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Research Article

Multiple Shoot Formation and Efficient Root Induction in *Cissus quadrangularis*

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ABSTRACT
A standard protocol for the fast *in vitro* multiplication and plantlet regeneration of a medicinal plant, *Cissus quadrangularis* from different *in vitro* grown explants (shoot tip, nodal explant and *in vivo* shoot tip) is described. All the three explants were cultured on MS medium supplemented with Zeatin alone or in combination with BAP and BAP alone or in combination with adenine sulphate for multiple shoot formation. Of the three explants tested, *in vitro* nodal explant produced the maximum number of shoots (5 shoots) and the maximum mean shoot length (2.86 cm) were observed on MS medium supplemented with 0.5 mg L^{-1} Zeatin after 30 days of inoculation. Multiple shoots were obtained from the *in vitro* shoot tip explant cultured on MS medium supplemented with Zeatin alone at low concentration and BAP in combination with adenine sulphate. Shoot tip cultures of field grown *C. quadrangularis* proliferated speedily *in vitro* on MS medium + BAP. For efficient root induction, the *in vitro* formed shoots were rooted on MS medium supplemented with or without IAA and IBA. The rooted *in vitro* raised plantlets were acclimatized in pots containing sterile soil having vermicompost at equal quantity and successfully transferred to green house with high survival rate.

Key words: Multiple shoot induction, Zeatin, BAP, auxins, shoot tip, nodal explants , *in vivo* shoot tip

INTRODUCTION

Cissus quadrangularis Linn. (fam: Vitaceae) commonly known as bone setter due to its bone fracture healing property is a rambling shrub, characterized by a thick quadrangular fleshy stem, found throughout the hotter parts of India, Sri Lanka, Malaysia, Thailand and West Africa (Udupa et al., 1970).The stem is used for the treatment of eye and ear diseases, irregular menstruation, asthma, piles, tumors, and fractures of bones, stomach ulcer, wounds and scurvy (Kritikar et al., 2000). It is useful in treating gastrointestinal disorders such as colic and dyspepsia (Williamson et al., 2002). The plant has numerous bioactive compounds such as alkaloids, resveratrol, piceatannol, pallidol, parthenocissin , quadrangularins, ascorbic acid, carotene, phytosterol substances, calcium, flavonoids, vitamins, enzymes, nicotinic acid, tyrosin, and triterpenoids. *Cissus quadranguaris* contains a variety of terpenoid components, such as the balsam ketone, amyrin, onocer 7 ene 3 a 21diol (Bhutani et al., 1984). The stem contains two asymmetric tetracyclic triterpenoids, and two steroidal principles. The presence of β-sitosterol, δ-amyrin, δ-amyrone, and flavanoids (quercetin) having different potential metabolic and physiological effects has also been reported (Jakikasem et al., 2000). It has also been reported that water extract of *Cissus quadrangularis* is found to be more effective and works antagonistically to HepG2 cell proliferation as compared to methanol extract, which is used traditionally for the treatment of cancer (Opoku et al., 2000). In addition, Resveratrol is an effective anti-cancer agent of natural chemicals from *C. quadrangularis* that can trigger the human tumor cells, CD95 signaling-dependent cell death (Clement 1998). Thus, the plant is in great demand for the production of traditional and modern medicines. Due to uncontrolled exploitation of this medicinal plant to meet its ever increasing demand for food, medicines and scientific research, it is being threatened.

Cissus quadrangularis requires warm tropical climate and are mainly propagated by stem cuttings during June and July. Propagation through seeds is unreliable because of non seed viability . The traditional propagation method by ‘cuttings’ limits the number of propagules. Therefore, the application of a reliable, *in vitro* clonal propagation system would provide an optional method of propagation to meet the pharmaceutical needs and for effective conservation of this precious medicinal plant. Moreover, the plant multiplication can be carried on throughout the year irrespective of season , and the stocks of germplasm can be maintained for several years.

To the best of our knowledge, no work in the field of tissue culture has been reported. In the present investigation we are reporting the protocol for an efficient *in vitro* micro propagation protocol for the multiplication of *Cissus quadrangularis* for the first time.

