INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

HIGH FREQUENCY SOMATIC EMBRYOS INDUCTION FROM THE RHIZOME EXPLANT OF *PANAX PSEUDOGINSENG* WALL. USING THIN CELL LAYER SECTION.

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ABSTRACT: An efficient protocol of somatic embryogenesis using transverse thin cell layer (tTCL) culture has been developed from rhizome explants of *P. pseudoginseng*, with potential for highfrequency somatic embryos production of this important medicinal plant. tTCL (0.5, 1.0 and 3.0 mm) of rhizome explants were cultured in Murashige and Skoog (MS) and Schenk and Hildebrandt (SH)media supplementedwith different concentrations of2, 4-dichlorophenoxyacetic acid (2, 4-D) to check the response on callus induction. Two types of media (MS and ½ MS) were used for induction of somatic embryogenesis. For callogenesis, 0.5mm tTCL displayed a better response in MS medium (84.32%) compared to same tTCL studied in SH medium (48.24%) in presence of 2,4-D.The frequency of somatic embryo formation was significantly higher (56.66%) in MS mediumincorporated with2.5mg/l each of BAP and 2,4-D, compared with that of ½ MS medium (40.00%), however the highest number of embryos (20.60) were obtained in ½ MS supplemented with 2.5mg/l each of KN and 2,4-D.Maximum conversion of somaticembryos into normal plantlets (28.33±0.30) was obtained in ½ MS with 1.0 mg/l of GA_3. Finally plantlets were transferred to a mixture of black garden soil, compost and leaf litter in the ratio of 2:1:1with survival rate 70%.

Key words: *Panax pseudoginseng*, Somatic embryogenesis, 2,4-D- dichlorophenoxyacetic acid. Medicinal plant, Conservation.

Abbreviations BAP: 6-Benzylaminopurine;2,4-D: 2,4-Dichlorophenoxyacetic acid; GA_3: Gibberellic Acid; KIN: Kinetin; FAA: Formalin Aceto-Alcohol solution; MS: Murashige and Skoog (1962); 1/2MS- half strength MS medium; SH: Schenk and Hildebrandt; SE: Somatic Embryo.

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INTRODUCTION

Most species of *Panax* are important medicinal herbs widely used as tonic medicine, it is a potential anticancer agent, helps in reducing blood pressure and has an anti-hyperglycemic tendencies (Awang1999). The roots are said to be analgesic, anti-inflammatory, antiseptic, astringent, diuretic, haemostatic, styptic, tonic and vulnerary. They are used in treatment of contused wounds, soft tissue injuries and all kinds of bleeding, both internal and external, like haematuria, nose bleeding, haematemesis, uterine bleeding etc (Bonfill et al. 2002). *Panax quenquifolius* L, commonly known as American ginseng (AG), is one of the ten most commonly used herbal medicines in the United States (Kim et al. 2006). It has been used in traditional medicine in China and other countries for the treatment of various diseases (Zhang et al. 2004), including psychiatric neurologic diseases as well as diabetes mellitus, due to the presence of a wide range of saponins and sapogenins (Li TSC 1995).

Phytochemical analyses of some other species of the genus *Panax* have revealed to synthesize the same class of biologically active saponins (ginsenosides) as syntheized by *P. ginseng* (Mathur et al. 2003). These congener species include *P. quinquefolium*L. (American ginseng), *P. pseudoginseng* Wall. (Sanchi ginseng), *P. japonicas* C.A. Meyer and *P. notoginseng* (Burkill) F.H. Chen. These species are increasingly used as substitutes for traditional ginseng prescriptions (Ngan et al. 1999). In recent years ginseng has become a popular tonic and health food in Western countries. Therefore the demand for the plant has increased dramatically worldwide. Ginseng is very expensive because of its long-term conventional (5–7 years) and troublesome production cycle. *Panax Pseudoginseng* from North East India is one of the most commercialized plant, noted for higher proportions of ginsenoside (Mathur et al.2003). The species are over-exploited from the natural habitats making its existence on the verge of extinction. As a result, propagation methods of these medicinal plants by plant tissue culture and particularly by somatic embryogenesis have been investigated.

The use of tissue culture to propagate elite individuals has proved to be an alternative strategy to improve yields through selection and cloning of elite materials (Robert et al. 1987, 1992, 2004). However, the cost of *in vitro* micropropagated plants is still high compared to those naturally propagated and more efficient and cost-effective methods are needed for largescale production for commercial planting (Proceedings by joint FAO/IAEA, Vienna, 2002). Previous studies with Korean and American ginsengs, callus formation and embryogenesis were obtained on calli derived from root explants after 2 months and 6 months respectively (Whei–Lan et al., 2002).In similar studies with *P.ginseng*, somatic embryos were obtained in root-derived calli after 3-8 months of culture (Chang and Hsing 1980; Ahn and Kim 1992). Nhut et al., 2012, induced somatic embryos from the TCL culture of *in vitro* root explants with the embryogenesis rate of 53.3%. Thus, somatic embryogenesis has been used as an important experimental tool for large- scale propagation, (Kharwanlang et al. 2016).

