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Asymbiotic seed germination and micropropagation of *Acampe rigida* (Buch.-Ham. *ex* J. E. Sm.) P. F. hunt through *In vitro* culture

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Abstract
Seeds of an endangered epiphytic orchid *Acampe rigida* were aseptically germinated on agar solidified MS, PM, MVW and KC media supplemented with sucrose, glucose and lactose separately. Higher germination was achieved on sucrose containing PM medium. Highest mean increased seedlings length was achieved on liquid PM with 0.5 mg/l NAA + 1.0 mg/l BAP followed by semisolid PM medium with 1.0 mg/l IAA + 1.0 mg/l BAP. The nodal segments directly produced the highest number of multiple shoot buds (9.02 ± 0.47/segment) on MS medium + 0.5 mg/l NAA + 1.0 mg/l BAP followed by 8.76 ± 0.62 shoot buds/segment produced on MS + 1.0 mg/l IAA + 2.0 mg/l BAP. Leaf segments underwent proliferation and produced maximum greenish PLBs (Protocorm Like Bodies) on MS medium supplemented with 1.0 mg/l Pic + 2.0 mg/l BAP followed by yellowish PLBs on MS + 1.0 mg/l IBA + 2.0 mg/l BAP. The highest mean increased individual shoot bud length was achieved on liquid PM (2.56 ± 0.20cm) with 1.0 mg/l IAA + 1.0 mg/l BAP followed by on agar solidified MS (2.53 ± 0.15cm) with 0.5 mg/l NAA + 1.0 mg/l BAP medium. The highest number of SPSs (Shoot Primordia Like Structures) formation took place in liquid MS + 0.5 mg/l Pic + 1.0 mg/l BAP. The increase in length as well as the number of roots developed seed originated and shoot bud derived seedlings were more on MS medium + 1.0 mg/l NAA followed by that on half strength MS0 medium + with 1.5% (w/v) sucrose containing rooting media. This efficient protocol of *Acampe rigida* could be used in orchid industry for the *in vitro* germination, SPSs production and micropropagation.

Keywords: *Acampe rigida*, *in vitro* germination, PLBs, SPSs

Introduction

Orchids are the most commercially important plants in the world. The Orchidaceae is one of the largest plant family having 35,000 species [1]. In Bangladesh, orchids are reported by 177 species with a variety under 70 genera [2] distributed throughout the country especially in Chittagong, Chittagong Hill Tracts, Cox's Bazar, greater Sylhet, Gazipur and Sundarbans mangrove forest [3]. Orchids grow in nature through seeds but in absence of appropriate hosts most of the seeds do not germinate in adequate percentage [4]. A single orchid capsule or pod contains millions of seeds. The seeds of orchids have neither functional storage organs, nor a true seed coat. Thus lacks of metabolic machinery do not let them germinate; only 0.2- 0.3% gets germinated in nature. This barrier may be overcome by adopting *in vitro* tissue culture technique [5-6]. The frequency of callus like bodies and protocorm like body (PLBs) production in orchids are influenced by many factors, such as genotypes, type of explants and composition of media [7]. Nutrient composition is considered as the major sources of variation in plant tissue culture.

Acampe rigida (Buch.-Ham. *ex* J. E. Sm.) P. F. Hunt is an epiphytic herb, stem very stout, erect, often branched, up to 60 cm tall, densely leafy. Inflorescence erect, flowers crowded, fleshy, pale lemon yellow, barred and spotted, crimson, fragrant and long lasting. Lip white, sparsely spotted purple, mid lobe of lip ovate, obtuse, sac broad, conical with an erect, dorsal hairy plate in the cavity. Flowering and fruiting at April to May [8]. In Bangladesh, it rarely occurs in the Sundarbans [9-10] and Cox's Bazar forest areas. The flowers and the whole plant are showy and are loved by orchid enthusiasts.