MATERIAL AND METHODS

Plant material and sterilization: Healthy shoot tip explants of *Cissus quadrangularis* were collected from plants grown at Ayurvedic College, Patiala, Punjab and Kulish samriti van, Jaipur, Rajasthan. Before use the excised shoot tip explants were taken in a conical flask and

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Fig. 1a-f **(a)** multiple shoots induced from *invivo* shoot tip on 2,4-D-1 mg L^{-1} +Kn-2 mg L^{-1} **(b)** Single Shoot proliferation on BAP-1 mg L^{-1} from *invivo* shoot tip explant (c) single shoot proliferation from invitro shoot tip on Zeatin-0.5 mg L^{-1} **(d)&(e)** multiple shoots induced on BAP-5 mg L^{-1} + adenine sulphate0.5 mg L^{-1} on invitro shoot tip **(f)** multiple shoots induced on BAP-3 mg L^{-1} +Zeatin-1 mg L^{-1} on invitro shoot tip explant

washed thoroughly with running tap water for 20 min. After that the explants were soaked in 5% (w/v) liquid detergent (teepol) for 5 min, then washed under running tap water for 10 min to remove all the remaining detergent. The shoot tip explants were then sterilized with 0.1% bevistin for 12 min, after the explant were rinsed with sterilized distilled water thrice, the explant were then surface sterilized by treating them with 0.1% solution of mercuric chloride for 2 min. After surface sterilization of explant, mercuric chloride was removed and the explants were washed 4-5 times with sterile distilled water immediately to remove all he traces of mercuric chloride. The sterilization procedure was done in front of the running laminar air flow cabinet. The sterilization method described above was used for *in vivo* explants only.

Culture medium and culture conditions: The basal culture medium used for the present study comprised MS (Murashige and Skoog, 1962) medium supplemented with 2% (w/v) sucrose. This basal medium was supplemented with various growth regulators – BAP, Zeatin, Adenine sulphate, Kinetin, 2,4-D and NAA at varying concentration either singly or in combination. The pH of the medium was adjusted to 5.7-5.8 with 1N NaOH and 1N HCl before gelling it with 0.8% (w/v) agar

