A. In greenhouse small plantlet (2-3 cm) transferred to plastic small pot

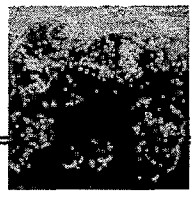
B. The regenerated plantlet (5-6 cm) transferred to plastic bag in greenhouse

C. Hardened *in vitro* plantlets ready for transfer to field

D. Field transferred *in vitro* grown plantlets showing vigorous growth

Photoplate - 3.3





successful acclimatization of *Centella* plants on field with 95% survival rate. Among 120 plantlets transferred to the field generated in the sand-soil-FYM potting mixture, as many as 99.16% (119) were found to survive. While plants (388) generated from other potting mixture were also transferred and successfully survived on the field. The established plants did not exhibit any morphological variation similar to those of the mother plant (Plate-3.3D).

The *in vitro* raised plants and conventional grown plants were uprooted from the field for leaf and root harvesting. A significantly whole plant consisted broad and higher number of leaves (10.5 ± 1.02; fw: 9.9 ±1.3gm) with numerous roots (12.1 ± 0.05; fw 7.5 ± 1.2 gm) per plant (Plate-3.4B) was noted when compared to the conventional grown plant stock with leaves (5.1 ± 0.01; fw: 3.4 ± 0.01 gm) and roots (5.1 ± 1.4; fw 2.3 ± 0.2 gm) respectively (Plate-3.4A). The net biomass of the tissue culture plant was higher than the normal plant (Table-6).

Table- 6: Biomass of different organ from normal and tissue culture plants of *Centella asiatica.* Data scored after 3 weeks.

	Normal plant		**Tissue culture plant**	
Plant Part	Morphology characters	Fresh weight (gm) per plant	Morphology characters	Fresh weight (gm) per plant
Leaves	Less number (5.1), small and green	3.4 ± 0.01	Numerous (10.5), large and dark green	9.9 ± 1.3
Roots	Short and slender with few lateral branches	2.3 ± 0.2	Long and slender	7.5 ± 1.2
Stem	Slender	0.9 ± 2.1	Stout	2.3 ± 0.6
Total		**6.6**		**19.7**

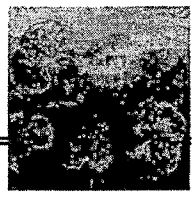
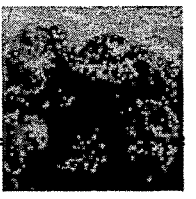


Plate-3.4

A. Conventional grown plants

B. *In vitro* raised plants

Photoplate - 3.4

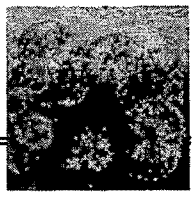


Roja and Heble (1996) has reported similar observations in *Rauwolfia serpentina* where net biomass of tissue culture plants was 170.73 gm compared to 77.28 gm of normal plant. Satheeshkumar and Bhavanandan (1988) also observed the same results in increase in biomass of *in vitro* plants of *Plumbago species.*

Phytochemical analysis

The thin layer chromatography was carried out based on routine Plant Drug Analysis (Wagner et al, 1984). The chromatograms produced by market sample, mother plant and tissue cultured raised plants were found to be identical with a Rf value of 0.60 confirming the presence of asiaticoside. Asiaticoside appeared violet band under UV light.

The protocol described here for the micropropagation of *Centella asiatica* facilitates the effective and efficient propagation with increased number of shoots, nodes and roots of this valuable medicinal plant, which can be used to provide quality-planting material.



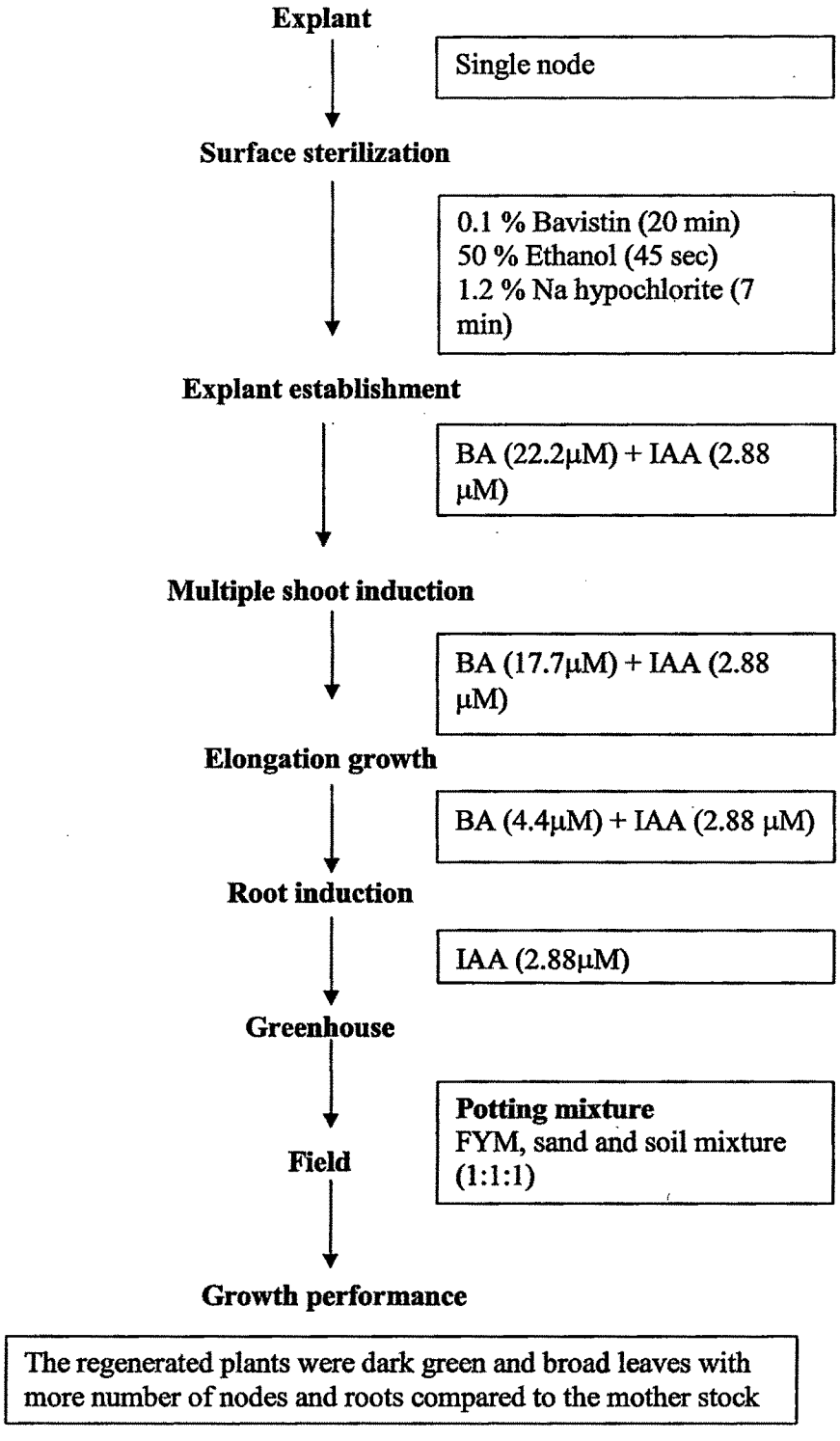


Figure - 2: Schematic representation of *in vitro* multiplication of *Centella asiatica* from nodal explants

MICROPROPAGATION OF *WITHANIA SOMNIFERA*

अश्वगन्धा कटूष्णा स्यात्तिक्ता च मदगन्धिका।

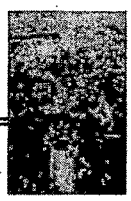
वल्या वातहरा हन्ति कासश्वासक्षयव्रणान्।

(राजनिघण्टु)

अश्वगंधानिलश्लेष्मश्वित्रशोथक्षयापहा।

बल्या रसायनी तिक्ता कषायोष्णाऽतिशुक्रला।।

(भा. प्र.)



Introduction

Withania somnifera (L.) Dunal, a species indigenous to India, is one of the most extensively used medicinal plants in traditional systems of medicine, Ayurvedic and Unani medicines (Roja et al, 1991). The use of ashwagandha in Ayurvedic medicine extends back to over 3000 - 4000 years to the teachings of an esteemed Sage (Rishi) Punarvasu Atriya. It has been described in the sacred texts of Ayurveda, including the Charaka and Sushruta Samhitas, where it is widely extolled as a tonic especially for emaciation in people of all ages including babies, enhancing the reproductive functions in both men and women.

Table-1: Vernacular nomenclature of *Withania somnifera* (L.) Dunal

Bengali	Ashvagandha
English name	Winter cherry
Gujarati	Ashwagandha
Hindi	Asgandh
Indian	Ashwagandha, Aaskand, Punir
Kanand	Hiri maddina-gadday, Amangura
Malayanum	Amukkuram, Pevetti
Marathi	Tlli, Kanchuki
Sanskrit	Ashwagandha
Tamil	Amukkara, Amukkira
Telgu	Ashwagandha, Pannerugadda

Family: Solanaceae

Withania somnifera, a constituent of Ayurvedic formulas and known as Ashwagandha, is considered as the premiere herb for all negative conditions associated with ageing. Modern herbalists classify ashwagandha as an adaptogen ie. a

plant with multiple actions that counteract the effects of stress and in turn promote wellness (Bone, 1996). Tribal people of Africa use *Withania* in fevers and inflammatory conditions (Duke, 1985). The traditional name - Ashvagandha seems to have derived from the sanskrit name ashwagandha which means smelling like a horse. In Indian scenario, it is known as Indian ginseng due to its properties similar to that of Ginseng. Ethanolic or aqueous extract of the root alone or in combination with other herbal materials is used in many commercial herbal formulations under various trade names such as Mentat, Geriforte, Pepcaps, 30-plus, Stresscom, Ashwagandharisht, Ashwagandhadi-churan, Ashwagandha-rasayanrist, and Ashwagandha-ghrit etc.

Morphology and Distribution

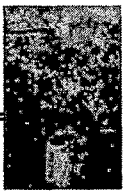
Withania somnifera is an erect, perennial, branched, tomentose, evergreen undershrub of 60-120 cm height. Leaves are simple, opposite, petiolate, elliptic-ovate to broadly ovate, entire, exstipulate, acute, oblique, glabrous and upto 10 cm length. Flowers are greenish or lucid yellow in axillary fascicles. The fruit is small, globose-berry, orange in color but turns red at maturity. The seeds are small, light, flat and light yellow in color. The fleshy roots are cylindrical on drying. The crop can grow over a wide variety of soils in sub-tropical, low rainfall regions but prefers well-drained, light-sandy loam, medium fertile soils of 6-8 pH. It is cultivated throughout the drier parts of India. It is a native of India, Pakistan and Sri lanka. In India, it is found growing all over Northwestern and Central India.

Medicinal properties

Ashwagandha's prime property is uniquely more beneficial for calming the mind, relieving arthritis and building sexual energy. It possesses adaptogenic, anti inflammatory, anti-tumor, anti-stress, anti-arthritic, anti-oxidant, immunodulatory, hemopoetic, radio-sensitizing, rejuvenating properties, tonic for general debility and also appears to exert positive influence on the endocrine, cardio-pulmonary and central nervous system (Budhiraja et al, 1987; Chopra et al, 1958; Suffness and Douros, 1982). It is generally used as anabortifacient, amoebicide, bactericide and contraceptive (Asthana and Raina, 1989).

It has got a wide range of applications in the treatment of various physiological disorders. It is a recognized folk remedy for a number of diseases viz arthritis, asthma, bronchitis, adenopathy, anthrax, candidiasis, cold, cough, cystitis, debility, diarrhoea, dropsy, dyspepsia, fever, gynecopathy, hiccups, hypertension, inflammations, lumbago, marasmus, nausea, piles, psoriasis, ringworm, scabies, senility, sores, syphilis, tuberculosis, tumors, typhoid, uterosis and wounds. Tuberous roots are astringent, bitter, acrid, somniferous, thermogenic, stimulant, aphrodisiac, diuretic and tonic. They are useful in conditions of leucoderma, constipation, insomnia, tissue building and nervous breakdown. The leaves are bitter and are recommended in fever, painful swelling and opthalmitis.

Part used: Leaves and roots



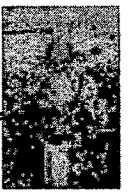
Ayurvedic properties:

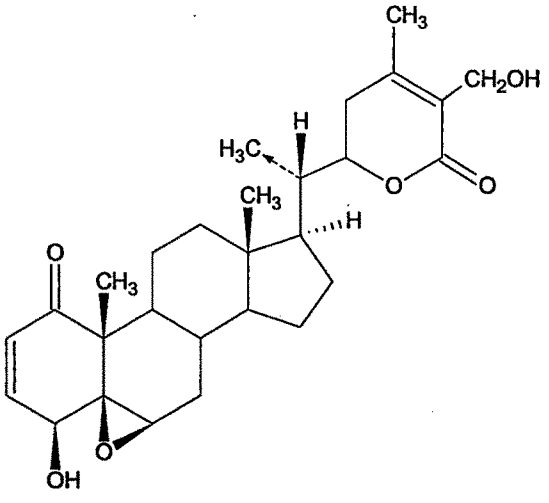
Sr. No.	Ayurvedic Properties	Description
1	Rasa (taste)	Tikta (bitter), Kasaya (astringent)
2	Gunna (properties)	Laghu (light, easy to digest), Snigdha (unctuous)
3	Veerya (potency)	Osna
4	Vipaaka (properties after digestion)	Katu (Pungent)

(Anonymous, 1999a; Anonymous, 1976)

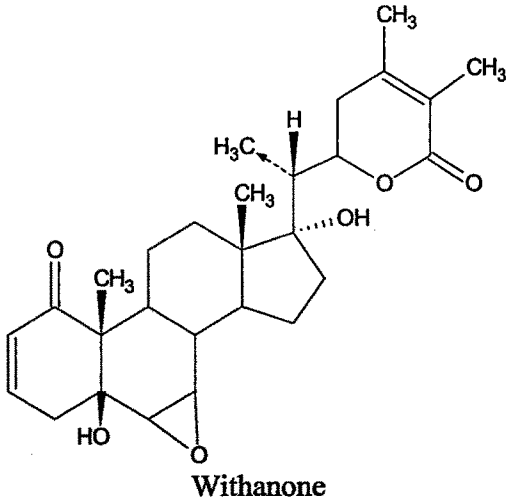
Active constituents

The species contains tropane alkaloids and withanolides, which account for the multiple medicinal applications (Tyler et al, 1981; Wanger et al, 1994) (Fig.1). Withanolides are a class of medicinally important compounds extracted from the genus, and the withaferin A is very important in commercial market for its tumor inhibitory activity (Kannan et al, 2005). The major biochemical constituents are steroidal alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII) and withanolides with a glucose at carbon 27 (sitoindoside IX and X). Withaferin A, chemically characterized as 4β, 27–dihydroxy-5β-6β-epoxy-1-oxowihtha-2, 24-dienolide, is one of the main withanolidal active principles desired for a non-ulcerating anti-inflammatory/chemotherapeutic drug (Sangwan et al, 2004). Leaves contain withanolides like Withaferin A that exhibit antibacterial, and antitumor properties (Jaffer et al, 1998; Devi 1996). It also contains starch, reducing sugars, hentriacontane, glycosides and dulcitol. The plant is also rich in iron and amino acids (roots and green berries).

