A Micropropagation system for *Cissus quadrangularis* - a valuable medicinal plant

Dissertation submitted in partial fulfillment for award of the degree of

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Submitted by

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CANDIDATE'S DECLARATION

I hereby declare that the work presented in dissertation entitled, **"A micro-propagation system for *Cissus quadrangularis* - a valuable medicinal plant"** in partial fulfillment for the award of degree of Master of Technology in Biotechnology from Thapar University, Patiala, is an authentic record of my own work done during the period of one year from August 2013 to July 2014, under the guidance of Dr. Manju Anand, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma to any other University or Institute.

Date: 18-07-2014
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CERTIFICATE

This is to certify that the dissertation entitled, **"A micropropagation system for *Cissus quadrangularis* - a valuable medicinal plant"** submitted by Pargat Singh in partial fulfillment of the requirements for the award of degree of Master of Technology in Biotechnology to Thapar University, Patiala, is an authentic record of student's own work carried out by him during the period of one year from August 2013 to July 2014, under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate to any other University or Institute.

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ABBREVIATIONS

Aº	Angstrom
BAP	Benzylaminopurine
BMS	Basal Murashige and Skoog's medium
ºC	Degree Celsius
MS	Murashige and Skoo's medium
CM	Coconut Milk
IAA	Indole 3- Acetic acid
IBA	Indole 3-bnutyric acid
Kn	Kinetin
NAA	Naphthalene Acetic acid
µM	Micromolar
mM	Millimolar
mg	Milligram
PGR	Plant growth regulator
Zn	Zeatin
2, 4-D	2, 4-dichloro phenoxy acetic acid
W	Watt

CONTENTS

List of Tables

List of Tables

List of Figures

ABSTRACT

The present study was carried out on a valuable medicinal plant *Cissus quadrangularis* Linn. (family Vitaceae) with a view to develop a standard protocol for its *in vitro* multiplication. This plant is commonly known as "Hadjod" in Hindi because of its ability to promote healing of the fractured bones and is known to have a number of other pharmacological effects like bone healing, anti inflammatory, analgesic, anti osteoporotic, antimicrobial, antiviral, antiulcer, antioxidant, and anti obesity properties. The different vegetative parts i.e. nodal explants, stem and leaves were excised from an elite field grown mature plant and thereafter planted on variously supplemented Murashige and Skoog's medium for multiple shoot proliferation, callus induction and direct as well as indirect organogenesis.

Cissus exhibited of multiple shoot proliferation from nodal segment on MS medium supplemented with Zn (2.28 µM - 9.12 µM) or fortified with BAP (4.44 µM - 8.8µM) alone in conjunction with Kn (4.65 µM - 9.13 µM). Out of all the combinations tried, best results, however, were obtained on MS medium supplemented with Zn (4.56 µM), where 8-10 shoots were formed after 7 weeks of culturing. *In vitro* formed shoots were excised and rooted on a separate root inducing full strength basal MS medium. Regenerated shoots thus formed were carefully excised and then rooted on basal MS medium. Thick, white coloured roots started appearing from basal end of regenerated shoot after 3 weeks which elongated further after 7 weeks. The rooted *in vitro* plantlets of *Cissus quadrangularis* were subjected to acclimitization through successive hardening stages so that these could be successfully transferred to field conditions.

Direct rooting was observed from leaf and stem segments on MS medium supplemented with different concentrations of IBA, 2,4-D, NAA either alone or in combination with Kn. From stem segment, best rooting was observed on IBA (4.90 µM) supplemented medium within 20 days of inoculation. Nearly four roots were formed which were thick, white without root hairs. Direct rooting was also observed on MS medium containing BAP (8.8 µM) along with Kn (9.30 µM) after 4 weeks, roots were thin and white in colour without hair but were much longer. In case of leaf segment MS medium fortified with NAA (10.74 µM) and Kn (2.325 µM) found to be the best where direct rooting occurred after 3 weeks. Roots were again thick, short and white in colour without root hairs.

Callus formation occurred from stem and leaf segments when planted on different concentrations and combinations of auxins and cytokinins. Stem segments callused on MS medium supplemented with different concentration of NAA (5.37µM - 21.48 µM) and Kn (2.325 µM - 9.30 µM). However, best results were obtained on NAA (10.74 µM) with Kn (4.65 µM).Callus formation started after 2 weeks of inoculation and the segment transformed into a mass of callus after 7 weeks. The callus was yellowish white, highly friable and showed the presence of significant amount of starch granules. Root differentiation from callus was observed after 4 weeks. Roots formed were thick, small and white in colour. Callus formation was also noted on BAP (8.88 µM - 17.76 µM) supplemented medium but the rate of callus formation was slow. Callus was hard, compact and greenish brown in colour.

Likewise callus formation from the leaf explant was observed on MS medium supplemented with NAA (5.37 µM) and Kn (2.325 µM) after 2 weeks. Root differentiation from leaf calli occurred more prominently on NAA (21.48 µM) and Kn (2.325 µM) containing medium after 6-7 weeks of culturing. The roots developed were similar to those developed from stem callus i.e. thick, small and white in colour.

The calli formed from stem and leaf explants were more or less identical in morphology. The calli obtained from these parts were heterogenous being composed of cells having different shapes and sizes. Histogenetic differentiation in the form of tracheids was observed in all the calli.Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls.

No shoot differentiation could be effected from the calli on the various media tried.

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DISCUSSION

The present investigation was undertaken on an important medicinal plant *Cissus quadrangulariss* Linn. with an aim to develop an efficient, reliable and reproducible protocol for its clonal propagation under *in vitro* conditions.

Cissus quarangularis is a valuable medicinal plant having diverse medicinal properties. *Cissus quarangularis* is not propagated through seeds because flowering and seed formation is very rare, also the flowers are sterile and plant does not set seeds. Its constant use in medicines, scientific studies, food and lack of its conservation strategies has caused its significant depletion from the forests, and now it is being threatened. To keep its supply continuous, for the production of herbal medicines, there is an urgent need to develop an efficient protocol for micropropagation of *Cissus quarangularis* for commercial cultivation. Till date no definite protocol is available for its *in vitro* multiplication although a few fragmented reports are available.

Micropropagation protocols have been developed for a wide range of medicinal plants like *Piperlongum* (Soniya and Das, 2002.), *Spilanthes acmella* (Saritha *et al.*, 2002), *Caper* (Carra *et al.*, 2007), *Chlorophytum borivillianum* (Debnath *et at*., 2007), *Glycyrrhiza glabra* (Arya *et al*., 2009), *Ferula assafoetida* (Zare *et al*., 2010), *Artemisia annua* L. (Ganesan and Paulsamy, 2011), *Lippia nodiflora* (Evelyne *et al*., 2011), *Bacopa monnieri* (L.) Pennel (Mehta *et al.*, 2012).

Micropropagation through enhanced axillary shoot proliferation is a reliable technique for clonal propagation as it enables to retain the clonal fidelity and prevents somaclonal variation in the cultures unlike the callus tissue. In the present investigation, multiple shoot proliferation was achieved using nodal segment. The axillary shoot proliferation from the cultured explants was remarkably influenced by the type and concentration of the growth regulator used. For shoot proliferation, cytokinins are one of the most important factors affecting the response (Sterk *et al.* 2003, Mishra *et al.*, 2005, George *et al*., 2008, Warrier *et al.*, 2010). Cytokinins used in plant cell culture regulate cell division, stimulate axillary and adventitious shoot proliferation, regulate differentiation and stimulate protein and enzyme activity (Gross and Parthier, 1994). A wide range of cytokinins like Zn, BAP and Kn have been employed in shoot proliferation (Bhojwani and Razdan, 1983, Viji *et al*., 2010). However a wider survey of literature suggested that BAP is the most reliable and

effective cytokinin for shoot proliferation. The effectiveness of cytokinin especially BA in promoting axillary shoot proliferation in many medicinal plants is well documented e.g. *Stevia rebaudiana* (Debnath, 2008), *Verbesina encelioides* (Karnawat *et al*., 2011) and *Rorippa indica* (Ananthi *et al*., 2011).

In the present investigation, axillary shoots were induced from the nodal segments on MS medium supplemented with various concentrations of Zn (2.28 µM-18.24 µM) and the best results were obtained on Zn (4.56 µM), where 3-4 shoots were formed after 2 weeks of culturing and number increased to 8-10 after 6 weeks. Garg and Malik (2012) has reported the formation of maximum of 5 shoots from the nodal segments of *Cissus quadrangularis* on MS medium fortified with Zn (0.5 mg/l). In the present study, multiple shoot proliferation was also observed when nodal segments were cultured on MS medium supplemented with BAP (8.88 µM) along with Kn (9.30 µM) where only 2-3 shoots were formed after 3 weeks of culturing.

Direct root induction from the inter-nodal segment was observed on MS medium supplemented with different concentrations of IBA, 2, 4-D, NAA either alone or in combination with Kn. Likewise synergism between BAP and kinetin also promoted root induction. Best rooting was observed on IBA (4.90 µM) supplemented medium within 20 days of inoculation. Nearly four roots were formed which were thick, white without root hairs. Likewise, Pandey *et al*. (2002) achieved rooting from callus of *Aconitum balfourii* when it was planted on different concentrations of IBA.

Similarly direct root induction on leaf segments was tested on MS medium by incorporating different concentrations of IBA, 2, 4-D, BAP, NAA either alone or in combination with Kn. Out of these hormonal combinations, best results were observed on culture medium supplemented with NAA (10.74 µM) along with Kn (2.325 µM) without any intervening callus formation. However with higher concentrations of NAA (21.48 µM) and Kn (9.30 µM) fortification, rooting was more but callus induction occurred simultaneously along with rooting.

In the present study optimal callusing from leaf explant was observed on MS medium containing NAA (5.37 µM) and Kn (2.325 µM) where as best callusing from stem explants was obtained on MS medium fortified with NAA (10.74 µM) and Kn (4.65 µM). Jayanti and Mandal (2001) reported callusing from leaf explants of *Tylophora indica* on MS medium with 2, 4-D (9.78 µM) and Kn (4.65 µM). Singh *et al*. (2009) achieved highest callus formation from leaf explants of *Rauvolfia*

serpentine on MS medium containing BAP (2.5mg/l) + IAA (2.0 mg/l). Similarly Ganesan and Paulsamy (2011) observed optimum callus induction from leaf explants of *Artemisia annua* L. on MS medium containing the NAA at 0.9 mg/l. Nathawat (2011) successfully carried out *in vitro* callus induction in *Cissus quadrangularis*a from stem explants on MS medium supplemented with NAA (2.5 mg/l and BAP (0.5 mg/l). Mehta *et al*., (2012) recorded best callus induction in leaf petiole explants of *Bacopa monnieri* on MS medium supplemented with 0.25 mg/l 2, 4-D + 0.5 mg/l Kn and 0.25 mg/l 2,4-D + 0.1 mg/l BAP. Kalpana *et al.* (2013) reported the callus from stem segments of *Cissus quadrangularis* on MS medium having higher concentrations of 2, 4-D (4.0 mg/l). Many medicinal plants have been successfully propagated *in vitro* by adventitious shoot initiation. But in present study, no shoot differentiation could occur on any tried hormonal combination but rhizogenesis and xylogenesis was observed from the calli raised from different explants on different media.

The callus formed from leaf and stem on the medium was more or less identical in morphology. The calli obtained from all the parts were heterogenous being composed of cells of different shapes and sizes. Histogenetic differentiation in the form of tracheids was observed in all the calli. Tracheids occurred singly or in groups and possessed reticulate thickenings on their walls. Tracheids were seen in the early stages of callus formation. Firstly only few tracheids could be seen which multiplied with the active proliferation of the callus. It seems there is a correlation between cell division and vascular differentiation. This contention gets support from other reports which suggests that cell division must precede the formation of vascular elements and that no vascular differentiation occurs in the absence of cell division.

Root formation was obtained from the callus developed on the medium supplemented with NAA (21.48 µM) and Kn (2.325 µM). Initially a few roots were formed but on further proliferation more and more roots appeared. These roots were small, thick, fleshy and brownish white in color.