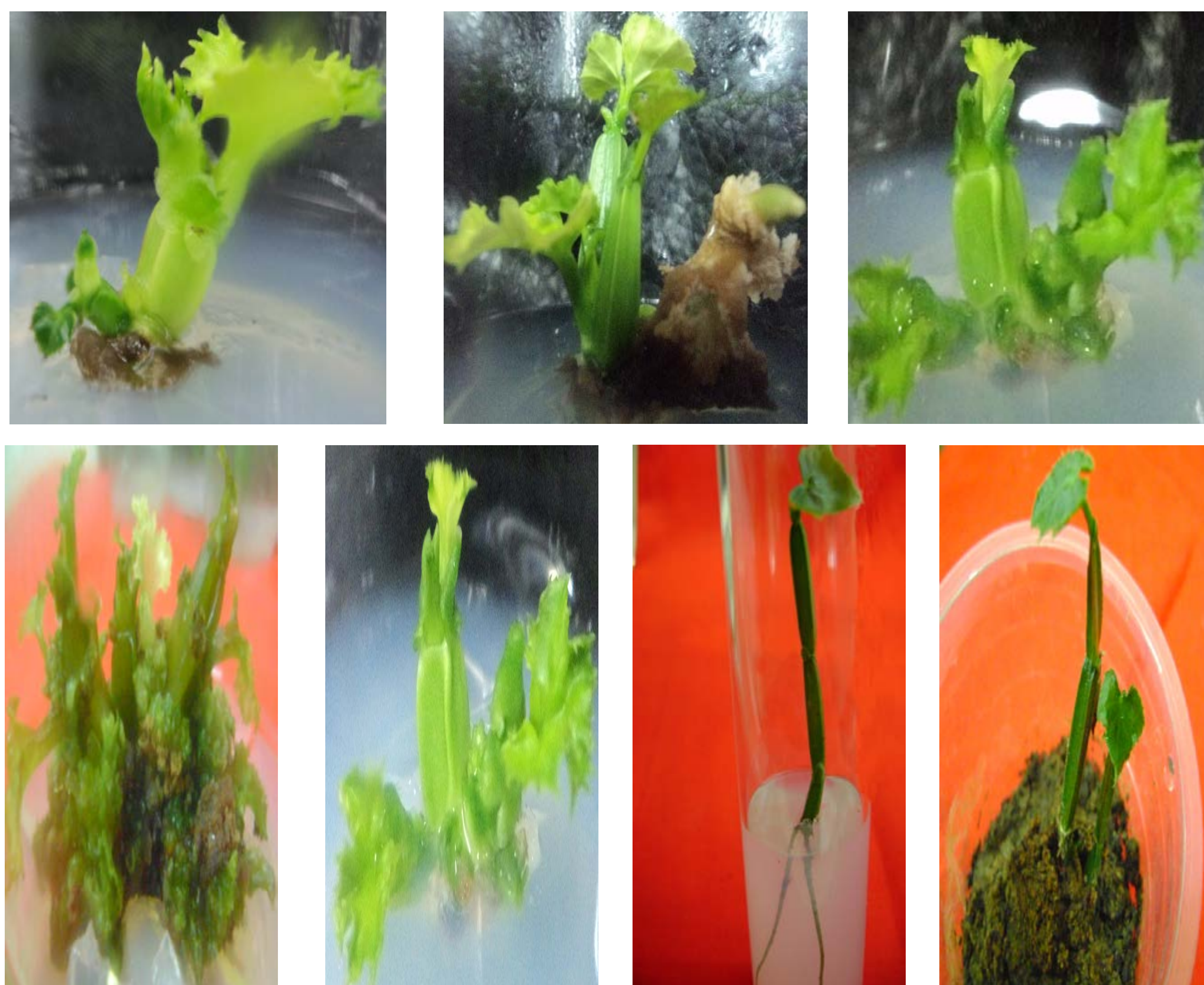

Fig.2g-m **(g)** shoots proliferated from *invitro* nodal segment on BAP-1+Zeatin-1 mg L^{-1} **(h)** Multiple shoots induced on Zeatin-.5mg L^{-1} **(i)&(k)** multiple shoots induced on Zeatin-.5 mg L^{-1}**(j)** Multiple shoots formation from invitro nodal on zeatin-1 mg L^{-1} **(l)** Rooted shoot on BMS **(m)** acclimatized plant

Table-1 Effect of plant growth regulators on shoot regeneration from *invitro* nodal segment of *C.quadrangularis* in MS medium after 30days of culture 8 test tubes were inoculated for each concentration

Sr. No.	PGR conc. (mg L^{-1})	% of explants Regenerated-directly	No. of shoots/ explant	Time taken toinitiate shooting (days)	Mean Length of the shoots (cm)	Degree of shoot Ing
1	Zeatin- 0.5	87	3	4	2.86	+++
2	Zeatin – 1	87	5**	5-7	2.43	+++
3	Zeatin -2	87	4	10-15	2.14	+++
4	BAP-1 + Zeatin – 1	75	2	7-10	1.41	++
5	BAP-3 + Zeatin – 1	62	2	15-20	1.7	++
6	BAP-2 + Adenine sulphate 8	62	1	7	2.1	++
7	BAP-6+KN-1	62	2	10-15	2.14	++
8	BAP- 2	62	2	20	1.5	++
9	BAP- 4	62	2	20	1.4	+
10	BAP- 16	62	2	20	1.8	++

+ poor shooting, ++ moderately good shooting, +++ satisfactory shooting, **highest number for *invitro* nodal segment explant . PGR= Plant Growth Regulator

Table-2 Effect of plant growth regulators on shoot regeneration from *invitro* shoot tip segment of *C.quadrangularis* in MS medium after 40 days of culture, 8 test tubes were inoculated for each concentration

Sr. no.	PGR conc. (mg L^{-1})	% of explants Regenerated-directly	No. of shoots/ explant	Time taken to initiate shooting (days)	Mean Length of the shoots (cm)	Degree of shooting
1	Zeatin-0 .5	75	2	15	1.33	++
2	Zeatin – 2	62	2	10	1.40	++
3	BAP-1+ zeatin – 1	75	4	25	2.17	++
4	BAP-3 + zeatin – 1	75	4***	20-25	2.75	+++
5	BAP-5 + Adenine sulphate 0.5	75	3	20	4	+++
6	NAA-4	50	1	25-30	3.1	+

+ poor shooting, ++ moderately good shooting, +++ satisfactory shooting, ***highest number for *invitro* shoot tip explant

Table-3 Effect of plant growth regulators on shoot regeneration from *invivo* shoot tip segment of *C.quadrangularis* in MS medium after 40 days of culture, 8 test tubes were inoculated for each concentration