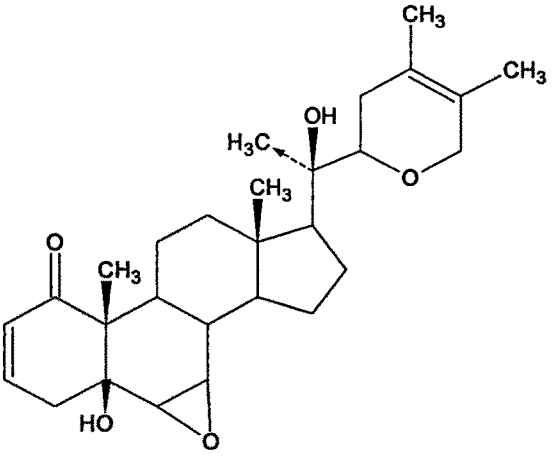




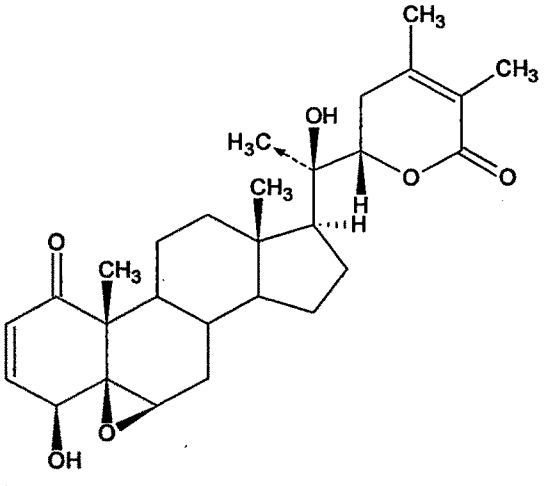
Withaferin A



Withanone



Withanolide A

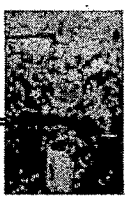


Withanolide D

Figure-1: Structure of few active compounds

Conventional practice for propagation

Conventionally, *Withania* is propagated through seeds. Planting is done late in rainy season during August-September. It grows well in semi-tropical areas receiving 500-700 mm rainfall. It requires sandy loam or light red soil having 7.5-8.0 pH with

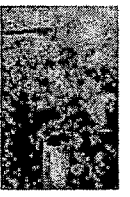


good drainage. A seed rate of 10-12 kg/ha is sufficient. A light shower after sowing ensures good germination but excessive rain affects the germination adversely.

The plant starts flowering and bears fruits from December onwards. Harvesting is done at 150-180 days after sowing ie from January to March. The maturity of plant is judged by drying out of leaves and yellow red berries. The entire plant is uprooted for roots, which are separated from the aerial parts by cutting stem 1-2 cm above the crown. The roots are then cut transversely into small pieces (7-10 cm) or dried as a whole in the sun. Berries are hands plucked separately. They are dried and crushed to take out the seeds.

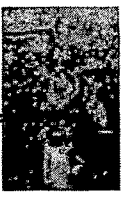
***In vitro* approaches**

In vitro techniques have been employed earlier in W. somnifera with limited success (Roja et al, 1991; Rani and Gover, 1999; Deka, 1999; Kulkarni et al, 2000). Roja et al, (1991) reported shoot regeneration from axillary meristem explants. Sen and Sharma, (1991) reported regeneration from germinating seeds and shoot tips of *W. somnifera*. Baburaj and Gunashekaran, (1995) and Abhyankar and Chinchanikar, (1996) reported shoot differentiation in *W. somnifera* from leaf explants. Kulkarni et al, (2000), Vadawale et al, (2004) and Sivanesan and Murugesan (2005) reported regeneration from various explants of ashwagandha on high and different concentration of growth hormones. There are only few reports on callus-mediated regeneration in Ashwagandha. Baburaj and Gunashekaran, (1995), Rani and Grower, (1999) and Pawar et al, (2001) reported regeneration from leaf callus. While Manickam et al, (2000) reported shoots from stem callus. On the analytical side, *W. somnifera* has also been studied in the context of withanolides and the



immunosuppressive phytochemicals (Furmanowa et al, 2001). Ray and Jha (2001) reported that withaferin A was produced in shoot cultures of *W. somnifera*.

The highly significant and practical fact about ashwagandha is that most tonics like ginseng, require special growing conditions and several years to develop their tonic properties (ginseng requires 7 years) while ashwagandha is unique as a tonic herb, ready for harvest after only one year of growth. So if ashwagandha is used more then it would relieve some of the threat of extinction from the wild or other highly popular herbs such as wild ginseng (*Panax quinquefolium*), golden seal (*Hydrastis canadensis*), suma (*Pfaffia paniculata*) and lady's slipper (*Cypripedium pubescens*) for instance. Moreover, viability of stored seed is limited to one year, poor germination and success rate of vegetative propagation is very low (Kulkarni et al, 2000). Owing to a number of therapeutic principles found in this plant, it is in demand for the alkaloid in the international market (Rawal, 2003). Therefore, there is a need to generate an *in vitro* protocol for large-scale multiplication of Ashwagandha for quality planting material.



Material and Methods

Plant material

Juvenile shoots (8-9 cm long) were collected from two-month old plants of *Withania somnifera* growing in the Botanical Garden of the Maharaja Sayajirao University of Baroda. Shoots were cut into single axillary node explants (size 1 cm) and leaves were discarded.

Sterilization

The explants were thoroughly washed under running tap water (30 min) and 0.2% (v/v) aqueous detergent Labklin LR (S. D. Fine, India) for 15 min followed by repeated rinsing with distilled water. Subsequently, explants were pre-treated (20 min) with 0.1% (w/v) fungicide-Bavistin (Carbendenzim; BASF, India) and serial rinsing with distilled water. Further surface-sterilization of explants was carried out under aseptic conditions in a Laminar Air flow hood (Lab Services, India).

Pretreated explants were surface sterilized with 50% (v/v) ethanol (30 sec) and 0.05 % (w/v) $HgCl_2$ (3 min). Finally, the explants were rinsed thoroughly (three times) with sterilized distilled water.

Culture medium and conditions

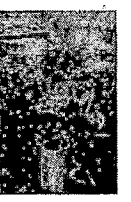
The sterilized explants were transferred to sterile petridishes and trimmed under aseptic condition and inoculated on agar based (0.8%; w/v; Qualigens, India) semi-solid MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of 6-benzyl adenine (0.5- 3.21μM). The cultures were incubated and maintained in the culture room with 25°C temperature under a 16-hr

photoperiod(50μEm^{2}s^{-1} irradiance) provided by cool white fluorescent tubes (Philips, India). The single nodes excised from the *in vitro* raised shoots were further subcultured on MS media for further multiplication. To study the effect of physical nature of the medium on the growth, axillary nodes were cultured in liquid and solid medium gelled with 0.8% agar.

The subcultures were carried out at regular interval of three weeks in culture bottles containing 30 ml of medium. Five explants were inoculated in each culture bottle for subcultures. Number of subculture cycles as well as the medium quantity was also standardized for commercial angle.

Induction of roots and acclimatization

Regenerated shoots (4-5 cm) were excised and transferred to half and full strength agar based MS medium with different concentrations of IBA (0–2.88μM) for root initiation. Shoots with well developed roots, were removed from the culture vessels, washed gently with water and transplanted in the plastic bags (10cm) containing a mixture of sand, soil and farmyard manure (1:1:1). Experiments with various other potting mixtures (like sand-soil, sand-soil-cocopeat and sand-soil-FYM) were also tried out. The plantlets were kept in the net house for acclimatization (2-4 weeks) before their subsequent transfer to the field. During hardening humidity was maintained by sprinkling water regularly throughout the day. Plants were gradually exposed to the normal conditions and transferred to the Medicinal Garden of GSFC Science Foundation for cultivation.



Post harvest

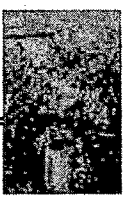
The plants were uprooted from the field after four months of their growth in the field. The adhered soil was removed from the harvested roots. The root crown was then carefully separated from stem and dried under shade (8-10 days). Later the dried roots were powdered for phytochemical analysis.

Phytochemcial analysis

Qualitative analysis was carried out through thin layer chromatography. The dried roots of mother plant, tissue culture raised plants and market sample were subjected to the phytochemical analysis. Known amount of dry powder of all the three samples were dissolved in 1ml of ammonia (25% in water) and shaken vigorously. Subsequently, 10 ml methanol was added and sonicated (10 sec). Then mixture was heated on a water bath (3 min) and filtered. Filtrate was evaporated to dryness and reconstituted in 1ml methanol. Samples were spotted on Silica gel GF 254 precoated TLC plates (0.2mm thickness). Solvent system used was Toluene-ethylacetate-formic acid (50:15:5). The phytochemical detection was carried out by immersing TLC plate in sulphuric acid reagent (1 sec), dried in stream of cold air and further heated to 110° C (2 min). The developed spots were observed under UV lamp.

Statistical analysis

Experiments were set up in a Randomized block design (RBD) and each of three repeated experiments had 10 replicates. Observations were recorded on the frequency (number of cultures responding for axillary shoot proliferation, nodal callus and root development) and the number of shoots per explants, type of response,



shoots length, roots per shoot and root length. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means.

Results and Discussions

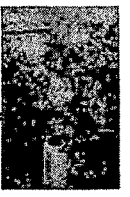
Initial establishment of sterile cultures proved rather difficult due to heavy bacterial contamination and sensitivity of the explants. To avoid bacterial contamination, nodal explants were dipped (10 min) in the antibiotic mixture containing streptomycin, rifampillicin and ampillicin (100 mg/l each). Further, a quick dip (15 sec) of the explants in 50% ethanol followed by treatment with low concentration (0.05%) of $HgCl_2$ (3 min) proved sufficient for contamination-free cultures.

The effect of explant position on the shoot was studied. It was observed that the axillary buds located at the middle position of the shoots gave maximum and faster response for bud sprouting (Table-2).

Table- 2: Effect of explant position on the shoot for bud-break response in Ashwagandha

Position of the explant on the shoot	Bud-break (%) Mean ± SE
Upper	35 ± 1.5
Middle	86 ± 1.2
Lower	63 ± 1.7

The bud break from the axillary node explants occurred on MS medium supplemented with 2.21μM BA (Plate-4.1A) after 6-7 days of inoculation. Nodal segments cultured on MS basal medium with BA alone gave rise to single shoot only. BA alone in the medium was not sufficient to induce multiple shoot buds indicating the necessity for using combination of auxin and cytokinin. Synergistic effect of BA and IAA showed the strongest effect on multiplication of axillary buds. The



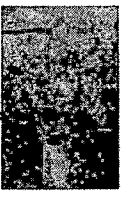
combination of BA and IAA has been reported also in *Boungainvillea* (Sharma et al, 1981), *Catha edulis* (Elhag, 1991) and *Plumbago rosea* (Harikrishnan and Molly, 1996).

Earlier, formation of 4-5 shoots have been reported in Ashwagandha from nodal explants on MS medium with 8.88 µM BA worker (Roja et al, 1991) and 3.32 µM BA, 1.16 µM KN and 0.98 µM IBA (Vadawale et al, 2004). Similarly, Sen and Sharma (1991) had reported shoot formations from germinating seeds.

Table-3: ANOVA and effect of BA and IAA concentration on responding explants and number of shoots/node in *Withania somnifera*

Source of variation	**Degree of freedom**	**Mean square**	**Number of shoots/node**	
Treatment	4	47.97	2.37**	3.32*
Error	45	0.034	1.39**	1.59*
Total	49			
Plant growth regulators (µM)		**Response (%)**	**Number of shoots /node (Mean ± SE)**	
IAA	BA			
00	00	0	0	
00	1.1	0	0	
00	2.21	25	1.1 ± 0.21	
0.57	1.1	55	3.1 ± 0.51	
0.57	**2.21**	**90**	**8.4 ± 0.13**	
0.57	3.21	75	4.3 ± 0.21	
1.14	1.1	45	2.0 ± 0.42	
1.14	2.21	20	2.3 ± 0.13	
1.14	3.21	2	1.2 ± 0.21	

- Mean are significantly different from each other (P<0.05*) (P<0.01 **)



The present study reports induction of multiple shoots on the MS medium supplemented with lower concentration of 2.21 μM BA and 0.57 μM IAA within 3-4 weeks (Plate-4.1B) (Table-3). Kannan et al, (2005) had demonstrated that in addition of BA in combination with NAA or IAA is essential for the induction of multiple shoot buds in same species. Further this results are in agreement with the results of Ghosh and Banerjee, (2003) in *Rauwolfia tetraphylla*. Multiple shoot formation from morphogenic cultures has been achieved among others in several species of Solanaceae (Macek 1998; Alvarez et al, 1993; Baddaoli et al, 1996; Ikenagat et al, 2000; Rejitha et al, 2002; Jaseela and Nair, 2004).

Analysis of variance revealed significant effect of treatments ($p<0.01$) for cultures showing axillary shoot proliferation and number of shoots/node. The frequency of responding explants increased markedly with an increase in the concentration of BA upto 2.21 μM. Maximum 8-9 shoots/node with 90% frequency was attained on MS medium containing 2.21 μM BA and 0.57 μM IAA (Plate-4.1C). An increase in the level of IAA beyond 1.14 μM resulted in profuse callus formation at the cut ends. Shoot cultures were multiplied further by transferring nodal segments excised from *in vitro* formed shoots on MS liquid and solid medium containing a combination of 2.21 μM BA and 0.57 μM IAA. Subculturing of nodal segment harvested from the *in vitro* derived axenic shoots in both solid and liquid medium enhanced continuous production of healthy shoots with good elongation growth (4-5 cm) in 8 days. Both the media favored initial elongation. However, solid medium (Plate-4.1D) was found to enhance the multiplication faster (15-18 days) with (8 shoots) as compared to the liquid medium (22-24 days) with (6 shoots).