However, no shoot differentiation could be effected from the leaf or the stem calli on any of the media combination tried.

Rooting and acclimatization of micro shoots

Root induction at base of regenerated shoots is an important and indispensable step to establish the tissue culture raised plantlets to the soil. In the present study the *in vitro* regenerated micro-shoots were carefully excised and transferred on BMS

medium alone or supplemented with different auxins for root initiation. Best root formation occurred on BMS medium where 100% rooting occurred after 21 days. Similar best rooting was recorded on BMS medium in *Tylophora indica* (Kaur *et al*. 2011). Mehta *et al*. (2012) reported best rooting in plantlets of *Bacopa monnieri* implanted on MS medium supplemented with 2.0 mg/l IBA.

The ultimate success of any *in vitro* protocol depends upon transfer and establishment of these plants in the field successfully. Since i*n vitro* raised plants are produced under controlled conditions of humidity, light and temperature so they need to be acclimatized before transferring them to field conditions through various hardening stages. After the complete plantlet formation, the rooted regenerants were carefully rescued from the cultures vessels and were transferred to plastic pots containing potting mixture consisted of steriled soil and vermicompost in the ratio of 1:1, then following various stages of hardening shifted to green house with 90% survival rate.

The present study demonstrates that an efficient, reliable and reproducible protocol has been established for the micropropagation of *Cissus quadrangularis* through axillary shoot proliferation of nodal segment. It has not been possible to induce *de novo* adventitious shoot formation either directly from the explants or indirectly through the callus. It is opined that cells in plant are undoubtedly totipotent but some vital hormonal and nutritional factor or their combination for differentiation could not be discovered by us during the stipulated period of this project.

RESULTS AND OBSERVATIONS

Objective: To develop a reliable and reproducible standard protocol for large scale propagation of *Cissus quadrangularis.*

Different vegetative explants like nodal segments, leaves and stem segment were collected from field grown elite plant of *Cissus quadranularis*. After initial disinfection with bavistin (0.1% w/v) for 10-15 minutes, explants were subjected to surface sterilization with 0.1% (w/v) mercuric chloride for 2-3 minutes for nodal segments and 3-4 minutes for leaves and internodal segments in laminar airflow bench. Explants were trimmed after sterilization to remove unwanted or dead portions. These were then planted on MS medium supplemented with various auxins and cytokinins either alone or in various combinations.

Nodal Explant Culture

Nodal segment, 4-5 mm in size having single node with one dormant lateral bud were collected and after surface sterilization they were cultured on MS medium supplemented with various concentrations and combinations of growth regulators either alone or in combination with each other. The axillary shoot proliferation from the cultured explant was remarkably influenced by the type and concentration of the growth regulator used. Multiple shoot formation was induced from nodal explants on MS medium supplemented with different cytokinins i.e. Zn, BAP and Kn in different concentrations either alone or in combination with each other and the auxin, 2, 4-D.

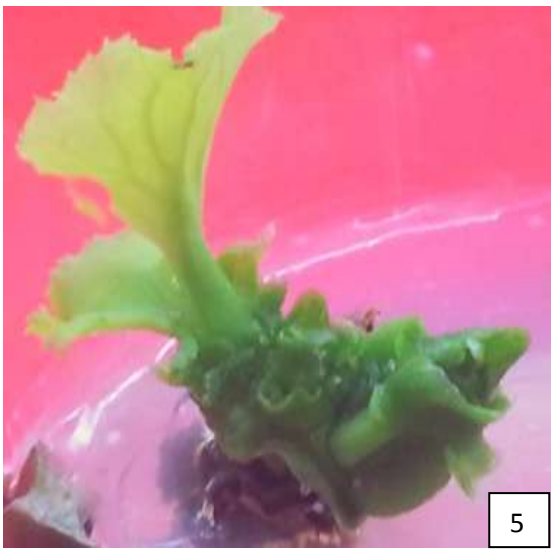


Fig. 4 Bud sprouting on MS + Zn (4.56 µM) after 10 days of inoculation. Fig. 5 Formation of 3-4 shoots after 15 days of culture.

Out of various cytokinins tested, best multiple shoot proliferation occurred on MS medium augmented with different concentrations of zeatin (2.28 µM - 9.12 µM) forming 4-12 shoots depending upon the concentration of zeatin. Out of various concentrations of zeatin used, best results were, however, obtained on 4.56 µM zeatin where initial bud break occurred after 10 days of inoculation (Fig. 4) forming 3-4 shoots from the axillary position after 15 days (Fig. 5). These shoots grew further forming well developed leaves (Fig. 6 and 7). These shoots multiplied further leading to the formation of 2-3 groups of shoot initials (Fig. 8) which sprouted into green leafy shoots after 28 days (Fig.9).When these microshoots were subcultured on the fresh medium containing zeatin (4.56 µM), nearly 8-10 shoots were formed from a single nodal explant after 6-7 weeks of culturing. Lower and higher concentrations of Zeatin were not effective in inducing multiple shooting.

Figs. 6&7 Further proliferation of shoots with well developed leaves after 4 & 6 weeks.

Multiple shoot formation was also observed on MS medium supplemented with BAP (8.88 µM) alongwith Kn (9.30 µM) where initial bud sprouting occurred after 12 days forming, 2-3 shoots after 2-3 weeks of culturing (Fig. 10, 11 & 12). Out of the various auxins tested, 2, 4 –D (9.74 µM) was able to promote multiple shoot proliferation (3 shoots) from the nodal segment. Response of various Plant growth regulators (PGR) on shoot proliferation from nodal segment is depicted in table 2 & figure 13.

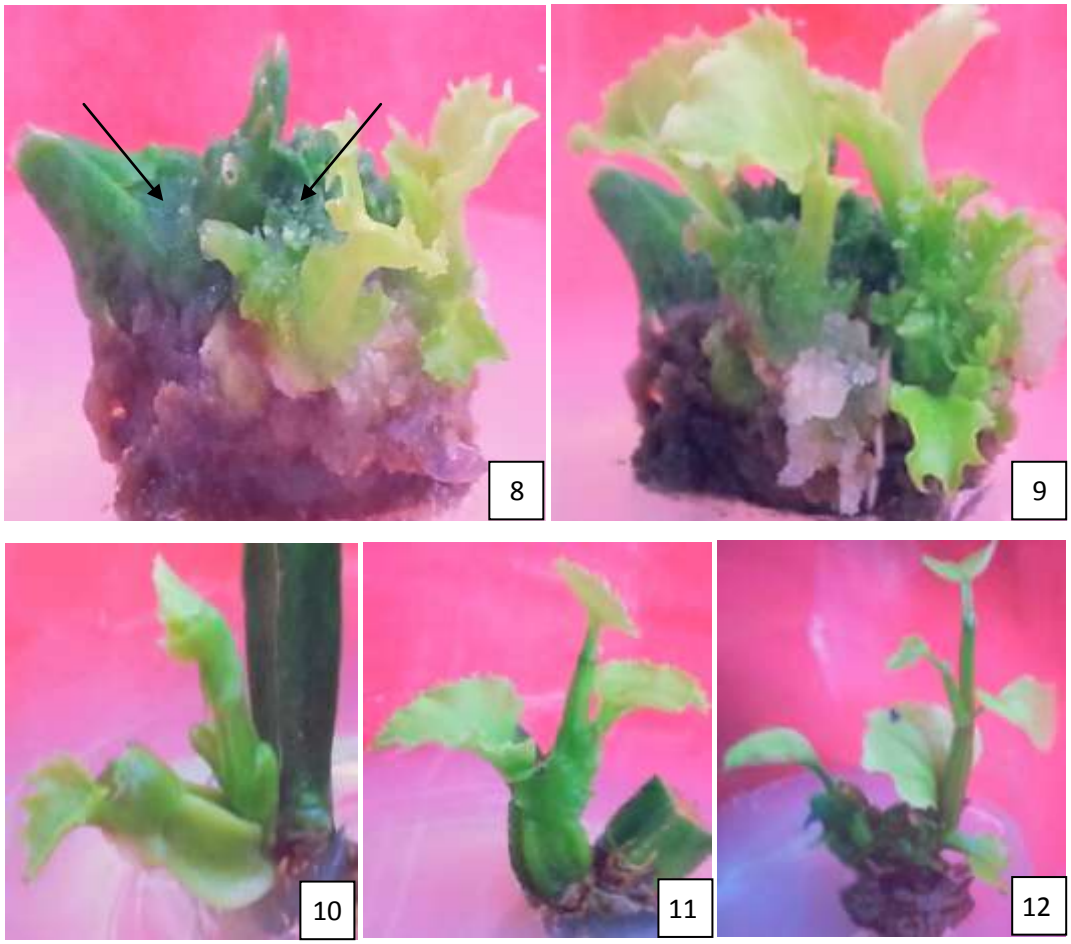


Fig. 8 Shoots alongwith 2-3 groups of shoot initials (arrows) at basal region on MS + Zn (4.56 µM) after 21 days of inoculation. Fig .9 Green leafy shoots formed after 28 days. Fig. 10 Bud break on BAP (8.88 µM) + Kn (9.30 µM after 12days of culturing. Figs. 11 & 12 Shoot formation after 21 days and 2-3 elongated shoots after 28 days respectively.

Table 2: Responses of various PGRs on shoot proliferation from nodal segment

S. No.	Plant Growth Regulators	% of Explants Regenerated	No. of shoots/ explants	Time taken for bud break (Days)
1	MS+Zn(2.28µM)	80	2-3	10-12
2	MS+Zn(4.56µM)	80	8-10	10-12
3	MS+Zn(9.12µM)	80	2-3	12-15
4	MS+BAP (4.44µM)	60	1-2	16-18
5	MS+BAP (8.88µM)	60	1-2	16-18
6	MS+BAP (8.88µM)+Kn(9.30µM)	80	3-4	12-13
7	MS+BAP (4.44µM)+Zn(4.56µM)	60	1-2	14-15
8	MS+2,4-D (4.53µM)	60	1-2	20-21
9	MS+2,4-D (9.06µM)	60	2-3	20-21

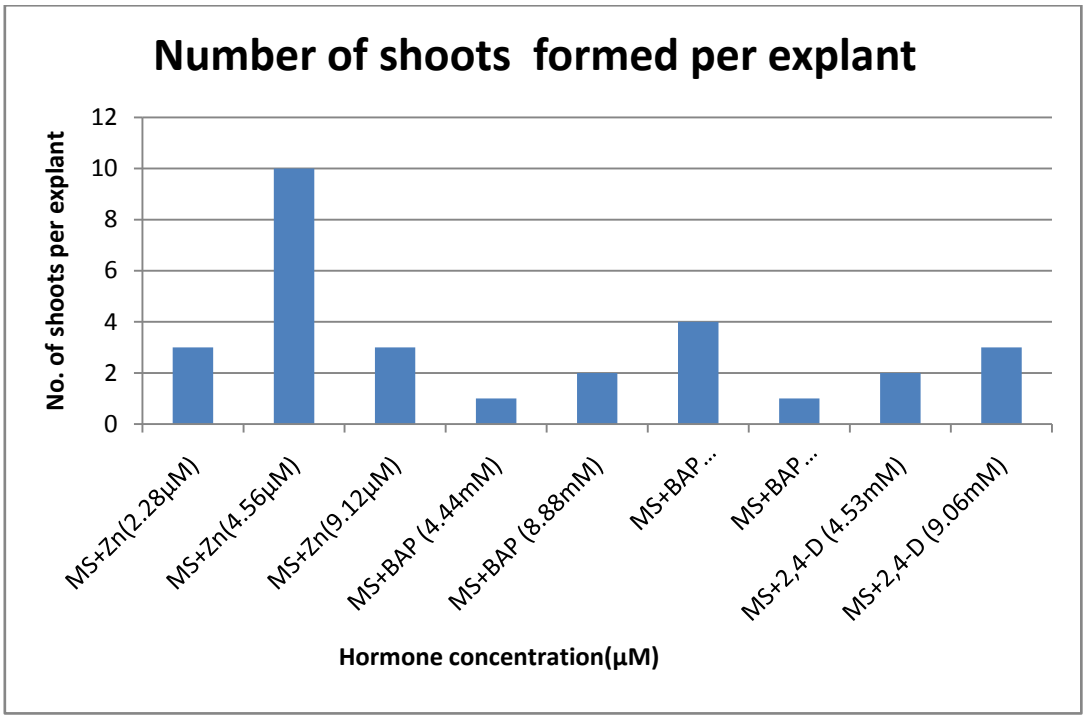


Fig . 13 No. of shoots formed per explant

Rooting of micro-shoots

Regenerated micro-shoots were carefully rescued from the culture bottles and test tubes under aseptic conditions and placed on sterile glass plate. Then each of these shoots were carefully separated and inoculated upright in the BMS medium alone or supplemented different auxins (Fig.14). Best root formation occurred on BMS medium where 100% rooting occurred after 21 days. Successive stages of root growth

Fig. 14 Inoculation of regenerated shoot on BMS. Fig. 15 Initiation of root primordium (arrow) from the basal end of regenerated shoot after 21 days of inoculation. Fig.16 Further growth of root primordium into well developed root after 28 days.

are shown in figures.15, 16 and 17).The roots grew further leading to the formation of well developed roots measuring 15-16 cm in length after 45-50 days (Fig.18).The

roots were long, white and devoid of root hairs. Complete plantlets with elongated shoot and root systems were formed after 50 days. Table 3.depicts percentage rooting on BMS medium and MS medium supplemented with different concentration of IBA.