Sr. no.	PGR conc. (mg L^{-1})	% of explants Regenerated directly	No. of shoots/ explant	Time taken to initiate shooting (days)	Mean Length of the shoots (cm)	Degree of shooting
1	BAP-1	62.5	1	20	4.5	++
2	BAP-2	50	1	10	1.62	+
3	NAA -1 + KIN-2	-	-	-	-	-
4	NAA -1 + KIN-0.5	-	-	-	-	-
5	2,4-D-1+ KIN- 2	50	3	20	1.12	+

+ poor shooting, ++ moderately good shooting, +++ satisfactory shooting

and dispensed in 35 ml aliquots into 150 ml conical flasks prior to autoclaving at 121°C and 15 lbs pressure for 20 min. The surface disinfected explants were implanted horizontally on the culture medium. The *in vitro* explants viz nodal segment and shoot tip explant were excised from *in vitro* grown plantlets and the explants were directly inoculated horizontally on the culture medium. Culture (also of *in vitro* rooting) in all experiments were incubated in a growth chamber maintained at 26±2°C under a 16/8- h (light/dark) photoperiod with light provided by cool white fluorescent tubes.

Shoot initiation and multiplication: For multiple shoot proliferation the sterilized shoot tip explants (*in vivo*) and *in vitro* explants- shoot tip and nodal segment were excised from *in vitro* grown plantlets and were cultured on MS medium supplemented with various plant growth regulators benzylaminopurine (1 mg L^{-1}, 2 mg L^{-1} ,4 mg L^{-1},8 mg L^{-1} and 16 mg L^{-1}), Zeatin (.5- 2 mg L^{-1}), Adenine sulphate (.5 mg L^{-1} and 8 mg L^{-1}) and Kinetin (.5 mg L^{-1} and 2 mg L^{-1}) either individually or in combination, such as BAP alone or in combination with Zeatin and adenine sulphate. All of the cultures were sub cultured on to the fresh medium after every 15 days. Percentage of the explants produced shoots; number of shoots per explant, mean length of shoot and time taken to initiate shooting were recorded after 4 weeks of culturing.

Rooting of regenerated shoots: For root induction, microshoots raised (4-5 cm) were excised individually and transferred to either Basal MS medium or medium supplemented with auxins such as Indole Butyric Acid (IBA; 1 mg L^{-1}), Indole-3-Acetic Acid (IAA;1-2 mg L^{-1}). Data were recorded for different parameters including percentage of shoots to which root initiated, time taken to initiate rooting and mean length of the root after 30 days of transfer on to the rooting medium.

Acclimatization: Plantlets with well developed roots and shoots were removed from the culture medium and after washing the roots gently under running tap water; the plantlets were transferred to plastic pots which contained autoclaved garden soil and vermin compost in equal volume. The potted plantlets were maintained inside the plant growth chamber set at 26±2°C for 3-4 weeks and watered every three days with distilled water. Hardening of potted plants for 3-4 weeks in a growth chamber (fig.n) was found to be essential. Afterwards, these plants were transferred to pot containing normal soil and maintained in a green house under normal dry length conditions.

All the experiments were conducted with 8 replicates per treatment. The experiments were repeated two times. The

Table-4 Effect of different concentration of IBA, IAA in MS medium and BMS on root induction in regenerated shoots: 10 test tubes were inoculated for each concentration.

Sr. no.	Hormone conc. (mg L^{-1})	No. of shoots subcultured	% of Shoots to which root initiated	Time taken to initiate rooting (days)	Mean Length of the roots (cm)	Degree of root ing
1	BMS	10	80*	12	6.13	+++
2	IAA-1	-	-	-	-	-
3	IAA-2	-	-	-	-	-
4	IBA-1	10	50	20	4.6	++

+ poor rooting, ++ moderately good rooting, +++ satisfactory rooting, *highest % of shoots to which root initiated

cultures were observed periodically and morphological changes were recorded at regular intervals.

RESULTS AND DISCUSSION

In the present study, the morphogenetic response of *in vitro* and *in vivo* explants to various cytokinins or plant growth regulators (BAP, Zeatin, Adenine sulphate) were observed (Tables-1-3) .. Several treatments were tried to regenerate multiple shoots from different *in vitro* and *in vivo* explants. The percentage of response varied with the type of growth regulator used as well as its concentration.