Thin cell layer (TCL) technique was first introduced to plant organogenesis studies by Tran Thanh Van in 1970's. Thin cell layer (TCL) technique employs various explants of small size from different plant organs excised either longitudinally (lTCL, containing one tissue type) or transversely (tTCL, containing small number of cells from different tissue types) (Tran Thanh Van, 1980).In order to increase the efficiency in culture initiation for somatic embryogenesis, tTCL technique was carried out in the present study. This technique has advantages by maximizing the use of starting material, minimizing the undesirable contamination and inreducing the time period. TCL being thin can directly contact to the culture medium enhancing faster nutrient and hormone uptake. As a result, rapid and uniform development of explants to different structures including callus, primary shoot/roots, and embryos were observed.Keeping in mind the importance of TCL and its application in somatic embryogenesis via callus formation, we report high frequency somatic embryos induction from the rhizome explant of *Panax pseudoginseng* Wall. using TCL.

MATERIALS AND METHODS
Explant Source
Young plants, four to five years old, were collected during monsoon season (May-September) from Phek district of Nagaland, India. The rhizome was thoroughly washed with detergent, labogen for 10 min. Explant was surface disinfected with 70% ethanol for 5 min, followed by surface sterilization with 10% sodium hypochlorite (4 %available chlorine, Himedia) with few drops of tween-20, Himedia) for 10 min, and with mercuric chloride (0.1% w/v) for 2 min, followed by rinsing four to five times with sterile distilled water.

Culture Media and Environment
The Murashige and Skoog (MS) (1962) and Schenk and Hildebrandt (SH) (1972) basal medium comprised of mineral nutrients, vitamins along with 30g/L sucrose and 0.8% agar. Plant growth regulators in various combinations were added to the medium. The pH of the medium was adjusted to 5.8 prior to autoclaving at 121℃. All the cultures were maintained at 24±2℃ in a culture room under cool white fluorescent lamp for 16-h photoperiod, except that explants for inducing callus were incubated at 24±2℃ in the dark.

Callus Induction
Transverse thin cell layer sections (0.5, 1.0 and 3.0mm thickness) of rhizome serve as initial explants for callus induction. The explants werecultured on Schenk and Hildebrandt (SH) (1972), and Murashige and Skoog (MS)(1962) medium supplemented with different concentrations (0.5, 2.5, 5.0, 7.5, 10.0 mg/l) of 2,4-dichlorophenoxy acetic acid (2,4-D) with 3% sucrose and 8g/l of agar (Himedia).Each treatment consisted of ten replicates and the experiment was repeated three times. All the cultures were kept in the dark for 8 weeks in the same medium, till the callus proliferated into good amount.

Somatic Embryos Development and Plantlet Regeneration

To check the response on the induction of callus into somatic embryos, 0.5g of morphogenic culture (callus) was transferred onto MS and 1/2MS basal medium supplemented with various concentrations (0.25-10.0 mg/l) of 6-benzyladenine (BAP) with 2.5 mg/l of 2,4-Dand various concentrations (0.25-10.0 mg/l) of kinetin (KN) with 2.5 mg/l 2,4-D separately. Controlled cultures were maintained on medium lacking growth regulators. The experiment was repeated three times, with 10 replicates (0.5g of callus) for each concentrations. After 10 weeks of inoculation, the compact callusturned into a white loose and fragile embryogenic callus and the frequency of somatic embryogenesis inductioni.e, the percentage of explants producing SEs was recorded accordingly. The mean number of embryos per explants (0.5g of callus) was recorded after 3 months of subculture, and the embryo forming capacity index (EFC) was calculated accordingly as, EFC= [(mean no.of SEs per explant) X (% of explants forming SEs)] ÷100. The embryos obtained were transferred to a germinating medium, 1/2MS and MS medium augmented (after autoclaving) with gibberellic acid (GA_3) at 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L. GA_3 was filter sterilized and added into the medium that had cooled to about 50–60 ℃. The cultured were kept in the same described culture condition. The frequency of conversion of somatic embryos into plantlets was calculated as [(number of plantlets per 0.5g callus)/(number of somatic embryos per 0.5g callus)]. Normal plantlets with both roots and shoots, were regenerated within 4 to 5 months after sub culturing the somatic embryos in the same medium. Each experiment was repeated three times. The well developed plantlets were transferred to glass house for hardening.

Histological observation and Scanning electron microscopystudies

SEs were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 h at 4 ℃, dehydrated through a graded ethanol series for 10 min each and embedded in saturated paraffin wax (Himedia), at 60℃. Sections of7µm were cut using a rotary microtome (LeicaRM2125RT). Paraffin wax was removed by xylene prior to rehydration of the tissues in a graded ethanol series, it was then stained with 0.05 % toluidine blue and mounted in DPX. The sections were observed and photographed using a Leica light Microscope.