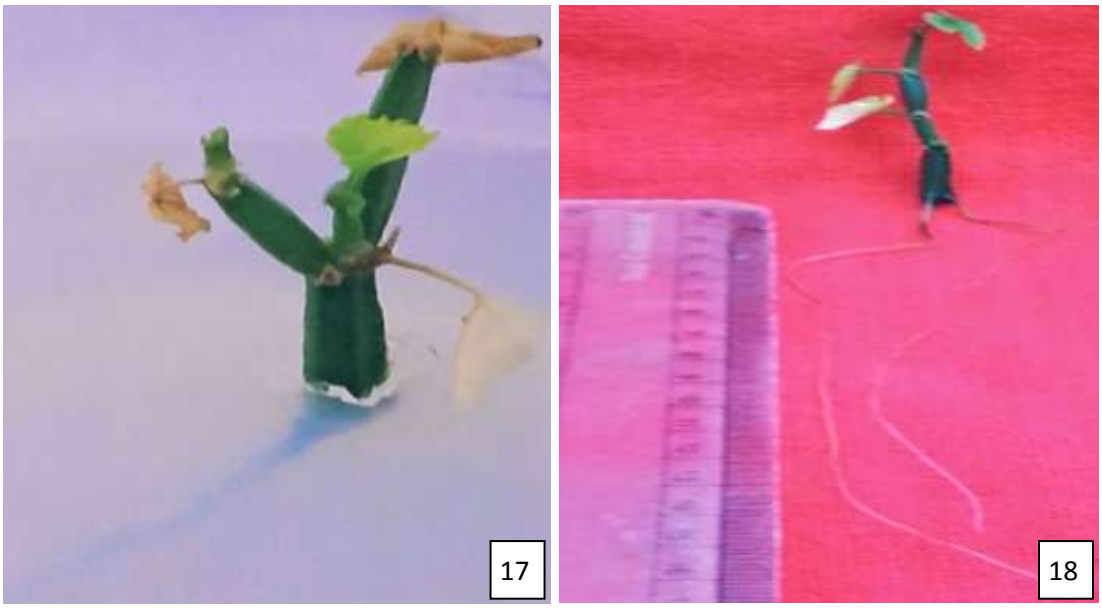


Fig.17 Well developed root from root primordium after 35 days. Fig. 18 A plantlet with well developed shoot and root system after 40 days.

Table 3: Rooting on BMS and MS medium supplemented with different concentration of IBA

S. No.	Growth regulators	% of shoots in which rooting initiated	Time taken to initiate root(days)	Degree of rooting
1	BMS	100	15	+++
2	MS+IBA-1	50	20	++
3	MS+IBA-2	0	0	0

Acclimatization and transfer of plantlets to the soil

After the complete plantlet formation the regenerants were acclimatized through successive hardening stages to transfer them to the field. The rooted plantlets were carefully rescued from the cultures vessel with extreme care by using forceps, keeping the roots intact and avoiding any mechanical injury to the plantlets. Roots were then washed with tap water to remove any remains of agar sticking to them. Plantlets were then transferred to plastic pots containing potting mixture consisted of steriled soil and vermicompost in the ratio of 1:1 (Fig.19) and, then covered with poly

bags having holes and kept under the growth room condition for 15 days (Fig.20). The plants were carefully monitored and watered regularly. After 3 weeks the plants with newly formed leaves were transferred to the poly bags having the same potting mixture and kept in the growth room for another 2 weeks (Fig.21).Thereafter the plants were shifted to green house bench and attempts are underway to establish these palntlets in the natural environment.

Fig.19 Plantlet transferred to plastic pot with potting mixture and kept under culture room conditions. Fig. 20. Plantlet in pot covered with polythene to maintain internal humidity. Fig. 21 Plantlet transferred to poly bag having same potting mixture after 3 weeks.

Stem culture

Stem segments, 5-6 mm in length were cultured on MS medium supplemented with different plant growth regulators (IBA, 2, 4-D, NAA, BAP and Kn) in different concentrations singly or in combinations.

Direct root induction from stem segment

Direct root induction from the inter-nodal segment was observed on MS medium supplemented with different concentrations of IBA, 2, 4-D, NAA either alone or in combination with Kn. Likewise synergism between BAP and kinetin also promoted root induction. Out of various auxins tested, best rooting was observed on IBA (4.90 µM) supplemented medium within 20 days of inoculation. Nearly four roots were formed which were thick, white without root hairs (Fig. 22). In case of 2,4-D (4.53 µM-9.06 µM) supplementation, 1-2 roots were formed after 15 day of inoculation and the roots were thin, white and without root hairs (Fig.23).

Out of the various combination of BAP (4.44 µM-8.88 µM) used in conjunction with Kn (2.325 µM-9.30 µM), optimal rooting occurred on MS medium supplemented with BAP (8.88 µM) and Kn (9.30 µM), where 2 roots appeared after

28 days of culturing which increased to four with passage of time. Again roots were thin, white without root hairs but were much longer (Fig.24). Direct root formation was also observed on MS medium supplemented with higher concentrations of NAA (10.74 µM) with Kn (2.325 µM), where a single root was formed after 25 days. The root was long, white and without any root hair (Fig. 25). In all above mentioned hormonal media, no callus formation took place, only rooting occurred.

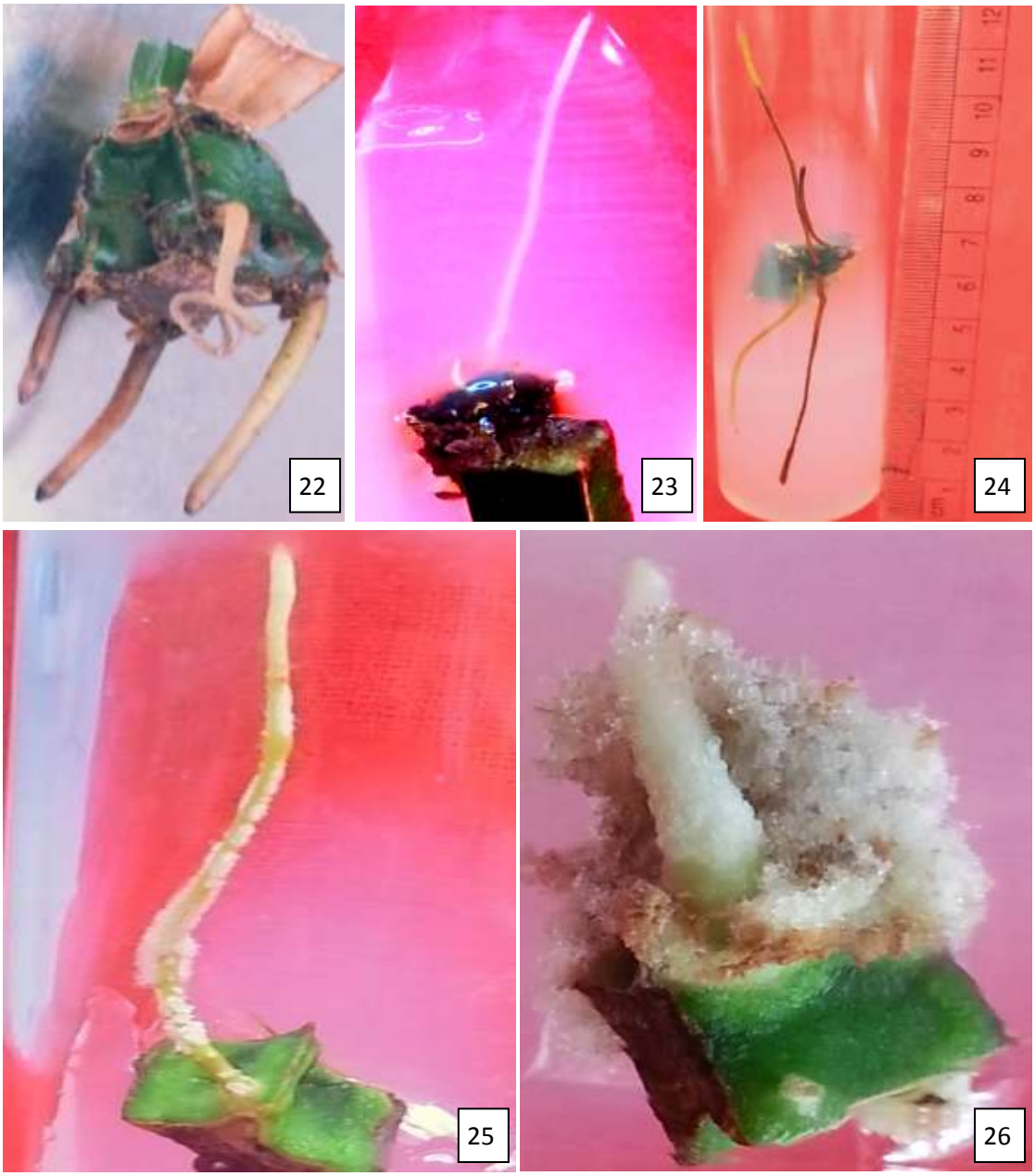


Fig. 22 Direct rooting from stem segment on MS + IBA (4.90 µM) after 20 days of inoculation. Fig. 23 On MS + 2, 4-D (9.06 µM) after 15 days of inoculation. Fig. 24 On MS+ BAP (8.88 µM) + Kn (9.30 µM) after 28 days. Fig. 25 On MS+NAA (10.74 µM) + Kn (2.325 µM) after 25 days. Fig. 26 On NAA (10.74 µM) with Kn (9.30 µM) within 25 day of inoculation.

However the incorporation of NAA (10.74 µM) along with higher concentration of Kn (9.30 µM) resulted in simultaneous callus formation along with rooting after 25 day of culturing. Roots were thick, short, white without hairs (Fig.26). Effects of growth hormones on direct root induction from stem segment are shown in Table 4.

Table 4: Effects of different PGRs on direct root induction from stem segment

S. No.	Plant Growth Regulators	Time taken to initiate root (Days)	No. of roots	Root characters
1	MS+ IBA(4.90µM)	20	3 - 5	Thick, white, without hairs
2	MS+ IBA(9.80µM)	25	0	----------
3	MS+2-4-D(4.53µM)	15	1 - 2	Thin, white without hairs
4	MS+2-4-D(9.06µM)	25	1	Thin, white without hairs
5	MS+BAP(4.44µM)+KN(9.30µM)	28	1 - 2	Thin, white, long without hairs
6	MS+BAP(8.88µM)+KN(9.30µM)	28	2-4	Thin, white, long without hairs
7	MS+NAA(5.37µM)+KN(2.325µM)	25	0	----------
8	MS+NAA(10.74µM)+KN(2.325µM)	25	1	Thick, white, long without hairs
9	MS+NAA(10.74µM)+KN(9.30µM)	25	2-3	Thick, white, short without hairs

Out of all above mentioned hormonal supplementation, IBA (4.90 µM) proved to be a best choice for direct root induction from leaf segment.

