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Development of an efficient protocol for *Robiquetia spathulata* (Bl) J.J. Sm. an Endangered Orchid Species Through *in vitro* Techniques

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Abstract

Robiquetia spathulata (Bl) J.J. Sm. is an epiphytic endangered orchid species of Bangladesh and PM (Phytamax - Arditti, 1977) basal medium was best for seeds germination than KC (Knudson, 1946), MS (Murashige and Skoog, 1962), MVW (Modified Vacin and Went, 1949) media. Sucrose was highly favourable for germination as sources of carbohydrates than glucose and lactose. The highest mean increased length of seed originated seedlings (3.68 ± 0.22cm) and individual shoot bud (3.37 ± 0.18cm) were achieved on 0.8% (w/v) agar solidified MS medium fortified with 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP. Nodal segments directly produced highest average number of multiple shoot buds (8.36 ± 0.45/segment) *via* organogenesis sprouted on MS medium + 1.0 mg/l IAA + 2.0 mg/l BAP; Whereas, leaf segments underwent proliferation *via* embryogenesis and produced maximum percent of greenish PLBs on MS medium supplemented with 1.0 mg/l Pic + 2.0 mg/l BAP. The increase in length as well as the number of roots developed seed originated and shoot bud derived seedlings were best on half strength PGRs free MS medium. The highest number of SPSs formation took place in liquid MS + 0.5 mg/l Pic + 1.0 mg/l BAP. This efficient protocol is trustworthy way for the *in vitro* germination, micropropagation, development of seedlings and SPSs production of *Robiquetia spathulata.*

Keywords: micropropagation; MSBs; PGRs; PLBs; SPSs.

1. Introduction

Orchids belong to the Orchidaceae family are distributed generally in the tropical parts of the world by 880 genera and about 26567 species [1] and few are in the arctic region. Orchidaceae is rich with diversity of species in Asia, represented by more than 1300 species in India, 579 species in Bhutan [2], 450 species in Nepal [3] and 187 species in Bangladesh [4].

Robiquetia spathulata (Bl) J.J. Sm. is an epiphytic orchid species widely distributed in India (Assam, Sikkim, Himalayas), Bangladesh, Bhutan, South China, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, Indonesia, Singapore, Philippines and protected by CITES. Flowering time May to July [2, 5] and inflorescence of this orchid looks very attractive, so it has good horticultural value. Haphazard collections by orchid lovers, habitat devastation, over-exploitation for orchid collectors and medicinal uses are the key factors that have come down at endangered label [6], so it is high time to perform efficient strategies to preserve in nature. Therefore, highly exploited species right away require *ex situ* conservation by tissue culture method. Throughout the last few years tissue culture system have been widely exploited for the mass scale propagation of many orchid species [7, 8].

Orchids naturally regenerate through seeds but in deficiency of suitable hosts, the seeds do not germinate sufficiently. A single orchid capsule/pod contains millions of seeds which have lack of endosperm or functional storage foods [8]. True seed coat not present, so lack of metabolic machinery [9] do not let them germinate, only 0.2-0.3% gets germinated in environment [9], these troubles may be overcome by adopting in vitro tissue culture method [10, 11]. The frequency of protocorm like body (PLBs) production in orchids is influenced by many factors, such as genotypes, type of explants and composition of media [12]. Nutrient composition is considered to be main source of distinction in plant tissue culture. Diverse culture media have been used for capable seed germination in orchid tissue culture [13]. Among them, PM medium was found to be able for PLBs development and leaflet regeneration [14].

Nodal and leaf segments from aseptically raised seedlings were used for micropropagation. The innovation of tissue culture techniques added a new aspect to the production of quality plants in huge quantities and propagation of magnificent and rare orchids [15-21]. However, orchid tissue culture based very little work has been done in Bangladesh. Therefore, the present study was undertaken to expand an effective *in vitro* propagation protocol for *R. spathulata.*

2. Materials and Methods

The mature pods of *Robiquetia spathulata* were collected from the trunk of *Dalbergia sissoo* tree at forest area of Roangchari, Bandarban, Bangladesh during October to November and stored in normal refrigerator at 5 0C before inoculation to develop a sustainable protocol for *in vitro* seed germination, micropropagation and SPSs development.