Scanning electron microscopy (SEM) was used for morphological observation of somatic embryos formation. The embryos were fixed as described earlier, washed in phosphate buffer for 1h, fixed in 1% osmium tetroxide for 1h and dehydrated through an increasing series of acetone. Dehydrated tissues were dried in a HCP-2 (Hitachi) critical point-drier. The dried samples were positioned horizontally to brass stubs with double-sticky tape, coated with gold in a JFC-1100 (JEOL) ion sputter Coater and observed using a JEOL, JSM-6360 SEM at 20 kV.

Acclimatization and Ex vitro plant establishment

Plantlets with well developed rootswere gently washed under running tap water to remove adhering medium. Subsequently they were transferred to the pots containing sterilized mixture of black garden soil, compost and leaf litter in the ratio of 2:1:1. The plantlets were maintained under greenhouse condition with a temperature of 25 ± 2 ℃. The plantlets were irrigated twice a day. Plantlets acclimatized successfully to *ex vitro* conditions with the survivability of 70%.

Statistical analysis

Statistical calculations were carried out according to the analysis of variance (ANOVA), and the results were examined according to tukey's test. In all the cases, values represented means of ten replicates per treatment, repeated three times.

RESULTS

Determination of potentiality tTCLof rhizome for callus induction

All the three different sections of thin cell layer (0.5,1.0,3.0 mm thickness) showed the initiation of callus after 1 week of inoculation in both the media tested. Callus started developing in the surface of the explants and proliferation of the callus continued in the explants with 0.5mm of thickness (fig 1A) in MS medium, with the exception in the SH medium, in which the explants became necrotic or showed less signs of active growth. The growth of the callus stopped in the other sections (1.0mm and 3.0mm) in both the media, where they eventually turned brown and died off. The frequency of the explants which produced calli, was highest (84.32%) in the MS medium with 2.5 mg/L 2,4D (table 1).

Table 1: Effect of 2,4-D on callus induction from the rhizome segment (0.5-1.0 mm)

Basal medium	PGR (mg/l) 2,4-D	Callus induction (%)*
0	0	0.0[e]
MS	0.5	62.38 ± 0.08[bc]
	2.5	**84.32 ± 0.12[a]**
	5.0	71.24 ± 0.10[b]
	7.5	60.00 ± 0.08[bc]
	10.0	52.48 ± 0.05[bc]
SH	0.5	18.44 ± 0.12[cd]
	2.5	34.00 ± 0.08[c]
	5.0	48.24 ± 0.10[c]
	7.5	27.32 ± 0.06[c]
	10.0	12.22 ± 0.12[d]

* Different letters within the column represent significant difference at P ≤ 0.05 by Tukey–test

Effect of growth regulators on embryogenic callus induction and somatic embryo development
Embryogeniccalli, (fig 1B) were obtained after 8 weeks of subculturing the callus in MS and ½MS media supplemented with 2,4-D, KIN and BAP in combination.The white and fragile embryogeniccallus was formed with the subsequent development to somatic embryos. In the present study the combined effect of 2,4-D with BAP and KIN used for the induction of SEs from the callus had a statistically significant impact on the frequency of embryogenesis after 3 monthsof culture. MS medium augmented with 2.5 mg/l 2,4-D and 2.5mg/l BAP (fig 4) gave the best response for highest frequency of SE induction (56.66%). ½ MS augmented with 2,4-D (2.5 mg/l) and KIN (2.5mg/l) displayed the best response with the highest mean number(20.60±0.80) of SEs (fig 5). EFC index which combines the frequency of SE induction and the mean number of embryos per 0.5g of callus, was observed to be highest (7.64) in ½ MS with BAP (fig 4) and lowest (0.14) in ½ MS with KIN (fig 5). The callus transferred to MS and ½ MS with only 2,4-D, did not produce SE, the callus turned brown and eventually died off.
Different stages of embryos were observed after 3months of culture.Globular embryos were induced from the embryogenic calli followed by the asynchronous development of the embryos. Lately, cotyledonary embryos were formed. The swollen light yellowish SEs were formed and the process is asynchronous as various stages of development of embryos were seen in the same explant (fig 2 A and B).When the embryos were subcultured in MS and ½ MS basal medium (with the required composition of both the macro and micro elements along with the vitamins without any additional supplements) devoid of/same plant growth regulators, turned brown and died eventually after 4 to 5 months. Hence ½ MS medium with alternative growth regulator ie GA$_3$was used for regeneration of somatic embryos.

Plant regeneration and acclimatization
 The embryos germinated in ½ MS medium in presence of GA$_3$ (fig 3A) and the highest response (28.33±0.30) was seen with 1.0mg/l of GA$_3$ (table 2), wherein complete normal plantlet was obtained after 4 to 5 months of culture in the same medium (fig 3B). The well developed and healthy plantlets were successfully acclimatized under shaded greenhouse conditions (fig3C). The regenerated plantlets showed no apparent morphological variations from the naturally growing plants. The shoots of regenerated plantlets withered away during winter, and new shoots were developed in the next spring. The survival rate was 70% after 9 months of transfer.

