Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN 2320-3862
JMPS 2016; 4(5): 165-170

Received: 21-07-2016
Accepted: 22-08-2016

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Effect of plant growth regulators and explants sources on somatic embryogenesis of matured tissue of the anticancerous medicinal plant *Plumbago rosea*

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Abstract
Plant regeneration was achieved from indirect somatic embryogenesis in *Plumbago rosea*. Embryonic callus induced from matured leaf, petiole and inter node explants grown on Murashige & Skoog (MS) medium supplemented with 2,4-dichloro-phenoxy acetic acid (2,4-D, 4.0 mgL-1) alone or in combinations with indole-3-acetic acid (IAA, 0.5 mgL-1), yeast extract (YE, 100.0 mgL-1), coconut water (CW, 10% v/v) and casein hydrolysate (CH, 100 mgL-1). Maximum callus induction was obtained from internode explants cultured on media containing 2,4-D (4.0 mgL-1), IAA (0.5 mgL-1), CW (100.0 mgL-1) and YE (100.0 mgL-1). Frequency of callus development, their nature and texture also differed based on their sources and effect of growth regulator concentrations. Frequency of globular embryo formation and a varied germination was recorded from explants source and nutritional media fortified with lower level of 2,4-D. Maximum maturation of globular embryo was recorded in 2,4-D (2.0 mgL-1), IAA (0.5 mgL-1) and YE (100.0 mgL-1). Subsequently germinated embryo developed into normal plants after being transferred to half strength of MS liquid medium. Our whole findings indicated that internode is the best tissue source for optimal somatic embryogenesis of *P. rosea.* Direct formation of somatic embryos also induced young *in vitro* grown plantlets (9 days old) and root segment of 5.0 cm when cultured in the medium containing kinetin (Kn, 2.0 mgL-1) and 2-naphthoxyacetic acid (NAA, 0.5 mgL-1) respectively. Hence, attempts to induce direct somatic embryogenesis have been achieved up to embryo regeneration and maturation.

Keywords: Inter-nodal explants, growth regulators, complex additives, mature tissue, embryonic callus

1. Introduction
Plumbago rosea L. (Plumbaginaceae) is an important medicinal plant and exploited source of plumbagin; the alkaloid (2-methyl-5 hydroxy-1, 4-napthaquinone) is synthesized in the roots of *Plumbago* species. The alkaloid contains a broad spectrum of pharmaceutically important metabolites such as anticancer (Krishnaswamy and Purushothaman, 1980; Jayaraman, 1987; Parimala and Sachdanandam, 1993; Devi *et al*., 1994) [23, 18, 32, 14], anti-microbial (Didry *et al*., 1994) [13], and lieshmanicidal (Kayser *et al*., 2000) [21] and insecticidal properties (Kubo *et al*., 1983) [24]. It also has antifeedant, growth regulatory and sterilant effects on insect pests (Gujar, 1990) [15]. The species is highly endemic in nature and included under the threatened flora (Biswas *et al*., 1998) for conservation. It can be propagated by off sets or small cuttings. Propagation through seed is difficult due to poor seed settings and germination. Methods of *in vitro* propagation have been shown to be useful tool for conservation of many threatened plant species. Plant regenerated through somatic embryonic pathway is an important consideration in developing a tissue culture protocol for transformation. At present, somatic embryogenesis has become an essential tool for the improvement of various systems (Cardosa *et al*., 2012; Sivanesan *et al*., 2011; Nuno-Ayala *et al*., 2012; Ratanasanobon and Seaton, 2010; Baskaran and van Staden, 2012) [5, 34, 38, 3]. Several investigators have concluded that casein hydrolysate itself is more effective for plant culture than the addition of the major amino acids. It can be a source of calcium, phosphate, several microelements, vitamins and most importantly a mixture of 18 amino acids. CH can be the excellent sources of reduced nitrogen, as they contain a relatively large amount of glutamine and overcome the shortage of glutamine when there is insufficient phosphorus for adequate biosynthesis (Khaled and Suliman, 2013) [19].

The present study reported the efficacy of different complex substances like casein hydrolysate (CH), yeast extract (YE), coconut water (CW), and the auxin including 2,4-dichloro-phenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA) in induction of somatic embryogenesis.

