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Research article

CONSERVATIVE EFFORTS THROUGH EMBRYO CULTURE OF A VULNERABLE PLANT *PHYLLANTHUS INDOFISCHERI* BENNET.

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ABSTRACT: Indian gooseberry is having high medicinal importance and is widely collected from *Phyllanthus emblica* Linn and *Phyllanthus indofischeri* Bennet, species belonging to family Euphorbiaceae endemic to Peninsular India and vulnerable globally. Poor and inconsistent germination of seeds, excessive fruit collection, unsustainable collection of fuel wood, and habitat loss leads to the low regeneration of this species. With an objective to establish the species population in the natural and allied habitats, our present investigation is carried on the conservation of this plant. Since conventional propagation methods were not effective, *in vitro* propagation of this plant was attempted for the first time. Our present study involves the effective procedures for *in vitro* conservation of *Phyllanthus indofischeri* through embryo culture. Embryos from the mature seeds were used as explants. Although germination was achieved on MS medium containing various growth regulators, maximum response was observed on MS medium fortified with BAP (2.0)+IBA (2.5)mg/l, BAP 2mg/l followed by GA_3 0.1 mg/l and observed for healthy plantlets formation.

Key words: Embryo culture, *Phyllanthus indofischeri*, Gibberllic acid, Conservative Efforts.

INTRODUCTION

Indigenous people living in and around forests mainly depend on Non-timber forest products (NTFP's) such as fruits, seeds, roots, etc. Due to over – exploitation of these NTFP's and lack of knowledge on their distribution or population status, may lead the species vulnerable to extinction [1]. In addition to this climatic change from the last few decades, high levels of soil and water pollution leads to the threatening of many plants species, which now considered as rare, vulnerable and endangered [2]. Among the various conservation methods, *in-vitro* propagation is generally undertaken to enhance the biomass and conserve the germplasm especially when the population numbers are low in the wild [3] for the maintenance of Plant genetic resources, *in-vitro* propagation methods are essential and they are becoming increasingly important for conservation of rare and endangered plant species [4]. From the ancient times in India Aonla or Indian gooseberry is considered as wonder fruit for its medicinal and theraupeutic properties [5]. It is highly nutritious and is the richest source of vitamin C (400-1300 mg/100g) among the fruits next only to Barbados cherry. Amla is the richest source of pectin, which is mostly useful in making jam and jellies. Medicinally, it acts as coolant, refrigerant, diuretic and laxative. It is the basic constituent of Chyavanaprash and Amrit Kalash, the Ayurvedic medicinal preparations. It is also used in tannin and dyeing industries. . Amla is widely collected from *Phyllanthus emblica* Linn (Euphorbiaceae) and *phyllanthus indofischeri* Bennet a species endemic to Peninsular India and globally vulnerable. The genus phyllanthus (Euphorbiaceae) is a favourite choice of rural people because of its immense medicinal properties like antidote, against liver diseases, antiviral properties, antioxidant, hepatoprotective , anti inflammatory and strong inhibitory effect against neurogenic diseases [6-8]. Plant tissue culture techniques were successfully used for the micropropagation of members of the genus *Phyllanthus* has been reported for the species such as *Phyllanthus emblica, P. urinaria, P. amarus, P. abnormis, P. caroliniensis, P. tenullus, P. niruri, P. stipulatus, P. beddomie* [9-13]. Due to the commercial importance of these fruits, and preferred marble green colour with less dark spots due to fungal attacks make these *Phyllanthus indofischeri* more vulnerable than the fruits of *Phyllanthus emblica*. Fruit harvesters lured by money cut the trees or their major branches to maximize collection. In Andhra Pradesh and Karnataka the fruiting branches along with tulsi (*Ocimum sanctum*) were worshipped during a religious festival called Utthana dwadasi, looped branches with young fruits were sold in local markets on that day. Although, if seeds were collected from the fruits, they do not even exposed to favourable conditions owing to seed dormancy.

These seeds may require special treatments like stratification, scarification, soaking in water, growth regulators *etc.* for overcoming dormancy [14]. Some times, embryo culture is the best alternative through which we can avoid the seed dormancy. The aseptic isolation and *in vitro* growth of an embryo under optimum culture conditions is called embryo culture and this helps in overcoming the post fertilization barriers of hybridization in woody plant species [15] Due to above factors the *Phyllanthus indofischeri* population is facing threat and warranting the need for amicable conservation measures. However, to date there was no report on the in vitro propagation of *Phyllanthus indofischeri*. Hence, the present investigation was undertaken to standardize a suitable protocol for the mass multiplication of this vulnerable plant through embryo culture.