Earlier Debnath et al., (2007) aimed at obtaining multiple shoots from various explants in *Chlorophytum borivilianum* and the number of buds produced per shoot was influenced by auxin and cytokinin concentrations.

Vijay,. Virk and Nagpal (2010) investigated the effect of explants type and different plant growth regulators on callus induction and plantlet regeneration in *Citrus jambhiri* Lush. Leaf segments, nodal segments and root segments excised from *in vitro* raised seedlings were used as explants. Regenerated shoots were rooted on MS medium supplemented with different plant growth regulators and best response (71%) was observed with NAA (0.5 mg/L).

Effect of different plant growth regulators on the regeneration of *Cissus* plantlets from *in vitro* nodal explant: In *in vitro* nodal explants at all the concentration of BAP (1 mg L^{-1}, 2 mg L^{-1}, 4 mg $L^{-1}$and 16 mg L^{-1}) and Zeatin (0.5-2 mg L^{-1}) alone facilitate shoot bud differentiation. A number of explants responded to regenerate multiple shoots in different concentrations of BAP and Zeatin alone and BAP in combination with Zeatin or Adenine sulphate. A morphogenetic response of *in vitro* nodal explants to various concentration of plant growth regulators are shown in Table-1. Of the two cytokinins tested, Zeatin alone was more effective than BAP in shoot proliferation as well as multiple shoot formation. On MS basal medium, without any growth regulator supplement., there was no sign of shoot proliferation even after four weeks. In *in vitro* nodal explants the highest percentage of cultures that regenerated shoots was 87% in MS medium supplemented with Zeatin (0.5-2 mg L^{-1}) (figs.i,j & k).The highest mean number of shoots per explant was recorded on the medium supplemented with 1 ppm Zeatin and the mean length of the longest shoot was 2.86 cm in 0.5 ppm Zeatin after 30 days of inoculation. The mean length of the shoot declined with increase in Zeatin concentration up to the optimal level (2 mg L^{-1}). Evelyne Priya and Ravindhran (2011) used nodes and shoot tips as the explants in *Lippia nodiflora* for the initiation of multiple shoots and cultured on Murashige and Skoog (MS) medium supplemented with cytokinin Benzyladenine (BA) and Kinetin (KIN). The maximum number of shoots was produced in BA 3.0 mg/l. An average of 14.66 ±1.30 shoots was produced from each explant.

Karuppusamy and Kalimuthu (2010) investigated rapid *in vitro* multiplication and regeneration from nodal explants of *Andrographis neesiana.* Nodal explants obtained from 30-d-old aseptic seedling were developed. High frequency of direct shoot proliferation was induced in nodal explants cultured on Murashige and Skoog's medium supplemented with thidiazuron. Amongst the various cytokinins tested (BAP, kinetin, thidiazuron and 2-isopentyl adenine), thidiazuron proved to be most effective.

Kumar *et al.* (2011) studied multiple shoot regeneration from nodal explants of Ashwagandha (*Withania somnifera* (L.) Dunal. Several experiments were done with nodal explants in an attempt to improve the process of regeneration of multiple shoots in *in vitro.* The optimum medium for multiple shoot formation (i.e., 38 shoots per explants) was Murashige and Skoog medium with BAP (1.5 mg/l) and IAA (1.5 mg/l). Gibberellic acid (0.15mg/l) was effective in enhancing the maximum shoot elongation. 72% of *in vitro* raised shoots responded for rooting on MS medium fortified with IBA (5.0 mg/l) and the rooted plants were hardened and transplanted to the soil. The plants showed 80-90% survival during transplanting.

Effect of different plant growth regulators on the regeneration of *Cissus* plantlets from *in vitro* shoot tip explant: In *in vitro* shoot tip explants, the highest percentage of shoot tip explant regenerated shoots was 75% in MS media having different concentration of BAP in combination with Zeatin or adenine sulphate and Zeatin alone. After 40 days of inoculation the highest mean number of shoots per explant was recorded 4 in the medium containing BAP-3 ppm + Zeatin-1 ppm (fig. f) and BAP-1 mg L^{-1} + Zeatin-1 mg L^{-1} (Table-2). Although the mean length of the longest shoots was recorded as 4 cm in the medium fortified with BAP-5 mg L^{-1}+ Adenine sulphate-.5 mg/l (figs.d&e) the lowest percentage of shoot tip regenerated shoots was 60 % in MS medium supplemented with NAA-4 mg L^{-1}. The least mean

number of shoots was also recorded in the same medium composition. Palanivel et al., (2009) reported multiple shoot formation and efficient root induction in groundnut by using BAP in combination with NAA and Kin as well as IAA.