In previous work, a report on somatic embryogenesis of *P. rosea* from juvenile leaf tissues (4 week old *in vitro* grown plants) was established by Das and Rout (2002) [11] in the presence of benzyl amino purine (BAP), IAA, α-naphthalene acetic acid (NAA), kinetin (Kn) and gibberellic acid (GA3) fortified media at various stages and effect of acetylsalicylic acid and ammonium Komariah *et al*. (2004) [22] for optimum embryogenesis. This study was undertaken to select the optimum growth regulator concentration and best tissue source for efficient and successful embryogenesis from three different field collected matured tissues. Studies of Pinheiro *et al*. (2014) [34] also established the protocol for induction of somatic embryogenesis from nodal segments and show to be the most suitable explant for this process in *Anthurium andraeanum* cv. Eidibel. among five explant types.

In case of direct somatic embryogenesis, the preliminary information on regeneration, growth, differentiation, and development were investigated and this is the first report of the species.

2. Methodology

2.1 Materials and sterilization

Plant materials and sterilization of three different types of explants leaf, petiole and internodes were collected from five year old established plants of experimental garden NEIST, Jorhat, Assam. For establishment of embryonic cultures, explants were washed thoroughly in 5% Teepol solution for 20 min and then washed several times in single distilled water. They were thereafter disinfected by aqueous mercuric chloride solution (HgCl2) (0.1% w/v) followed by several rinses in sterile distilled water to remove the traces of HgCl2. Sterilized explants were cut into segment, internode (8 to 10 mm), leaf (1 cm) and petiole (1.0 to 1.5 cm) and implanted into sterilized media containing various combinations of phytohormones. For basal media preparation, constituents of MS medium were added to agar-agar (0.8 gL-1, Hi-Media) and autoclaved at 121°C at 15 lbs for 15 min with pH adjusted to 5.8 prior autoclaving.

2.2 Media composition

Induced primary somatic embryonic calli were sub cultured for further production of embryonic callus and their subsequent germination. Medium for induction of somatic embryonic callus, their proliferation and germination was conducted by transferring the somatic embryos into a low concentration of 2,4-D alone or in combination of IAA and different complex additives. In defining the media composition, 6 different media containing 2,4-D (4.0 mgL-1) alone or in combination with IAA (0.5 mgL-1) and three complex substances, namely, CW (10% v/v), YE (100 mgL-1) and CH (100 mgL-1) were tested separately for induction of embryonic callus and marked as stage-I (Table 1). For maturation of somatic embryos, callus of embryonic character were transferred to a nutritional support, having both IAA and individual complex substances with incorporation of reduced level of 2,4-D (2.0 mgL-1) and marked as stage II (Table 2). Calli were incubated for two months period in this medium. For germination of somatic embryos, half strength of MS medium without any phytohormones and full MS supplemented separately either with Kn or GA3 (0.5 and 1.0 mgL-1) were tested.

2.3 Direct embryogenesis

In direct embryogenesis, two different types of explants, namely, young plantlets (6, 9, 12, 15, and 18 days old) and root segments (3.0 to 4.0 cm) from *in vitro* plantlets grown in half strength of MS basal media were tested as explants sources. These were separately inoculated in MS medium fortified with NAA (0.2 to 0.5 mgL-1) along with Kn (0.5 to 3.0 mgL-1), respectively.

2.4 Incubation condition

All cultures were maintained in culture room at 25 ± 2°C and 70 to 75% relative humidity. Light source was provided by florescent tubes (40 Watts), emitting at an intensity of 3000 lux approximately at culture levels for 16 h light and 8 h dark in a 24 h cycle.

2.5 Histology

For histological studies, fresh tissue were isolated and fixed in 1:1 acetic acid: ethyl alcohol mixture and kept overnight; this was followed by staining with 2% aceto orcein and normal HCl solution (9:1). After proper staining, small pieces of tissues were transferred to a grease free slide and squash the tissue in 45% acetic acid by exerting uniform pressure followed by sealing the slide with paraffin, then observed under microscope.

2.6 Statistical analysis

Data represented were the average of 10 replicates. The frequency of globular embryo induction was calculated by the percentage of induced callus in each culture and calculating them with the mean of 10 such replicates. The whole experiment was repeated twice.