Histological and SEM analysis
The histological study was carried out to analyzed the ontogeny of the SEs. The study revealed the successive stages of development of the SEs including the globular, late globular and cotyledonary embryos (fig 2E as shown in arrows). The SEs were observed tobe initiated from the outer layer of the embryogenic calli. There is no apparent vascular connection between SEs and the masses of callus.SEM analysis further confirmed the early development stages of the SEs. (fig-2C) wherein the globular and the late globular stage of the embryos were observed in the study. The asynchronous development process took place where numerous globular embryos and other different stages of development were induced in the same explants (fig-2D).
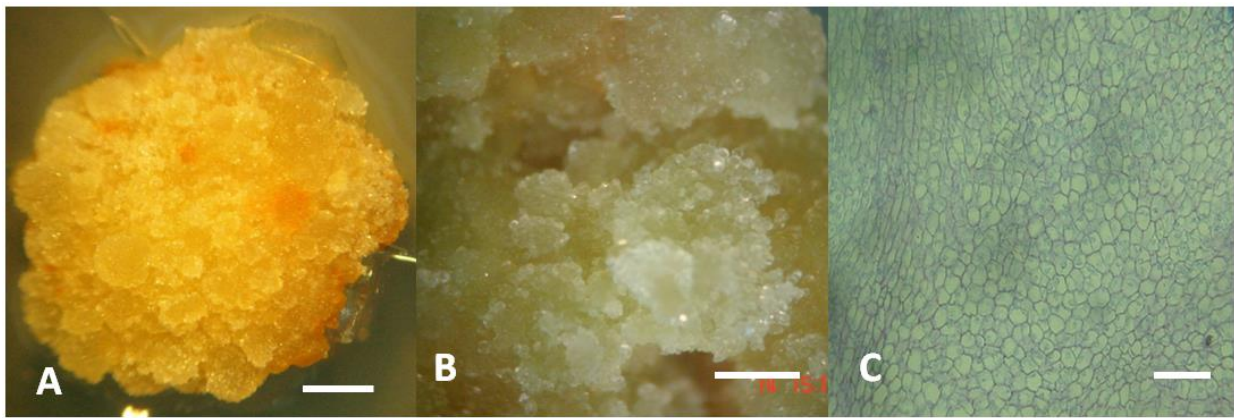
Fig-1: Callogenesis in P.pseudoginseng (A) callus from Ttcl of rhizome (B) Embryogenic calli (C) Histological section of embryogenic calli Bar 5mm
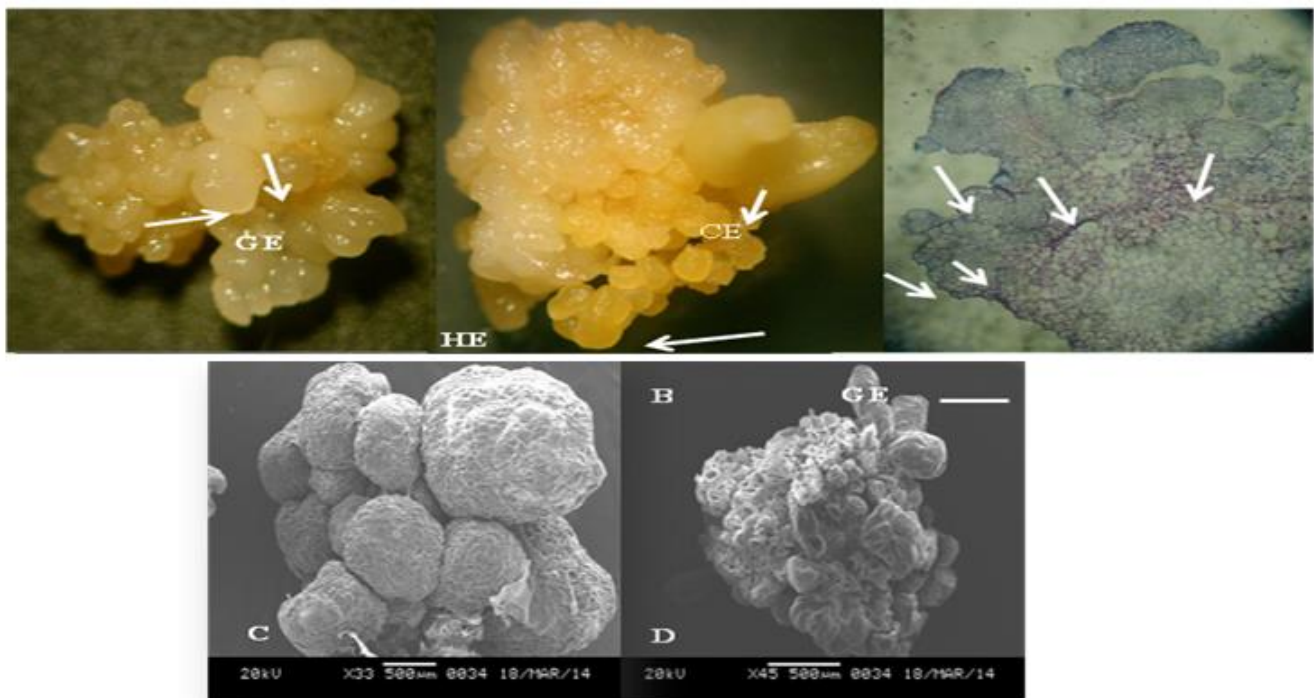