Effect of different plant growth regulators on the regeneration of *Cissus* plantlets from *in vivo* shoot tip explant: Multiple shoots were induced from the explant cultured on MS medium containing 2,4-D-1 mg L^{-1}+ Kin-2 mg L^{-1}, but these failed to elongate. On the other hand, when MS medium containing BAP at low concentration (1 mg L^{-1}) was used, the sprouting period or shoot differentiation period was lengthened, but percentage of regenerated explants increased and shoots/explant were substantially reduced. Only one shoot was formed which attained a height averaging 4.5 cm within 40 days of culturing on the same media. There was a progressive increase in shoot length with decreasing BAP concentration (Table-3).

In vitro shoot proliferation and rooting capacity of avocado cv. Fuerte was tested by using two apical and axillary buds with varying concentrations of BAP and IBA on MS media. Explants showed different responses towards shoot proliferation with highest shoot number by axillary buds (2.50). On the contrary the apical buds gave positive results for shoots length (2.16 cm) over axillary buds. In axillary buds 1.0 mg l^{-1} BAP gave best rate of shoot multiplication (4.80), whereas 1.5 mg l BAP favored the good shoot length development (4.06 cm) with apical buds (Zulfiqar, *et al*., 2009)**.**

Induction of bud sprout was obtained from shoot tip and nodal explants derived from *in vitro* grown plants of *Accia chundra* on the Murashige and Skoog (MS) basal medium supplemented with 6-benzylaminopurine (BA) (1.0 mg/l) and 20 mg/l adenine sulfate (Ads) (Rout et al., 2008).

Rooting of regenerated shoots: 4-5 cm long shoots derived at the completion of the shoot multiplication cycle were excised and transferred to MS basal medium with or without plant growth regulator. Root formation from the basal cut portion of the shoot was observed 12 days after transferring to rooting medium. IBA at low concentration in half strength MS medium was effective for rooting and the best rooting was achieved in the basal MS medium without auxins. The optimal rooting efficiency for shoots (80%) as well as the highest root length (6.13 cm) was obtained on the same medium, where rooting was initiated after 12 days of culture. Debnath et al., (2007) transferred elongated shoots to ½ Ms medium having auxins for rooting at various concentrations (0.5-2.5 mg/ l). Palanivel et al., (2009) reported maximum rooting in excised cotyledonary node derived in vitro shoots when transferred to full strength MS medium fortified with different concentrations of IAA. Low concentration of IAA induced 50% of the excised shoots produced rooting in *Lippia nodiflora.* They used 0.1, 0.5, 1.0 and 1.5 mg/l of auxin . Half strength MS medium containing IBA of 1.0 mg/l produced the maximum number of roots (10.3 ± 4.00) in *Lippia nodiflora* (Evelyne Priya, and Ravindhran, (2011).

Hardening: To optimize the hardening procedure, plantlets with well developed roots and shoots were transferred to the plastic pots containing sterile soil and vermicompost at equal volume and hardened as per the procedure described in "Material and Methods"(fig.n). The hardening survival was 80% in 1:1 (soil/vermicompost). Very high survival rate of in vitro plants shows the efficiency of the procedure and could be easily adopted for large scale multiplicatuion.

Arya et al., (2009) reported that only 20% plants survived when planted during the rainy season in *Glycyrrhiza glabra* but in contrast 80-90% plantlets survived when hardened and acclimatized before transferring to field.

Different workers have used different hardening procedures. Thus, Evelyne Priya, and Ravindhran, (2011) employed hardened rooted plants and grew them in sand: cocopith (1:1) with good results.

Present investigations demonstrate fast *in vitro* multiplication and plantlet regeneration of a *Cissus quadrangularis* from different *in vitro* grown explants (shoot tip, nodal explant and *in vivo* shoot tip). The protocol is extremely useful in transformation studies and also in the selection of desirable genotypes with elite traits. The present work also shows that nodal explants have great morphogenetic potential for *in vitro* micro propagation , which will be of immense use in transformation studies to secure desirable genotypes for multiplication.

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