Plate-4.1

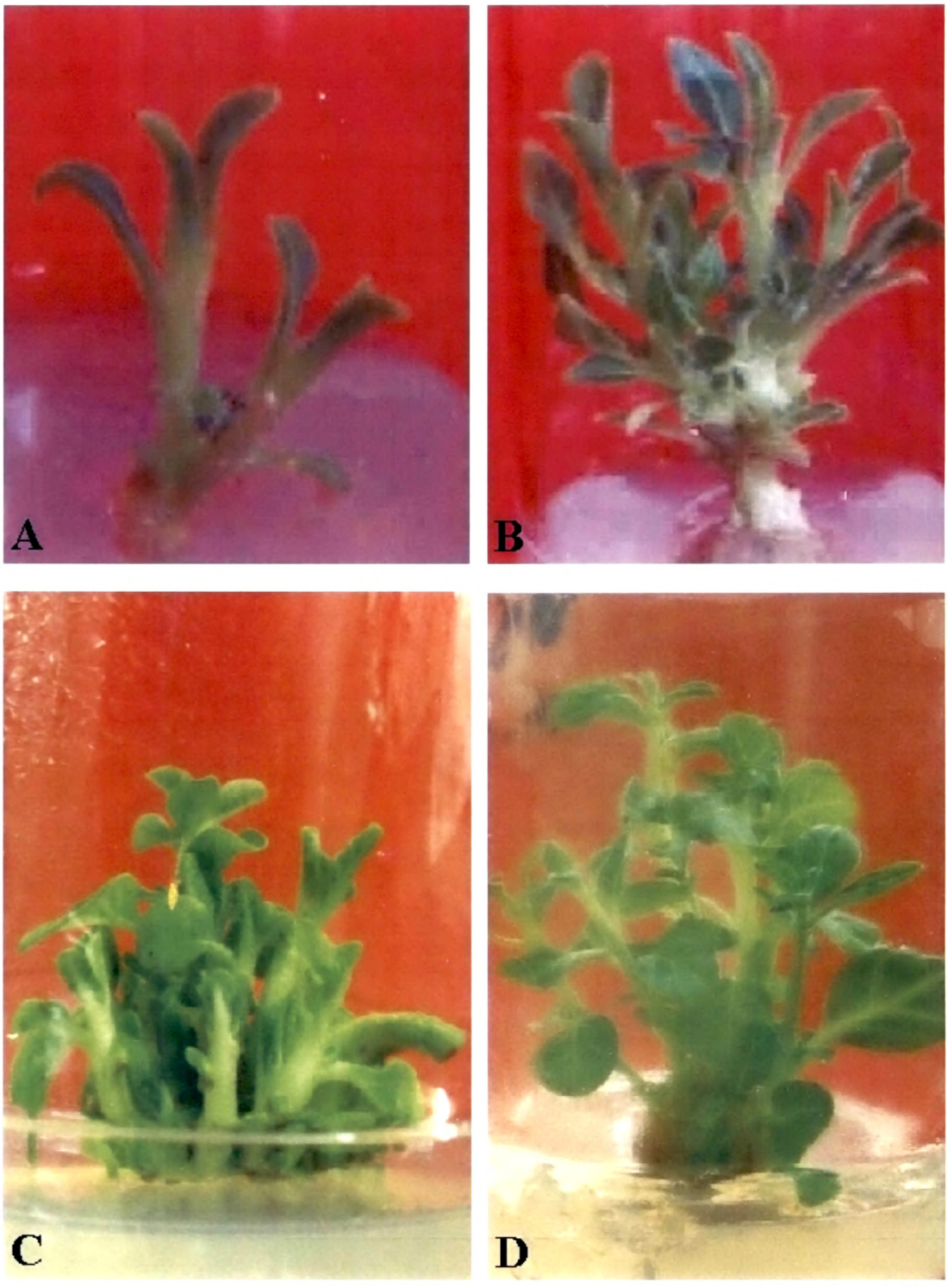
A. Bud break from nodal explant

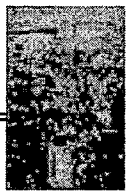
B. Induction of multiple shoot formation

C. Shoot formation on MS medium containing 2.21 μM BA and 0.57 μM IAA

D. Enhanced shoot formation on semi solid medium

Photoplate - 4.1





One serious disadvantage of using liquid media for shoot growth and multiplication is that shoots, which are perpetually submerged in liquid cultures, may become hyperhydric and will then be useless for micropropagation (George, 1993; Debergh, 2000). Ebrahim and Ibrahim (2000) reported that the solid medium should be used to overcome the production of vitrified shoots of *Maranta leuconeura* and to insure obtaining vigorous plants with higher chlorophyll content.

The cultures were subcultured till ten passages to study the rate of multiplication with commercial angle (Fig.2). Percent shoot development as well as number of shoots per node retained the highest values (90%; six to eight shoots per node) upto seven subcultures (Plate-4.2A, B, C). Later subsequent passages caused decrease in number of shoots formed from the nodal explant.

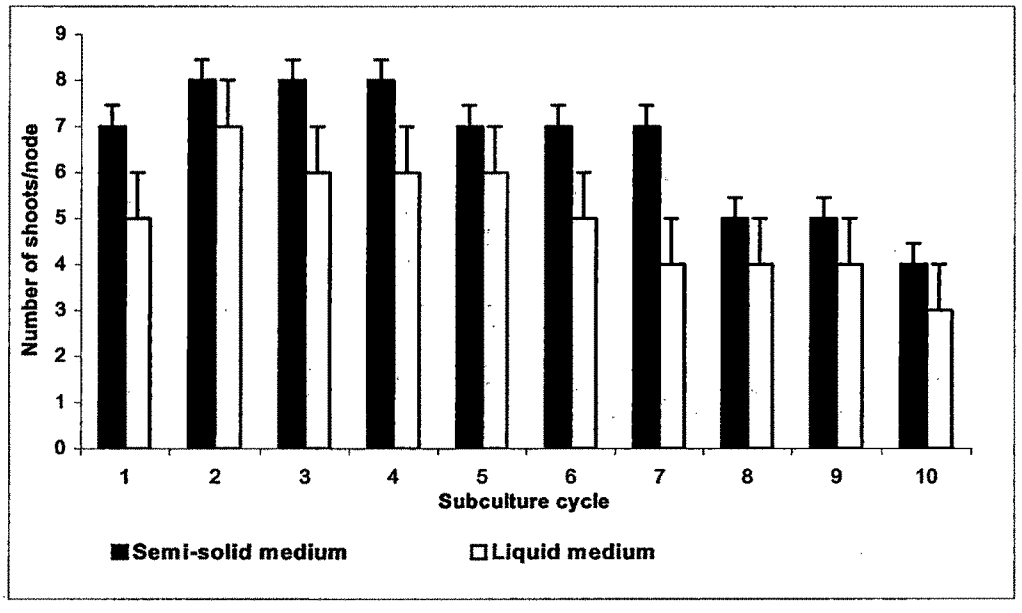
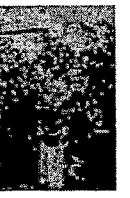


Figure-2: Rate of Shoot formation from *in vitro* nodal explants during different subcultures of Ashwagandha

Plate-4.2

A, B & C. Shoot formation from nodal and rhizome explants during different subcultures of *W. somnifera*

Photoplate - 4.2

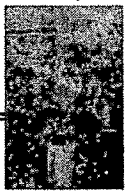


Callus formation

Copious callus formation was observed from the base of nodal explants on higher concentration of BA and IAA. Callus was separated and transferred to the medium supplemented with BA (0.0-4.4 μM) and IAA (0-1.71 μM). The callus was compact and creamish white coloured callus, which led to the proliferation of large number of shoots within three weeks. Initiation of shoot buds was noticed on the callus (Plate-4.3A, B)

Profuse callus resulted in formation of shoots on MS medium supplemented with 3.21 μM BA and 1.14 μM IAA through organogenesis (Plate-4.3C). This proliferating callus (250-300 mg) on separation resulted in the formation of around 80-100 shoots culture. According to Marks and Simpson (1994), callus formation at the basal cut ends, may be due to the action of accumulated auxin, which stimulates cell proliferation, especially in the presence of cytokinin. Callus formation at the basal cut ends of nodal explant on multiplication medium is in agreement with the results reported (Rani and Grower, 1999) in the same species on medium with very high concentration of 8.87 μM BA. Callus formation response was more (94%) on medium supplemented with 3.21 μM BA and 1.14 μM IAA (Table-4).

Shoot formation from the callus occurred on the same medium after 2 weeks. Maximum number of 25 shoots (1-2 cm) was observed from the callus (Plate-4.3D). This high morphogenic efficacy of node callus may be due to the passage of some internodal components from the pre-existing axillary buds that are essential to evoke caulogenesis (Martin, 2002). Number of shoots formed from the callus was found much higher than the nodal explant. Elongation, rooting and hardening of regenerated shoots was achieved successfully through callus (Plate-4.3E, F, G) Baburaj and



Gunasekaran (1995) have also reported callus induction from leaf explants of *W. somnifera* on MS medium supplemented with 10.7 µM NAA and 2.32 µM KN.

Table-4: Effect of different combinations of BA and IAA on frequency (%) and number of shoots/node through internodal callus

Plant growth regulators (µM)		**Response (%)**	**Number of shoot/ culture (Mean ± SE)**
BA	IAA		
1.1	0.57	22	4.2 ± 0.42
2.21	0.57	69	5.4 ± 0.11
3.21	0.57	70	11.1 ± 0.31
3.21	**1.14**	**94**	**24.9 ± 0.21**
3.21	1.55	70	15.2 ± 0.11
4.4	1.55	55	7.21 ± 0.15

Media volume was also standardized for commercial angle. According to our experiment, less quantity of medium (15-30 ml) with 5 explant in culture bottle resulted in less number of shoots and callus proliferations (Table-5).

Table-5: Standardization of media volume for shoot and callus formation

Media Volume (ml)	**Shoots/ 5 explants/subculture**	**Shoot Elongation (cm)**
15	16 + callus initiation	2-3
20	22 + callus (50 mg)	2-3
30	29 + callus (100 mg)	2-3
40	40 + callus (250-300 mg)	4-5
50	40 + callus (250-280 mg)	4-5

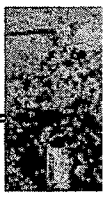


Plate-4.3

A & B. Initiation of shoot buds from the callus obtained at the base of the node

C. Shoot formation from the callus

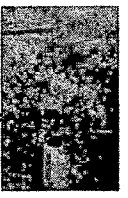
D. Multiple shoot formation on MS medium supplemented with 3.21 μM BA and 1.14 μM IAA from the callus

E. Elongation of shoot

F. Root formation

G. Hardening of regenerated shoots generated through organogenesis

Photoplate - 4.3



Further media got exhausted soon and shoots had to be transferred to fresh medium for further growth. While 40 ml, solid medium was found sufficient for 5 explant to generate 40 shoots (4-5 cm) and large amount of callus proliferation (250-300 mg).

While more amount of medium (50 ml) did not enhanced more shoots or callus. Liquid medium was not suitable for commercial angle, as callus formation was not proper and delayed in multiplication of shoots.

Root Induction

Elongated shoots of 4-5 cm length were separated and cultured individually on half / full strength MS medium with / without auxin. There was no root formation on basal half and full strength MS medium. However, when it was supplemented with IBA (0.98-2.46 µM) moderate to profuse rooting occurred in 50-100 % of the cultured shoots (Plate-4.4A). The frequency of cultures showing root regeneration, number of roots/shoot and mean root length showed significant effect in half and full strength MS medium. Maximum response (100%) of root formation with most number of roots/shoot (25 in total) and highest root length (9.8 cm) was observed on full strength MS medium containing 2.46 µM IBA (Plate-4.4B). The formation of roots often increases in proportion to the concentration of auxin applied, but when the concentration becomes supra-optimal, callus formation is promoted, and roots have a abnormal appearance and their average length, and subsequent shoot growth may be decreased (George, 1996). In fact, the presence of auxin is necessary only in the induction as well as in the initiation phases of rhizogenesis, which occur in the first days after transferring the plants to the rooting medium (Grattapaglia and Machado, 1998). While Manickam et al (2000) and Sivanesan and Murugesan (2005) have

reported root formation on higher levels 9.84 µM IBA with relatively much less number of roots per shoots, 5.1 and 16 respectively. Similarly, Vadawale et al (2004) observed only 9.09 roots per shoot with 3.69 µM IBA.

Comparatively, full strength MS medium was found much better than half strength MS medium for root formation (Fig.-3) on *in vitro* derived shoots (Plate-4.4C).

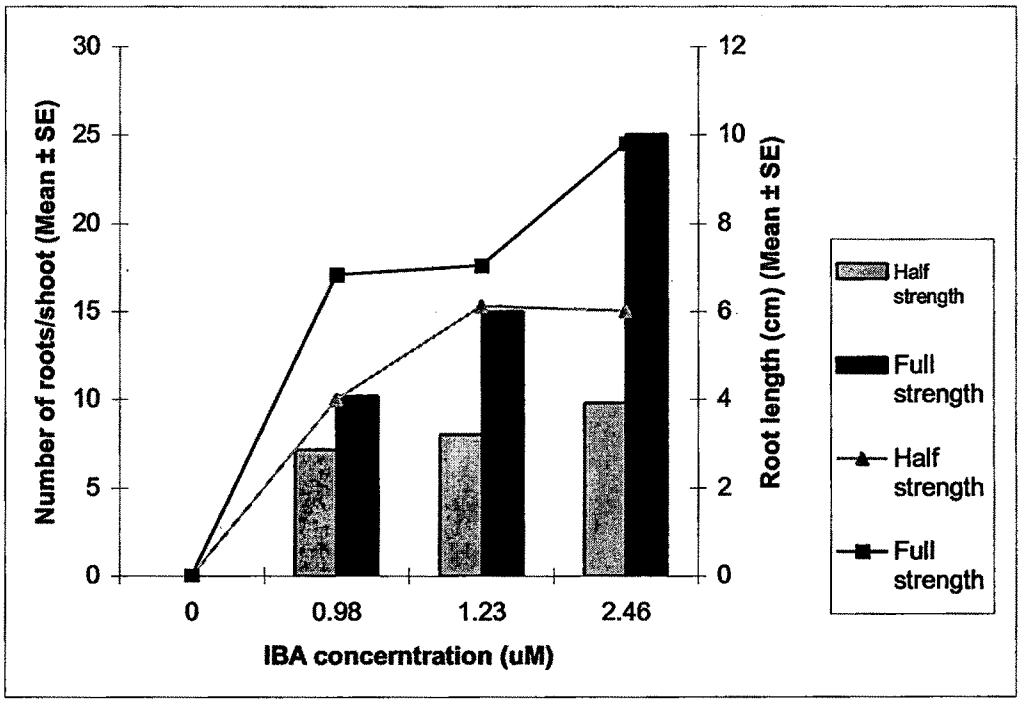


Figure - 3: Effect of half/full strength MS medium containing IBA on root induction

The efficacy of IBA in induction of roots on of regenerated shoots has been established in various ornamental and medicinal plants (Hoque et al, 1998; Hossain et al, 2000; Islam et al, 2001; Epstein et al, 1993; Shibli et al, 1999). It has been further reported that IBA is superior to IAA/NAA for its more stable nature (Hutchinson, 1981; Litz and Jaiswal, 1990). Moreover, Nickell (1982) demonstrated slow

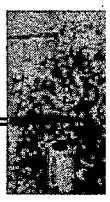


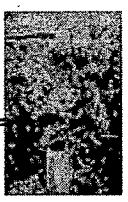
Plate-4.4

A. Root initiation

B. Root formation on the *in vitro* shoot of *W. somnifera*

C. Profuse root formation on full strength MS medium supplemented with 2.46 μM IBA

Photoplate - 4.4



movement and slow degradation of IBA, which facilitates better induction of roots.

Acclimatization of regenerated plantlets

The rooted shoots were transferred from the culture vessels to plastic bags containing various potting mixtures (Table–6) (Plate-4.5A) and maintained in the Agri-net house. The equi-volume potting mixture containing farmyard manure – soil - sand showed the highest survival (98%) (Plate-4.5B) while establishment of plantlets in other potting mixtures like cocopeat- sand - soil was 75% and sand - soil was 65% only. Enough humidity was maintained by regular spray of water on and near plants. After 2-3 weeks of acclimatization, the plantlets (10-12 cm) were transferred to the Medicinal Garden of GSFC Science Foundation and were successfully established in the soil. Among 150 plantlets transferred to soil, as many as 144 plants showed to survived (Plate-4.5C).