Fig-2: Somatic embryogenesis (A) Globular Embryos (B) Asynchronous development of SEs (C) SEM micrographs globular SEs (D) Asynchronous SEs micrographs (E) Histological section showng asynchronous development of SEs (Shown in arrows). Bar-1 mm (GE: Globular, HE: heart shaped, CE: cotyledonary embryos.
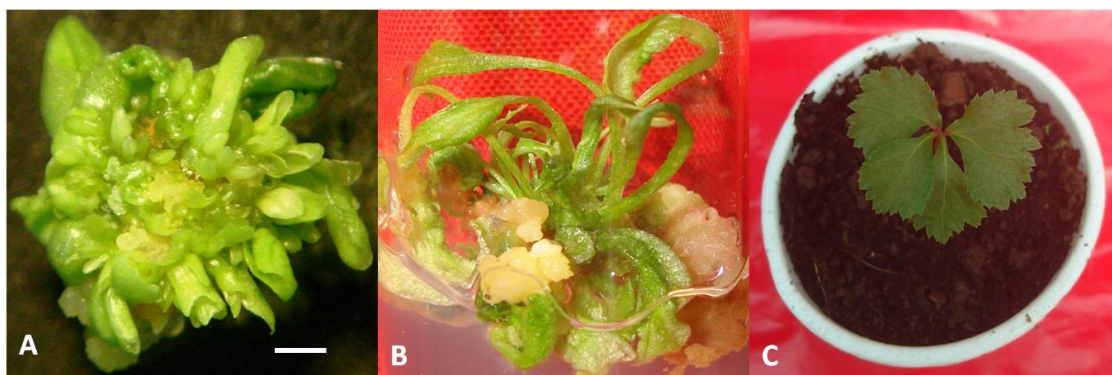

Fig-3: Plant regeneration of SEs in (A) Germinating embryos (B) Regenerated plantlets (C) hardened plant, Bar-1mm.
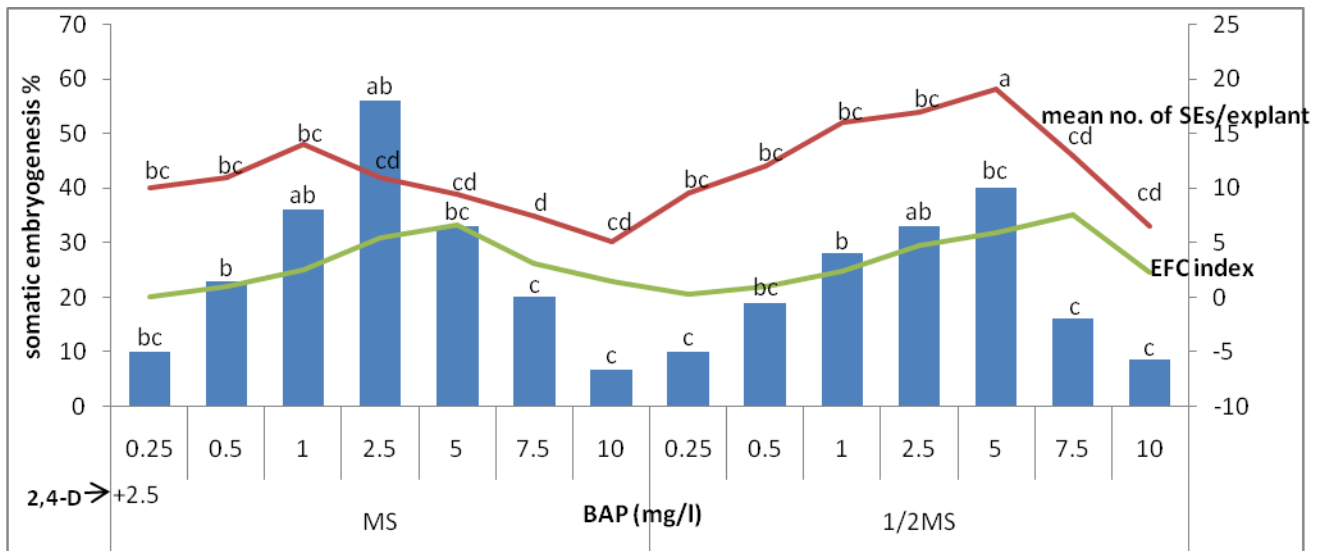
Fig. 4: Effect of different concentrations of BAP (0.25-10.0mg/l) in combination with 2,4-D (2.5mg/l) in MS and ½ MS medium on the induction of somatic embryos from the derived callus (0.5g). Different letters represent significant difference at P ≤ 0.05 by Tukey–test.
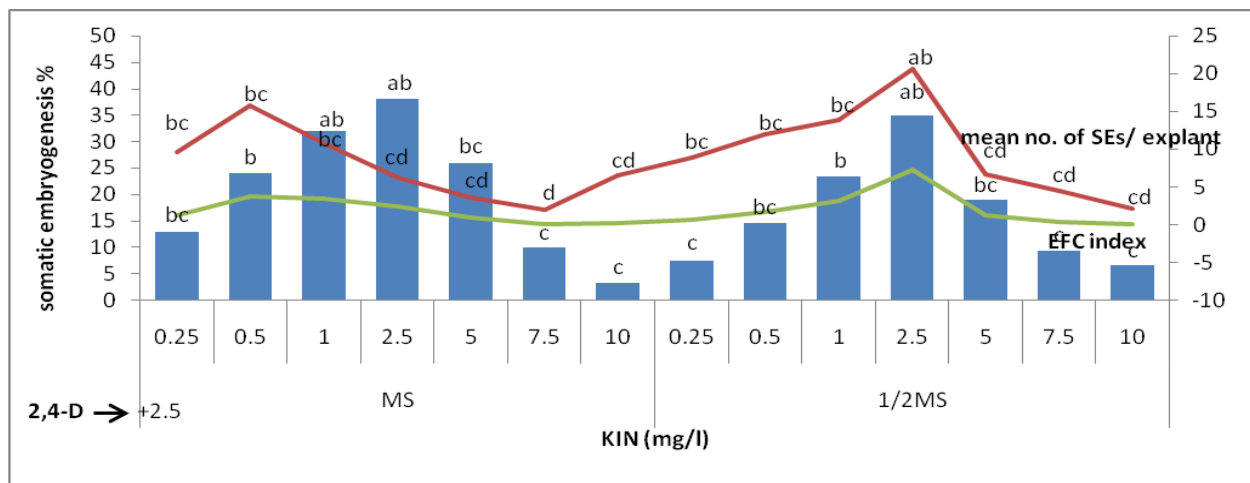