Callusing

Different Auxins (NAA, IBA, and 2, 4-D) and Cytokinins (Kn and BAP) alone or in combination were supplemented in various concentrations for establishing

callus from the stem segments. Supplementation of NAA (5.37 µM - 21.48 µM) along with Kn (2.325 µM - 9.30 µM) was found to be most effective for callus induction. Although addition of NAA (5.37 µM - 21.48 µM) to the medium induced callus successfully, but the addition of Kn enhanced the callus formation to a great extent. The best callusing response was obtained on MS medium fortified with NAA (10.74 µM) and Kn (4.65 µM). Callus formation started at the cut ends of stem segments after 14-15 days of culturing (Fig.27), proliferated further (Fig.28) and after 6-7 weeks the entire segment turned into a mass of yellowish white and friable callus (Fig.29). The callus, however, turned brown on subsequent sub-culturing.

Fig. 27 Callus initiation from the stem segment at cut ends on MS+ NAA (10.74 µM) + Kn (4.65 µM) after 15 days of inoculation. Fig. 28 Further proliferation of callus after 28 days. Fig. 29 Yellowish white friable callus formed after 6-7 weeks. Fig. 30 Compact callus formed on MS+BAP (17.76 µM) after 8 weeks.

Callus induction from stem segment was also observed on BAP (4.44 µM-17.76 µM) supplemented medium after 28 days of inoculation but the rate of callus formation was slow. On BAP (17.76 µM) incorporated medium, callusing initiated 25-28 days and after 8 weeks, the whole stem segment turned into a mass of callus which was compact, hard and greenish brown in colour (Fig.30). Effects of different growth hormones on callus induction from stem segment after 5 weeks of inoculation is shown in table 5.

Table 5: Effects of different PGRs on callus induction from stem segment

S. No.	Plant Growth Regulators	% of Explants formed callus	Time taken to initiate callus (Days)	Degree of callus
1	MS+NAA(5.37µM)	60	18	+
2	MS+NAA(10.74µM)	60	18	+
3	MS+NAA(21.48µM)	65	15	+
4	MS+NAA (5.37µM)+Kn(2.325µM)	90	15	++++
5	MS+NAA (10.74µM)+Kn(2.325µM)	75	15	++
6	MS+NAA (21.48µM)+Kn(2.325µM)	65	18	+
7	MS+NAA (5.37µM)+Kn(4.65µM)	80	15	+++
8	MS+NAA (10.74µM)+Kn(4.65µM)	100	15	+++++
9	MS+NAA (21.48µM)+Kn(4.65µM)	70	18	++
10	MS+NAA (4.44µM)	70	18	++
11	MS+NAA (8.88µM)	80	15	+++
12	MS+NAA (17.76µM)	80	15	+++

"+" very less growth,"++" less growth,"+++" average growth,"++++" good growth and "+++++" very good growth

Study of callus

The stem callus formed on NAA (10.74 µM) and Kn (4.65 µM) fortified medium found to be highly friable, cells were dispersed easily into single cells or group of cells when placed in water. Microscopic study established its heterogeneous nature. Cells were spherical, ovoid and elongated having different sizes (Fig.31). A magnified cell having numerous starch granules scattered throughout the cytoplasm is

shown in fig. 32. Stem callus study revealed the differentiation of xylem in the form of tracheids, which were present singly or in groups forming nodules (Fig.33). Magnified tracheids showing reticulate thickenings are shown in fig. 34.

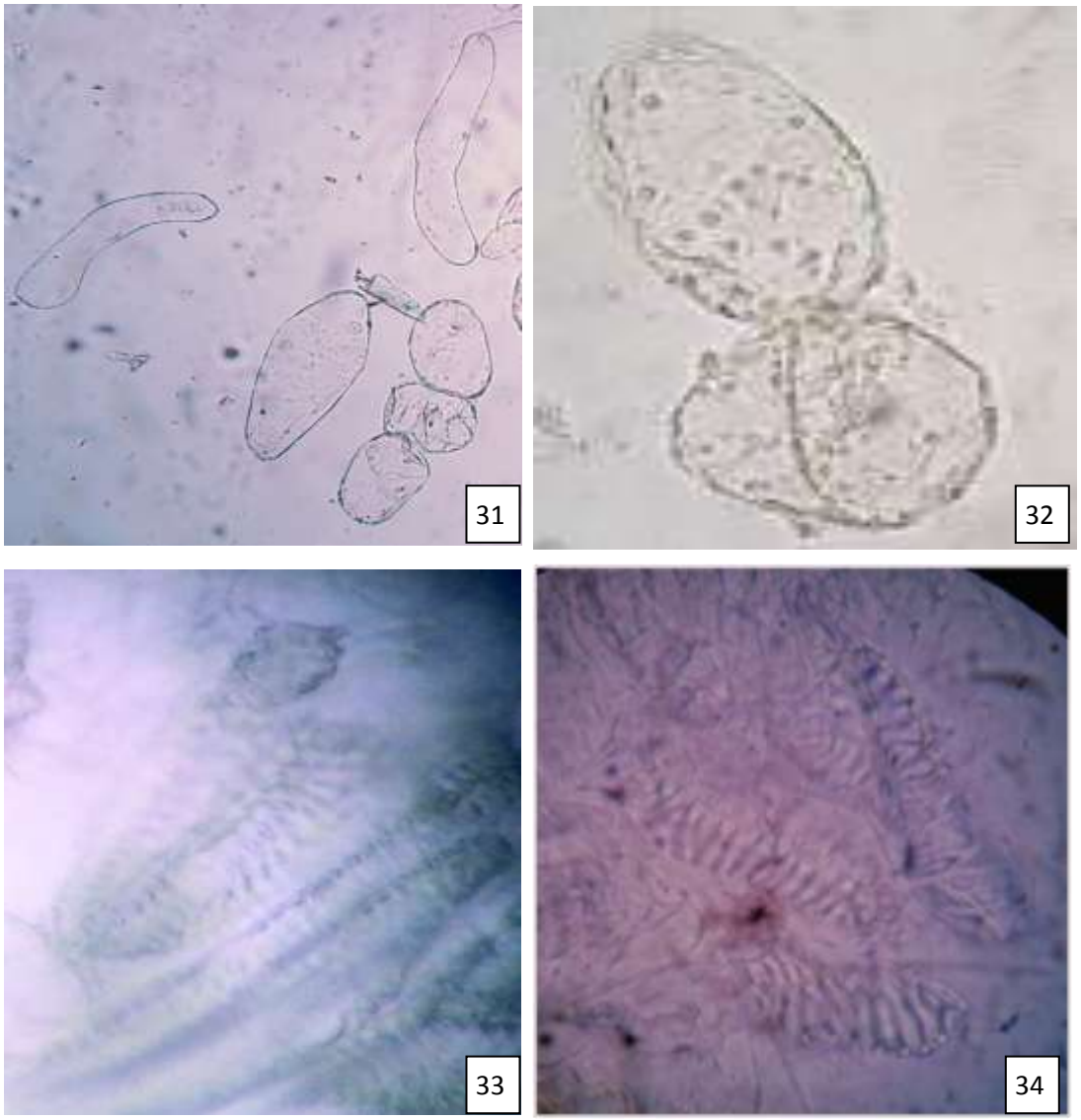
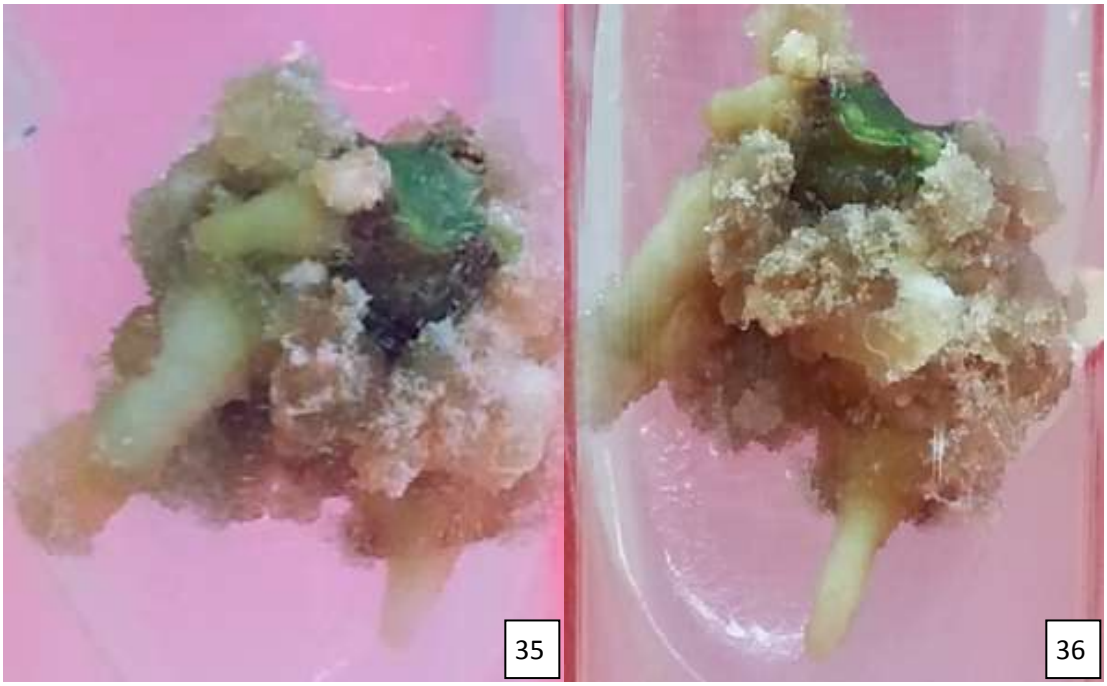


Fig. 31 Group of cells having different shapes and sizes isolated from stem callus. Fig. 32 A magnified callus cell showing presence of starch granules. Fig. 33 Group of tracheids isolated from stem callus seen at 40 X. Fig. 34 Magnified picture of tracheids at 100X, showing reticulate type of thickening.

Root Differentiation:

Root differentiation was observed from the callus on NAA (10.74-21.48 µM) and Kn (4.65 µM) augmented medium. In the start, 2-3 roots were formed from the callus after 28 days of inoculation (Fig.35 & 36) but with further proliferation more roots were differentiated. These roots were small, thick, and white without hairs. No differentiation of roots was seen IBA or IAA supplemented medium.



Figs. 35 & 36 Root formation from stem callus on MS + NAA (21.48 µM) + Kn (4.65 µM) after 28 days of inoculation.

Shoot Differentiation

In the present investigation, no shoot differentiation could be effected on any of the media combination tried.

Leaf Culture

To raise the leaf culture, leaf pieces of 4-5 mm size were cultured on MS medium supplemented with different growth regulators either alone or in conjugation with each other.

Direct rooting from leaf segment

Direct root induction on leaf segments was tested on MS medium by incorporating different concentrations of IBA, 2,4-D, BAP, NAA either alone or in combination with Kn. No response was observed on medium containing IBA (4.90 µM-9.80 µM) or 2, 4-D (4.53 µM- 9.06 µM) or BAP (4.44 µM-8.88 µM) either alone or in combination with Kn (2.325 µM-9.30 µM). Rooting was induced on the medium having NAA (5.37µM-21.48 µM) and Kn (2.325 µM -9.30 µM) within 15 day of culturing. The roots were thick, small, white without root hairs. Prominent rooting was observed on culture medium supplemented with NAA (10.74 µM) along with Kn (2.325µM) without any callus formation (Fig.37) but with higher concentrations of NAA (21.48 µM) and Kn (9.30 µM) fortification, rooting was more but callus

induction occurred simultaneously with rooting (Fig.38). Table 6 gives the details of effects of growth regulators on direct root induction from leaf segment.

Fig. 37 Direct rooting from leaf segment on MS + NAA (10.74 µM) + Kn (2.325 µM) after 18 days of inoculation, devoid of any callus. Fig 38 Direct rooting on MS + NAA (21.48 µM) + Kn (9.30 µM) simultaneous with callus.