MATERIALS AND METHODS

Plant material

Phyllanthus indofischeri is a monoecious tree grows up to 12m tall, leaves are alternate, sub-sessile, oblong, 1-2.8 x 0.5-1.3 cm, flowers fascicled, in leaf less portion of branchlets male and female flowers are different. Fruits drupaceous, globose, 2.5-4 cm across, marble green; seeds 6; grey colour. The fruits of *Phyllanthus indofischeri* were collected from the Nallamala forest of Bramhamgari mattam located at Kadapa district, Andhra Pradesh state. The voucher specimens were deposited in S.K. University Herbarium, Anantapur.

Sterilization protocol

Fresh and healthy fruits were collected and dried for a period of 2 weeks. Seeds were isolated and thoroughly washed under continuous flashing of running tap water for 5 min, then washed with commercial detergent Tween-20 for 3 min and finally surface sterilized with $HgCl_2$ (0.1% w/v) for 5-10 min followed by rinsing with autoclaved distilled water for 3-5 times to remove any trace of mercuric chloride. Lastly, the seeds were soaked overnight.

Isolation of embryos

The sterilized and soaked seeds were transferred to the petriplate having the autoclaved filter papers. By using the scalpel hard seed coat was cracked, mature embryos were carefully isolated by splitting the kernel longitudinally without any injury to embryo.

Culture media and *in vitro* culture conditions maintained

In the present study MS, B5, WPM media were tested on mature embryos of *Phyllanthus indofischeri*. Among the different media used, the highest plantlet development was observed with Murashige and Skoog's medium or MS medium (1962) [16]. Hence for further experiments this medium is opted. The explants were inoculated on MS medium containing 3.0% sucrose (w/v) and 0.8 % agar fortified with various concentrations (0.5-4.0 mg/l) of auxins (Indole butyric acid(IBA), Naphthalene acetic acid (NAA)), cytokinins (Benzyl Amino Purine (BAP), Kinetin (Kn) and Gibberllic acid (GA_3)(0.05-0.4 mg/l) alone and in combinations. The pH of the medium was adjusted to 5.6±2 before autoclaving at 121°c for 20 minutes. The culture tubes were incubated at 25±1°C with a photoperiod of 16h at 2000-3000 lux of cool white fluorescent light. Cultures were initiated in 150 x 25 mm glass tubes.

RESULTS AND DISCUSSION

The present investigation was carried out with an aim to devise a suitable protocol to obtain a high frequency of plantlet regeneration of *Phyllanthus indofischeri*. The germination of seeds of *Phyllanthus indofischeri* seems very difficult under natural conditions. However, there were no results obtained with basal MS medium. The present study showed the necessity to use elite germplasm ie., embryos to get the viable plantlets. *In vitro*, effects of different phyto hormones concentrations like Gibberllic acid (GA_3), Auxins (IBA, NAA) and cytokinins (BAP, Kn) was observed alone and in combination fortified with MS medium. In all the cases the embryo germination was observed, and found that the percentage of germination frequency has varied (Table.1 and Table.2). Role of BAP and GA3 on multiple shoot induction from leaf bits and internodes of *P. amarus* was reported by R. Chitra et al., (2009) [17].

MS medium supplemented with GA_3 was used for culturing of this vulanerable plant *Phyllanthus indofischeri* embryos in different concentrations ranging from 0.05 mg/l to 0.4 mg/l and the results were obtained. Among the different concentrations GA_3 tested, highest frequency of germination (84%) was obtained with 0.1 mg/l followed by 0.2 mg/l (72%) and 0.05 mg/l (56%). It indicates that a moderate dosage of GA_3 is effective where as increased or decreased concentrations show's inhibitory effect on germination. (Table.1).

The first visible change noted during incubation was the separation of the cotyledons from each other (Fig: a, b& c) followed by the growth of the radicle and hypocotyl. The cultures then developed into complete plantlets (Fig.d). GA_3 is known to play an important role in germination by initiating the mobilization of nutrient reserves stored in the endosperm. The addition of GA_3 in growth medium has often successfully induced germination (Graph.1).