Full strength KC [22], MS [23], MVW [24] and PM [25] media with different carbohydrates sources (sucrose, lactose, glucose) was used for *in vitro* seed germination of *Robiquetia spathulata.* MS and PM based (solid and liquid) eighteen types of elongation media were prepared using with different concentrations and combinations of Plant Growth Regulators (PGRs). 0.8% (w/v) agar was added in solid media but liquid media comprises all elements except agar. Sixteen types of micropropagation media were prepared using MS basal media with various concentrations and combinations of PGRs. Half strength MS and nine types of full strength auxin supplemented MS media were prepared for well-developed root system. Medium pH was in step at 5.8 by using 0.1N NaOH or HCl earlier to gelling with agar. Media was heated till the agar was dissolved. Hormones *viz.* Kinetin (Kn), 6-benzyl Amino Purine (BAP), Picloram (Pic), Napthelene Acetic Acid (NAA), Indole Acetic Acid (IAA) and Indole Butaric Acid (IBA) were newly prepared. 100 ml of the media were dispensed into 250 ml culture bottles and autoclaved at 121^{o}C for 20 minutes at 15 lbs pressure. Culture bottles with inoculated seeds were kept in a growth room at 14/10h stable light and dark environment at 24±2 ^{o}C. Cultures were monitored frequently and the data was recorded.

Collected mature pods were initially washed under running tap water to eliminate the outside particles attached to it. Then they were washed with detergent containing 2-4 drops of Tween-20 for 15 min, and then rinsed with tap water waiting the detergent was washed away. In inoculation chamber the pods were surface sterilized using 0.1% mercuric chloride solution for 10 minutes followed by 70% ethanol for 30 sec and double distilled water for 3-4 times. Surface sterilized pods were dehydrated on double layer Whitman filter paper to absorb surplus surface water and dissected longitudinally with the help of sterilized surgical blade and forceps to expose the powdery seeds. The powdery seeds were scooped, inoculated and spread on the surface of agar gelled media under aseptic state. The seeds were permitted to germinate and distinguish into protocorms and seedling development.

Nodal and leaf segments were grown aseptically on the PGRs supplemented agar solidified medium. Nodal segments produced directly multiple shoot buds (MSBs) *via* organogenesis; whereas, leaf segments proliferated and produced Protocorm Like Bodies (PLBs) *via* embryogenesis. With different concentrations and combinations of PGRs (BAP, Kn, NAA, IAA, IBA and Pic.) containing 0.8% (w/v) agar solidified & liquid MS and PM elongation media were prepared for enhancing elongation of seedlings. MSBs and PLBs were subcultured in the elongation media and thereafter, transferred to rooting media for induction of well-developed root system. In elongation media, Shoot Primordia Like Structures (SPSs) were developed at the basal zone of the seedlings; after that these SPSs were subcultured on PGRs supplemented media where they produced plantlets. Thereafter, those plantlets were subcultured on elongation and rooting media respectively. Eventually, the seedlings were cultured in low nutrient supplemented or growth retardant containing media to check their growth and by this way save them for long time for further used as research materials.

Half strength MS0 and nine different types of 0.8% (w/v) agar solidified MS medium containing with 3% (w/v) sucrose and three kinds of auxins *viz.* IAA, IBA, NAA were used for generation of strong and stout root system. The competence of the media in terms of enhancing the progress of root system was calculate based on the increase in number and length of roots that developed within 30d of culture in rooting media.

Well developed rooted plantlets were taken out of the culture vessel and transferred to outside the culture room subsequent consecutive phases of adaptation. The mouth of the culture vessels was kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. Later on, those were kept outside of the culture room for 12h. Finally, the seedlings were taken out of the culture vessels and rinsed with running tap water for elimination of agar attached to the roots. Then the seedlings of *R. spathulata* were transferred to pots containing pit moss, saw dust, black coal and coconut coir (0.5:1:1:1). Transplanted seedlings were watered frequently for about 2-3 months till the seedlings were recognized and grew well used for as *ex situ* conservation.