Fig. 5: Effect of different concentrations of KN(0.25-10.0 mg/l) in combination with 2,4-D (2.5 mg/l) in MS and ½ MS medium on the induction of somatic embryos from the derived callus (0.5g). Different letters within each column represent significant difference at P ≤ 0.05 by Tukey–test.

Table 2: Effect of different concentrations of GA$_3$ on regeneration of somatic embryos in ½ MS.

Plant growth regulator	Mean number of regenerated plantlets*
0.0	0 [d]
0.2	5.0±0.22 [bc]
0.5	8.33±0.30 [bc]
1.0	**28.33±0.30** [a]
1.5	16.66±0.33 [b]
2.0	11.66±0.30 [bc]
2.5	5.0±0.22 [bc]
3.0	3.33±0.21 [c]

* Different letters within the column represent significant difference at P ≤ 0.05 by Tukey–tes

DISCUSSION

The present study is the first report of somatic embryogenesisin *P.pseudoginseng* through tTCL from the rhizome explants. In this procedure, three different sections (0.5, 1.0 and 3.0mm), showed their capacity to generate callus. The thinness section (0.5mm) proved to be the best explants source as all the cell are uniformly exposed and attached more closely to the medium. The original concept of totipotentiality (totipotency) which Haberlandt himself conceptualized almost 75 years earlier—that was revolutionary about TCLs. Rather, it was the capacity to more strictly control the outcome of an organogenic ''programme'', not so much by the contents and additives of the medium or the surrounding environment, but rather by the size of the explants itself, that captivated the minds and attention of plant tissue culture scientists since 1973 (Silva, 2012). Thus, in our present study the few mm (0.5) thick tTCL section showed to be most suitable explants for the induction of callus.Another conclusion drawn from the present study is theadvantage ofTCL culture that reduces the time interval required to generate the desired callus or somatic embryoseespecially with this medicinal plant where the leaching of the secondary metabolites took place that leads to the browning and dying of the explants.

Induction of callus was significantly different among the two different media, in which MS medium resulted in highest induction percentage compared to SH medium. This result also indicated that differences in the combinations of nutrients and their different concentrations contributed to the callus formation potential of the explant. The effect of growth regulators in callus induction have been studied in various plant species.The potential of 2,4-D had been reported as the most efficient growth regulator *Eleutherococcus sessiliflorus, Gymnema silvester, Holestemma ada-kodien, Paspalum scrobiculatum, Andrographis paniculata* (Choi et al., 2002, Kumar et al., 2002, Martin, 2004).In our study 2,4-D was found to help in induction of callus within a week of culture. The combined effect of 2,4-D, BAP and KIN for the induction of embryogenic calli and the subsequent formation of somatic embryo formation, as seen in the present study, was in accordance with the micropropagation of *P.assamicus* (Kharwanlang et al., 2016)and similarly with *Anthurium andreanum* 'Tera' as reported by Beyramizade et al, 2008. Our findings contradict with the study in monocot, in case of ginger as reported by Lincy et al, 2009 and Guo and Zhang, 2005 where the callus became embryogeniconly in the presence of BAP and complete removal of 2,4-D from the medium favoured somatic embryogenesis. Similar combination of hormones i.e., BAP and 2,4-D has resulted in embryo formation from primary culture stage in *Pelargonium x domesticum* (Haencsh, 2007). Likewise, same combination of growth regulators induced embryogenic callus in *Acacia arabica* (Nanda & Rout, 2003) and in *Ocimum basilicum* (Gopi & Ponmurugan, 2006).Another important conclusion from the present study is the time required for the induction of somatic embryos was cut short to about 3 months as compared to the previous work carried out by Whei–Lan et al., 2002 and Chang and Hsing 1980; Ahn and Kim 1992, where somatic embryos were obtained after a period of 6 months and 3-8 months respectively.