Table 1: The frequency of plantlet development from embryos placed on MS medium with different plant growth hormones (%)

S.No	Plant Growth Harmone	Harmonal concentration (mg/l)	No.of embryos cultured	No.of embryos responded	Response of embryos (%)
1.	GA_3	0.05	25	14	56
		0.1	25	21	84
		0.2	25	18	72
		0.3	25	12	48
		0.4	25	Nil	Nil
2.	BAP	0.5	25	11	44
		1.0	25	16	64
		2.0	25	20	80
		3.0	25	13	52
		4.0	25	08	32
3.	Kn	0.5	25	09	36
		1.0	25	17	68
		2.0	25	12	48
		3.0	25	08	32
		4.0	25	10	40
4.	IBA	0.5	25	09	36
		1.0	25	11	44
		2.0	25	08	32
		3.0	25	06	24
		4.0	25	07	28
5.	NAA	0.5	25	10	40
		1.0	25	07	28
		2.0	25	16	64
		3.0	25	14	56
		4.0	25	Nil	Nil

Table 2: The frequency of plantlet development from embryos placed on MS media with different Phyto hormonal combinations (%)

S.No	Hormonal combination& Conc. used (mg/l)	No.of embryos cultured	No.of embryos responded	Response of embryos (%)
1.	**BAP(2.0)+ IBA(0.5)**	40	16	40
	BAP(2.0)+ IBA(1.5)	40	25	62.5
	BAP(2.0)+ IBA(2.5)	40	35	87.5
	BAP(2.0)+ IBA(3.5)	40	22	55
2.	**BAP(2.0)+ NAA(0.5)**	40	09	22.5
	BAP(2.0)+ NAA(1.5)	40	13	32.5
	BAP(2.0)+ NAA(2.5)	40	15	37.5
	BAP(2.0)+ NAA(3.5)	40	11	27.5
3.	**Kn(1.0)+ IBA(0.5)**	40	14	35
	Kn(1.0)+ IBA(1.5)	40	30	75
	Kn(1.0)+ IBA(2.5)	40	13	32.5
	Kn(1.0)+ IBA(3.5)	40	17	42.5
4.	**Kn(1.0)+ NAA(0.5)**	40	18	45
	Kn(1.0)+ NAA(1.5)	40	20	50
	Kn(1.0)+ NAA(2.5)	40	24	60
	Kn(1.0)+ NAA(3.5)	40	16	40

Effect of Gibberllic acid on Embryo Culture

Effect of auxins on embryo culture

The MS medium was fortified with different concentrations of IBA and NAA ranging from 0.5 mg/l to 4.0 mg/l. IBA showed a maximum response of 44% with 1.0 mg/l and minimum response of 24% with 3.0 mg/l. Healthy and viable plantlets were observed in all concentrations of IBA.

The other auxin employed in the present investigation for rooting was NAA (Fig.e), showed maximum response of 64% with 2.0mg/l and minimum response of 28% with 1.0mg/l. Among the two auxins IBA is prominent and showed response at all concentrations whereas, the NAA did not show any response at high concentrations e.g., 4.0 mg/l, but the highest germination percentage was obtained with NAA (Graph. 2). Callus proliferation was observed from nearly 8.3% embryos with auxins. In tissue culture auxins play a crucial role in root induction. Elongation phase of root is highly sensitive to auxin concentration so, as the concentration of auxin increased it will be detrimental for the plant growth.

Effect of cytokinins on embryo culture

MS medium supplemented with BAP and Kn were utilised in this study with a concentration ranging from 0.5 mg/l to 4.0 mg/l. BAP with a concentration of 2.0 mg/l shown maximum germination percentage (Fig.f), of 80% with well developed shoot system followed by 64% with 1.0 mg/l. The shoot length varied from 2.0- 2.5 cm and the number of shoots produced was 2-3. The MS medium augmented with Kn 1.0 mg/l showed a maximum response of 68% and minimum response of 32% with 3.0 mg/l. The mean shoot length was 1.7-2.0 cm and number of shoots produced was 1-2. Plantlets that are produced at lower concentrations of BAP (0.5-2.0 mg/l) were normal and had the tendency to elongate while at higher concentration (3.0-4.0 mg/l) the plants were thinner and weaker (Graph.2). The other cytokinin Kn being a mild cytokinin was perhaps shown good response of germination.