Like most of the orchids, *Acampe rigida* species is naturally propagate by seeds or mericlones. However, because of the difficulties involved in natural germination of the seeds along with the high human pressure on the natural populations through use of the flowers, it is necessary to develop alternative propagation methods [11]. The species is incapable of apomixis or autogamy, a fact that should be considered for conservation purposes when a propagation method is selected [12].

Asymbiotic methods of germination under *in vitro* conditions are an alternative tool for the efficient propagation of orchids [13-15]. Knudson [16] first successfully propagated orchids (*Cattleya* Lindl. and *Laelia* Lindl.) using *in vitro* germination and the micropropagation technique by Morel [17] is routinely used for mass multiplication of economically important orchids. Since that time, the reports in the literature indicate that *in vitro* germination is now a significant option for propagating orchid species, although marked differences among species are expected [15, 18].

Micropropagation of orchid could also be done with the use of aseptically grown seedlings. In fact, the discovery of tissue culture techniques added a new dimension to the production of quality plants in large quantities and propagation of exquisite and rare orchids [19-24]. However, very little work has been done for the germination, micropropagation and conservation of this species.

The objective of the current study was to develop an effective *in vitro* asymbiotic germination, micropropagation,

SPSs (Shoot Primordia Like Structures) production and seedlings development protocol for the orchid *Acampe rigida* with an aim to propagate this species for conservation as well as for commercial use.

Materials and Methods

Immature green capsules of *Acampe rigida* (Buch.- Ham. *ex* J. E. Sm.) P. F. Hunt collected from Bandarban, Chittagong Hill Tracts (CHT), Bangladesh were used as materials for this study.

1. Surface sterilization of plant materials: Immature green capsules of *Acampe rigida* were washed with running tap water to remove dust particles and then washed with sterile distilled water for 3 - 4 times. The fruits were then rubbed with savlon socked cotton and washed 2 - 3 times with distilled water. Then they were treated with 0.2% (w/v) $HgCl_2$ for 5 minutes for surface sterilization and thereafter washed 2 - 3 times with double distilled water in a laminar airflow cabinet. Finally surface disinfection was done by treating with 70% ethanol for 1 minute and then washed for 2 - 3 times with double sterile distilled water.

2. Culture medium and incubation: Four basal media *viz.* KC [25], MS [26], MVW [27] and PM [28] were used by supplemented with three types of carbohydrate source *viz.* lactose (disaccharide), sucrose (disaccharide) and glucose (monosaccharide) for seed germination *in vitro*. Eighteen types of modified media were prepared using MS and PM basal media (solid and liquid) with different concentrations and combinations of PGRs for enhancing elongation of seedlings of Agar of 0.8% (w/v) was used in solid media only. Sixteen types of modified media were prepared using MS basal media by supplemented with different concentrations and combinations of PGRs to extensive study of micropropagation. Half strength MS media and full strength auxin supplemented MS media were used for well-developed root system. Agar (0.8% w/v) was used as a gelling agent to solidification of media. pH of the media was adjusted at 5.8 in case of MS and 5.4 in KC, MVW and PM by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture and about 50 ml of media was dispensed into 100 ml each culture vessel and autoclaved at 121^0C for 20 minutes at 15 lb/cm^2 pressure. All cultures were maintained at 25±2^0C under 350-500 lx illumination for 14 hours photoperiod using white fluorescent tubes and 10 hours dark.

3. Seed culture: Surface sterilized immature green capsules were kept on sterilized petri dish containing sterilized filter paper for drying. They were cut longitudinally with the help of sharp sterilized surgical blade. The immature seeds were scooped out with the help of sterilized spatula and transferred to and spread over the surface of lactose, sucrose, glucose supplemented KC, MS, MVW and PM basal medium. Sub-culturing was carried out every six weeks into fresh medium and ten replicates were used for each treatment. The initiation and rate of seed germination was recorded regularly. The entire experiment was performed in aseptic condition in laminar air flow hood.

4. Culture of nodal segments: The nodal segments of 1 - 2 cm were grown aseptically on the plant growth regulators (PGRs) supplemented medium.