Table-6: Acclimatization and survival of regenerated plantlets of *Withania somnifera* in agri-net house and open field

Potting Mixture	Proportion	Survival (%) (Mean ± SE)	
		Agri-Net House	**Field**
Sand + Soil	1:1	65.03 ± 0.12	60.21 ± 0.10
Sand + Soil + FYM	**1:1:1**	**98.01 ± 1.20**	**95.12 ± 0.01**
Cocopeat + Sand + Soil	1:1:1	75.09 ± 0.09	69.20 ± 0.12

The plantlets did not show any morphological variation when compared to those of the source plants. The observed survival 95% of regenerated plants on the field is much comparable to the earlier reports with 60-90% survival rate (Rani and Grower, 1999; Rani et al, 2003; Vadawale et al, 2004; Sivasesan and Murugesan, 2005; Kannan et al, 2005). To our knowledge, this is the first report of a highest rate

Plate-4.5

A. The plantlet (2-3 cm) were transferred to plastic small bag in Agri-net house

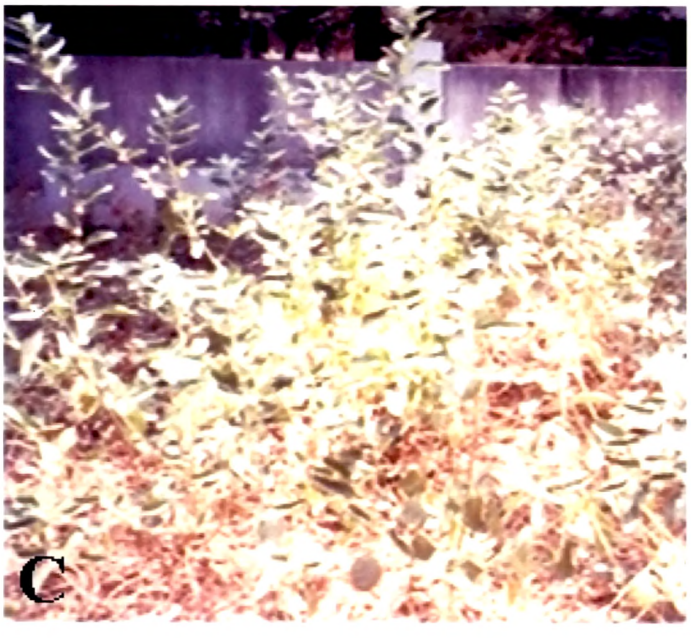
B. The hardened plantlet (5-6 cm) in plastic pot ready for transfer to field.

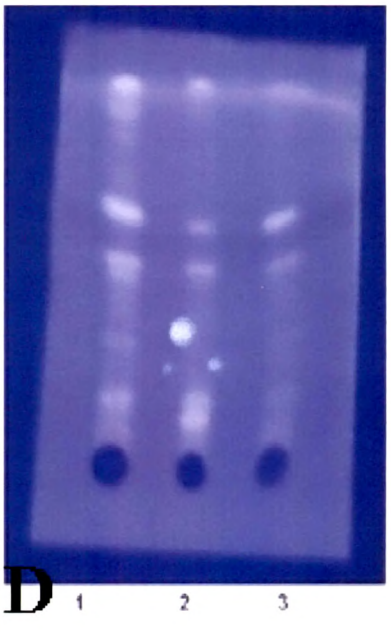
C. Field transferred *in vitro* grown plantlets showing vigorous growth

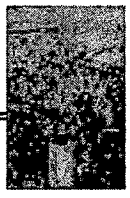
D. Thin layer chromatography

1. Normal plant
2. Tissue culture plant
3. Market sample

Photoplate - 4.5





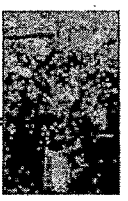


of survival for micropropagated plants of *Withania somnifera* in Agri-net house and subsequent transfer to the field.

Phytochemical analysis

Sangwan et al, (2004) reported wide variations in the content of active principles in the commercially available *Withania* products as quantified by HPLC. Hence, it is necessary to maintain stringent levels of phytochemicals in herbal products. The chromatograms (TLC) developed in the present study for donor (1), micropropagated plants (2) and market samples (3) were found to be very identical (Plate-4.5D). The spots also included normal β-sitosterol under UV lamp. The chromatogram developed is in line with the one reported by Mahadevan et al, (2003) for two commercial polyherbal formulations in *Withania somnifera.*

The micropropagation protocol of *Withania somnifera* has been successfully established. This effective and efficient protocol with increased number of shoots and roots of a valuable and commercially viable medicinal plant can be used to provide quality-planting material for large-scale cultivation. This would, in turn, help to sustain the ever-increasing demand in the present scenario of herbal market.



Explants

Node, callus

Surface Sterilization

0.1% Bavistin (20 min)
50% Ethanol (30 sec)
0.05% $HgCl_2$ (3 min)

Multiple Shoot Induction

2.21 µM BA + 0.57µM IAA

Root Induction

2.46µM IBA

Net-house Hardening

Potting mixture
FYM- Sand - Soil (1:1:1)

Field Transfer

No morphological change & variation in levels of secondary metabolite compared to the mother stock.

Growth Performance

The regenerated plants did not show any morphological change and variation in levels of secondary metabolite when compared with the mother stock

Figure-4: Schematic representation of *in vitro* multiplication of *Withania somnifera*

MICROPROPAGATION OF *ALOE VERA*

कुमारी भेदिनी शीता तिक्ता नेत्त्या रसायनी।

मधुरा बृंहणी बल्या वृष्या वातविषप्रणुत्।

गुल्मप्लहियकृद्वृद्धि कफ ज्वर हरी हरेत्।

ग्रन्थ्याग्नि दग्ध विस्फोट पित्तरक्तत्वगामयान्।।

(भावप्रकाश)

Introduction

Aloe vera L: *Nature's all purpose healer.* It has acquired one of the highest levels of recognition for herbs among the public due to a wide range of applications from laxative to gentler moisturizer, herbal beverages and dietary supplements making it an excellent herb just above anything. Rightly said that *Aloe* is cure for all diseases.

Synonyms: *Aloe indica* Royle, *Aloe littoralis* Koening, *Aloe vera* Tourn. ex Linn., *Aloe barbadensis* Mill

Table-1: Vernacular nomenclature of *Aloe vera* L.

Hindi	Musabhar, Ghikanvar
Bengali	Ghirta Kumari
English name	Indian Aloe, Barbodos Aloe, Jafarabad Aloe, Curcacao Aloe
Gujarati	Kuwar patto
Kanand	Kolasoare, komarika, Maulisara
Malayanum	Kattavazha
Marathi	Korphad
Oudia	Kumari, Musahaboro
Sanskrit	Ghirta-kumari, Kumari, Ajara, Amara, Bhrngesta, Vipulasrava, Brahmhaghni, Taruni, Grhankanya, Grhakumari, Bala, Mrdu, Saruna, Atipittala, Kapila, Vistari, Vira,
Tamil	Chirukattalai, Kattalai
Telgu	Kalabanda

Family: Liliaceae

Aloe vera L enjoys a long history of lay acceptance as possessing healing or curative qualities (Gjerstad and Riner, 1968). The first document of *Aloe* was found in the city of Nippur, where Sumerian clay tablet included *Aloe vera*, among the plants of great healing power written around BC 2200. It is assumed that around BC 1550 Papyrus Ebers, an Egyptian, documented the detailed discussion of *Aloe*'s medicinal values, which included twelve formulas for mixing *Aloe* with other agents for both internal and external treatment. It is also believed that Alexander, the Great, had conquered Socotra Island, just to obtain *Aloe*, reportedly at the request of Aristotle. The use of *Aloe* plant is as old as the human civilization. In the first century AC, the Greek physician Dioscorides used *Aloe* for mouth infections, sores, and wounds and as a purgative. In the10th century, *Aloe* was used in England and during the 17th century, the East India Company frequently purchased *Aloe* from the king of Socotra. Further, the history says the people of Egyptians, Assyrians, and Mediterranean countries used the dried latex of *Aloe* plant and also the gel against worm infections to relieve headache, sooth chest pains, burns, ulcers, skin diseases and allergies. It was even observed that *Aloe* was placed as one of the commodities of cosmetic for the Egyptian first ladies (queens). The Bible also mentions the use of *Aloe* (John19: 39). Pliny, a Roman scholar had suggested the use of plant for preserving the dead bodies from decay. Even today, the Egyptians hang an *Aloe* plant over the door of a new house to provide a long and fruitful life for its occupants. In late 1940's, *Aloe* was used in the treatment of radiation burn victims after Hiroshima and Nagasaki, which led to the shimmering of healing properties of *Aloe*. Truly said by the Egyptians *Aloe* "The plant of immortality" (Crosswhite et al, 1984).

Morphology and Distribution

Aloe is a valuable ornamental and medicinal genus. It is a xerophytic, succulent and perennial plant with multiple tuberous roots and many fibrous supporting roots. It has turgid large green leaves in a rosette pattern. Leaves are few, sessile, densely crowned in the short stem, wide dilated bases, spreading below, then ascending. The leaves are 20-60 cm long, glaucose-green, narrow to long lanceolate, acuminate, and smooth except for the spiny teeth on the margin and have a thick smooth cuticle. The mature plant produces long inflorescence (50-100cm long). Flowers are of shades varying from orange-red to yellow corolla, cylindrical, bisexual with epi-calyx and six stamens, protogynous, ovary superior. Buds in the inflorescence are in dense raceme terminating the scapes. The plant is generally propagated through suckers. Its cultivation is easy and can flourish in the driest and poorest quality of soils. The plant can be separated into two basic products: gel and latex. *Aloe vera* gel is the leaf pulp or mucilage obtained as a thin clear jelly from the parenchymal tissue that makes up the inner portion of the leaves thought to have emollient and moisturizing effects and therapeutic properties (Tyler, 1993; Rund, 1996; Anonymous, 1976). The gel contains carbohydrate polymers, such as glucomannans and pectic acid, plus various other organic and inorganic compounds. *Aloe* latex commonly referred to, as *Aloe* juice is a bitter yellow exudate from the pericyclic tubules just beneath the epidermis of the leaves.

Aloe has naturalized throughout the tropics and warm regions worldwide. The exact origin of *Aloe* is rather uncertain, yet it is suspected to have come from South, East or North Africa (Rund, 1996). The introduction of several species of *Aloe* in India, East and West Indies, Europe and other tropical countries has been reported by

Grindlay and Reynolds (1986). It is planted in Indian gardens and also found in a semi-wild state in many parts of India.

Medicinal properties

The *Aloe* leaf gel is known to possess important biological properties such as anti-inflammatory, antibacterial, antitumor, antiallergic, infected wound healing by immuno enhancement and general tonic effect. It is purgative, aphrodisiac, alexiteric, useful in eye diseases, enlargement of spleen, liver complaints, vomiting, erysipelas, asthma, leprosy, jaundice and strangury (Kirtikar and Basu, 1975). It is also reported to exhibit antifungal activity against four common post harvest fruit pathogenic fungi (Saks et al, 1996).

It has been used as laxative, gentler moisturizer, herbal beverages and dietary supplements. The juice and liquid supplements stimulates the immune system to relieving stomach disorders to aiding digestion. Juice of the leaves is used on burns, colic, skin diseases, constipation, abdominal tumours, lumbago and flatulence. The exudate is used for helminthiasis and anthelmintic conditions in children. In addition, it is used in painful inflammations (Brueton, 1995), chronic, ulcers and opthalamia. The exudate is used for local applications. It is used for the treatment of eczematous skin under the name of musabbar. Pulp mixed with honey and turmeric is recommended in coughs and colds (Raina, 1982). A sweet confection prepared from the pulp of the leaves is also given in piles.

Part used: Whole plant, dried juice of leaves, pulp and root

Ayurvedic properties

Sr. No.	Ayurvedic Properties	Description
1	Rasa (taste)	Tikta (bitter), Katu (pungent), Madhura (sweet)
2	Gunna (properties)	Guru, Snigdha (unctuous), Picchila
3	Veerya (potency)	Sita (cooling)
4	Vipaaka (properties after digestion)	Katu (pungent)

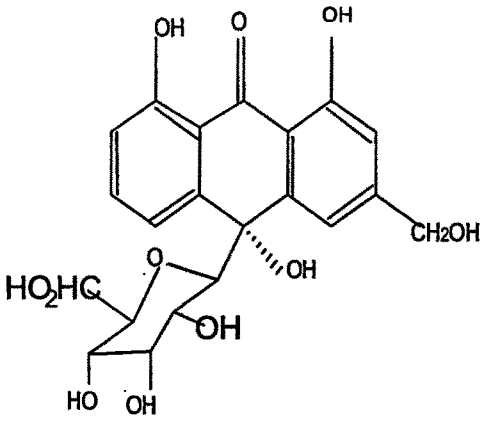
(Anonymous, 1999a; Anonymous, 1976)

Active constituents

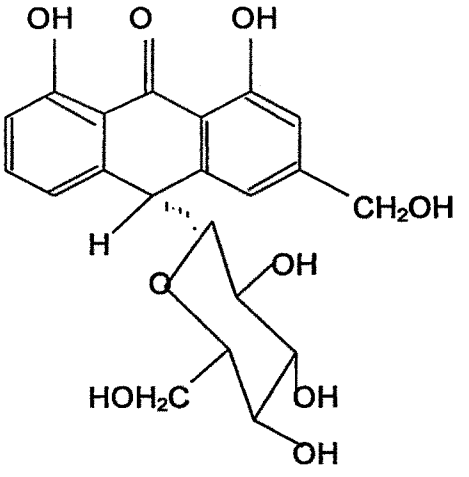
Leaves have therapeutic and laxative properties. Anthraquinones, especially aloin is the principal active constituent of *Aloe*, which is the mixture of glycosides (Mapp Rk et al, 1970). Aloin content varies from 10 to 30% depending on the species. Apart from aloins A (barbaloin) and B (isobarbaloin), there are hydroxyanthracene derivatives (25-40%). Barbaloin is the main glycoside in aloin, which is water-soluble and isobarbaloin is unstable compound. Further (3-4%) 7-hydroxyaloins A and B, Aloe-emodin, resins, Aloesin and its a glycone Aloesone, chromone derivatives and chrysophanol are also present.

Acemannan, a mannose-containing polysaccharide, has been reported as the main active substance present in *Aloe vera* filet (Hart et al, 1989). Acemannan, commercially known as Carrysin, is a linear polysaccharide composed of (1,4)-linked mannosyl residues, with C_2 or C_3 acetylated and some side chains of mainly galactose attached to C_6. In addition to polysaccharide, it is composed of simple sugars,

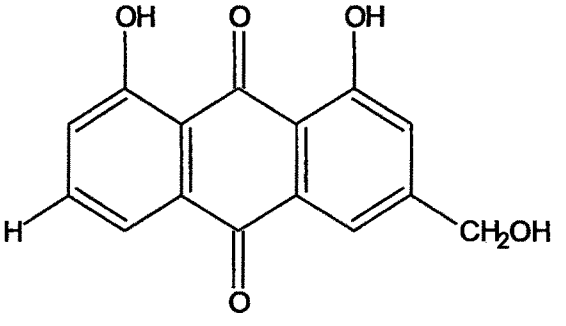
glycoproteins, lipids, proteins, enzymes, amino acids, vitamins, mineral and sterols. Mucilage is the movable layer between the more solid inner gel fillet and the stiff outer rind.