Effect of auxins and cytokinin on embryo culture

In the present study, MS medium fortified with GA_3 found to be very effective in germination, but it promotes the formation of abnormally long cotyledons which was very distinctive in the seedlings, in some species even, it is reported that the addition of GA_3 to the medium produces elongated and narrow leaves. Although, the germination percentage of GA_3 was effective, abnormally long cotyledonary growth, stunted growth of radicle, poor in-vitro root formation discourages the usage on our study.

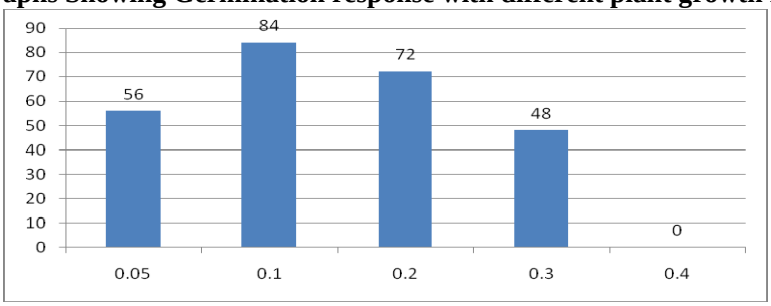
Among the auxins and cytokinins the BAP 2.0 mg/l found to be more effective (Fig. f) followed by Kn (1.0 mg/l) so these were maintained as standard and different combinational media were prepared by varying the concentration of auxins. Among the combinations BAP(2.0 mg/l)+IBA(2.5 mg/l), Kn(1.0 mg/l)+IBA(1.5 mg/l), Kn(1.0 mg/l)+NAA(2.5 mg/l), BAP(2.0 mg/l)+NAA(2.5 mg/l), shows the germination percentage of 87.5, 75, 60 and 37.5, respectively (Table 2) (Fig:g),. The results suggested that the exogenous supply of auxin and cytokinin were important for the plantlet formation. Highest number of shoots were observed in the combinations of Kn (2.0 mg/l)+ GA_3 (0.5 mg/l) in *P. emblica* as was reported by Govinda nayaka (2006), 4.43 µM BA + 0.54 µM NAA combination in *Holostemma annulare* (Roxb.) was reported by Sudha *et al.,* (1998) using nodal explants in both studies. In embryo culture maximum number of shoot development was reported in *Croton scabiosus* Bedd. using BAP (0.5 mg/l)+ Indole Acetic Acid (IAA) (3.0 mg/l) by Salamma and Rao (2013).

Table 3: Response of embryos on different phytohormones alone and in combinations

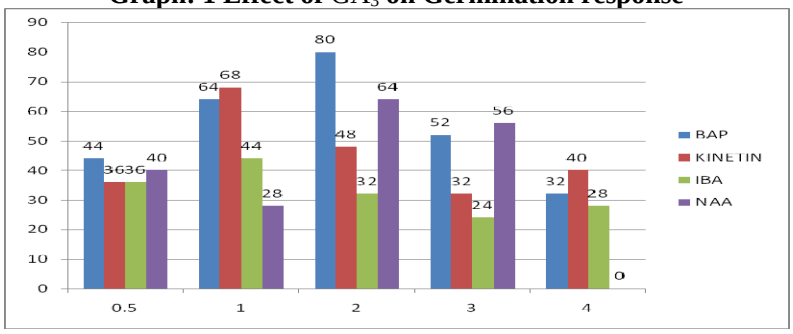
				Response of embryos				Percentage of response			
S.No	**Phyto hormone (mg/l)**	**Total no. of embryo's cultured**	**Total no. of embryo's responded**	**EP**	**ES**	**ER**	**EC**	**EP**	**ES**	**ER**	**EC**
1.	GA_3 (0.1)	125	64	58	04	Nil	02	90.6	6.25	Nil	3.12
2.	BAP (2.0)	125	68	45	10	08	05	66.1	14.7	11.7	7.35
3.	Kn (1.0)	125	56	37	08	07	04	66.0	14.2	12.5	7.14
4.	IBA(1.0)	125	41	18	08	10	05	43.9	19.5	24.3	12.1
5.	NAA(2.0)	125	47	22	07	11	07	46.8	14.8	23.4	14.8
6.	BAP 2.0)+ IBA (2.5)	200	98	61	19	13	05	62.2	19.3	13.2	5.10
7.	BAP(2.0)+ NAA(2.5)	200	84	46	17	14	07	54.7	20.2	16.6	8.33
8.	Kn(1.0)+ IBA(1.5)	200	96	53	15	19	09	55.2	15.6	19.7	9.37
9.	Kn(1.0)+ NAA(2.5)	200	79	42	16	15	06	53.1	20.2	18.9	7.59