5. Culture of leaf segments: Leaf segments of 0.5 - 1.0 cm size were collected from the plantlets grown aseptically on the PGRs supplemented medium. On the medium leaf segments from leaf explants were subcultured on the same fresh medium. Culture temperature and light intensity were similar to that mentioned earlier.

6. Culture of shoot primodia like structures (SPSs): In elongation, huge quantities of SPSs were produced in some hormone supplemented liquid and solid media at the basal zone of the seedlings. These SPSs were then subcultured on PGRs supplemented media where they produced plantlets. Thereafter those plantlets were subcultured on elongation and rooting media respectively.

7. Rooting: Half strength MS0 and auxins supplemented IAA, IBA, NAA nine different modified MS medium were used for induction of strong and stout root system. The efficiency of the media in terms of enhancing the development of root system was assessed based on the increase in number and length of roots that developed within 30d of culture in rooting media.

8. Transplantation: In rooting medium the plantlets grew further and produced well developed root system. Those were taken out of the culture vessel and transferred to outside the culture room following successive phases of acclimatization.

Results and Discussions

The seeds of *Acampe rigida* were germinated on MS, PM, MVW and KC media supplemented with different sources of carbohydrates *viz.* sucrose, glucose and lactose (Tables-1). Percent germination on PM was better than the other media (Fig.1a). Similar result was also noted by Dasgupta and Bhadra [29]; Bhadra *et al.* [30]; Bhadra and Bhowmik [31]; Bhowmik and Rahman [32]; Bhowmik and Rahman [33] in *Cymbidium aloifolium, Dendrobium aphyllum, Arundina graminifolia, Calanthe densiflora, Robiquetia spathulata* respectively. PM media is highly enriched with vitamins and organic additives than other media. Addition of vitamins and additives into the medium was reported to be enhanced for seed germination and seedling growth of many orchids. Peptone in media enhances the germination rate and also favours the healthy protocorm development. It has also been proved that peptone is useful in inducing differentiation in the protocorms on PDA medium. Pant and Gurrung [34] reported that MS medium was found best for germination of *Aerides odorata orchid seeds.* Sugar is an important component of any kind of nutrient medium used in tissue culture studies. Generally sucrose is used in the medium but in some cases other carbohydrates such as lactose, glucose, maltose, fructose, dextrose, galactose, mannitol, cellulose, inulin, mannose have also been used [35-36]. Carbon source has also great role for *in vitro* orchid seed germination. Among three carbon sources, the percentage of seed germination was higher in sucrose containing medium than in two glucose and lactose containing media.

Following germination protocorms developed and turned into mini seedlings and continued further growth. Eighteen kinds of solid and liquid elongation media were prepared using MS and PM basal media (Table-2) with different concentrations and combinations of PGRs (BAP, Kn, NAA, IAA, IBA and Pic.) for enhancing elongation of seedlings.

0.8% (w/v) agar was also used in solid media but in liquid media no agar was added so that the medium remained liquid. The efficiency of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture on elongation media. 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 liquid PM medium (2.57 ± 0.21cm) with 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP (Fig.1b) followed by 0.8% (w/v) agar solidified PM medium (2.47 ± 0.16cm) supplemented with 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP (Fig.1c). Comparison of the results of liquid and solid media revealed that liquid culture was better than 0.8% (w/v) agar solidified condition. The overall results indicate that PM based medium was better than MS based medium for enhancing elongation of shoot system of the seedlings. Similar findings have been recorded in many other studies on orchids [31, 37-41].