Aloin A



Aloin B



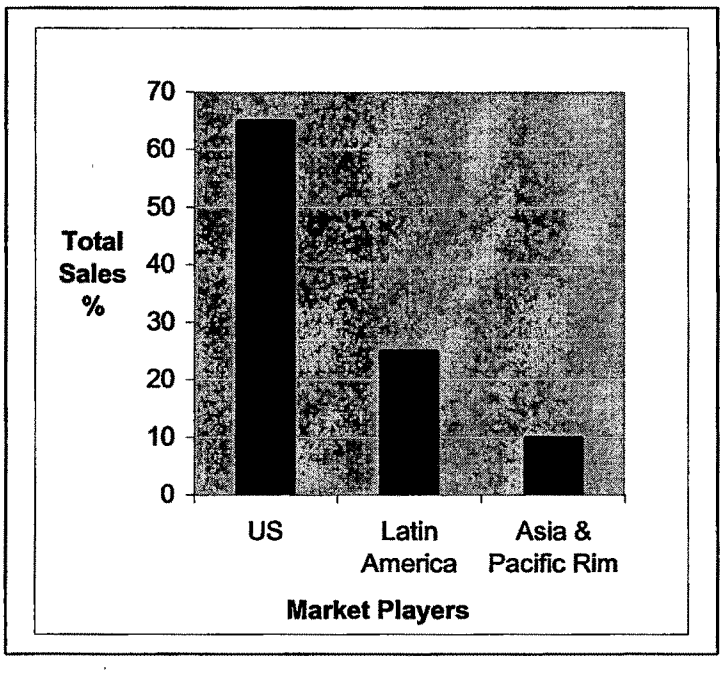
Aloe emodine

Fig-1: Structure of active compounds

Trade

The present trend of people diverting back to nature has made the herbal trade a booming business worldwide today. The overwhelming curing response of the *Aloe* towards many health problems has raised scientists as well as the consumers, to know more and more of *Aloe*'s benefits. The demand for *Aloe* derivatives is increasing compare to other leading herbals, which have led to severe difficulties in the recent past and lost the market. While *Aloe* appears to be virtually unaffected by the negative trend as other herbal products due to its multipurpose use and new and new products developing in market. As we know the commercialization of *Aloe vera* is a success story.

The Bar graph (Fig.2) denotes the relative raw material sale estimated by the International *Aloe* Science Council (IASC) by different major countries in near future.



Presently the raw material value of *Aloe* is currently $ 70-90 million globally and shall increase by 35% within the next five years. In the finished product market of *Aloe* is estimated to be over $ 35 billion globally in near future.

Figure-2 Raw material sales of *Aloe vera* (Estimated by IASC)

Today the value of China's *Aloe* industry is approximately $12 million but a ten-fold growth is anticipated within the next five to eight years. The Chinese demand for derivatives alone estimated to reach $2.4 billion by 2010. This means that China will soon become one of the major suppliers worldwide. The trend of using Green Foods and further organically developed has a great demand in the market of Japan. *Aloe vera* leaf food and beverage products are very popular in Japan at the present time. They used on large scale for various aspects of intestinal health and to moisturize the skin. Today many products, apart from therapeutic products are been made from *Aloe* (cream, hair lotion, balm, health drink, skin gel, shave gel, hair cleaner, richner, face pack, tooth cleaner, soap, etc.,) and the demand for *Aloe* for fresh gel or as formulation is increasing more and more. Though within last 10 years, large amount of research in prominent labs all over the globe on *Aloe*'s medicinal properties has taken a great pace, adulteration in *Aloe* business is also becoming a serious problem. As it is easily carried out through the addition of excess water to gels or maltodextrin to powders.

Cultivation

Aloe vera is cultivated on limited scale in India (Madhyapradesh, Andhra pradesh, Rajasthan and Gujarat), while in the United States, most of the *Aloe* is grown in the Rio Grande valley of South Texas, Florida and Southern California. Globally *Aloe* can be found cultivating in Mexico, the Pacific Rim countries, South America, the Caribbean, Australia and Africa also.

Conventional propagation is generally done through root suckers or rooted plantlets (side tillers) or very less by seeds. Large amount of male sterility is observed

due to irregularities during pollen mitosis and failure in fruit setting (Sapre, 1975). Further the rate of regeneration through seeds is also very low. The ideal time of plantation is after rainy season. Healthy sucker or side tillers can be planted closely at a distance of 50x 40x30 cm under low soil fertility and water stress condition. Around 60,000 – 65,000 rooted healthy plantlets are sufficient for the planting on a hectare of land.

Harvesting and processing

Aloe leaves are harvested from mature mother plant (10-15 months old) by sharp blades of knives. Three harvests can be done in a year. Yield can be obtained upto five years after planting. A spray of Ridomil – MZ or dithan Z 78 is recommended on the plants to avoid fungal infection after harvesting.

Freshly harvested plants are generally allowed to wilt and lose moisture in the field before transplanting. 45-55 tonnes of thick fleshy leaves per hectare can be obtained. Care must be taken in preparing the leafy plant material for drying or distillation. A concrete floor under shade can be used for drying the leaves.

Aloe is processed using the hand-filleted technique or whole leaf procedure. Hand filleted processing removes the inner gel while avoiding the yellow latex found next to the rind. Whole leaf extracts for consumption are prepared after sterilization and blending by grinding the entire leaf and then removing the bitter yellow component (Aloin) by charcoal filtration.

Commercial *Aloe* is obtained by evaporating the yellow exudates, which drains from the leaves cut from the plant. The juice is concentrated by boiling and then poured into tins.

The enormous demand globally for *Aloe* products, slow conventional propagation, widespread male sterility and variations in amount of active components in different varieties of *Aloe* has provoked for an urgent need to mass propagate the elite species through biotechnological approaches to act as the seedling material. To overcome this problem, plant tissue culture, a promising tool of biotechnology is one of the alternative ways, which can render in generating large-scale propagation of elite plant species. The present study was therefore undertaken with the objectives of the micropropagation of *Aloe vera* and to analysis the secondary metabolites.

***In vitro* approaches**

Little work has been done on *in vitro* culture of *Aloe* species, as establishment of primary cultures has been the major constraint probably because of the exudation of phenolic substances from the explant into the culture medium resulting in the death of the explant. Linsmaier and Skoog, (1965) reported shoots regeneration from callus on medium supplemented with 0.2mg/l 2,4 D and 1mg/l KN. While Sanchez et al, (1988) reported *in vitro* studies on leaves of *Aloe barbendensis*, Natali et al, (1990) reported shoots from meristem tips on MS medium supplemented with 1.1 µM 2,4-D and 2.2µM BA and Roy and Sarkar, (1991) reported callus formation on reduced 2,4-D and increased KN concentration. Shoot regeneration from the axillary node has been reported earlier (Meyer and Staden, 1991) using IBA. Similarly Richwine et al, (1995) reported the induction of shoots using zeatin. Chaudhuri and Mukundan, (2001) reported shoot formation (20 shoots) on 44.4µM BA + 160 µM AS + 0.49µM IBA. While Liao et al, 2004 studied the effects of three factors 8.88 µM BA, 0.3 mgl^{-1} NAA, 30 g l^{-1} sucrose and 0.6 g l^{-1} PVP on shoot induction of *A. vera* var. *Chinensis*.

Materials and Methods

Plant material

The suckers of size (4cm) from one-year-old plant of *Aloe vera* were collected from the Botanical Garden of the Maharaja Sayajirao University of Baroda. The rhizome and axillary node was used as source of explants.

Sterilization

The rhizome explants were excised (2cm) and were thoroughly washed under running tap water (30 min) and treated with 0.2% (v/v) aqueous detergent (Teepol) (15 min) followed by repeated rinsing with distilled water. Subsequently, the explants were treated (30 minutes) with (0.1%, w/v) Bavistin (Carbendenzim; BASF, India) and followed by through rinsing with distilled water. Then the explants were surface sterilized with 70% (v/v) ethanol (1 min) and 0.03 % (w/v) $HgCl_2$ (4 min). Finally the explants were washed thoroughly (three times) with sterilized distilled water.

Initiation of cultures

The rhizome explants (0.8cm) was excised and inoculated on MS (Murashige and Skoog, 1962) medium supplemented with different concentration of plant hormones BA (2.2- 44.4 µM), IBA (0.49–1.96 µM) and IAA (1-2.88 µM) with 3% sucrose. The pH of each medium was adjusted to 5.8 prior to autoclaving. The medium and other necessary tools were sterilized by autoclaving at 121°C (15 min). The cultures were incubated in the culture room with 25 ± 1°C temperature under a 16-hr photoperiod (50µEm^{2}s^{-1}irradiance) provided by cool white fluorescent tubes (Philips, India).

The axillary nodes were also excised from *in vitro* cultures and were cultured on MS medium supplemented with growth hormones for multiple shoot generation. Subsequently, the generated shoots were subcultured at every three-week interval on the same medium.

Induction of roots and acclimatization in the soil

Regenerated shoots (3-4 cm) were excised and transferred to half and full strength agar based MS medium with different concentrations of IAA (0.0–2.88 μM) for root induction. Well developed shoots, which formed roots, were removed from the culture vessels, washed gently under running tap water and planted in the plastic pots then to plastic bags containing a mixture of sand, soil and farmyard manure (1:1:1). Experiments with various other potting mixtures were also tried out. The plantlets were kept in the greenhouse for acclimatization (2-4 weeks) before their subsequent transfer to the field. Plants were gradually exposed to the normal conditions and transferred to Medicinal Garden of GSFC Science Foundation.

Phytochemcial analysis

Qualitative analysis was carried out through thin layer chromatography. The aloin from leaf of tissue culture raised plants and mother plant were taken and subjected to phytochemical analysis. 1mg of aloin in 1ml of methanol was dissolved and shaken vigorously. Samples were spotted on Silica gel GF 254 precoated TLC plates (0.2mm thickness). Solvent system used was ethyl acetate - methanol - water (100:16.5:13.5). The plate was dried and observed under UV lamp.

Percentage of barbaloin was also calculated for the mother plant and *in vitro* generated plants as per the method mentioned in Indian Herbal Pharmacopoeia. The powdered sample (0.3 gm) moistened with 2ml methanol by adding 80 ml of water and warming at 60°C. it was cooled, filter into volumetric flask and dilute to 1000ml with water. 10ml of this solution was added to 1ml of 600g/l solution of ferric chloride and 6ml of HCl. Then the solution was heated and refluxed (4 hrs). Then it was transferred to a separating funnel, which was rinsed with water (4ml) and NaOH (4ml). Subsequently washed three times by ether (20ml). Then the organic phase was diluted with ether and evaporated (20ml) carefully to dryness on a waterbath. The residue was dissolved in 10ml of 5g/l solution of magnesium acetate in methanol. The absorbance was measured at 512nm using methanol as the compensation liquid.

Confirmation test for anthraquinone were also carried out by the following methods:

1. Borax test: In 10-ml solution, 0.5 gm of borax powder was heated.
2. Bromine test: In 5ml sample solution, 5ml of bromine solution was added.
3. Modified Borntrager test: In 0.1 gm of sample, 2 ml of 5% solution of ferric chloride and 5ml of dil. HCL was heated on boiling water for 5 minutes. It was cooled and gently shaken with organic solvent. Organic solvent layer was separated and an equal volume of ammonia was added.
4. Nitric acid Test: In 5ml of solution, 2ml of concentrated nitric acid was added.

Results and Discussions

Rhizome and axillary node of *Aloe* grown in the Botanical Garden were used as explants for initiation of cultures. A severe problem of fungal contamination was noticed during the establishment of explants in cultures. Use of a fungicide helped in reducing the problem of contamination and about 95% explants remained contamination free. The explants treated 30 minutes with (0.25%; w/v) bavistin gave the best results for maximum control of fungal contamination (Table-2).

Table-2: Effect of increasing concentration of Bavistin in pretreatment solution on the rate of contamination in *Aloe vera*

Concentration of Bavistin (%; w/v)	Contamination (%) Mean ± SE
0.00	99 ± 9.2
0.15	66 ± 3.5
0.2	16 ± 4.6
0.25	0.0 ± 0.0

The exudation of phenolics (browning) hinders the growth of the culture and was the major hindrance in establishing *in vitro* cultures of *Aloe vera* (Plate-5.1A, B). Addition of anti-oxidants such as ascorbic acid and PVP to the culture medium has been reported in many plants such as *Aloe vera* (Roy and Sarkar, 1991; Liao et al, 2004), *Madhuca latifolia* (Singh et al, 1992) and *Anacardium occidentale* (Sudripta et al, 1999) for alleviating the browning problem. In present study, *Aloe* explants seldom became brown when ascorbic acid (0.08 gl^{-1}) was included in the medium. Further cultures were transferred to the fresh medium within two weeks for better response (Plate-5.1C).

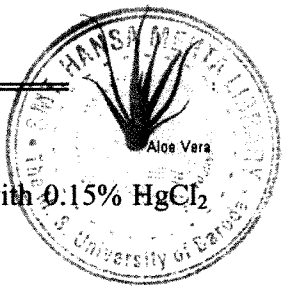
Different concentration of $HgCl_2$ was used for surface sterilization. The best

Plate- 5.1

A & B. The exudation of phenolics

C. Response of ascorbic acid in the medium

Photoplate - 5.1



results for maximum contamination free cultures were established with 0.15% $HgCl_2$ and 4 minutes (Table-3).

Table-3: Effect of $HgCl_2$ on control of contamination in *Aloe vera*

Concentration of $HgCl_2$ (%) (4 minutes)	Contamination (%) Mean ± SE
0.02	100 ± 2.6
0.05	66 ± 1.5
0.08	46 ± 2.1
0.1	15.6 ± 1.3
0.15	0 ± 3.05
0.2	**

** explants got burned.

Initially rhizome enlarged in the MS medium supplemented with 4.4μM BA, 2.88 μM IAA and 0.98 μM IBA. Large number of shoot bud initiation was observed within a period of 3 weeks of inoculation in the same medium (Plate-5.2A). To our knowledge, there are no published reports for multiplication of *Aloe vera* using rhizome explants. Almost 100% of the rhizome explants responded for the growth in the medium containing 4.4 μM BA, 2.88 μM IAA and 0.98 μM IBA. A large number of shoot buds (35 shoots) was observed on the rhizome explant (Table-4) (Plate-5.2B). The sprouted buds developed into young shoots (2-3 cm) with 2 leaves within 2 weeks. The shoots were isolated, cultured on the same medium and later the axillary nodes were excised from the *in vitro* cultures for multiplication. The remaining rhizome was again cultured in the fresh medium for initiation of other shoot buds.

Initiation of number of shoot buds from the node was observed after 2 weeks on MS medium supplemented with BA, IAA and IBA (Plate-5.2C). Chaudhari and

Mukundan, (2001) had reported shoot induction in *Aloe vera* on high concentration of growth hormones 44.4μM BA, 160 μM AS and 0.49μM IBA using shoot tip explants.