In vitro culture experiments were carried out methodically with the help of adequate no of explant. Different growth parameters were considered to record data on morphogenic changes of explant under diverse situation. The data on various parameters from unlike experiments were recorded after requisite days of culture. All experiments were conducted in triplicate and data were presented as mean ± standard error (mean ± SE). Standard deviation (SD) was calculated with Microsoft Excel 2013. In the table the mean ($\bar{x}$) data of different replication of each treatment are accompanied by standard error of mean (SE) which was calculated as follows:

Standard error (SE) = $\frac{SD}{\sqrt{N}}$ Where, SD = Standard deviation, N = Number of observation

3. Results and discussion

Seeds of *Robiquetia spathulata* were aseptically cultured on 0.8% (w/v) agar solidified KC, MS, MVW and PM media with three different sources of carbohydrates *viz.* sucrose, glucose and lactose (Table-1). The whole results point out that PM was better [14] than the other three media in respect of the percentage of germination and required time for germination of this orchid species (Fig.1a). Maximum PLBs are green and some are greenish or yellowish green in colour. PM media is enriched with vitamins and organic additives which are enhanced for seed germination and seedling development of many orchids [26, 27]. Peptone in media enhances the germination rate and also favours the healthy protocorm development [21, 28]. MS medium was found best for germination of *Aerides odorata* orchid seeds [29]. Carbon source has also great role for *in vitro* orchid seed germination. Sugar is an important component used in tissue culture studies. Generally sucrose is used in the medium but in several cases other carbohydrates such as lactose, glucose, maltose, fructose, dextrose, galactose, cellulose, mannose have also been used [30, 31]. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than in two glucose and lactose containing media. Very poor response was observed on lactose containing all of the media [13, 14].

Germinated protocorms were transferred to elongation media to encourage fast elongation and improvement of growth (Table-2). The effectiveness of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture. Different hormone combinations and culture condition were found to be better for elongation of seed originated and multiple shoot buds originated tiny plantlets. The highest rate of elongation, mean increased seedlings length took place in 0.8% (w/v) agar solidified MS medium (3.68 ± 0.22cm) with 0.5 mg/l IAA + 1.0 mg/l BAP (Fig.1b) followed by liquid MS medium (3.22 ± 0.21cm) supplemented with 1.0 mg/l Pic + 1.0 mg/l BAP (Fig.1c). Comparison of the results of liquid and solid media revealed that solid culture was better than agar solidified condition [13, 32]. The overall results indicate that MS based medium was better than PM based medium for enhancing elongation of shoot system of the seedlings [28, 33-37]. Similar findings were noted in *Aerides multiflora* [38], *Calanthe densiflora* [21], *Cymbidium cyperifolium* [14], *Dendrobium palpebrae* [32], *Erythrodes humilis* [13] and *Micropera obtusa* [39]. But in some cases, liquid culture was best for elongation [40, 41]. In liquid medium, more surface exposure facilitate nutrient absorption probably facilitated more uptakes of nutrients thereby contributing to better and prolific growth of seedlings and solid media limited this exposure [14, 21, 38-41].

In vitro seedlings derived nodal and leaf segment were used as explants source of *R. spathulata* for rapid micropropagation [28, 35, 42, 43]. The nodal explants were grown on 0.8% (w/v) agar solidified MS media fortified with a variety of combinations and concentrations of PGRs and produced multiple shoot buds *via* direct organogenesis (Table-3). The effectiveness of a medium was evaluated on the basis of number of shoot buds formed from each explant. The nodal segments of *R. spathulata* undergo direct organogenesis and maximum number of shoot buds (8.36 ± 0.45/segment) were produced when cultured on 0.8% (w/v) agar solidified MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP (Fig.1d) followed by 7.94 ± 0.46/segment on MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP [8, 35, 43]. In *Vanilla planifolia,* MSBs were formed from auxiliary bud explants on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA [44]. Leaf segments underwent proliferation and produced seedlings *via* PLBs. Embryogenesis was induced in leaf segments and maximum percent of greenish PLBs were produced on MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l Pic + 2.0 mg/l BAP (Fig. 1e) followed on MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 2.0 mg/l Kn.

Enormous quantities of SPSs were produced in some hormone supplemented media at the basal region of the seedlings. Low concentration of auxins (IAA, NAA, Pic) and high concentration of cytokinin (BAP) was more effective for SPSs development. On the whole, liquid media were more effective than agar solidified media. The highest number of SPSs formation took place in liquid MS + 0.5 mg/l Pic + 1.0 mg/l BAP (Fig.1f) and agar solidified PM + 0.5 mg/l NAA + 1.0 mg/l BAP. Most of the SPSs were greenish and few were yellowish. MS based media was better than PM based media for induction of SPSs [36, 45, 46].