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3. Results and Discussion

Source of plant growth regulator is an important factor for impacting somatic embryogenesis and plant regeneration. In most cases, successful plant regeneration needs a mixture of the different auxin and cytokinin. Present study recorded on somatic embryogenesis of *P. rosea* from matured internodal tissues as best tissue and optimum concentration with auxin like 2,4-D, IAA and complex additives. Accordingly, induction of efficient high quality embryonic callus is a pre requisite for achieving efficient plant regeneration. Most of the media were tested at stage-I and all types of explants induced embryonic callus after 4 weeks of culture. However, the nature of the callus type and frequency of callus induction varied between different treatments and type of explants cultured (Table 1). A similar result regarding cones of interior *spruce* was reported by Robert *et al*. (1989) [39] on a varied nature and frequency of embryonic callus induction in different treatments associated with different type of explants during study of somatic embryogenesis. In their report, Robert *et al*. (1989) [39] observed that different tissue types within the same plant differ in their response during *in vitro* callus formation. Chakravarthi *et al*. (2009) [6] also reported that embryogenic and organogenic responses were significantly affected by explants type in all concentrations. Among the various explants cultured, internode explants responded more promisingly towards induction of embryonic callus (100.0±0.0) (Figure 1A) followed by petiole and leaf explants (87.2 ± 6.5) and (60.1± 2.6), respectively. Media containing

combinations of 2,4-D (4.0 mgL-1), CW (100.0 mgL-1), YE (100.0 mgL-1) and IAA (0.5 mgL-1) have shown the maximum response towards induction of embryonic callus from almost all type of explants cultured than media containing 2,4-D alone which showed initiation of embryonic callus. In general, 2,4-D is one of the most important hormones inducing somatic embryogenesis widely used in horticultural plants Aiqing *et al*. (2011) [1]. Contrary to the observed effect, IAA, YE and CH alone failed to induce any callus (data not shown). Several authors reported the importance of 2,4-D for induction of somatic embryonic callus in various plant species; namely, Raghavan (2003) [35] in zygotic embryo culture from various species of Brasicaceae, Choi *et al*. (2002) [8] in *Eleutherococcus sessiliflorus*, Kumar *et al*. (2002) [25] in *Gymnema sylvestre*, Vikrant (2003) in *Paspaleum scrobiculatum* and Martin (2003) [4] in *Holostemma ada kodien*. Comlekcioglu *et al*. (2009) [9] also described the effects of medium of two different explants on somatic embryogenesis of snake melon C*ucumis melo* var. *flexuosus*

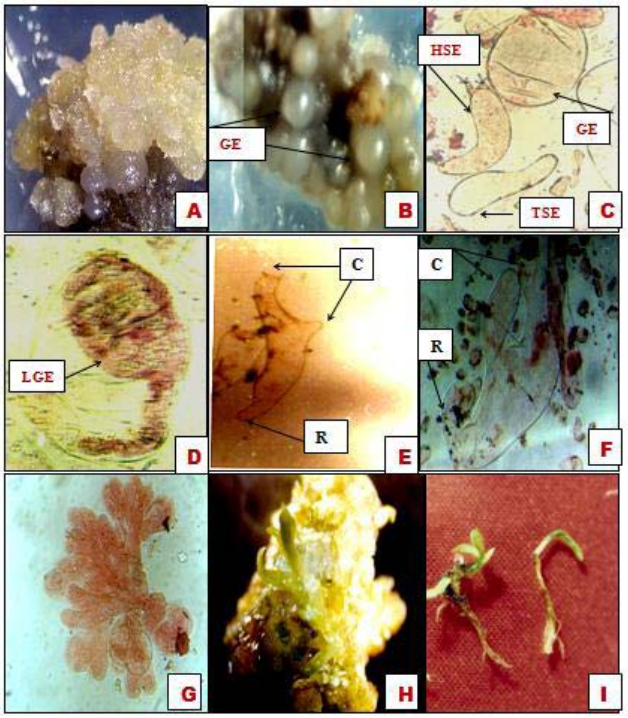

Fig 1: Description

A and B: High frequency somatic embryonic callus and globular somatic embryos of inter node explants on MS medium supplemented with (2,4-D 4.0 + CW 100.0 +YE 100.0 + IAA 0.5 mgL-1). C: Views of embryos at different stages. GE: Globular embryo, HS: heart shaped, TSE: torpedo shaped embryo. D: late globular embryo; E and F: cotyledonary embryo showing two distinct cotyledons with root meristem (C: Cotyledon, R: Root); G: secondary embryogenesis from calli of leaf grown on MS medium fortified with 2,4-D (2.0 mgL-1); H: emerging shoots from germinating embryo on MS medium supplemented with 2,4-D 2.0 mgL-1 + YE 100.0 mgL-1 + IAA 0.5 mgL-1; I: Embryonic plantlet having healthy root shoot system. Photo magnification is 400X.