Table – 4: Effect of various combination of auxin and cytokinin on bud sprouting on rhizome explant of *Aloe vera*

BA (μM)	IAA (μM)	IBA (μM)	Number of shoots/ explant Mean ± SE
0.0	0.0	0.0	1.02 ±0.2
2.2	0.0	0.0	1.7 ± 1.02
2.2	0.57	0.0	6.6 ± 3.5
2.2	1.14	0.98	4.0 ± 2.6
4.4	1.14	0.98	15.0 ± 5.1
4.4	**2.88**	**0.98**	**35.0 ± 1.02**
6.6	1.14	0.98	10.1 ± 0.13
6.6	2.88	0.98	15.5 ± 6.3

Earlier shoot regeneration from the axillary node in the same species has been reported by Meyer and Staden, (1991) using IBA. While Roy and Sarkar, (1991) and Natali et al, (1990) reported very few shoots regeneration on medium containing 2,4–D and KN. Aggrawal and Barna (2004) reported only 5 shoots from shoot tip explants in the liquid medium containing 22.2 μM BA and 0.2 mg/l IBA and 10 mg/l citric acid. Sanchez et al (1999) had reported indirect shoot regeneration from leaf through callus intervention on MS medium with 2,4 D and Kinetin. Earlier induction of shoots using zeatin in *Aloe sps* has also been reported (Richwine et al, 1995). Liao and Tan, (1999) generated shoots on MS medium containing 8.88μM BA and NAA (0.3 mgl^{-1}), in *A. vera* var. *Chinensis*. This indicates that hormonal requirement for axillary shoot formation appears to be different for the same species.

Plate-5.2

A. Bud break from rhizome explant of *A. vera*

B. Numerous shoot formation on MS medium supplemented 4.4 µM BA, 2.88 µM IAA and 0.98 µM IBA

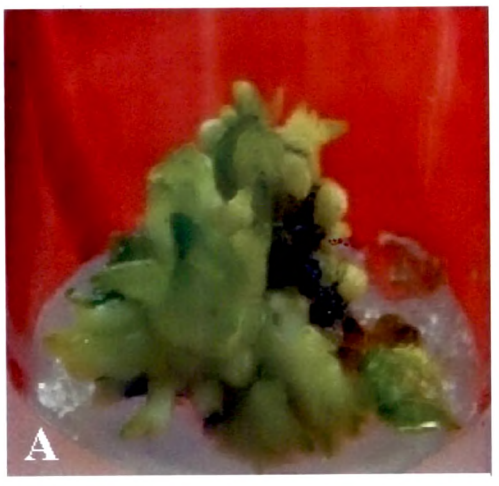
C. Bud break from nodal explant of *A. vera*

D. Induction of multiple shoot formation from nodal explant

E. Shoot elongation

F. Root induction

Photoplate - 5.2



In present study MS medium supplemented with 4.4 µM BA, 2.88 µM IAA and 0.98 µM IBA showed the maximum multiplication with 12-13 shoots (Plate-5.2D). In contrast, a genus of a related family (Liliaceae), *Chlorophytum*, required a very high concentration of 22.2 µM BA for shoot regeneration (Purohit et al, 1994) while another genus *Curculigo orchioides* required very less concentration of 0.44µM BA (Wala and Jasrai, 2003) for shoot regeneration.

Further increase in the concentration of BA had no effect on the number of multiple shoots. In contrast, in *Crinum* (Amaryllidaceae), the enhanced level of BA had stimulating effect on the total number of regenerated plantlets (Slabbert et al, 1993). These results confirmed that some plant species have enough levels of endogenous hormones and does not require high levels of exogenous growth regulators for plant regeneration (Hussey, 1982).

The elongation of shoots (3-4 cm) was observed within a week time (Plate-5.2E). The shoots (4 cm) were separated from the *in vitro* culture for root induction. Literature indicate that cytokinin inhibit while auxin promote root induction. The inhibitory effect of cytokinin free bases on rooting has been reported (Drewes and Van Staden 1989). The shoots were cultured individually on half and full strength MS medium with or without auxin for root induction. No root formation was observed on half and full strength MS basal medium, but when supplemented with IAA (0.0-2.46 µM) moderate to profuse rooting occurred in 95-97% of the cultured shoots.

The maximum root formation response was observed on half strength MS media containing 1.14 µM IAA (Plate-5.2F). Agarrwal and Barna, (2004) have reported (2.8) roots per shoot on full strength MS basal medium in *Aloe vera*. The

maximum number of roots per shoot (4) with (3.5 cm) was achieved within the period of 12 days.

Regular subcultures through induction had no adverse effect either on rate of proliferation or on the quality of the plantlets obtained from both the explants (Plate-5.3A). *In vitro* shoots were excised from the rhizome and nodal explants and cultured on the same medium for further multiplication. The number of shoots per subculture was standardized from commercial point of view. Multiplication rates (12-14 shoots) from the axillary node and (32-35 shoots) from rhizome remained constant even after 7 subcultures (Table-5).

Table-5 Effect on number of shoots from rhizome and axillary explants during different subcultures on *in vitro* cultures of *Aloe vera*

Number of Subculture	Average number of shoots /rhizome culture	Average number of shoots/node
1	35	12
2	36	13
3	34	13
4	34	13
5	33	12
6	33	12
7	32	11
8	29	10

In vitro shoots with roots were subjected for acclimatization. The plants were gently removed from the medium, washed with 0.5% bavistin and transferred to Greenhouse in plastic pots (Plate-5.3B) and bags containing the potting mixture sand, soil and farmyard manure (1:1:1). 100% survival rate was observed in the Greenhouse (Plate-5.3C). After 2 weeks of acclimatization, the hardened plants (10-12 cm) were

Plate- 5.3

A. Subculture of regenerated shoots of *A. vera*

B. In greenhouse small plantlet (2-3 cm) transferred to plastic small pot

C. The hardened plantlet (5-6 cm) in plastic pot

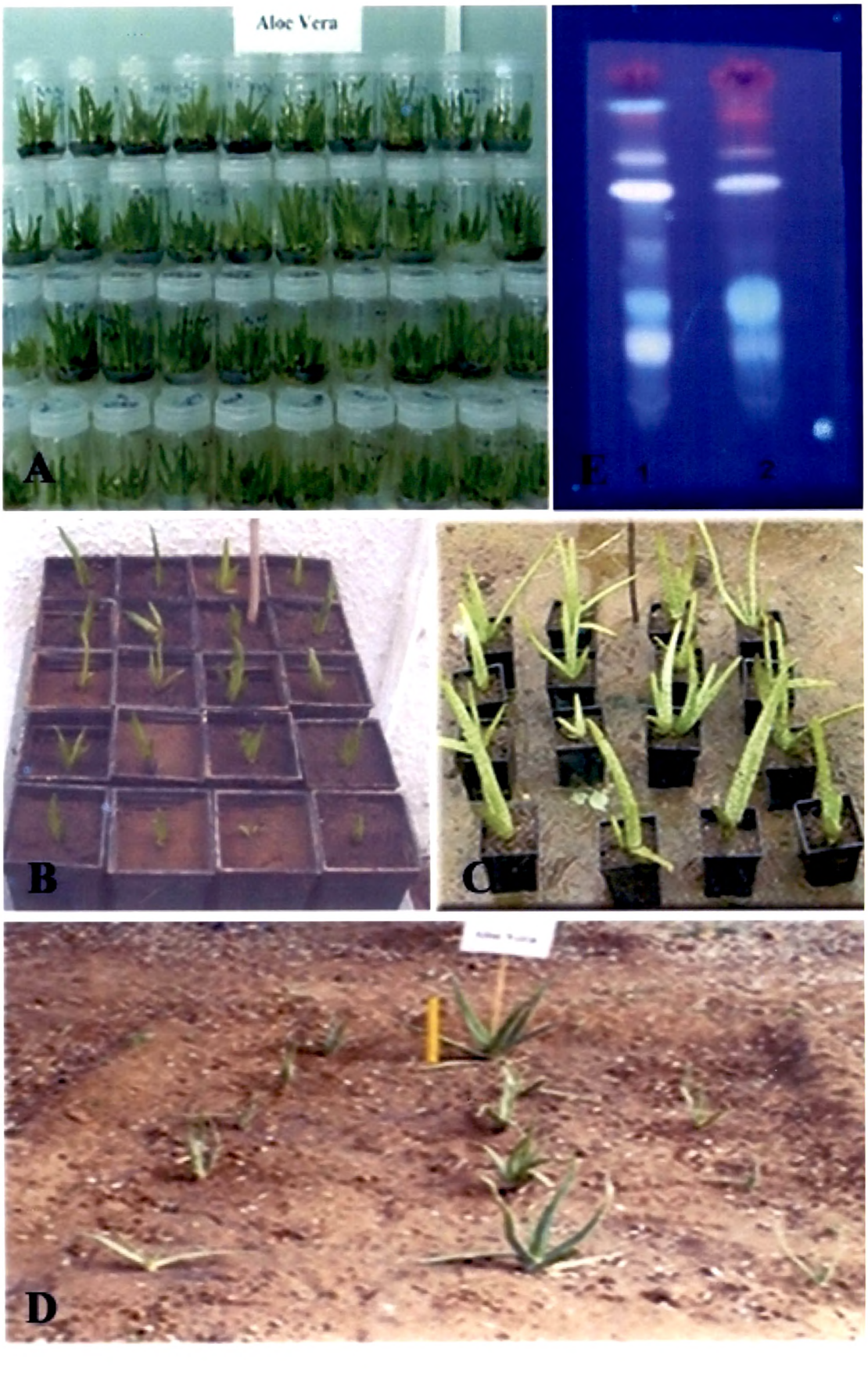
D. Field transferred *in vitro* grown plantlets showing vigorous growth

E. Thin layer chromatography (Yellow spot : Aloin)

   1. Tissue culture plant

   2. Normal plant

Photoplate - 5.3



successfully established in the soil of Medicinal Garden of GSFC Science Foundation (Plate-5.3D). Among 300 plantlets transferred to soil, as many as 291 (97%) were found to survive on the soil in comparison to the 65-92% survival of plantlets recorded in the experiments of previous workers (Agarrwal and Barna 2004; Liao et al, 2004). No morphological variation of any nature was observed among the *in vitro* raised plants when compared with the mother stock.

Phytochemical analysis

Identity and confirmative test for anthraquinone in *Aloe vera* were also carried out by the various methods. Aloin positively responded to the test for anthraquinone glycosides with modified Borntrager test by producing pinkish red colour. Borax test resulted in formation of green fluorescence due to the presence of *Aloe* emodine, nitric acid test resulted in reddish orange colour and bromine test by producing pale yellow precipitate of tetrabromalion.

Qualitative analysis was carried out through thin layer chromatography. The aloin from leaf of tissue culture raised plants and mother plant was subjected to phytochemical analysis. 1mg of aloin was dissolved in 1ml of methanol and shaken vigorously. Samples were spotted on Silica gel GF 254 precoated TLC plates (0.2mm thickness). Solvent system used was ethyl acetate- methanol – water (100:16.5:13.5). The plate was dried and observed under UV lamp. The phytochemical analysis reveals the presence of Aloin, Aloe emodine, monosaccharides, glucosides, sterols triterpenes. Aloin appeared as brownish spot at Rf value 0.45, which changed to yellow color under UV lamp (Plate-5.3E).

Analytical method and percentage of hydroxyanthracene derivatives (barbaloin) was also calculated as per the method mentioned in Indian Herbal Pharmacopoeia for the mother plant and *in vitro* generated plants. We observed percentage of hydroxyanthracene of the mother plant (5.68) and *in vitro* generated plant (5.88).

Thus, *Aloe vera* could be successfully multiplied *in vitro* from the rhizome and axillary node.

Explant

↓ Rhizome and axillary node

Surface sterilization

↓ (0.25%) Bavistin (30 min)
(0.15%) $HgCl_2$ (4 min)

Multiple shoot induction and proliferation

↓ BA (4.4μM) + IAA (2.88μM) + IBA (0.98μM)

Root Induction

↓ Half MS + IAA (1.14μM)

Greenhouse

↓ Potting mixture: FYM, Sand and Soil (1:1:1)

Field

↓ 300 plantlets were transferred to the field

Growth performance

The regenerated plants did not show any morphological change when compared with the mother stock

Figure-3: Schematic representation of *in vitro* multiplication of *Aloe vera* L .

MICROPROPAGATION OF *PLUMBAGO ZEYLANICA*

चित्रकोग्निशमः पाके कटुः शोफकफापहः।

वातोदरार्शः ग्रहणीक्रिमिकण्डूतिनाशनः।।

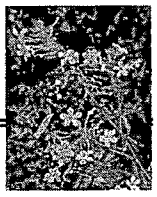
(राजनिघण्टु)

चित्रकः कटुका पाके वहनिकृत् पाचनो लघुः।

रूक्षोष्णो ग्रहणीकुष्ठशोथार्शः कृमिकासनुत्।

वातश्लेमहरो ग्राहि वातर्शः श्लेष्मपित्तहृत्।।

(भावप्रकाश)



Introduction

Plumbago zeylanica Linn (Chitrak) belonging to the family Plumbaginaceace is an important medicinal plant. It is native to Southern India and Malaysia and is now naturalized in much of South East Asia and in Africa. It is grown as a perennial herb in most parts of India, but on larger scale in the plains of West Bengal and Southern India.

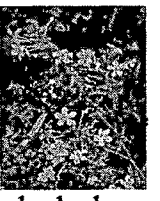
Table-1: Vernacular nomenclature of *Plumbago zeylanica* Linn.

Local name	Chitrak
English name	Ceylong leadwort
Sanskrit	Citraka, Agni, Anala, Dahana, Pathi, Vanhisamjnaka, Vyala, Osna, Jyoti, Pavaka, Sabala, Aruna, Hutasana, Sura, Satha, Jvala, Sardula, citrapali, Sikhi, Krsanudahana, Varuna, Citranga, Dvipi, Jyotiska, Hutabhunka
Hindi	Chitrak, Chita, Chitra
Gujarati	Chitaro, Chitrak
Bengali	Chitrak, Chita, Chitra
Kanand	Vaahini
Malayanum	Tumpukotuveli
Tamil	Venkotiveli Cittira mulam
Marathi	Chitraka, Chitramula
Telgu	Tellachitramulamu
Oudia	Chita mulo

Family: Plumbaginaceae

Morphology and Distribution

A perennial sub scandent shrubs, glabrous with white flowers in elongated terminal spikes. Fruit globose capsule and oblong pointed contained in viscid

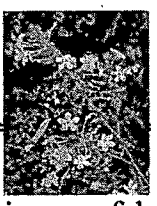


glandular persistent calyx. Found throughout India. Common in wastelands, hedges and in forests.

Medicinal properties

Plumbagin, an alkaloid, used primarily in research designed to exploit its properties as a superoxide generator, an antibiotic, an antineoplastic agent, germicide, stimulates muscular tissue in smaller doses and paralyses in larger ones. It is also used in treatment of cancer, rheumatoid arthritis, dysmenorrhea, and contusion of extremities.