Table 6: Effects of different PGRs on direct root induction from leaf segment

S. No.	Plant Growth Regulators	Time taken to initiate roots (Days)	No. of roots	Root characters
1	MS+ IBA(4.90µM)	25	0	----------
2	MS+ IBA(9.80µM)	25	0	----------
3	MS+2,4-D(4.53µM)	25	0	----------
4	MS+2,4-D(9.06µM)	25	0	----------
5	MS+BAP(4.44µM)+KN(9.30µM)	25	0	----------
6	MS+BAP(8.88µM)+KN(9.30µM)	25	0	----------
7	MS+NAA(5.37µM)+KN(2.325µM)	15	1	Thin, white, short without hairs
8	MS+NAA(10.74µM)+KN(2.325µM)	18	1-2	Thin, white, short without hairs
9	MS+NAA(21.48µM)+KN(9.30µM)	18	2 - 3	Thin, white, short without hairs

Callusing

For callus induction, leaf explants were cultured on MS medium supplemented with various concentrations of NAA, 2, 4-D, BAP singly or in conjunction with Kn.

Leaf segments, when inoculated on MS medium containing NAA (5.37 µM - 21.48 µM) resulted in callus formation to good amount, but the incorporation of Kn

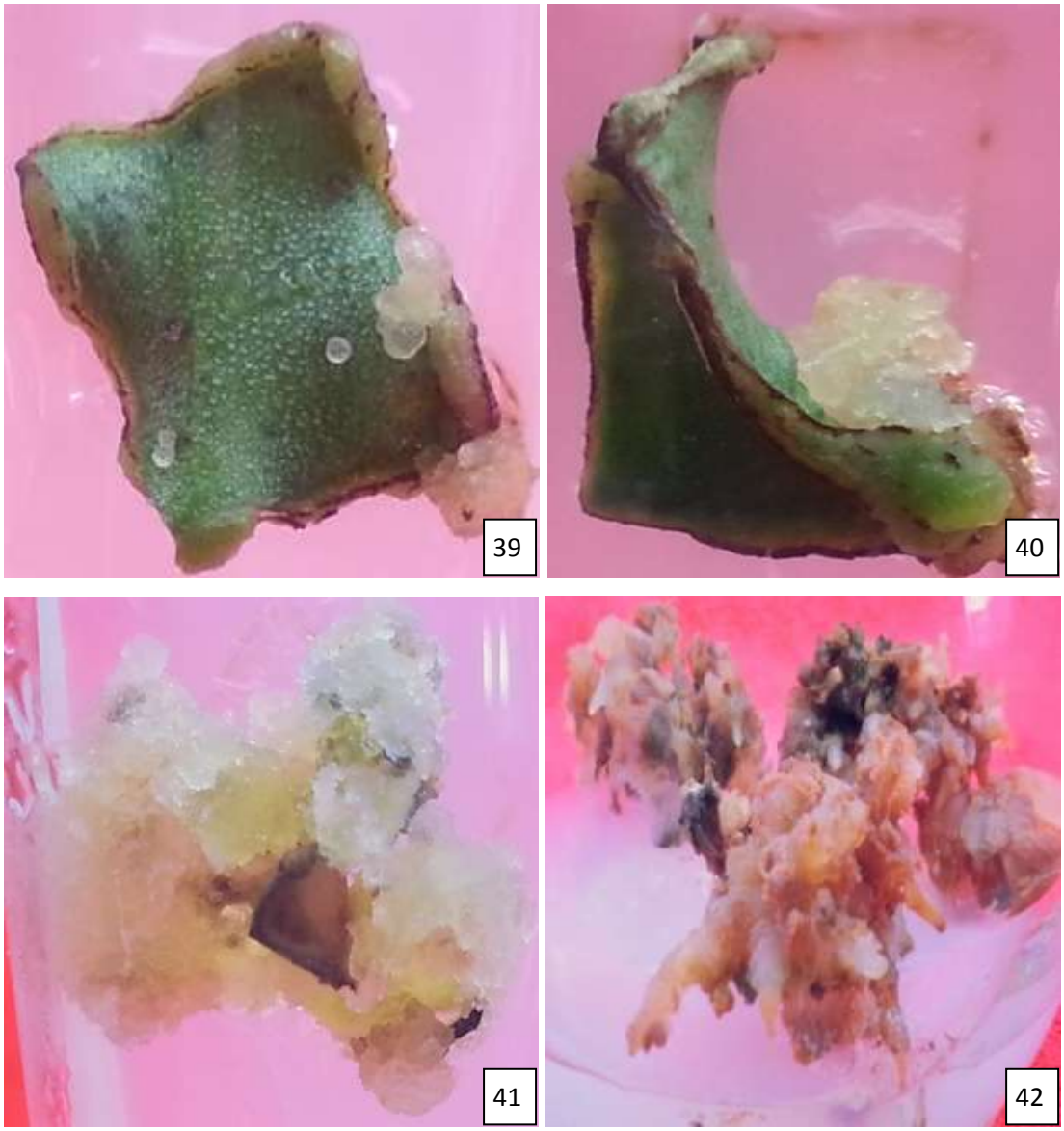


Fig. 39 Callus initiation from the leaf segment at cut ends on MS + NAA (5.37 µM) and Kn (2.325 µM) after 15 days of inoculation. Fig. 40 Further proliferation of callus after 28 days. Fig. 41 Whole leaf segment turned into yellowish white friable callus after 5 weeks. Fig. 42 Mass of callus after 10 weeks.

(2.325 µM-9.30 µM) with NAA evoked the best response. Optimal callusing was observed on MS medium containing NAA (5.37 µM) and Kn (2.325 µM) where callusing started from the cut ends after 15 days of culturing (Fig.39).The callus

proliferated further (Fig.40) and after 5 weeks, the whole leaf segment turned into a mass of yellowish white, soft and friable callus (Fig.41). The callus proliferated further when sub-cultured onto fresh medium and showed sustained growth (Fig 42). MS medium supplementation with 2,4-D (4.53 µM-9.06 µM) or BAP (4.44 µM-8.88 µM) alone and in combination with Kn (4.65 µM-9.30 µM) also resulted in callus formation but callus growth was very slow and poor. Different concentrations and combinations of growth regulators used and their effect on callus induction from leaf explants is depicted in table 7.

Table 7: Effects of different PGRs on callus induction from leaf explants

S. No.	Plant Growth Regulators	% of Explants formed callus	Time taken to initiate callus (Days)	Degree of callus
1	MS+NAA(5.37µM)	20	18	+
2	MS+NAA(10.74µM)	20	15	+
3	MS+NAA(21.48µM)	30	18	+
4	MS+NAA (5.37µM)+Kn(2.325µM)	100	15	+++++
5	MS+NAA (10.74µM)+Kn(2.325µM)	80	15	++++
6	MS+NAA (21.48µM)+Kn(2.325µM)	50	18	+
7	MS+NAA (5.37µM)+Kn(4.65µM)	80	15	++++
8	MS+NAA (10.74µM)+Kn(4.65µM)	80	18	++++
9	MS+NAA (21.48µM)+Kn(4.65µM)	60	18	++
10	MS+2,4-D (4.53µM)	0	21	0
11	MS+2,4-D (9.06µM)	0	21	0
12	MS+2,4-D (18.12µM)	20	21	0
13	MS+2,4-D (4.53µM)+Kn(4.65µM)	40	18	+
14	MS+2,4-D (9.06µM)+Kn(9.30µM)	40	18	+
15	MS+BAP (4.44µM)	20	21	+
16	MS+BAP (8.88µM)	40	21	+
17	MS+BAP (17.76µM)	30	18	+
18	MS+BAP (4.44µM)+Kn(4.65µM)	50	18	+
19	MS+BAP (8.88µM)+Kn(9.30µM)	50	18	+

"+" very less growth,"++" less growth,"+++" average growth,"++++" good growth and "+++++" very good growth.

Differentiation from the callus

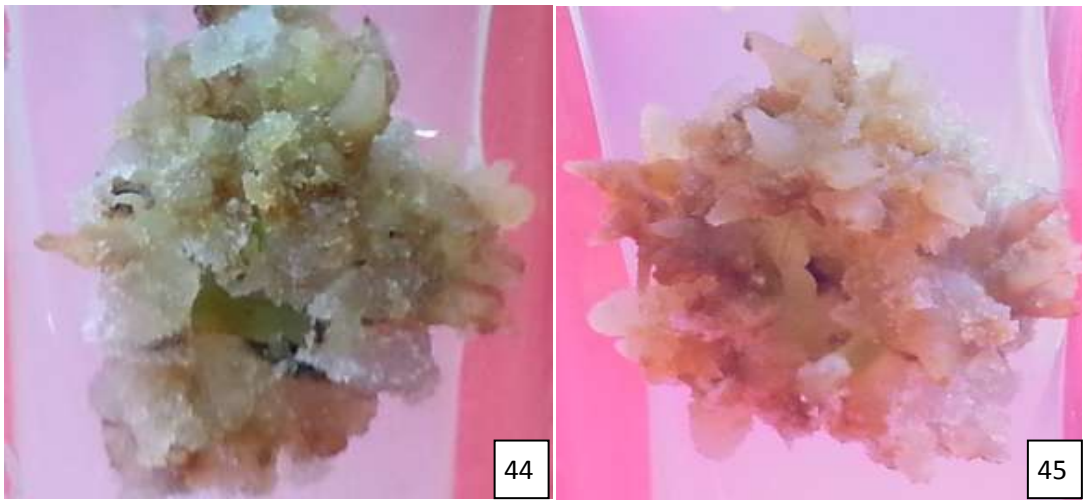
Histogenetic Differentiation

Histogenetic differentiation was observed in the form of tracheids which were present mostly in the form of groups to form nodules. The tracheids were variable in size and had reticulate type of thickenings on their walls (Fig.43).

Fig. 43 Group of tracheids seen from the leaf callus.

Rhizogenesis

Root differentiation from the callus formed on MS medium containing NAA (5.37 µM-21.48 µM) alongwith Kn (2.325 µM) was observed after 5 weeks of culturing. Best rooting response was obtained from the callus developed on the medium supplemented with NAA (21.48 µM) and Kn (2.325 µM). Initially a few roots were formed (Fig. 44) but on further proliferation more and more roots appeared (Fig. 45). These roots were small, thick, fleshy and brownish white in color.



Figs. (44 & 45) Root differentiation from leaf callus on MS + NAA (21.48 µM) + Kn (2.325 µM) after 4 and 6 weeks of culturing.

Caulogenesis

No shoot formation was observed from the leaf callus even after 16 week of culturing on different combination of auxins and cytokinins.

MATERIAL AND METHODS

Experimental material

The present investigation was carried out on a valuable medicinal plant *Cissus quadrangularis* Linn belonging to family Vitaceae. It is commonly known as "Bone setter" in English and “Hadjod” in Hindi because of its bone fracture healing properties.

Habit and habitat:

It is a succulent, perennial climber reaching a height of 1.5 m, having a thick, quadrangular jointed cactus like stem. Its general habitat is lowland and medium altitude dry grassland, deciduous bush-land and woodland often associated with termite mounds. It grows natively in hotter and dried regions of India such as Deccan Peninsula. It is also found on lower eastern slope of Western Ghats and is wide spread in the drier areas of the world such as Sri Lanka, Malaysia, Thialand, Java, Arabia and South Africa (Udupa *et al*., 1970).

Morphological Characters:

Cissus quadrangularis is a shrubby vine with glabrous and fleshy quadrangular 4-winged stem with constrictions at its nodes. Internodes are 8-10 cm long and 1.2-1.5 cm wide. Leaves are simple, alternate, fleshy 3-lobed ovate with serrated margins (Fig.1). The leaves measure about 8 cm long and 6 cm broad. Numerous tendrils grow out of the plant’s nodes. The flowers are also typical of the grape family are small, bisexual, tetramerous, greenish white occurring in short umbellate cymes opposite to leaves and come at the end of the summer. The flowers have lobed cup shaped calyx and greenish yellow petals with red tips. The fruits are small, round berries ±0.7 cm in diameter, red on ripening and one seeded (Fig.2) (Panda and Das, 2004 and Wealth of India, 2005). Flowering and fruiting time is May- June. Plant does not set seeds as flowers are sterile.