Gibberellic acid is often used to stimulate the germination of somatic embryos (Goerge, 1993). In this study the lower concentration (1.0 mg/l) of GA_3 promote the germination of SEs. With increasing in the concentration, the conversion of SEs into plantlets decreased. The lower concentration of GA_3 that promote the plantlet conversion in this experiment is similar to that used in other studies. For instance, mostly in the range of 0.3- 1.0 mg/l 0f GA_3, SEs germination were successful in *Vitis* sp. (Mullins ans Srinivasan, 1976), *Citrus* sp. (Kochba, Button, Spiegel-Roy, Bornman and Kochba, 1974) and *Panicum maxicum* (Lu and Vasil, 1982). In this current study, the embryos growing in basal medium did not germinate they eventually died off. However the correct concentration of GA_3 (1.0mg/l) was identified to produce the maximum number of healthy and normal plantlets.

The ontogeny of SEs were further analyzed by histological section which showed the different stages and the asynchronous development of SEs. The section of embryogenic callus consisted of dense cytoplasm and prominent nuclei. The globular embryos with differentiated protodermis, further developed into the advanced stages of SEs, and the SEs were observed tobe initiated from the epidermal layer of the explants tissue.

Acclimatization is the most crucial stage for the successful in vitro regeneration protocol. Plantlets with fully developed roots and shoots after removing from the nutrient media through gentle washing, were transferred into thermocol cups containing sterilized mixture of black garden soil, compost and leaf litter in the ratio of 2:1:1 for the purpose of hardening (fig 2). Hardened plants were then transferred to pots and kept in field conditions in a greenhouse. 70% of survival rate was recorded.

CONCLUSION

An efficient and cost effective protocol has been established for the conservation of this medicinally important plant via thin cell layer technique for somatic embryogenesis induction supported by histological and electron microscopy study.No morphological abnormalities were observed in the tissue culture raised plants. So far no tissue culture work had been carried out with *P. pseudoginseng* which is a medicinally important plant, except one report by Mathur et al., 2003, where the callus obtained were highly recalcitrant and failed to give response. Hence the present study provide a simple and effective protocol for the large scale production of this valuable medicinal plant.

ACKNOWLEDGEMENT

REFERENCES

Ahn I.O., B.D. Kim. (1992). Transition of cellular oxidation–reduction status in accordance with somatic embryogenesis during the cultures of ginseng (*Panax ginseng*). J Kor Soc Hort Sci: 33(2) 231–235.

Awang D.V.C.. (1999). The neglected ginsenoside of North American ginseng (*Panax quinquefolius* L). Journal of Herbs, Spices & Medicinal Plants: 7:103-109.

Beyramizade E., P. Azadi, M. Mii (2008). Optimization of factors affecting organogenesis and somatic embryogenesis of *Anthurium andreanum* Lind. 'Tera'. Propagation of Ornamental Plants, Vol.8(4) pp. 198-203, ISSN 1311-9109

Bonfill M., R.M. Cusido, J. Palazon, C. Morales (2002). Influence of auxins in organogenesis and ginsenoside production in *Panax ginseng* callus. Plant Cell Tiss. Org. Cult 68:73-78.

Chang W.C., Y.I Hsing (1980). Plant regeneration through somatic embryogenesis in root–derived callus of ginseng (*Panax ginseng* C. A. Meyer). Theor Appl Genet 57:133–135.

Choi Y.E., S.K. Ko, K.S. Lee, E.S. Yoon (2002). Production of plantlets of *Eleutherococcus sessiliflorus* via somatic embryogenesis and successful transfer to soil. Plant Cell,Tissue and Organ Culture, Vol.69, No.2, (May 2002), pp. 201-204, ISSN 1573-5044.

Gopi C., P. Ponmurugan. (2006). Somatic embryogenesis and plant regeneration from leaf callus of *Ocimum basilicum* L. J. Biotechnol., 126: 260-264.

Gou Y., Z.X. Zhang (2005). Establishment and plant regenerations of somatic embryogenic cell suspension cultures of the *Zingiber officinale* Rosc. Scientia Horticulturae 107, 90–96.

Haencsh K. (2007). Influence of 2,4-D and BAP on callus growth and the subsequent regeneration of somatic embryos in long-term cultures of *Pelargonium* x *domesticum*cv. Madame Layal. Plant Biotechnol., 10(1): 69-77.