Source of explants, size of explants, media composition, pH and other environmental factors may play a significant role in mass scale clonal propagation of orchids. *In vitro* seedlings derived nodal and leaf segment were used as explants source of *Acampe rigida* for rapid micropropagation [31, 39, 42-46]. The nodal explants were cultured on 0.8% (w/v) agar solidified MS media supplemented with various combinations and concentrations of PGRs and produced multiple shoot buds *via* direct organogenesis (Table-3). The efficiency of a medium was assessed on the basis of number of shoot buds produced from each explant. Direct organogenesis took place and that was dependent on combinations of the media. The nodal segments of *Acampe rigida* underwent direct organogenesis and maximum number of shoot buds (9.02 ± 0.47/ segment) were produced when cultured on 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP (Fig.1d) followed by 8.76 ± 0.62 shoot buds/ segment on MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP. Similar findings also noted by Bhadra and Hossain [39] in *Geodorum densiflorum*, Bhowmik and Rahman [32] in *Cymbidium aloifolium* and Kauth *et al*. [45] in *Calopogon tuberosus*. In *Vanilla planifolia,* multiple shoot buds were produced from axillary bud explants on MS medium supplemented with 2.0 mg/l BAP and 1.0 mg/l NAA by George and Ravishankar [47].

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 IBA + 2.0 mg/l BAP.

Comparative study of the efficiency of the media in liquid and solid form was another aspect of the present investigation. PLBs and SPSs derived seedlings underwent elongation when grown individually on PGRs supplemented agar solidified and liquid elongation media. The elongation rate was different depending on PGR supplements liquid and solid media and liquid culture was best for elongation. Such better effect of liquid medium was also reported by Hoque *et al*. [48] in *Cymbidium bicolor* and Watad *et al*. [49]. More surface exposure of cultured seedlings to liquid medium facilitated more uptakes of nutrients thereby contributing to better and prolific growth of seedlings.

In most of the cases low concentration of auxins (IAA, NAA, Pic) and higher concentration of cytokinin (BAP) was more effective for SPSs development at the base of the seedlings. On the whole, liquid media were more effective than agar solidified media. Most of the SPSs were greenish and a few were yellowish in color. MS based media was better (Fig.1f) than PM based media for induction of SPSs. Similar finding was noted by Bhadra and Hossain [39] in *Geodorum densiflorum*, Malabadi *et al*. [50] in *Vanda coerulea,* Sinha and Roy [51] in *Vanda teres*, Martin [52] in *Ipsea malabarica.*

The multiple shoot buds that produced from the cultured explants underwent elongation when further grown on elongation media. The results are summarized in Table-2. The highest mean increased individual shoot bud length (2.56 ± 0.20cm) was achieved on liquid PM medium with 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP (Fig. 1g) followed by on 0.8% (w/v) agar solidified MS medium (2.53 ± 0.15cm) with 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP medium (Fig. 1h) among solid & liquid culture. It is evident that elongation of shoot bud was better in liquid than 0.8% (w/v) agar solidified media. Further PM was found better than MS for elongation of shoot bud. Similar finding was noted by Bhowmik and Rahman [32] in *Calanthe densiflora.*

The elongated Shoot buds and seed originated seedlings produced roots in elongation media but those were weak and few in number. The increase in length as well as the number of roots developed seed originated and shoot bud derived seedlings were more on MS medium supplemented with 3% (w/v) sucrose and + 1.0 mg/l NAA (Fig.1i) followed by that on half strength MS0 medium + with 1.5% (w/v) sucrose containing rooting media (Table-4). A similar finding was noted that NAA was most appropriate in inducing roots in *Cymbidium* by Banerjee and Mandal [53]. The differing result was also noted by Nayak *et al*. [54] in *Acampe praemorsa*, Pant and Swar [55] in *Cymbidium iridioides* observed that IBA was effective for rooting. Gangaprasad *et al*. [56] in *Ipsea malabarica* and Barman *et al*. [57] in *Cymbidium* also reported that IAA and auxin supplemented medium was more efficient for induction of strong and stout root system. It is noted that low concentration of auxin is more suitable than high concentration for induction of well-developed root system.

Hardenings of *in vitro* raised seedlings were adjusted to outside natural environment through successive phases of acclimatization. It was possible to attain a considerable rate of survive of *in vitro* grown seedlings.

Conclusions

PM media was found superior then KC, MS and MVW media and sucrose supplemented media was better than glucose and lactose containing media for promoting germination of orchid seeds. Liquid condition was better in terms of promoting shoot elongation than its solid counterpart.