Fig.1. A plant of *Cissus quadrangularis*

Fig.2. Cissus plant with fruits

Medicinal importance

The entire plant of *Cissus quadrangularis* is of medicinal value and is known to have a number of pharmacological effects like bone healing, anti-inflammatory, analgesic, anti-osteoporotic, antimicrobial, antiviral, antiulcer, antioxidant, anti-tumor, antipyretic and anti-obesity properties (Singh *et al*., 1984, Deka *et al*., 1994, Murthy *et al*., 2003, Jainu *et al*., 2010, Swamy *et al*., 2006, Oben *et al*., 2006, Oben *et al*., 2006, Panthong *et al*., 2007, Austin and Jegadeesan, 2009, Balasubramanian *et al.*, 2010,Vijay and Vijayvergia, 2010).

Cissus has been used by common folk in India for promoting the fracture healing process. It has been prescribed in the ancient Ayurvedic texts as a general tonic and analgesic, with specific bone fracture healing properties. *Cissus quadrangularis* is used for obesity, diabetes, a cluster of heart disease risk factors called "metabolic syndrome," and high cholesterol. It has also been used for bone fractures, weak bones (osteoporosis), scurvy, cancer, upset stomach, hemorrhoids, peptic ulcer disease (PUD), painful menstrual periods, asthma, eye and ear diseases, malaria, and pain. It is also used in bodybuilding supplements as an alternative to anabolic steroids (Raj and Joseph, 2011).

Phytochemical Properties:

Cissus quadrangularis contains alkaloids, resvertol, piceatannol, palidol, partthenocissin, quadrangularins, ascorbic acid, carotene,phytosterol substances, calcium, flavanoids, vitamins, enzymes, nicotinic acid, tyrosin and triterpenoids (Joseph and Raj, 2011).

Cissus is reported to contain a number of steroids including β-sitosterol, daucosterol, β-amyrin, δ-amyrone, tarexerol and freidalin (Mishra *et al*., 2010, Bhutani *et al*., 1984, Mehta *et al*., 2001, Singh *et al*., 2007, Rao *et al*., 2011, Chi *et al*., 2010, Aswar *et al*., 2012). Of these δ-amyrone and freidalin are ketosteroids. Anabolic steroids (Mishra *et al*., 2010) and phytosterols (Shah *et al*., 2010) are also found to be present. Quercetin and kaempferol are the flavonoids which are isolated from Cissus (Singh *et al*., 2007, Thakur *et al*., 2009). Three unsymmetric tetracyclic triterpenoid namely, oncer-7-ene-3∞,21β-diol and oncer-7-ene-3β,21∞-diol (Bhutani *et al*., 1984, Mehta *et al*., 2001) as well as 7–oxo-oncer-8-ene-3β,21-∞-diol (Mishra *et al*., 2010) have been isolated and structurally characterized from *Cissus* (Bhutani *et*

al., 1984, Mehta *et al*., 2001). The plant extract serves as a rich source of calcium ions which when treated with CO_2 leads to formation of calcite crystals of highly irregular morphology (Sanyal *et al*., 2005). Stilbene derivatives which are Quadrangularins A, B, and C (Adesanya *et al*., 1999), resveratrol, piceatannol, pallidol (Singh *et al*., 2007) and parthenocissin are also present in the stem. Phytochemical analysis has revealed that the plant contain high amount of dietary antioxidants that include vitamin C, beta-carotene (Oben *et al*., 2007) and polyphenols.

Formulations of *Cissus*:

A number of formulations based on *Cissus* are available in different forms e.g. dry powder, syrup and capsules, to be administered for different ailments. These are manufactured by large number of companies like Amazon, GN labs, USP labs, Primaforce, etc under different brands. Most common formulation is 'Super Cissus', sold in 750 mg capsules, others are 'Mega Cissus' and "Primaforce's Cissus" which contain 400 mg per pill and 1000 mg per capsule respectively. An Ayurvedic preparation is "Laksha Gogglu" which is used for alleviating pain, reducing swelling and promoting healing of simple factures (Mishra *et al*., 2010).

Fig. 3: Different Products of *Cissus Quadrangularis*

Ethno veterinary usage

Cissus quadrangularis is fed to cattle as a galatologue to induce flow of milk. The whole plant is used in cases of fractures, sprains, rheumatism, irregular growth of teeth, broken horn, anthrax, haematuria, elephantiasis, dislocation of hip, various wounds and cracked tail.

Safety profile

The safety of *Cissus* extracts has been assessed in human, animal and cell culture systems and based on studies to date, *Cissus* extracts appear to be exceedingly safe and free of adverse effects at the doses commonly used.

The results of the studies in rats and mice (Attawish *et al*., 2002, Kothari *et al*., 2011, Aswar *et al.*, 2012), the cell culture study (Muthusami *et al*., 2011a, 2011b) and the three clinical studies in humans (Oben *et al*., 2006, 2007, 2008) taken together with the long history of traditional, medicinal use indicate that *Cissus* stem powder and dried extracts given daily for extended periods of time (up to 10 weeks in the humans and 90 days in rats) do not product serious adverse events. Safety studies in rat shows no toxic effects at dosages as high as 2000 mg/kg of body weight. The fresh juice of the plant may irritate the skin and cause itching.

Glassware

The glassware used for experimental work comprised of corning test tubes (25x125 ml), conical flasks (150 ml, 250 ml, 500 ml, and 1000 ml) and culture bottles (25 x 150 ml). In addition other glassware includes graduated measuring cylinders (100 ml, 1000 ml), petri-dishes, beakers and a range of pipettes (200 µ1, l ml, 2 ml, and 10 m1). Before use, the glassware was subjected to chromic acid solution (mixture of $K_2Cr_2O_7$ + H_2SO_4+ H_2O) followed by thorough washing with tap water. All the vessels were then washed thoroughly with teepol (1% v/v) detergent solution. Then they were cleaned under running tap water. The glassware was then inverted in a clean tray and left to dry in the oven. Plugs for the tubes and flasks were made out of absorbent surgical cotton wrapped in muslin. 5-10 ml water was then poured into each culture vessel which was tightly plugged. The glassware was then steam sterilized in an autoclave at a pressure of 15 lb/inch² at 121°C for 15 - 20 minutes.

Culture medium

The media formulation described as Murashige and Skoog (1962) referred as MS medium was selected as the optimal culture medium. Stock solutions of generally

4 times major elements, 100 times minor elements, 10 times organic constituents were prepared. These stock solutions were stored in a freeze chest at 4°C and were mixed in desired proportions only before use. None of the stock solutions were stored for more than 15 days. Stocks of the hormones were also made either 2X or 4X. They were also kept at 4°C.

Defined amount of all ingredients were mixed and volume was adjusted by distilled water. After incorporation of all the ingredients, 2% sucrose was also added along with all these supplements and various concentrations of plant growth regulators. pH of the medium was adjusted to 5.6-5.7 with 0.1N HCl or NaOH. The prepared medium was then gelled with 1% w/v agar-agar.

Definite aliquots of the medium were then added depending upon the capacity of the culture vessels. Generally 25m1 of medium was distributed into the test-tubes and 40m1 in culture bottles. Tubes were plugged with cotton plugs, steam sterilized at 15 lb/inch^2 (121ºC) for 15 minutes. After autoclaving, tubes were placed in slanting stands to give slanted surface to agar media. These were then left to cool and solidify.

Table 1: Composition of Murashige and Skoog's medium (1962)

Ingredients	Concentration (mg/1)
Major	
$(NH_4)NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor Elements	
$MnSO_4.4H_2O$	22.3
H_3BO_3	6.2
Kl	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.4H_2O$*	27.8
Na_2EDTA*	37.3

Organic constituents:	
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
PyridoxineHCl	0.5
Thiamine HCl	0.1
Sucrose	20,000

* $FeSo_4.4H_2O$ and Na_2EDTA are the alternative to the use of Ferric Na EDTA which is added freshly to the medium (i.e. 0.04gm/1).

Following are some of the supplement, which were used either singly or in combinations for shoot proliferation, callus induction and organogenesis.

1. Basal MS medium (BMS)
2. MS+BAP (4.44µM), MS+BAP (8.88µM), MS+BAP (17.76µM)
3. MS+NAA(5.37µM), MS+NAA(10.74µM), MS+NAA(21.48µM)
4. MS+BAP (4.44µM)+Kn(4.6,5µM), MS+BAP (8.88µM)+Kn(9.30µM)
5. MS+2,4-D (4.53µM), MS+2,4-D (9.06µM), MS+2,4-D (18.12µM)
6. MS+2,4-D (4.53µM)+Kn(4.65µM), MS+2,4-D (9.06µM)+Kn(9.30µM)
7. MS+NAA (5.37µM)+Kn(2.325µM), MS+NAA (10.74µM)+Kn(2.325µM)
8. MS+NAA (21.48µM)+Kn(2.325µM), MS+NAA (5.37µM)+Kn(4.65µM)
9. MS+NAA (10.74µM)+Kn(4.65µM), MS+NAA (21.48µM)+Kn(4.65µM)
10. MS+Zn (2.28µM), MS+Zn (4.56µM), MS+Zn (9.12µM)
11. MS+BAP (4.44µM)+Zn(2.28µM), MS+BAP (8.88µM)+Zn(4.56µM)
12. MS+2-4-D (4.53µM)+Kn(4.65µM), MS+2-4-D (4.53µM)+Kn(9.30µM)

Inoculations

All the experimental manipulations were carried out under strictly aseptic conditions in a laminar air flow bench fitted with a bactericidal U.V. tube (15W, peak emission 2637 A^0). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, scalpels, blade etc.), gas burner, lighter, tube containing

absolute alcohol etc. were also cleaned with alcohol. The fresh material to be inoculated was kept in a Petri-dish covered with a piece of black paper in order to protect it from the harmful effects of U. V. rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with U.V. rays kept continuously on for one hour.

Surface sterilization of explants

The explants like leaves, nodal and inter-nodal segments and shoot apices were taken from the healthy plant growing under the *in vivo* conditions. These were placed in bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (1% v/v) for another 10-15 minutes and then washed properly to remove the detergent. The explants were then treated with Bavistin (0.1 % w/v) for another 10-14 minutes to remove the fungal contamination and then washed properly to remove the fungicide.

Hands and arms which were to be used inside the inoculation chamber were scrubbed with alcohol before inoculation. The rims of the test tubes and the sides of the plugs were flame sterilized. Instruments (like forceps, scalpels, spatula etc.) were all sterilized by dipping in the alcohol and flaming a number of times. Care was taken to cool the instruments before putting into operation. The explants were further sterilized in the laminar air flow chamber with 0.1% mercuric chloride ($HgCl_2$) solution for 3-5 minutes depending upon the explants. Leaves were treated with 0.1% $HgCl_2$ for 2-3 minutes whereas nodal and internodal segments were treated with 0.1% $HgCl_2$ for 3-4 minutes. The explants were then thoroughly washed (4 - 5 washings) with sterilized distilled water to remove the traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions. The explants were then planted on variously augmented MS medium.

Cultural conditions

All the cultures were maintained in an air conditioned culture room at a temperature of 25 ± 4°C. The source of illumination consisted of 4 feet wide

fluorescent tubes (40 W) and incandescent bulb (25 W). The intensity of illumination was 50 µ mol/ m^2/s^1 at the level of cultures and 12 hours light regime was followed by 12 hours of darkness.

Acclimatization of plantlets

The micro-propagated plants (5-6 cm long, with 2-3 healthy roots) were hardened through successive weaning stages. The plantlets were rescued carefully from culture tubes and were washed under tap water to remove agar adhering to them. Plantlets were then transferred to plastic cups (of capacity 200 ml) containing soil : vermicompost in the ratio of 1:1. Initially they were covered with perforated plastic covers to maintain higher internal humidity and kept under growth room conditions for a period of 15-20 days. Young plantlets were taken out carefully from plastic cups and were shifted to poly bags containing the same potting mixture kept inside growth room for 2 weeks. The hardened plants were thereafter transferred to green house for another 2 weeks and efforts are going on to transfer these plants in open field conditions.