with 2,4-D, NAA, N6-benzylaminopurine (BAP) and 2-isopentenyladenine (2-iP) and culture conditions were investigated for somatic embryogenesis. Jevremovic *et al*. (2015) reported in his study, 2,4-D as the sole hormone for somatic embryogenesis for the endemic species *Iris reichenbachii*. The texture, nature of callus initiation and mode of origin were also found varied depending on the type of explants cultured. A varied average time requirement for callus initiation was reported by Morozowska *et al*. (2014) [31] in *Primula veris* L., a well-known medicinal herb in different explants type roots, cotyledons and hypocotyls. Callus induction in inter nodal segment originated from cut ends, then gradually expanded and the whole explant converted into callus masses. Nature of the callus were found to be loose, soft, slimy, and grey in colour, but growing activity of these calli were slow. Visual observation of these calli also showed high embryonic nature, confirmed by microscopical observation. Calli induced from leaf explant were compact, semi hard, loose and off-white in colour, but fast growing in nature. In this case, calli restricted to the periphery region were observed more in meristematic nature, whereas centrally located calli seems to be quite inactive. Similar type of callus growth and texture was also reported by Michaux *et al*. (1992) and Montoro *et al*. (1993) [30] in *Hevea brasiliensis*. In petiole explant, callus induction was found to be restricted to the basal region that contact with the medium. In these cases, callus texture was almost similar to the callus of leaf explants.

The experiments conducted in the present study clearly showed that formation of globular embryos in different explants cultured in different media was not same type; this might be due to differences in the uptake of nutrients and in recognition of different cell type. Sujhatha and Reddy (1998) also experimented with similar findings during their study on *Castor* species. After long incubation in the selected medium, calli became lost in their embryonic nature, where central necrosis was recorded and simultaneously secondary embryogenesis was induced towards the border side of this callus (Sujhatha and Reddy, 1998). This is agreement with our findings in induction of secondary embryogenesis (Figure 1G) from leaf source recorded after being kept in prolong period in the stage II medium. Our findings also concur with the reports of Charleen and Hazal (1992) [7] and Sellars *et al*. (1990) [40] on leaflets of peanut and immature zygotic cotyledons of pea, respectively. A similar type of induction of somatic embryos from *Manihot esculenta* with additional exposure to 2,4-D was also reported by Stamp and Henshaw (1987). These calli remained mucilaginous and watery, embedded in a matrix and translucent even after 3 months of incubation.

Maturation of somatic embryos have been recorded upon gradual withdrawal of 2,4-D from the culture medium (Table 2), which was also reported by Rangaswamy (1986) [37] and Beena and Martin (2003) [27] on angiosperm and *Ceropegia candelabrum*, respectively. Kumar and Chandra (2014) [26] reported a simple and reproducible method for effective utilization for production of elite plants and there by the conservation of *Swertia chirayita* valuable medicinal herb with reduced concentrations of 2,4-D in combination with Kn for high yield of somatic embryos. In *S. chirayita*, 1.0 or 1.5 mg/L of 2,4-D alone was also found to be better for somatic embryogenesis induction and production of somatic embryos in Balaraju *et al*. (2011) [2]. He *et al*. (2011) [17] recorded the highest frequency of somatic embryogenesis in *Gentiana straminea* when leaf explants were inoculated on MS medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L BA after 4 weeks of culture. Maturation of somatic embryos, recorded within 7 to 10 days of incubation from internode explants when cultured in media having low level of 2,4-D along with IAA and YE. The use of synthetic auxin 2,4-D for induction of