Table 1: *In vitro* germination of seeds of *Acampe rigida*

.Nutrient medium	Carbohydrate source with concentration	Number of culture vessels used	Number of culture vessels in which seeds germinated		Time (d) required for germination	Observation
			No.	%		
KC	2% (w/v) sucrose	10	06	60	35 - 40	Yellowish PLBs
	2% (w/v) glucose	10	04	40	35 - 38	Yellowish PLBs
	2% (w/v) lactose	10	—	—	—	No response
MS	3% (w/v) sucrose	10	08	80	32 - 36	Brownish PLBs
	3% (w/v) glucose	10	05	50	35 - 38	Yellowish green PLBs
	3% (w/v) lactose	10	04	40	40 - 45	Yellowish PLBs
PM	2% (w/v) sucrose	10	10	100	30 - 35	Yellowish green PLBs
	2% (w/v) glucose	10	05	50	35 - 40	Yellowish PLBs
	2% (w/v) lactose	10	04	40	38 - 42	Brownish green PLBs
MVW	2% (w/v) sucrose	10	08	80	35 - 40	Yellowish PLBs
	2% (w/v) glucose	10	05	50	38 - 40	Yellowish green PLBs
	2% (w/v) lactose	10	—	—	—	No response

'—' Indicates no germination

Table 2: Mean increased in length (cm) and SPSs development per seed originated and shoot bud derived seedlings of *Acampe rigida* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Solid media			Liquid media		
	Seedlings length (cm) after 30d of culture ± SE	Time required (days) for SPSs production and SPSs colour	Mean increased individual shoot bud length (cm) after 30d of culture ± SE	Mean increased seedlings length (cm) after 30d of culture ± SE	Time required (days) for SPSs production and SPSs colour	Mean increased individual shoot bud length (cm) after 30d of culture ± SE
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.93 ± 0.15	—	1.75 ± 0.12	1.86 ± 0.20	—	1.82 ± 0.17
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	2.37 ± 0.21	50-60 YS	2.14 ± 0.17	2.31 ± 0.24	35-45 YS	2.15 ± 0.21
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	2.42 ± 0.17	—	2.27 ± 0.13	2.45 ± 0.22	45-50 GS	2.43 ± 0.20
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.78 ± 0.29	—	1.82 ± 0.23	1.84 ± 0.31	—	1.92 ± 0.27
MS + 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	2.32 ± 0.19	55-60 GS	2.53 ± 0.15	2.26 ± 0.22	40-45 GS	2.17 ± 0.19
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	2.41 ± 0.22	—	2.36 ± 0.18	2.38 ± 0.26	45-50 GS	2.45 ± 0.21
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.87 ± 0.16	—	1.69 ± 0.12	1.98 ± 0.20	40-50 GS	1.81 ± 0.17
MS + 3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	2.08 ± 0.21	55-60 YS	2.01 ± 0.18	2.41 ± 0.24	35-40 GS	2.35 ± 0.21
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	2.33 ± 0.25	—	2.38 ± 0.21	2.44 ± 0.28	45-55 YS	2.41 ± 0.24
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.98 ± 0.18	—	1.82 ± 0.16	2.03 ± 0.21	—	1.95 ± 0.18
PM + 2% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	2.45 ± 0.20	50-60 GS	2.37 ± 0.17	2.25 ± 0.23	40-50 GS	2.38 ± 0.21
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	2.47 ± 0.16	—	2.52 ± 0.17	2.39 ± 0.25	50-55 GS	2.56 ± 0.20
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	2.05 ± 0.25	—	1.83 ± 0.20	2.12 ± 0.27	—	1.92 ± 0.24
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	2.31 ± 0.14	50-60 GS	2.25 ± 0.11	2.57 ± 0.21	40-45 GS	2.26 ± 0.14

PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	2.48 ± 0.16	—	2.41 ± 0.12	2.38 ± 0.20	50-60 YS	2.02 ± 0.17
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	2.01 ± 0.26	—	2.03 ± 0.21	2.05 ± 0.29	45-55 GS	2.11 ± 0.25
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	2.43 ± 0.28	45-50 GS	2.24 ± 0.23	2.18 ± 0.31	40-45 YS	2.28 ± 0.28
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	2.52 ±0.24	—	2.41 ± 0.19	2.22 ± 0.28	50-60 GS	2.46 ± 0.23

*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 multiple shoot buds/ PLBs from nodal and leaf explants of *A. rigida* when grown on 0.8% (w/v) agar solidified MS media supplemented with different PGRs.