The MSBs that formed from the cultured nodal explant undergo elongation when further grown separately on elongation media (Table-2). The highest mean increased individual shoot bud length (3.37 ± 0.18cm) was achieved on agar solidified MS + 0.5 mg/l IAA + 1.0 mg/l BAP (Fig. 1g) followed by liquid MS + 1.0 mg/l Pic + 1.0 mg/l BAP medium (Fig. 1h). It is evident that elongation of shoot bud was better in agar solidified media than liquid condition [13, 32]. Further MS was found better than PM for elongation of individual shoot bud [33-37].

Table 1: *In vitro* seeds germination of *Robiquetia spathulata.*

Nutrient medium	Carbohydrate source with concentration	Number of culture vessels used	Number of culture vessels in which seeds germinated		Required time (d) for germination	Remarks
			No.	%		
KC	2% (w/v) glucose	10	04	40	48 - 50	Green PLBs
	2% (w/v) lactose	10	03	30	50 - 52	Green PLBs
	2% (w/v) sucrose	10	05	50	46 - 48	Green PLBs
MS	3% (w/v) glucose	10	05	50	46 - 48	Yellowish green PLBs
	3% (w/v) lactose	10	04	40	48 - 50	Green PLBs
	3% (w/v) sucrose	**10**	**06**	**60**	**43 - 45**	**Green PLBs**
PM	2% (w/v) glucose	10	05	50	45 - 47	Yellowish green PLBs
	2% (w/v) lactose	10	03	30	50 - 52	Yellowish green PLBs
	2% (w/v) sucrose	**10**	**08**	**80**	**40 - 42**	**Green PLBs**
MVW	2% (w/v) glucose	10	04	40	52 - 54	Yellowish green PLBs
	2% (w/v) lactose	10	02	20	53 - 55	No response
	2% (w/v) sucrose	10	05	50	48 - 50	Greenish PLBs

Table 2: Mean increase in length (cm) and SPSs development per seed and shoot bud originated seedlings of *R. spathulata.*

Culture medium	PGRs Conc. (mg/l)	Solid media			Liquid media		
		Increase in length of seedlings after 30d of culture (Mean ± SE)	Required time (d) for SPSs creation and SPSs colour	Increase in length of individual shoot bud after 30d of culture (Mean ± SE)	Increase in length of seedlings after 30d of culture (Mean ± SE)	Required time (d) for SPSs creation and SPSs colour	Increase in length of individual shoot bud after 30d of culture (Mean ± SE)
MS + 3% (w/v) sucrose	1.0 IAA + 0.5 BAP	2.66 ± 0.21	—	2.45 ± 0.23	2.75 ± 0.19	—	2.23 ± 0.21
	0.5 IAA + 1.0 BAP	**3.68 ± 0.22**	45-50 (YS)	**3.37 ± 0.18**	3.04 ± 0.21	45-50 (YS)	2.75 ± 0.23
	1.0 IAA + 1.0 BAP	2.98 ± 0.24	—	3.12 ± 0.23	3.12 ± 0.26	—	3.01 ± 0.27
	1.0 NAA + 0.5 BAP	2.72 ± 0.14	—	2.68 ± 0.17	2.85 ± 0.13	—	2.24 ± 0.15
	0.5 NAA + 1.0 BAP	3.25 ± 0.19	**40-45 (GS)**	3.14 ± 0.17	3.11 ± 0.20	45-50 (GS)	2.71 ± 0.21
	1.0 NAA + 1.0 BAP	3.02 ± 0.13	—	2.92 ± 0.15	3.18 ± 0.16	50-55 (GS)	2.96 ± 0.18
	1.0 Pic + 0.5 BAP	2.68 ± 0.19	—	2.63 ± 0.21	2.84 ± 0.19	—	2.42 ± 0.22
	0.5 Pic + 1.0 BAP	3.42 ± 0.22	55-60 (GS)	3.25 ± 0.25	2.98 ± 0.23	**35-40 (GS)**	2.85 ± 0.26
	1.0 Pic + 1.0 BAP	3.15 ± 0.26	—	3.16 ± 0.22	**3.22 ± 0.21**	40-45 (GS)	**3.09 ± 0.24**
PM + 2% (w/v) sucrose	1.0 IAA + 0.5 BAP	2.87 ± 0.20	—	2.74 ± 0.19	2.66 ± 0.18	—	2.46 ± 0.16
	0.5 IAA + 1.0 BAP	3.53 ± 0.24	50-55 (YS)	3.08 ± 0.22	3.14 ± 0.26	45-50 (YS)	2.72 ± 0.23
	1.0 IAA + 1.0 BAP	3.26 ± 0.25	—	2.97 ± 0.20	3.09 ± 0.22	—	3.02 ± 0.19
	1.0 NAA + 0.5 BAP	2.56 ± 0.17	—	2.52 ± 0.14	2.93 ± 0.17	—	2.37 ± 0.15
	0.5 NAA + 1.0 BAP	3.08 ± 0.21	55-60 (GS)	3.01 ± 0.19	3.17 ± 0.20	45-50 (GS)	2.71 ± 0.17
	1.0 NAA + 1.0 BAP	3.15 ± 0.27	—	2.84 ± 0.24	3.13 ± 0.25	50-55 (GS)	2.95 ± 0.22
	1.0 Pic + 0.5 BAP	2.74 ± 0.24	—	2.72 ± 0.27	2.68 ± 0.21	—	2.46 ± 0.25
	0.5 Pic + 1.0 BAP	3.32 ± 0.20	50-55 (GS)	3.22 ± 0.19	2.83 ± 0.21	40-45 (GS)	2.82 ± 0.23
	1.0 Pic + 1.0 BAP	3.29 ± 0.23	—	3.02 ± 0.24	3.05 ± 0.20	50-55 (GS)	3.06 ± 0.22