Kharwanlang L., M.C. Das, S. Kumaria, P. Tandon (2016). Histological and SEM studies on somatic embryogenesis in rhizome-derived callus of *Panax assamicus*. Ban. 5(4): 93-99.

Kim O.K., Y.C. Kim, Y.E Choi, Y.K. Seong (2006). Optimization of direct somatic embryogenesis from mature zygotic embryos of *Panax ginseng* C.A Meyer. J Plant Biol 49:348-352.

Kochba J., J. Button , P. Spiegel-Roy, C.H. Bornman, M. Kochba (1974). Stimulating of rooting of citrus embryoids by gibberelic acid and adininesulphate. Annals of Botany 38:795-802.

Kumar H.G.A., H.N. Murthy, K.Y. Paek (2002). Somatic embryogenesis and plant regeneration in *Gymnema sylvestre*. Plant Cell, Tissue and Organ Culture, Vol.71, No.1, (October 2002), pp. 85–88, ISSN 1573-5044

Li T.S.C. (1995). Asian and American ginseng: A review. Hort Technology 5: 27–34.

Lincy A.K., A.B. Remashree, B. Sasikumar (2009). Indirect and direct somatic embryogenesis from aerial stem explants of ginger (*zingiber officinale* Rosc). Acta Bot. Croat 68 (1): 93-103.

Lu C.Y., I.K. Vasil (1982). Somatic embryogenesis and plant regeneration in tissue culture of *Panicum maximum* Jacq. American Journal of Botany 69:77-81.

Mathur A., A.K. Mathur, R.S. Sangwan, A. Gangwar, G.C. Uniyal (2003). Differential morphogenetic responses, ginsenoside metabolism and RAPD patterns of three *Panax* species. Genet resour Crop Ev 50:245-252.

Martin K.P. (2004). Plant regeneration protocol of medicinal important *Andrographic spaniculata* (Burm. F.) Wallich ex Nees via somatic embryogenesis. In Vitro Cellular & Developmental Biology-Plant, Vol.40. No.2, (March 2004), pp.204-209, ISSN 1054-5476

Monteiro M., C. Kevers, J. Dommes, T.Gaspar (2002). A specific role for spermidine in the initiation phase of somatic embryogenesis in *Panax ginseng* CA Meyer. Plant Cell Tiss. Org. Cult. 68: 225-232.

Mullins M.G., C. Srinivasan (1976). Somatic embryo and plantlets from an ancient clone of the grapevine (cv.Cabernet Sauvignon) by apomixes *in vitro*. Journal of Experimental Botany 27: 1022-1030.

Nanda R.M., G.R. Rout (2003). *In vitro* somatic embryogenesis and plant regeneration in *Acacia arabica.*Plant Cell Tiss. Org. Cult., 73: 131-35.

Ngan F., P. Shaw, P. But, J. Wang (1999). Molecular authentication of *Panax* species. Phytochemistry 50: 787– 791.

Nhut D.T., NG. P. Huy, H.X. Chien, T.C. Luan, V.T. Vinh and L. B. Thao (2012). *In vitro* culture of petiole longitudinal thin cell layer explants of vietnamese ginseng (*panax vietnamensis* ha et grushv.) and preliminary analysis of saponin content. Int J Appl Biol Pharm. 3 (3): 178-190.

Robert M.L., J.L. Herrera, J.L. Chan, F. Contreras (1992). Micropropagation of Agave spp. In: Bajaj YPS (eds), Biotechnology in Agriculture and Forestry, vol 19. High-tech and micropropagation III.II.9.Springer Berlin pp 306–329.

Savangikar V.A. (2002). Role of low cost option in tissue culture: Proceedings by joint FAO/IAEA, Vienna. Low cost option for tissue culture technology in developing countries, IAEA Australia. pp 11.

Tran K. Thanh Van (1980). Control of morphogenesis by inherent and exogenously applied factors in thin   cell layers. Int. Rev. Cytol. Suppl. 11A, 175–194.

Robert M.L., J.L. Herrera, F.Contreras, K.N. Scorer (1987). In vitro propagation of *Agave fourcroydes* Lem. (Henequén). Plant Cell Tissue Organ Cult 8:37–48.

Robert M.L., J.L. Herrera-Herrera, M.A. Herrera-Alamillo, A. Quijano, U. Balám (2004). Manual for the in vitro culture of Agaves. Technical paper Nº 38. United Nations Industrial Development Organization, Vienna, Common Fund for Commodities.

Silva J.A. (2012). The role of thin cell layers in regeneration and transformation in orchids. Plant Cell Tiss. Org. Cult. 113: 149-161.

Whei–Lan T., S. Tiffany, T. Min–Chiang (2002). Explant preparation affects culture initiation and regeneration of *Panax ginseng* and *Panax quinquefolius*. Plant Cell Tiss. Org. Cult. 68: 233-239.

Zhang Z.Y., J.J. Zhong (2004). Scale-up of centrifugal impeller bioreactor for hyperproduction of ginseng saponin and polysaccharide by high density cultivation of *Panax notoginseng* cells. Biotechnol Prog 20:1076-1081.