Extracts of the root have been reported to be a powerful poison (Premakumari et al, 1977). The root is pungent, diuretic, germicidal, vessicant, abortifacient and has a presence of an alkaloid Plumbagin. It is used as an anticancer drug (Kirtikar and Basu 1975; Modi 1961; Krishnaswamy and Purushottamam, 1980; Pillai et al, 1981; Jayaraman, 1987; Parimala and Sachdanandam, 1993), antimalarial (Likhitwitayawuid et al, 1998), antimicrobial (Didry et al, 1994), cardiotonic (Itoigawa et al, 1991) and anti-fertilityaction (Bhargave, 1984). It stimulates the secretion of sweat urine and bile and has stimulant action on the nervous system. Roots are specially used in treatment of rheumatism, skin disease, diarrhea, influenza, ulcers, leprosy, enlarged spleen, piles and anasarca. It is also used as an appetizer. Milky juice is used as application in scabies and unhealthily ulcers. Its paste is applied externally in leprosy. Coconut oil is processed with the root to a straw yellow colour is used as a hair tonic which stimulates hair growth just like cantharise oil in Kodiveli. Externally a paste of leaves and root is applied to painful rheumatic areas and itchy



skin problems. The paste acts as a counter-irritant. Tincture of root bark is powerful sudorific and antioperiodic.

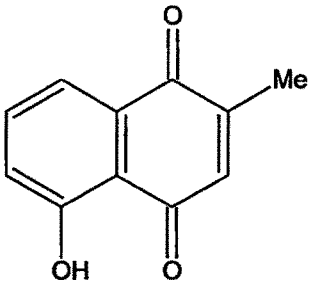
Part used: Seeds and roots

Ayurvedic properties

Sr. No.	Ayurvedic Properties	Description
1	Rasa (taste)	Katu (pungent)
2	Gunna (properties)	Laghu (light, easy to digest), Ruksa, Tiksa
3	Veerya (potency)	Osna
4	Vipaaka (properties after digestion)	Katu (pungent)

(Anonymous, 1999a; Anonymous, 1976)

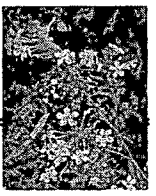
Active constituents



Plumbagin

Fig-1: Structure of active compound

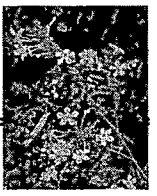
Plumbagin (2-methoxy-5-hydroxy-1,4-naphthoquinone), a natural and an orange–yellow naphthoquinone pigment, in small doses has stimulant action on the central nervous system, on muscle pain and on the secretion of sweat, urine and bile.



***In vitro* approaches**

Very less work has been reported on *in vitro* studies on *Plumbago zeylanica*. Rout et al, (1999) reported callus cultures from leaf and stem explants on 4.4 μM BA and 1.42 μM IAA and the same group Rout and Das (2002) reported direct plant regeneration from leaf explants on MS medium supplemented with 6.7 μMBA, 1.4 μM IAA and 370 μM Adenine sulphate. While Harikrishnan and Hariharan, (1996) reported shoot formation from the nodal explants of *Plumbago* rosea. Previous experiments to obtain this quinone *in vitro* showed that plumbagin was best synthesized and accumulated in *Plumbago zeylanica* (Heble et al, 1974), *Drosophyllum lusitanicum* (Nahalka et al, 1996), Drosera natalensis, D. capensis (Crouch et al, 1990) and Drosera gigantea (Budzianowski, 2000).

There are few reports on *in vitro* studies on *Plumbago* sps, despite the commercial interest in plumbagin. At present, the most exploited source of plumbagin is the roots of *Plumbago* spp. (*Plumbago europea, P. rosea, P. zeylanica*). The reports on cultivation and breeding are limited. Propagation through seed is unreliable due to poor germination and death of young seedlings under natural field conditions (Anonymous, 1989). Further, these plants grows quite slowly and it takes long time until the roots are suitable for use (Kitanov and Pashankoy, 1994). So alternative tool for mass multiplication of elite plant species is the tool of biotechnology-micropropagation. The present study was therefore undertaken with the objectives of the mass multiplication of *Plumbago zeylanica*.



Materials and Methods

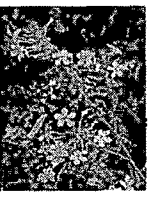
Explant

Healthy plants of *Plumbago zeylanica* were collected from Botanical Garden of the M. S. University of Baroda. These plants served as the source of explants. These plantlets were well maintained in the medicinal garden of GSFC Science Foundation. The plants were watered twice a day for healthy growth of plants. A day or two before explant collection, the elite plants were sprayed with 1.0 % (w/v) bavistin solution in order to reduce contamination. Axillary node and leaf were used as explant for experiments.

Sterilization

The explants were initially washed thoroughly under running tap water for 30 minutes to remove soil then treated with by (0.2%; v/v) aqueous surfactant Teepol (BDH, India) (15 min) followed by repeated rinsing with distill water. Subsequently then explants were pre-treated (20 min) with (0.1%; w/v) fungicide – Bavistin (Carbendenzim; BASF, India) and serial rinsing with distill water. Further surface sterilization was done under aseptic conditions in a Laminar Airflow chamber (Lab services, India)

A two-step sterilization procedure was followed. Pre-treated explants were surface sterilized with (70%; v/v) ethanol (1 min) and followed by (0.7%; w/v) $HgCl_2$ (4 min). The remnants of sterilizing agents were washed off by rinsing the explants 5 to 6 times with sterilized double distilled water. The explants were blotted dry for few minutes on a sterile filter paper under aseptic conditions.



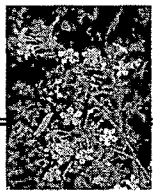
Culture medium and conditions

The explants were cut into appropriate size and placed aseptically onto MS (Murashige and Skoog, 1962) medium supplemented with different concentration of auxins and cytokinins with 3% (w/v) sucrose. For the multiplication, shoots generated from different explants were excised and sub-cultured as *in vitro* explants to MS medium supplemented with various concentrations of BA /KN and IAA. Cultures were incubated with at 25 ± 1°C with a relative humidity of 70% under a 14-hr photoperiod and 50 $\mu Em^{-2}s^{-1}$ irradiance provided by cool white fluorescent tubes (Phillips, India).

Induction of roots and acclimatization

In vitro well-developed regenerated shoots (3-5cm) of *Plumbago zeylanica* were isolated and were subjected to induction of roots on MS medium supplemented with different concentrations of auxins. Subsequently they were transferred to the Green house and then to field. Well-rooted plantlets of *Plumbago zeylanica* were removed from the culture vessels. Roots were gently washed to remove agar. The regenerated plantlets (4-6cm) were transferred to small sized plastic pots containing various potting mixtures in different ratios. Small plantlets (2-3cm) were transferred to portray with different potting mixtures. They were initially kept under greenhouse for 2-3 weeks. The plants were frequently subjected to mist irrigation with spraying bottle for 1-3 weeks. Later the hardened plants were transferred to Medicinal Garden of GSFC Science Foundation

Tissue culture raised as well as vegetative grown plants were compared for their morphology and growth parameters.



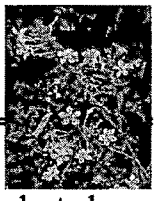
Phytochemical analysis

Qualitative analysis was carried out through thin layer chromatography. The shade-dried roots of *in vitro* raised plants and mother plants were crushed into powder form and were subjected to phytochemical analysis (Harborne, 1964).

Results and Discussions

The nodal explant were cultured on basal MS medium and in medium supplemented with cytokinins (BA or KN) and auxin (IAA). No bud formation was observed in basal medium. Bud break on the nodal segment was achieved on MS medium with 6.7 μM BA and 1.4 μM IAA (Plate-6.1A) within two weeks, while no bud formation was observed in the medium containing KN (Table-2). Although BA and KN are equally effective in inducing bud formation as reported, beneficial effect of the former compared to the latter are vouched for in several publications. But in contrast, the role of BA in bud breaking has been recorded for many other medicinal plants such as *Hedeoma multiforum* (Adolfina et al, 1997), *Plumbago rosea* (Harikrishnan and Hariharan, 1996; Das and Rout, 2002) and *Wedelia calendula* (Emmanuel et al, 2000). The study demonstrates the superiority of BA as a shoot inducing cytokinin in the *in vitro* induction of adventitious shoots from axillary buds of *Plumbago zeylanica.*

The explants inoculated in the MS medium containing BA and IAA developed small buds within 2 weeks of culture. However, no further growth occurred when maintained in the same medium. Hence they were transferred to MS medium with low concentration of BA (1.1-6.7 μM). The buds differentiated into shoots after two weeks of transfer (Plate-6.1B). Among the different concentration of growth hormones tested, 4.4 μM BA and 1.4 μM IAA elicited the maximum number of shoots (12 multiple shoot) from nodal explants (Table-3). Shoot multiplication through the formation of buds resulted into higher number of shoots in comparison to the cultures maintained in the MS medium with BA and IAA giving direct shoot initiation and multiplication. This results is in agreement with results of Chetia and

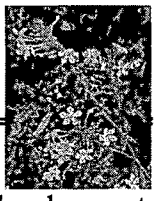


Handique, (2000) in *P. Indica.* Direct shoot regeneration from nodal explants have been reported earlier on higher concentration of growth hormones in *Plumbago* sps. (Selvakumar et al, 2001) on MS medium with 27.2 µM AS and 2.46 µM IBA. Similarly, Verma et al, (2002) also reported rapid propagation of *P. zeylanica* with maximum 4 multiple shoots per nodal segment with 8.87 µM BA and 0.49 µM IBA.

Table-2: Effects of different combinations of growth hormones on bud break through nodal explant of *P. zeylanica*

Concentrations (µM)			Response (%) (Mean ± SE)
BA	**KN**	**IAA**	
0.0	0.0	0.0	0
2.2	-	1.4	5 ± 0.06
4.4	-	1.4	45 ± 0.09
6.7	**-**	**1.4**	**90 ± 0.01**
6.7	-	2.8	75 ± 0.27
8.8	-	1.4	59 ± 0.31
8.8	-	2.8	55 ± 0.16
-	0.46	0.0	0
-	2.32	1.4	0
-	4.65	1.4	Callusing
-	4.65	2.8	Callusing

The present study exemplifies a positive modification of shoot induction efficacy on MS medium with low concentration of an auxin and cytokinin (Plate-6.1C). Our results are agreeable with Das and Rout, (2002) findings. Excision and



culture of the nodal segments from *in vitro* derived shoots facilitated the development of increased number of shoots. The elongation of shoots (3-4 cm) was observed in the same proliferation medium within two weeks of incubation (Plate-6.1D).

Table-3: Effects of different combinations of BA and IAA on MS medium on shoot formation through nodal explant of *P. zeylanica*

Concentrations (μM)		**Response (%)**	**Number of shoots/node* (Mean ± SE)**
BA	**IAA**		
0.0	0.0	0.0	0.0
2.2	0.5	10	1.5 ± 0.26
4.4	0.5	45	4.5 ± 0.16
4.4	**1.4**	**85**	**12.1 ± 1.34**
4.4	2.8	69	5.5 ± 0.21
6.7	1.4	58	4.8 ± 2.26
8.8	1.4	55	4.1 ± 1.20

On an average within two subcultures, single node explant generated 120 shoots in presence of 4.4 μM BA and 1.44 μM IAA. The shoot multiplication at this enhanced pace was also achieved in subsequent culture upto 5-6 cycles (Plate-6.2C).

Callus was initiated from young leaves on MS medium supplemented with BA (0.0 - 8.8 μM), IAA (0.0 - 2.88 μM) and AS (160 μM). The explants enlarged and developed calluses at the cut surfaces in the medium within two weeks of inoculation and this subsequently covered thee entire surface of the explants within 3 weeks of

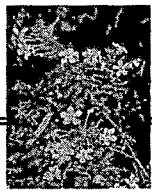


Plate-6.1

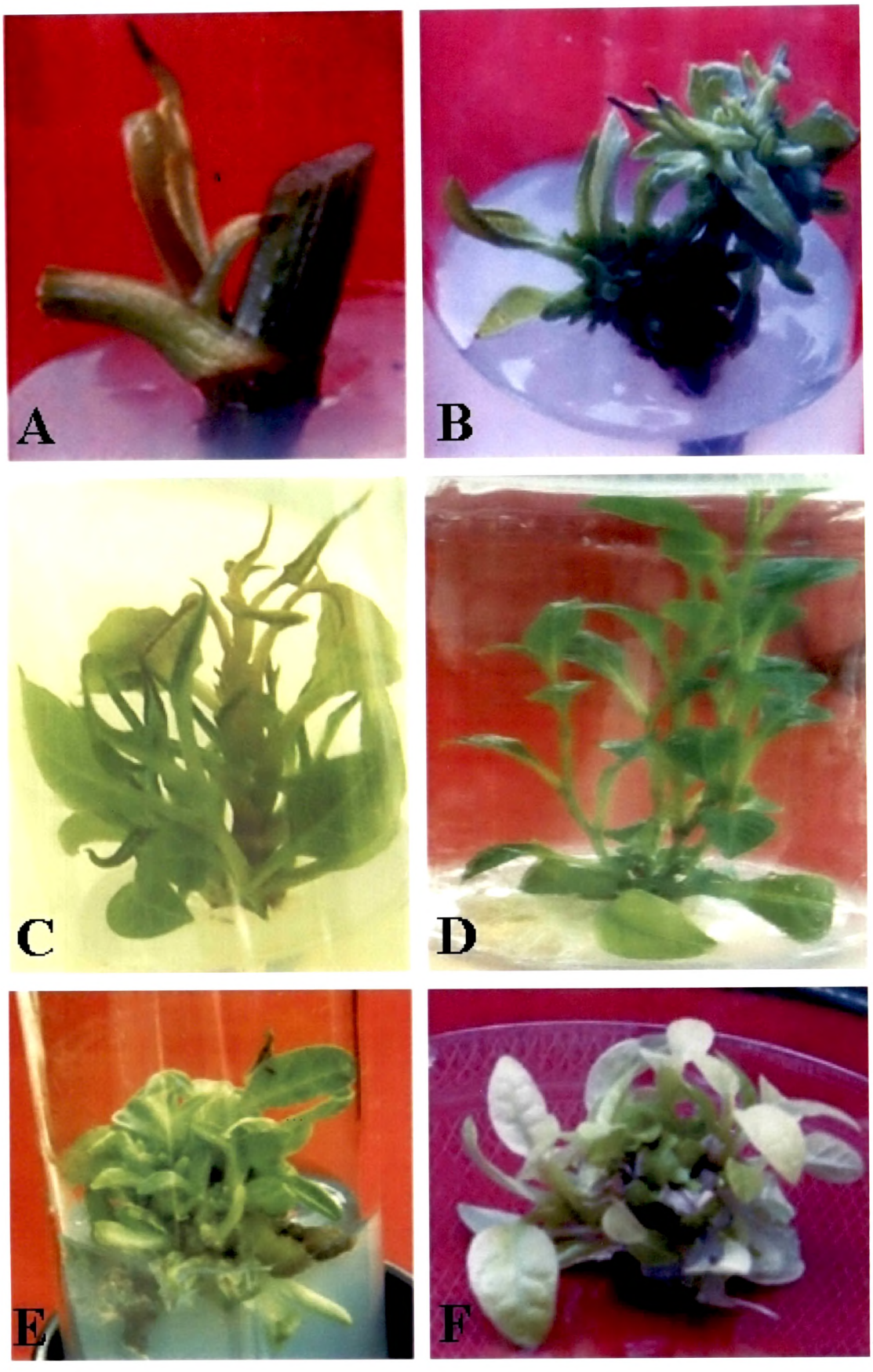
A. Bud break from nodal explant of *P. zeylanica*

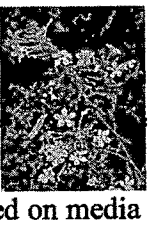
B. Numerous bud sprouts on MS medium containing BA (22.2 μM) and IAA (2.88 μM) within 2 weeks

C. Induction of multiple shoot formation on MS medium containing BA (4.4μM) and IAA (1.4μM)

D. Shoot elongation

Photoplate - 6.1

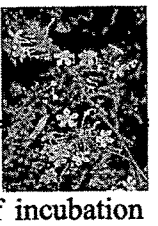




culture. There was no sign of callus formation when explants were cultured on media without auxin. White granular calluses developed on the surface of the explants. Callus formation from the leaf explants of *Plumbago zeylanica* is in agreement with results obtained by Rout et al, (1999) in the same species however varied in hormonal combinations where they obtained callus on medium supplemented with high concentration of growth hormones 11.42 µM IAA and 2.22 µM BA. Best callus (nodular) formation was observed on MS medium containing 6.7 µM BA, 1.42 µM IAA and 160µM AS (Table-4). Callus induction failed to grow on higher concentration of cytokinins. Callus generation from leaflets has been reported in *Plumbago rosea* of Plumbaginaceae family, *Arachis* and *Coronilla* of Fabaceae family.