Review of Literature

With an ever-increasing global inclination towards herbal medicine, there is a steep increase in demand for a huge raw material of medicinal plants. To meet the supply of raw material, medicinal plants (which are mostly wild), are being over harvested from phyto-diversity rich areas. Moreover, all parts of the plant (leaves, flowers, fruits, seeds, roots) are used for preparing plant based drugs and in some instances even young (immature) parts are being harvested. Further, in many medicinal plants, flowering and seed formation is rare and sometimes seeds are not viable. All this is resulting in the depletion of medicinal plant material which is to be replenished at same or higher rate to maintain regular supply.

Ideally, herbal plants should be grown under uniform environmental conditions and the planting material must have the same genetic make-up as of the selected high-yielding clones, which is possible when they are cloned through an *in vitro* strategy. In this situation biotechnological tools are handy and significant, for multiplication, genetic uniformity and improvement of medicinal plants through *in vitro* regeneration.

Beginning of micropropagation of medicinal plants:

After the discovery of kinetin, the major work on *in vitro* regeneration has been centered around tobacco (*Nicotiana tabacum*) tissue culture culminating in the first convincing demonstration of the control of differentiation of shoots or roots or both by the kinetin-auxin ratio followed by carrot (*Daucus carota*) tissue culture and demonstration of totipotency of plant cell with the regeneration of complete flowering plants of carrot from its phloem cells. The micropropagation of medicinal plants remained neglected till complete plants of *Rauvolfia serpentina* (L.) Benth. (Serpentine; Sarpagandha), a miracle drug plant of India, was produced from its somatic callus tissue, which grew *ex vitro* and fruited normally (Chaturvedi *et al.*, 2007). Now, micropropagation has emerged as a promising technique for rapid and large scale propagation of selected medicinal plants. Micropropagation, is infact, the miniature version of clonal propagation carried out under aseptic conditions. The technique of micropropagation is based on the concept of totipotency as proposed by Harberlandt. Every cell of the plant body is totipotent i.e. capable of giving rise to a new plant under proper nurture conditions. Micropropagation protocols have been

developed for a wide range of medicinal plants like *Piperlongum* (Soniya E V and Das M R, 2002), *Spilanthes acmella* (Saritha *et al*., 2002), *Caper* (Carra *et al*., 2007), *Chlorophytum borivillianum* (Debnath *et al*., 2007), *Glycyrrhiza glabra* (Arya *et al*., 2009), *Citrullus colocynthis* Linn.(Mahesh *et al*., 2010), *Aglaonema* (Mariani *et al*., 2011) and many more.

Micropropagation can be achieved by any of the following three techniques:

- Multiplication by enhanced axillary shoots proliferation
- Multiplication by *de novo* adventitious shoot formation
- By somatic embryogenesis

In 1st and 2nd techniques plantlet formation occurs via organogenesis through production of unipolar shoots which are further multiplied, followed by rooting in a multistage process. In contrast, somatic embryogenesis leads to the formation of bipolar embryos through steps that are often similar to zygotic embryogenesis. All the above three techniques have been adopted for the production of medicinal plants under *in vitro* conditions.

Multiplication by enhanced axillary shoot proliferation:

Micro-propagation through axillary shoot proliferation is an easy, safe and the most reliable technique for mass propagation because it ensures genetic uniformity of clones. This method assures the consistent, large scale production of true-to-type, pathogen free plants within a short period of time. Ehanced axillary branching method involves the abolition of apical dominance resulting in de-reppression and multiplication of axillary buds. A shoot tip and an axillary bud when grown under high cytokinin concentration, usually develop axillary shoots which can be subdivided into smaller clumps of shoots. These shoots can develop similar clusters after subculturing on fresh medium. This process can go on indefinitely and large number of plants can be raised starting from a single shoot tip or axillary bud.

Cytokinins alone or in combination with lower concentration of auxins play an important role in inducing axillary bud sprouting in several medicinal plants. The rate of multiplication has been extremely variable with genotype and cytokinin requirements. Banerjee *et al.,* (1999) reported in *Centella* that initial sprouting required the presence of BAP (2 mg/l) and IBA (0.1 mg/l), however, for induction of multiple shoots, higher concentration of BAP (3 mg/l) and lower concentration of NAA (0.05 mg/l) was required. Tiwari *et al.,* (2001) reported multiple shoots in

Bacopa on MS medium supplemented with auxins or / and cytokinins with or without coconut milk. Maximum number of plants was obtained on medium containing Kn/2-ip (0.1 mg/l) and Kin (1 mg/l) in shoot tip and nodal cultures respectively. Singh *et al*. (2009) developed an efficient protocol for *in vitro* regeneration of endangered medicinal plant *Rauvolfia serpentine.* Among the various combinations of BAP and IAA used, the frequency of shoot regeneration was highest, 75%o on BAP (2.5) +IAA (0.4) mg/l.

Evylene and Ravindhran (2011) reported multiple shoot proliferation from mature nodal segment of *Lippia nodiflora* on MS medium supplemented with BA (3.0 mg/L) and Kn(0.50 mg/L) where the maximum number of shoots (14.66 shoots) were produced from each explant. Das *et al.* (2011) achieved multiple shoots from single shoot tip of *Stevia rebaudiana* on MS medium having 3% sugar and 2mg/l Kn. Kumar *et al*. (2011) successfully regenerated multiple shoots (38 shoots per explant) from nodal explants of Ashwagandha (*Withania somnifera* L.) with BAP (1.5 mg/l) and IAA (1.5 mg/l) incorporated medium. Karnawat *et al*., (2011) induced multiple shoot regeneration from nodal segments of *Verbesina encelioides* on MS medium supplemented with 3 mg/l BAP. Mehta *et al.,* (2012) described a protocol for multiple shoot regeneration from nodal segment of *Bacopa monnieri* through auxiliary shoot proliferation on MS medium supplemented with 0.5 mg/l BAP + 2.0 mg/l Kn.

De novo formation of adventitious shoots:

De novo adventitious shoot formation through direct regeneration is considered to be the most reliable method of micropropagation because in this method, genetic uniformity is maintained among the progenies and is less prone to contamination because total numbers of stages of culturing are reduced significantly. Good number of medicinal plants have been successfully propagated *in vitro* by adventitious shoot initiation, directly from explants like root, stem, petiole, leaf lamina and flower parts or indirectly from the calli obtained from these explants. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots. *De novo* formation of adventitious shoots from various explants has been reported in a number of medicinal plants like *Panax ginseng* (Choi *et al*.,1998), *Withania somnifera* Dunal (Kulkarni *et at*., 2000), Sivanesan *et al*., 2005), *Chlorophytum borivillianum* (Debnath *et al*.,

2007), *Glycyrrhiza glabra* L.(Arya *et al*., 2009), *Embelia ribes* (Annapurna and Rathore, 2010), *Cassia angustifolia* (Siddique *et al*., 2010), *Tylophora indica* (Kaur et al. 2011) and many more.

Singh *et al*. (2009) reported highest callus formation from leaf explants of *Rauvolfia serpentine* on MS medium containing BAP (2.5mg/l) + IAA (2.0) mg/l. Naika (2007) developed and standardized indirect shoot formation protocol through stem callus culture for the medicinal climber *Clematis gouriana.* The explants induced optimized callus at the concentration of 1.0 mg l-1 BAP and 0.3 mg l-1 NAA. After initiation of callus, it was immediately transferred to MS medium containing 4.0 mg l-1 BAP and 0.5 mg IBA. Upon longer incubation for about 2-6 weeks on the same culture medium initiation of shoot buds from the callus mass was observed and regeneration of plantlets with higher frequency (mean of 11.1 ± 0.23 shoots per explants) was noted.

Zare (2010) achieved indirect shoot regeneration in *Ferula assa-foetida* (Asafetida), a medicinally important vulnerable species, by callusing on MS medium with 1 mg NAA and 2 mg BA and then transferring to MS medium containing 1-3 mg l-1 BA or Kn alone or in combination with 0.2 or 0.5 mg l-1 NAA which facilitated indirect organogenesis.

Nathawat (2011) reported i*n vitro* callus induction in *Cissus quadrangularis*a from stem explants on MS medium supplemented with NAA (2.5 mg/l and BAP (0.5mg/l). Ganesan and Paulsamy (2011) developed a reliable protocol for callus induction and organogenesis from leaf explants of *Artemisia annua* L. on MS medium containing BAP (0.5 mg/l and GA3 (1.0.mg/l) where higher no of shoots (20.67/callus) formed. Mehta *et al*. (2012) recored best callus induction in leaf petiole explants of *Bacopa monnieri* on MS medium supplemented with 0.25 mg/l 2, 4-D+ 0.5 mg/l Kn and 0.25 mg/l 2, 4-D+ 0.1 mg/l BAP. Kalpana *et al.* (2013) observed the callus from stem segments of *Cissus quadrangularis* on MS medium having higher concentrations of 2,4-D (4.0 mg/l). Yaacob (2013) successfully achieved optimum shoot regeneration in *Justicia betonica* Linn. by culturing internodal explants on MS medium supplemented with 1.5 mg /l NAA and 0.5 mg/l BAP.

Although indirect shoot regeneration from callus is considered a good method of multiplication, but the plants developed from the calli may not be true to elites

because of the incidence of aneuploidy and polyploidy associated with callus and the plants obtained from it. This becomes the cause of many morphological, physiological and genetic variation of the callus and raised plants. Also this approach of multiplication is not applicable to many important crop species. In cases where it is applicable, initial plant regeneration capacity of the tissue may decrease with passage of time. Even then callus is regarded a peculiar piece of material for rapid plant multiplication, as thousands of plants can be raised from a small piece of tissue.

Somatic embryogenesis:-

It is another alternative to traditional vegetative propagation method as it offers a rapid and large-scale propagation system. It involves the formation of a bipolar structure containing both shoot and root meristems, and developing in a manner similar to zygotic embryos. These embryo like structures have been given different names like accessory embryos, adventives embryos and embryoids. These embryoids can develop into fully functional plants under appropriate conditions. As somatic embryos already carry a pre-formed radical which develops root, so there is no need of rooting as is required in organogenesis.

Somatic embryogenesis was first reported by Steward *et al*., in carrot root tissue in 1958. Embryoids formation has been reported in tissue and organ cultures, derived from leaf petiole, root, floral parts etc of a number of medicinal plant like *Ginger* (Kackar *et al*., 1993), *Truf-type bermudagrass* (Chaudhury *et al.*, 2000), *Pimpinella tirupatiensis* (Parkash *et al.*, 2001), *Centella asiatica* L.(Martin, 2004), *Tylophora indica* (Chandrasekhar *et al.*, 2006), *Rauvolia serpentine* L(Singh *et al*., 2009), *Podophyllum peltatum* (Sundar and Jawahar, 2010), *Withania sominifera* (Sharma *et al.*, 2010), *Chrysanthemum* (Mani and Senthil, 2011), *Eclipta alba* L.(Devendra *et al*., 2011) and many more medicinal plants.

In the case of *Cissus quadranularis*, there is hardly any report regarding a definite micropropagation protocol for its propagation.

INTRODUCTION

Medicinal Plants: An overview

Plants have been important source of medicines for thousands of years apart from being source of food, fibre, flavour, wood etc. The application of plants as medicines date back to prehistoric period and the use and knowledge of medicinal plants is evidenced through ancient records of all major systems of medicine such as Ayurveda, Unani, Chinese medicine and Japanese Kampo (Natesh, 2001). According to World Health Organization (WHO), over 80% of the world's population, primarily those of developing countries relies on traditional medicine, largely plant base, for their primary healthcare . Medicinal plants also play an important role in the lives of rural people, particularly in the remote parts of developing countries with few health facilities. They serve as therapeutic agents as well as important raw materials for the manufacture of traditional and modern medicine.