somatic embryos on cultured explants can be traced to the work of Halperin and Wetherell (1964) [16] who showed that callus produced from any vegetative part of *Daucus carota* such as the root, petiole, inflorescence stalk reared in a medium containing a high concentration of 2,4-D of embryonic callus formed somatic embryos upon transfer to a medium with a reduced level of the auxin. The use of a defined medium and a single step transfer of callus or a cell suspension growing in a medium supplemented with moderate dose of 2,4-D, containing a reduced amount of auxin or none at all became widely popular in inducing somatic embryogenesis in a broad range of species (Thorpe and Stasolla, 2001; Raghavan, 2004; Diab, 2015) [36, 12]. Presently, embryos were more prominent, well organized, round in structure and dispersed all over the culture surface (Figure 1B). Microscopical preparation of these calli showed average 517.2 numbers of embryos per 100 mg callus which were globular to heart and torpedo shaped embryos (Figure 1C) which were gradually differentiated with a lateral notch (Figure 1D) as late globular embryo. Optimal induction of embryonic callus as well as maturation of somatic embryogenesis recorded when medium was fortified with yeast extract. In Stage II, media induction of optimal matured globular embryo (Figure 1E to F) recorded 82.2±6.8 having 2,4-D 2.0 + YE100 + IAA 0.5 mgL-1 from internode callus. Calli of petiole origin possesses 68.3±4.3 from media incorporated with 2,4-D 2.0+I AA 0.5 mgL-1. From leaf source, 50.7±7.6 was recorded cultured in the medium 2,4-D 2.0 mgL-1.

Table 1: Effect of different media combinations on embryonic callus formation from different explants

Stage I (mg L-1)	Petiole			Internode		Leaf segment
	%C	% EC %C		%EC	%C	%EC
MS+2,4-D 4	100.0±0.0	80.7±2.8	61.7±4.4	62.7±5.5	10.5±1.6	41.4±6.6
MS+2,4-D 4+ CH 00	64.3±6.0	40.8±6.2	100.0±0.0	60.5±6.1	20.7±2.5	52.9±7.4
MS+2,4-D 4+ YE100	63.9±3.2	62.8±6.1	100.0±0.0	66.2±5.4	20.4±3.8	21.5±2.3
MS+2,4-D4+CW100+IAA0.5	45.0±0.0	30.6±6.6	53.0±0.0	20.5±6.2	10.7±2.0	38.1±7.3
MS+2,4-D4+CH100+IAA0.5	99.1±1.2	80.3±4.8	98.6±1.8	82.0±3.6	10.7±4.4	10.6±1.8
MS+2,4-D4+YE100+IAA 0.5	73.2±7.3	87.2±6.5	100.0±0.0	100.0±0.0	10.2±2.9	60.1±2.6

Values are expressed as mean ± SE; C, Culture forming embryonic callus; EC, embryonic callus.

Table 2: Effect of different media and plant growth regulators on frequency of globular embryo from different type of explants

Stage II (mg L-1)		Petiole		Internode		Leaf
%C		FGE %C		FEG		%C FGE
MS+2,4-D 2.0	20.4±6.3	30.9±4.6	10.9±3.7	22.1±7.5	11.4±5.6	50.7±7.6
MS+2,4-D 2.0+CH 00	10.3±2.0	23.6±5.7	10.5±2.7	11.1±3.4	11.2±3.7	10.5±1.9
MS+2,4-D 2.0 +YE100	53.8±7.7	41.6±5.8	55.0±7.4	22.4±6.1	46.4±8.3	21.6±4.9
MS+2,4-D2.0+IAA0.5	22.1±4.7	68.3±4.3	54.2±5.0	33.8±6.1	11.8±3.8	11.6±2.5
MS+2,4-D2.0+YE100+IAA0.5	37.1±7.3	20.3±3.7	100.0±0.0	82.2±6.8	42.1±7.0	13.6±4.6
MS+2,4-D2.0+CH 100+IAA0.5	72.3±7.5	12.4±3.3	23.4±5.8	21.1±5.6	22.3±5.4	35.4±7.1

Values are expressed as mean ± SE; C, Culture forming globular embryonic callus; FGE, Frequency of globular embryonic callus.

Calli originated from various explants sources also express variations and varied germination percentage. Only from internode origin in half strength of MS medium supported maximum germination (Figure 1H) of 70% somatic embryos after 2 weeks of incubation. Complete plant with root and shoots formation was recorded within 8 and 9 weeks old cultures (Figure 1I). In embryo germination in *P. rosea*, suitability of half strength of MS medium was reported (Das and Rout, 2002) [11]. In case of leaf calli, embryo germination was observed in 10% from GA3 0.1 mgL-1 fortified medium. In cases of this study, embryo germination was recorded in GA3 incorporated medium attained only up to leafy cotyledon stage instead of formation of the whole plant. This finding was similar with Mathur *et al*. (2002) [28] who recorded in three different *Panax* spp. Rangaswamy (1986) [37] also reported that, the medium with gibberellins had faster maturation and also enhancement of planting efficiency in diverse groups of angiosperms. Likewise, Craiz *et al*. (1997) [10] reported favorable action of GA3 towards somatic embryogenesis. Petiole origin supported towards enhancement of callus of and germination was recorded 40%. Half strength of media when supplemented with Kn for germination observed rhizogenic callus. Regenerated plantlets were transferred to the pots containing soil and sand (1:1) after proper hardening, which were well grown and acclimatized within 25 days.