Table-4: Effects of growth hormones on callus induction of *P. zeylanica*

Concentrations (µM)			**Callus induction (%) (Mean ± SE)**
BA	**IAA**	**AS**	
0.0	0.0	0.0	0.0
2.2	1.42	160	1.5 ± 0.26
4.4	1.42	160	21.9 ± 0.02
6.7	1.42	160	34.5 ± 0.16
6.7	**2.88**	**160**	**89.2** ± 0.21
6.7	2.88	340	78.1 ± 2.26
8.8	1.42	160	56.5 ± 0.01
8.8	2.88	340	44.8 ± 0.23
0.0	0.0	160	0.0



Leaf callus developed underwent organogensis after 3 weeks of incubation onto various regeneration media containing different concentrations of BA, IAA and AS (Plate-6.2A). The highest number of shoots from the leaf callus was observed on MS medium with 4.4 µM BA, 1.42 µM IAA and 160 µM AS (Table-5). On an average 30 shoot were noted in callus cultures (Plate-6.2B). Subsequent subcultures upto 6 cycles) of organogenic callus resulted in an extensive proliferation and an enhanced rate of caulogenesis, with more than 35 shoots. Our results are consistent with the earlier report on Ashwagandha indicating that cytokinin and auxin influenced shoot bud regeneration in plants (Chaplot et al, 2005).

Table-5: Effects of growth regulators on MS medium containing 160 µM AS on shoot bud regeneration from leaf callus of *P. zeylanica*

Concentrations (µM)		**Response (%)**	**Number of shoots/culture (Mean ± SE)**
BA	**IAA**		
0.0	0.0	-	-
2.2	1.4	-	-
2.2	2.8	25	2.08 ± 1.75
4.4	**1.4**	**93**	**30.16 ± 1.43**
4.4	2.8	85	22.02 ± 1.02
6.7	1.4	75	15.09 ± 1.02
6.7	2.8	67	9.54 ± 1.12
8.8	1.4	60	6.36 ± 1.43

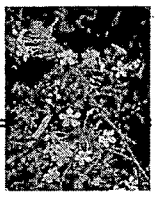


Plate-6.2

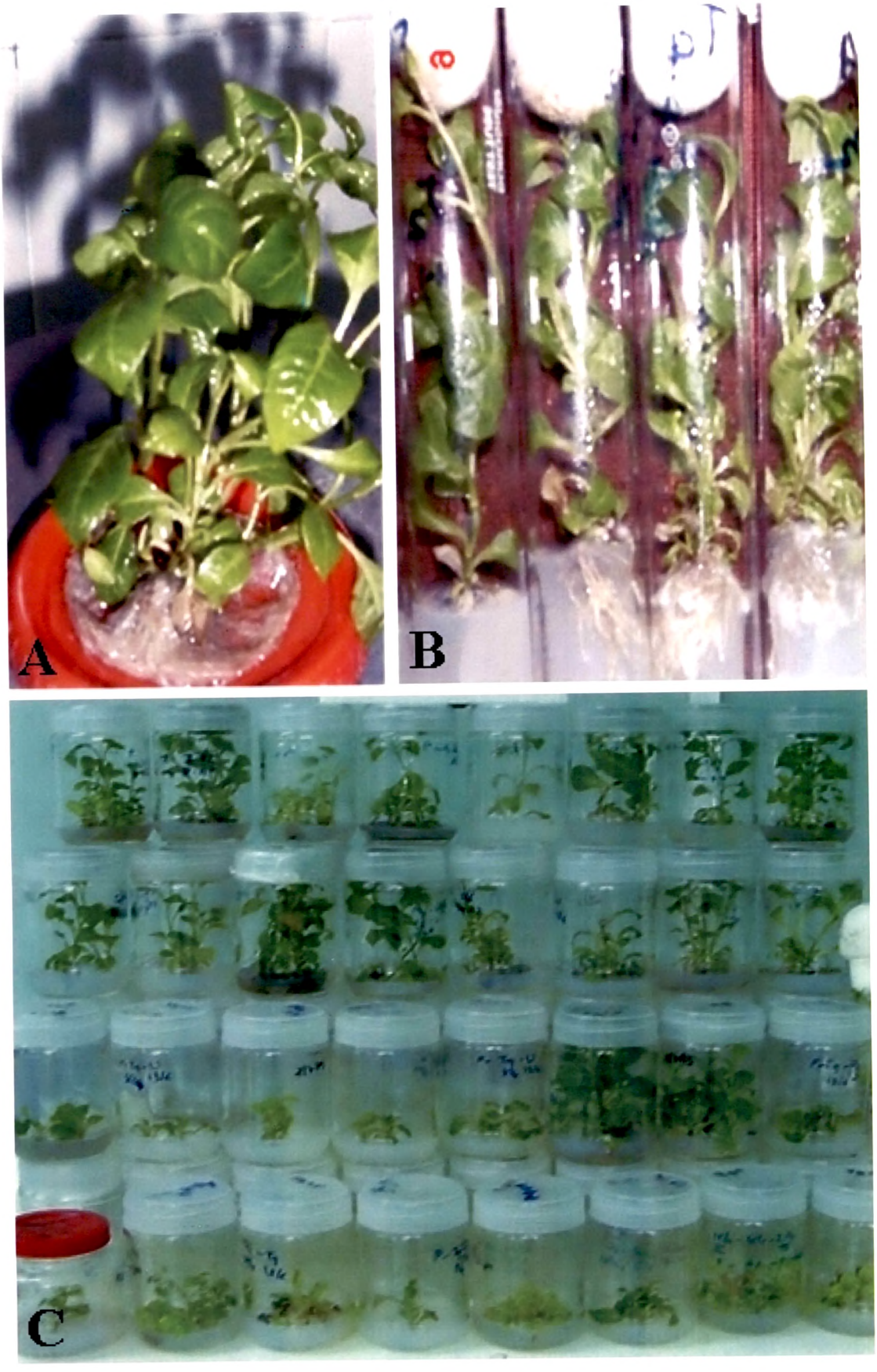
A. Initiation of shoot from leaf callus

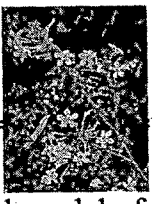
B. Shoot formation from callus on MS medium with BA (4.4μM), IAA (1.4μM) and AS (160μM)

C. Subculture of regenerated shoots

D. Elongation and rooting shoots from callus

Photoplate - 6.2





Well-developed shoots (3-4 cm) generated through axillary buds and leaf callus were excised and cultured on MS medium with different concentrations of auxins for root induction. Root induction was found to be more prominent in the medium containing 0.57μM IAA and 1.2μM IBA alone. These results are in accordance with that of Manickam et al, (2000) on *Withania somnifera* and Segio et al, (2000) on *Anethemis nobilis*. Roots elongated upto 12-13 cm within 15 days of incubation period (Plate 6.3A). Earlier workers (Rout et al, 1999; Selvakumar et al, 2001; Verma et al, 2002) had reported less number of roots (4-5 roots) on half strength MS medium containing 0.57μM IAA, 4.92μM IBA and 0.49μM IBA respectively. While profuse rooting was observed on full strength MS medium supplemented with IAA and IBA alone, the best result (15 roots) was obtained on MS medium with 1.2 μM IBA within 10 days (Fig.-2).

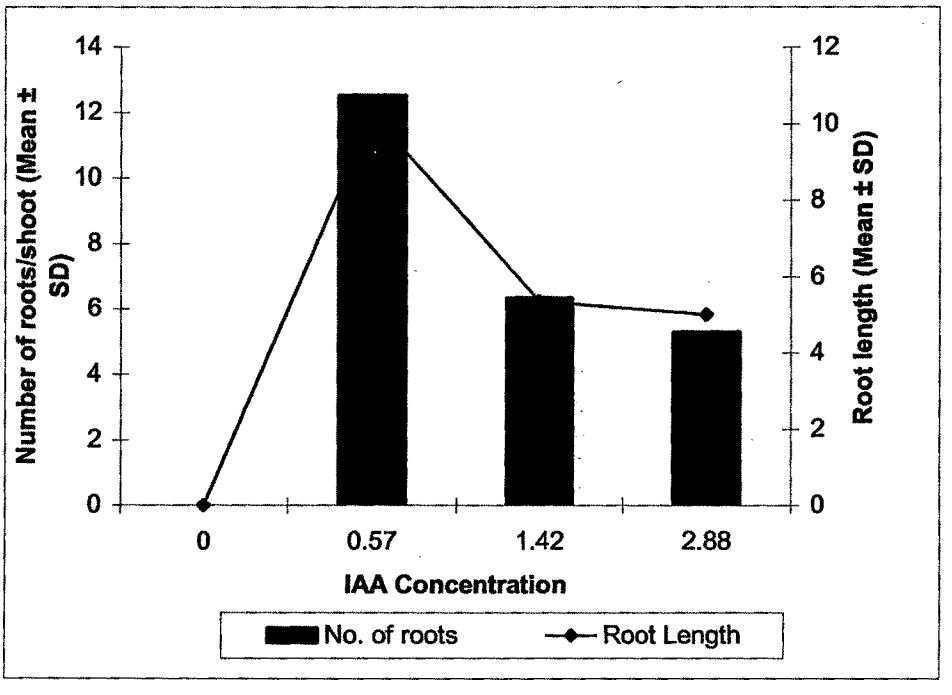


Figure-2: Root induction

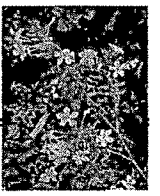


Plate-6.3

A. Root induction on full strength medium supplemented with IBA (1.2 μM)

B. Vigorous root formation within a period of two weeks

C. Induction of roots on regenerated shoots on MS medium containing IBA (1.2μM)

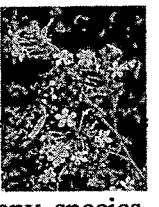
D. Hardened plants ready for transplantation to field in potting mixture (1:1:1, Sand + Soil + FYM)

E. Field transferred *in vitro* grown plantlets showing vigorous growth

F. Harvested roots of *in vitro* raised plants in the process of drying

1. Normal root
2. Tissue culture root

Photoplate - 6.3



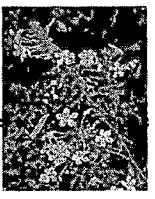
The potency of IBA in root induction has been reported in many species (Epstein et al, 1993). The slow movement and slow degradation of IBA facilitates its localization near the site of application and thus functions better in inducing roots (Nickell, 1982). Maximum frequency (97%), number of roots/shoot (around 15) and mean root length (13.41cm) was achieved within 10 days when shoots were cultured on MS medium with IBA (Plate-6.3B).

The ultimate success of *in vitro* propagation lies in successful establishment of plants in the soil. The regenerated plantlets were transferred to plastic pots containing sterilized potting mixture - sand, soil and farmyard manure (1:1:1) and transferred to net house for acclimatization (Plate-6.3C). Normally in absence of greenhouse facilities, *in vitro* plantlets loose tremendous amount of water through leaf surfaces with poorly deposited epicuticular wax and due to poorly developed or non-active stomatal system (Wardley et al, 1983). This problem was taken care of by regular sprinkling of water and irrigating the regenerated plantlets twice in a day. The rooted shoots demonstrated 100% survival rate in net house (Plate-6.3D). However, a 96% transplantation success of *in vitro* hardened plantlets in the field (Table-1) was observed in comparison to the 65-90% survival of plantlets recorded (Plate-6.3E) in the experiments of previous workers (Rout et al, 2002; Selvakumar et al, 2001). The high survival rate of *in vitro* plants of *P. zeylanica* indicates that this procedure could be easily adopted for large-scale multiplication and cultivation. The *in vitro* propagated plantlets resembled with the general growth and morphological characteristics of the donor plants.

The *in vitro* raised plants and seed grown plants were uprooted from the field for root harvesting. A significantly higher number of roots (19.0 ± 0.6) and fresh

weight (153.1 ± 2.4 gm) per plant was noted (Plate-6.3F_2) when compared to the seed generated stock with roots (5.1 ± 1.4) and fresh weight (47.3 ± 0.2 gm) respectively (Plate-3F_1). Similar observations have been reported by Satheeshkumar and Bhavanandan, (1988) in *Plumbago* rosea with higher number of roots and fresh weight per plant (18.0 ± 0.5; 137.4 ± 3.4 gm) compared to conventional rooted cuttings (14.0±1.7; 47.9 ±1.6 gm) respectively. Further qualitative analysis through the thin layer chromatography, emulated the presence of plumbagin (yellow spots) on the silica gel plate with R_F 0.76 on mobile phase- Petroleum ether: ethylacetate (7:3) in both *in vitro* generated and seed grown plants.

Thus, we have established a reproducible protocol for *Plumbago zeylanica* by using nodal and leaf explants. This protocol can be exploited for conservation and commercial propagation of this medicinal plant in India.



Explant

↓ Node, Leaf callus

Surface sterilization

↓ (0.1%) Bavistin (20 minutes)
(50%) Alcohol (1 minute)
(0.1%) $HgCl_2$ (2 minutes)

Initiation

↓ BA (6.7 µM) + IAA (1.4 µM)

Multiple shoot induction

↓ BA (4.4µM) + IAA (1.42 µM)

Elongation

↓ BA (4.4µM) + IAA (1.42 µM)

Rooting

↓ IAA (1.42µM) / IBA (1.2µM)

Net-house

↓ Farmyard manure, sand and soil mixture (1:1:1)

Field

↓ 95% survival rate

Plantlets obtained

Figure-3: Schematic representation of shoot multiplication from single nodal and leaf callus explants of *Plumbago zeylanica*