About 20,000 to 35,000 species of plants are used as medicines, pharmaceuticals, cosmetics and neutraceuticals by different ethnic groups over the entire world (Trivedi, 2006). Recent estimate suggests that over 9,000 plants have known medicinal applications in various cultures and countries (Majid *et al*., 2012). In India, it is reported that the traditional healers use 2500 plant species, out of which 100 species of plants serve as regular source of medicine (Pei, 2001). Over the past few years, herbal remedies have regained a wide recognition and are making a comeback as the drugs obtained from plants are cheaper, exhibit a remarkable efficacy in the treatment of various ailments and are much safer with least side effects as compared to allopathic medicines (Siddiqui *et al*., 1995, Iwu *et al.*, 1999, Idu *et al*., 2007, Mann *et al*., 2008, Ammara *et al.*, 2009). The use of herbal medicines is growing in developed countries, presently 25% of the UK population use herbal medicine (Vines, 2004, Zhou and Wu, 2006). About 40% of compounds used in pharmaceutical industry are directly or indirectly derived from plants because the chemical synthesis of such compounds is either not possible and/or economically not viable (Stafford *et al.*, 1986, Rout *et al.*, 2000, Oksman-Caldentey and Inzé, 2004, Sidhu, 2010).

Medicinal plants are an integral component of research and development in the pharmaceutical industry. They constitute nearly 70% of the basis of modern

pharmaceutical products including 25% of drugs derived from different plants and many other are synthetic analogues built on prototype compounds isolated from them. World Health Organization, estimated that approximately one fourth of the 500 million prescriptions written in US each year contain a mention of leafy plant extracts or active ingredients obtained from or modeled on plant substances (Malik *et al*., 2012). The most popular analgesic, aspirin, was originally derived from species of *Salix* and *Spiraea* and some of the most valuable anti-cancer agents such as paclitaxel and vinblastine are derived solely from plant sources (Katzung *et al*., 1995, Pezuto *et al*., 1996)

Indian Biodiversity and Status of medicinal plants in India:

India is a gene rich country and accounts for 8% of the total global diversity. India is known to harbour a rich diversity of higher plant species (about 17000 species) of which 7500 are known as medicinal plants (Shiva, 1996) with concentrated spots in the region of Eastern Himalayas, Western Ghats and Andaman & Nicobar Islands. It stands tenth among the plant genetic resource rich countries and one of the world's top twelve-mega diversity nations (Khoshoo, 1995). Such a huge number of medicinal plant species has allowed the evolution of many systems of herbal medicine such as Ayurveda, Unani, Siddha, Homeopathy and Naturopathy (Vaidya and Devasagayam, 2007 and Chaturvedi *et al*., 2007). Ayurveda, the oldest medical system in Indian sub-continent has alone reported using approximately 2000 medicinal plant species, followed by Siddha and Unani (Kala, 2002). Though India has a rich biodiversity, about 90% of the medicinal plants used by industries are collected from the wild with 70% of the plant collection involving destructive harvesting because of the use of plant parts like root, bark, wood, stem and the whole plant. The growing demand is putting a heavy strain on existing resources, which pose a definite threat to the genetic stock and biodiversity of medicinal plants, causing a number of plants to be either threatened or included in the endangered category. The assessments done so far for the prioritized native medicinal species, have resulted in assignment of IUCN red list status to nearly 250 plant species with 44 species being critically endangered, 113 endangered and 87 vulnerable (Ved and Tandon, 1998 and Ved and Goraya, 2007).

Trade of Medicinal plants:

The international trade of medicinal plants is dominated by only a few countries. About 80 % of the world-wide imports and exports are allotted to only 12 countries with the dominance of temperate Asian and European countries. Whereas Japan and the Republic of Korea are the main consumers of *pharmaceutical plants*, China heads the list of the world's top 12 countries of export followed by three countries, namely the USA, Germany, and Hong Kong which are among the 12 leading countries of export and the 12 leading countries of import and stand out as important trade centres (Lange, 2004). Though Germany dominates the European trade, it ranks 3rd after Hong Kong as a world consumer.

According to WHO, the international market of herbal products is around $6.2 billion, which is growing at the rate of 15 to 25% annually and according to an estimate of WHO, the demand for medicinal plants is poised to grow to $5 trillion by the year 2050. Unfortunately, India's share in the global medicinal plants related export trade is just 0.5 percent. The Export-Import Bank of India, in its report for the year 1997, puts medicinal plants related trade in India at $.5.5 billion. Regarding the export of medicinal plants, India's contribution to the international market is comparatively low, because the country's natural resources are not being fully exploited. India's share in the global export of medicinal plants just 0.52 percent not withstanding it's having 15,000 species of such plants (Kumar and Janagam, 2011).

Medicinal plants, thus, offer remarkable opportunities to generate income and employment and play an important role in the economy of a country.

Scope for India:

The production and consumption of medicinal plant based products is going to grow at a significant rate both at domestic and international level. India has a good potential to participate in this trade and reap the benefits. So India must develop scientific cultivation, post harvest technology, processing, manufacturing, research and extension, patenting and marketing for medicinal plants and their products. The small and poor growers of these plants, mostly located in hills, mountains and inaccessible places must also be made more involved with the processes of commercial production and marketing of these products so that they can increase their earnings and are definitely not exploited. The state governments have to carry

forward this task with great zeal. Though economic importance of medicinal plants is well known, it is considered as a forestry sub-sector (non timber forest products) in India. Till Medicinal Plants Board was constituted in year 2000, no nodal agency was there to look into medicinal plants as an 'economic sector' and different organizations were dabbling with different aspects of medicinal plants without any focus and co-ordination thereby leading to paradox of simultaneous existence of under-utilization and overexploitation. Further, the lack of co-ordination has also led to critical research gap, that is, there is a regrettable absence of any research community working on socio-economic and policy aspects of medicinal plants, such as that which exists with regard to agro-technology, biotechnology etc. (Kala *et al*., 2006). Because of that India could not make deserving place in World trade of medicinal plants.

This is time to replan India's participation in the global market and to collect all the available information regarding medicinal plant development in the country in order to obtain a comprehensive overview which will provide the necessary insight for the co-ordination and effective action to get the economical benefits (Majid *et al*., 2012).

Medicinal plants and Plant Tissue Culture

With an ever increasing inclination towards plant based drugs, there is huge demand for medicinal plants. This over harvesting is creating heavy pressure on selected, high valued medicinal plant populations pushing a number of medicinal plant species on the verge of extinction. To maintain proper supply of plant based material for the drugs, these medicinal plants need to be replenished in desirable quantity and rate, for which supply of high quality planting material is needed. Major problem with medicinal plant species is their slow growth rates, low population densities and narrow geographical range.

Majority of the medicinal plants either do not produce seeds or their seed are too small to germinate in soil. Thus mass propagation of disease free planting material is generally a problem. Moreover the sexually propagated plants demonstrate a high degree of heterozygosity since their progenies are not true to type unless they have been derived from inbred lines. As a result, plants raised through seeds shows great variations in growth, habit, composition and overall yield. Similarly majority of the medicinal plants are not amenable to conventional vegetative propagation methods as

they have often proved cumbersome and the plants raised through these methods are invariably infested with one or more pathogens which deteriorates their quality and genetic vigor, thus limiting the multiplication of desired cultivars. Due to the shortage of high quality planting material, cultivation and domestication of medicinal plants is facing great problem. It is therefore imperative to adopt alternative methods having high multiplication rates to produce large number of plants of improved quality and shortened rotation. In this regard, micropropagation or *in vitro* propagation holds high potential for true to type, rapid and mass multiplication of selected high quality planting material under disease free conditions. *In vitro* propagation implies *in vitro* growth of plantlets sampled from any part of plant in defined, suitable nutritive culture medium. Micropropagation insures a good regular supply of high quality medicinal plants, using minimum space and time.

Advantages of Micropropagation:

1. Requires relatively small growing space.
2. The technique of micro-propagation is applied with the objective of enhancing the rate of multiplication. Through tissue culture over a million plants can be grown from a small, even microscopic, piece of plant tissue.
3. Shoot multiplication usually has a short cycle (2-6 weeks) and each cycle results in logarithmic increase in number of shoots.
4. Tissue culture gives propagules such as mini-tubers or microcorms for plant multiplication throughout the year irrespective of the season.
5. The small size of propagules and their ability to proliferate in a soil free environment facilitate their storage on a large scale and also allow their large scale dissemination by suitable means of transport across international boundaries.
6. Stocks of germplasm can be maintained for many years.
7. Pathogen free plants can be raised and maintained economically.
8. When plants of one sex are desired commercially, clonal propagation in dioecious is extremely important since seed progently yields 50% females and 50% male. For example, male plants of asparagus officinalis are more valuable than female plants. Propagation by stem cutting in male asparagus is not successful but can be achieved by tissue culture.

Techniques of Micropropagation

Micropropagation of plants can be achieved through three main techniques:-

1. Enhanced axillary shoot proliferation:

Micro-propagation through apical and auxillary shoot proliferation is the most common, reliable and applicable method for *in vitro* mass multiplication. Cells of the meristems are uniformly diploid and least susceptible to genetic changes. Hence, it is the most reliable technique for mass propagation since it ensures genetic stability of the clones.

2. *De novo* formation of adventitious shoots:

New adventitious shoots can develop either:

a) Directly from the explants like root, stem, petiole, leaf lamina and flower parts etc. or

b) Indirectly from callus cultures obtained from these explants. Plants obtained through calli may not be true elites because of high incidence of polyploidy and aneuploidy associated with callus cells and plants obtained from it.

3. Somatic embryogenesis:

It involves the formation of bipolar embryos from the somatic cells which can develop into fully functional plants under appropriate conditions.

Stages in micropropagation

Micropropagation involves 4 definite stages.

These are as follows:

Stage I: Isolation, Establishment and Stabilization of explants in aseptic cultures:

The objective of this stage is isolation, surface sterilization and aseptically establishment of explants on appropriate culture medium.

Stage II: Shoot multiplication or rapid somatic embryo formation using a defined culture medium:

The purpose of this stage is to maintain the culture in a stabilized state and multiply the micro-shoots to the number required for rooting. It can be achieved through any one of the following four methods:

(i) Multiplication through calli obtained from different organs and tissues and their subsequent sub-culturing leading to organogenesis.

(ii) Multiplication through direct induction of shoots on the explants.

(iii) Multiplication through growth and proliferation of existing apical shoots & adventitious buds.

(iv) Somatic or nonzygotic embryogenesis directly on the explants or in callus cultures.

Stage III: Rooting of regenerated shoots in *in vitro* conditions:

The function of this stage is to root the stage II micro-cuttings or shoot clusters for successful transfer to the soil. The process may involve:

- Elongation of shoots prior to rooting.
- Shoots are separated manually from clusters and transferred to a rooting medium supplemented with auxin or basal medium for root formation.

Stage IV: Acclimatization or hardening: (Transfer of plantlets to natural environment):

This stage involves the shift from a heterotrophic to an autotrophic condition.

- The climatic adaptation of a plant when shifted to a new environment is known as acclimatization.
- Plants which are produced under cultural conditions (high humidity, low light, constant temperature) when transferred to field conditions are required to be acclimatized.
- Acclimatization imparts some tolerance to moisture stress and a shift from heterotrophic to autotrophic nutrition. During hardening, plantlets develop cuticle and their stomata start functioning.
- Hardened plantlets are then transferred to glass or polyhouse bench and finally transferred to normal environmental conditions.

Rationale and Objectives

The present study is carried out on a medicinally important plant *Cissus quadrangularis* which is used in traditional medicine across the world for treatment of fractured bones, diarrhea, skin disorders, irregular menstruation, piles, tumors wounds and scurvy (Kritikar *et al*., 2000). Because of its tremendous importance, large-scale production of this plant under disease conditions is highly desirable.

Propagation of this plant is not possible through seeds as the flowers are sterile and the plant does not set seeds. It is propagated by vegetative means only. Apart from these, there are a number of constraints like edaphic factors, climatic and

seasonal dormancy which limit the propagation and conservation of this plant species through conventional methods. This valuable plant has not been studied much through tissue culture technique and no definite micropropagation protocol is available for its multiplication.

Keeping in view the medicinal importance and the difficulties associated with the propagation of this valuable plant, the present investigation has been conducted with the following objectives:

- To develop a reliable and reproducible standard protocol for large scale propagation of this plant in short duration of time and space.
- To raise disease free and genetically pure elite plants under *in vitro* conditions.