Shoot length recorded from 50 seedlings/shoot bud taking 5 at random from each of 10 culture vessels; GS=Greenish SPSs; YS=Yellowish SPSs; '–' Indicates no response.

Table 3: Development of MSBs/ PLBs from nodal and leaf explants of *R. spathulata.*

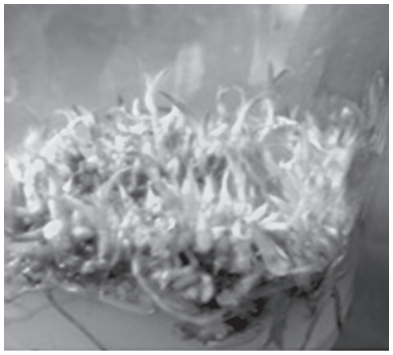
Combinations and concentrations of PGRs (mg/l)	Explants	% of induced MSBs/PLBs per segment	Required time (d) for sprouting of MSBs/ PLBs	Number of MSBs/ PLBs produced per segment (Mean ± SE)
0.5 IAA + 1.0 BAP	NS	65	32 - 35	7.23 ± 0.48
	LS	—	—	—
0.5 IAA + 1.0 Kn	NS	55	30 - 35	5.96 ± 0.48
	LS	—	—	—
1.0 IAA + 2.0 BAP	**NS**	**75**	**30 - 32**	**8.36 ± 0.45**
	LS	45	50 - 55	Green PLBs
1.0 IAA + 2.0 Kn	NS	60	30 - 33	6.74 ± 0.37
	LS	45	55 - 60	Green PLBs
0.5 IBA + 1.0 BAP	NS	55	33 - 36	6.45 ± 0.40
	LS	—	—	—
0.5 IBA + 1.0 Kn	NS	55	35 - 38	6.32 ± 0.48
	LS	—	—	—
1.0 IBA + 2.0 BAP	NS	60	33 - 36	7.04 ± 0.50
	LS	35	58 – 60	Yellowish PLBs
1.0 IBA + 2.0 Kn	NS	55	35 - 38	5.95 ± 0.39
	LS	—	—	—
0.5 NAA + 1.0 BAP	NS	65	30 - 35	7.15 ± 0.51
	LS	35	55 - 60	Green PLBs
0.5 NAA + 1.0 Kn	NS	55	35 - 38	5.68 ± 0.35
	LS	—	—	—
1.0 NAA + 2.0 BAP	**NS**	**70**	**30 - 32**	**7.94 ± 0.46**
	LS	—	—	—
1.0 NAA + 2.0 l Kn	NS	60	32 - 35	6.98 ± 0.53
	LS	—	—	—
0.5 Pic + 1.0 BAP	NS	55	33 - 36	5.89 ± 0.42
	LS	—	—	—
0.5 Pic + 1.0 Kn	NS	50	32 - 36	
	LS	—	—	—
1.0 Pic + 2.0 BAP	NS	60	30 - 35	7.01 ± 0.50
	LS	**55**	**45 - 52**	**Green PLBs**
1.0 Pic + 2.0 Kn	NS	55	32 - 36	6.02 ± 0.43
	LS	**50**	**50 - 55**	**Yellowish PLBs**