In case of direct somatic embryogenesis explants, namely, plantlets and root segments

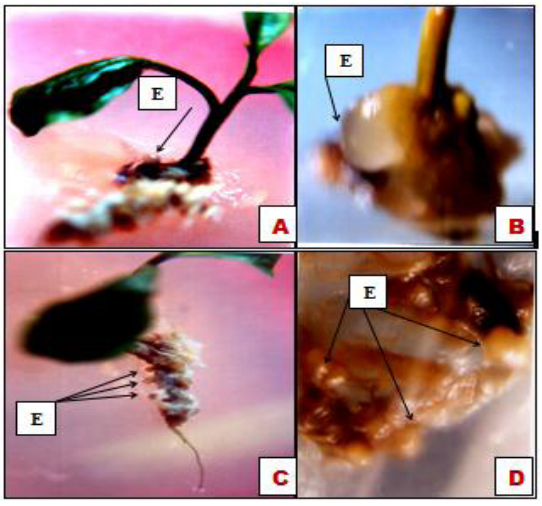

Fig 2: A: Direct globular embryo on root shoot junction of uncut cotyledon on MS medium supplemented with Kn2.0 + NAA0.5 mgl^{-1}. B: Enlarged view of globular embryo with cut cotyledon. C and D: Globular embryos hang on roots of plantlets and surface of the root segment. Photo magnification is 400X

when cultured in MS medium with Kn 0.5 to 3.0 + NAA 0.2 to 0.5 mgL-1, swelling of explants observed within 2 weeks of inoculation. These swelled roots gradually produced globular, embryonic type of structures after 20 days of incubation from media containing Kn 2.0 +NAA 0.5 mgL-1.

Plantlets are more embryogenic than root segments. Induction of direct embryos depends on the age of the plantlets. On 6 days old plantlets, round and transparent body appeared directly on the surface within 6 weeks of culture in root shoot junction (Figure 2A). These embryonic structures enlarged and developed into individual distinct, shiny, watery and globular shaped embryos (Figure 2B). Globular bodies are found to be hanged on the tip of the root hairs (Figure 2C). Chakravarthi *et al*. (2009) [6] recorded on 3 to 4 days old cotyledon explants effect on somatic embryogenesis in which older tissues were not responsive. Murthy and Saxena (1994) also observed the effect of age of the explants in *Arachis hypogea* where younger plantlets were higher in their embryogenic competence while the explants of older age failed to produce somatic embryos, which indicated that the physiological age of the plant is the most important in determining the embryogenic capabilities. Root segment also produced distinct embryos on the surface when cultured in the aforementioned media (Figure 2D). These well differentiated embryonic structures could easily be separated from the mother tissue. All cultures were sub cultured at a regular interval of 10 days. Separation of prominent globular embryos from mother tissue gradually lost their transparent nature and became deep grey in colour and finally dried. This indicated that the role of the shoots was the most important in the process of somatic embryogenesis. Similarly, Kim *et al*. (1990) and Gambley and Dodds (1991) established that the presence of cotyledon is important for the production of adventitious shoots in *Glycin max* and *A. hypogea*, respectively. Further investigation on direct embryogenesis is needed to determine the regeneration of plantlet of this important medicinal plant species.

4. Conclusion

In this study, it was established that matured field grown intermode segment could be capable of optimum indirect somatic embryogenesis of *P. rosea* than leaf tissue by incorporation of different auxin and complex substances. Preliminary investigation of direct embryogenesis from young *in vitro* grown plantlet and from root segment was also recorded as a first report of the species.

5. Acknowledgement

I am very much grateful to acknowledge my Director, Rain Forest Research Institute, Jorhat, Assam for his support, help and encouragement from all sides, which enable for ahead my research works.

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