Generally both shoot and root developments were observed in plantlet development from embryos. However, some embryos shown shoot development without roots, root development without shoot or callus development. The results were obtained after 50 days were tabulated (Table-3) regarding single and combinational concentration of hormones. Profuse root formation on MS+NAA (3.0 mg/l). Nearly 60% of the embryos were able to form the plantlets, among total inoculated embryos, 16% embryos were shoot forming embryos, 9% were root forming embryos and callus proliferation was observed from nearly 8.3% embryos (Fig:h).

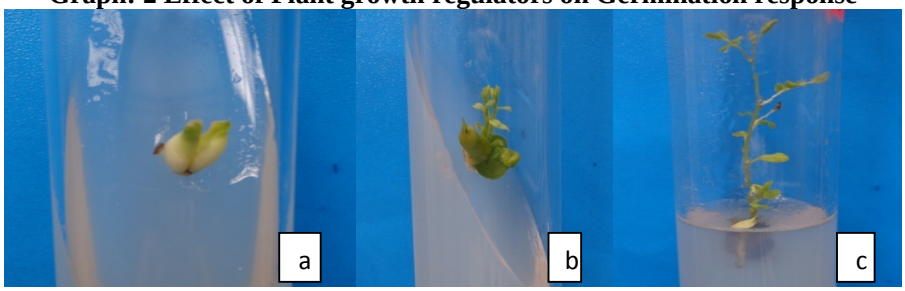
Graphs Showing Germination response with different plant growth regulators

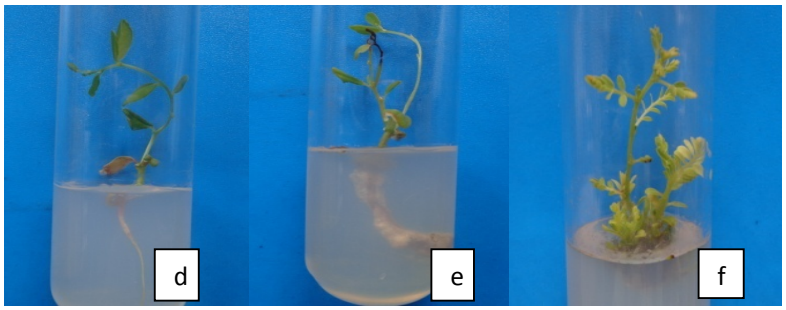


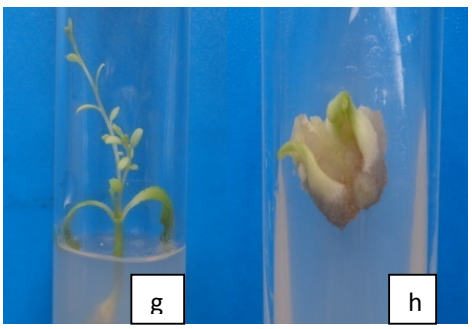
Graph: 1 Effect of GA_3 on Germination response



Graph: 2 Effect of Plant growth regulators on Germination response







Figures: *In vitro* embryo culture of *Phyllanthus indofischeri*

Fig:a, b, c: Stages of regenerative patterns of embryos placed on MS medium augmented with plant growth hormone GA_3 (0.1 mg/l) d: Complete plantlet formation on MS + GA_3 (0.1 mg/l), e: Profuse root formation on MS+NAA(3.0 mg/l), f: Multiple shoot formation on MS+BAP(2.0 mg/l), g: Plantlet formation on MS+BAP(2.0 mg/l)+ IBA(2.5 mg/l), h: Embryos forming callus on MS+IBA (3.0 mg/l).

CONCLUSION

In summary, the results of this research showed an efficient method for the in-vitro production of *Phyllanthus indofischeri* by using the embryos as explants. High concentration of BAP (2.0 mg/l) in combination with IBA (2.5 mg/l) showed maximum response. To the best of our knowledge our study was the first to report regarding micropropagation of this medicinally important and globally vulnerable plant. At sustainable basis, for further mass propagation of this species these studies will helpful and had laid foundation for *in vitro* conservation of *Phyllanthus indofischeri.*

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