Based on observations recorded from 20 cultured segments in each medium; NS= Nodal Segment; LS= Leaf Segment; '-' indicates no response.

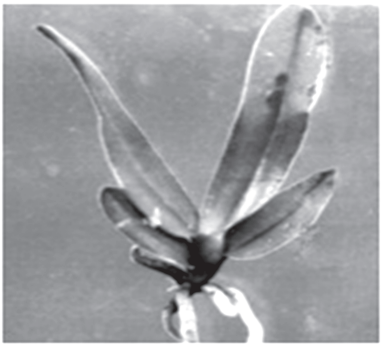
Table 4: Mean increase in length (cm) and number of roots per seed originated and shoot bud derived seedling of *R. spathulata.*

Culture medium			Average increase in length and number of roots per seed originated seedling		Average increase in length and number of roots per shoot bud derived seedling	
			Length (Mean ± SE)	No. of roots/ seedling (Mean ± SE)	Length (Mean ± SE)	No. of roots/ seedling (Mean ± SE)
½ MS0			**4.01 ± 0.24**	**2.22 ± 0.17**	**4.12 ± 0.25**	**2.25 ± 0.13**
Conc. of Auxin (mg/l)	IAA	0.5	3.72 ± 0.21	1.89 ± 0.14	3.81 ± 0.22	1.92 ± 0.15
		1.0	**3.68 ± 0.23**	**2.04 ± 0.14**	**3.99 ± 0.22**	**2.11 ± 0.16**
		1.5	3.56 ± 0.23	1.82 ± 0.12	3.77 ± 0.23	1.87 ± 0.12
	IBA	0.5	3.72 ± 0.26	1.76 ± 0.11	3.85 ± 0.25	1.83 ± 0.10
		1.0	3.65 ± 0.28	1.61 ± 0.09	3.62 ± 0.27	1.62 ± 0.11
		1.5	3.53 ± 0.27	1.57 ± 0.11	3.47 ± 0.23	1.54 ± 0.09
	NAA	0.5	3.61 ± 0.25	1.85 ± 0.11	3.54 ± 0.22	1.93 ± 0.11
		1.0	3.68 ± 0.23	1.92 ± 0.13	3.71 ± 0.21	2.04 ± 0.13
		1.5	3.45 ± 0.21	1.73 ± 0.12	3.32 ± 0.25	1.81 ± 0.11

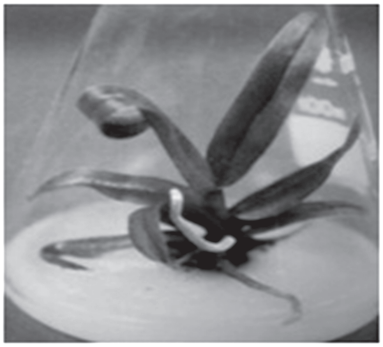
From 50 seedlings/ shoot buds taking five at random from each of ten culture vessels.



Immature seeds germinated on agar solidified PM medium

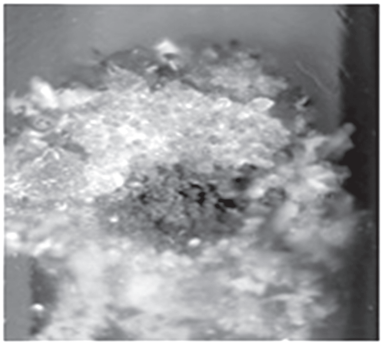


Elongated an individual seedling on liquid MS + 1.0 mg/l Pic + 1.0 mg/l BAP



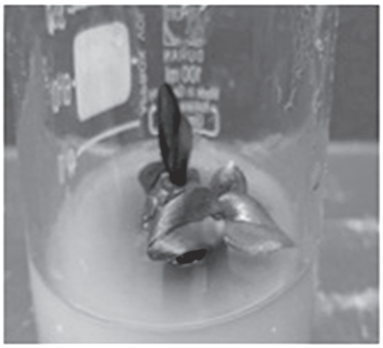
Elongated an individual seedling on agar solidified MS + 0.5 mg/l IAA + 1.0 mg/l BAP

MSBs sprouted from nodal segment on agar solidified MS + 1.0 mg/l IAA + 2.0 mg/l BAP

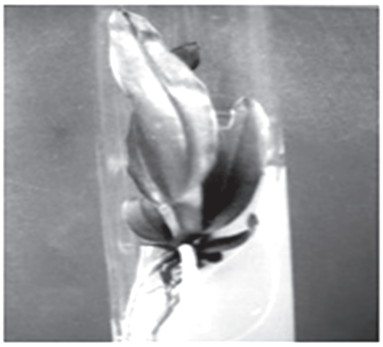


Development of PLBs from leaf segment on agar solidified MS + 1.0 mg/l Pic + 2.0 mg/l BAP

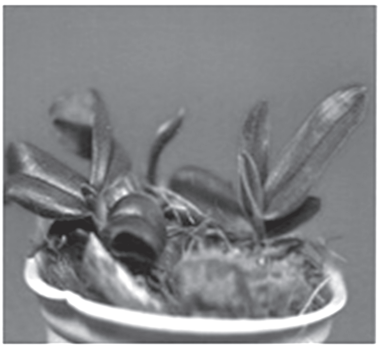
Development of SPSs at the base of the shoots in liquid MS + 0.5 mg/l Pic + 1.0 mg/l BAP



Elongated seedlings from MSBs on agar solidified MS + 0.5 mg/l IAA + 1.0 mg/l BAP



Development of well developed root system on agar solidified ½MS + 1.5% (w/v) Sucrose



In vitro developed R. spathulata plantlets are growing in pot outside of the culture room

Figure 1: In vitro seed germination, elongation, SPSs production, multiple shoot buds and seedling development of *Robiquetia spathulata.*

The elongated shoot buds and seed originated seedlings produced roots in elongation media but those were poor in length and number. Half strength MS0 and nine different types of PGRs (IAA, IBA, NAA) supplemented MS media were used for initiation of strong and stout root system (Table-4). The competence of the rooting media was evaluated based on the increase in length and number of roots created for every plantlet within 30d of culture. Increased in length as well as the number of roots developed seed originated and shoot bud derived seedlings were more on half vigor PGRs free MS medium with 1.5% (w/v) sucrose [14] containing rooting medium (Fig.1i) followed by MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l IAA [47] . In some cases, IAA and auxin supplemented medium was more capable for initiation of strong and stout root system [13, 21, 38, 49]. It is noted that low concentration of auxins are more appropriate than high concentration for generation of well

developed root system [32, 39, 48].

Hardening of *in vitro* raised seedlings is a significant aspect of plant tissue culture. Usually *in vitro* grown seed and shoot buds derived seedlings cannot adjust directly to outside natural environment. For that reason it is considered important to formulate efficient protocols for quick hardening technique. With this idea a number of mature *in vitro* developed seedlings (65%) were adjusted to outside natural atmosphere through consecutive phases of adjustment.

4. Conclusion

PM media was found superior then KC, MS & MVW media and sucrose supplemented media was better than glucose & lactose containing media for promoting germination of this orchid seeds. For comparing the efficiency in terms of enhancing seedling, solidified media was better in terms of promoting shoot elongation than its liquid counterpart. Increased in length of root and root numbers of was higher in shoot bud derivative seedlings than that of seed originated seedlings. Tissue originated plants has a greater value in commercial firm and *ex situ* conservation. This clonal propagation method created deep attention among the orchid growers and had a remarkable impact on the expansion of orchid industries.

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