Combinations and concentrations of PGRs	Explants	% of induced multiple shoot buds/ PLBs per segment	Time (d) required for sprouting of multiple shoot buds/ PLBs	Number of multiple shoot buds/ PLBs produced per segment (Mean ± S.E.)
0.5 mg/l IAA + 1.0 mg/l BAP	NS**	70	30 - 35	8.12 ± 0.54
	LS***	—	—	—
0.5 mg/l IAA + 1.0 mg/l Kn	NS**	75	32 - 36	8.25 ± 0.45
	LS***	—	—	—
1.0 mg/l IAA + 2.0 mg/l BAP	NS**	75	30 - 35	8.76 ± 0.62
	LS***	—	—	—
1.0 mg/l IAA + 2.0 mg/l Kn	NS**	65	35 - 38	7.93 ± 0.46
	LS***	40	45 - 50	Green PLBs
0.5 mg/l IBA + 1.0 mg/l BAP	NS**	55	40 - 42	4.67 ± 0.35
	LS***	—	—	—
0.5 mg/l IBA + 1.0 mg/l Kn	NS**	50	40 - 42	4.15 ± 0.29
	LS***	—	—	—
1.0 mg/l IBA + 2.0 mg/l BAP	NS**	60	35 - 40	5.48 ± 0.31
	LS***	45	48 - 52	Yellowish PLBs
1.0 mg/l IBA + 2.0 mg/l Kn	NS**	55	34 - 38	5.40 ± 0.36
	LS***	35	45 - 50	Yellowish PLBs
0.5 mg/l NAA + 1.0 mg/l BAP	NS**	80	30 - 35	9.02 ± 0.47
	LS***	—	—	—
0.5 mg/l NAA + 1.0 mg/l Kn	NS**	65	35 - 38	7.85 ± 0.46
	LS***	—	—	—
1.0 mg/l NAA + 2.0 mg/l BAP	NS**	40	34 - 38	4.01 ± 0.32
	LS***	—	—	—
1.0 mg/l NAA + 2.0 mg/l Kn	NS**	40	35 - 40	4.18 ± 0.33
	LS***	—	—	—
0.5 mg/l Pic + 1.0 mg/l BAP	NS**	50	35 - 38	5.06 ± 0.29
	LS***	—	—	—
0.5 mg/l Pic + 1.0 mg/l Kn	NS**	45	32 - 35	4.85 ± 0.35
	LS***	—	—	—
1.0 mg/l Pic + 2.0 mg/l BAP	NS**	55	35 - 40	6.12 ± 0.45
	LS***	55	48 - 52	Green PLBs
1.0 mg/l Pic + 2.0 mg/l Kn	NS**	55	32 - 35	6.07 ± 0.43
	LS***	40	45 - 50	Green PLBs

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

Table 4: Mean increased in length (cm) and number of roots per seed originated and shoot bud derived seedlings* in ½ MS0 and auxin supplemented MS rooting media.

Culture medium			Average increased length and number of roots per seed derived seedling		Average increased length and number of roots per shoot bud	
			Mean length (cm) ± S.E.	Mean no. of roots/ seedling ± S.E.	Mean length (cm) ± S.E.	Mean no. of roots/shoot bud ± S.E.
½ MS0			3.95 ± 0.22	3.18 ± 0.17	3.96 ± 0.22	3.27 ± 0.18
Auxin (mg/l)	IAA	0.5	3.46 ± 0.20	2.34 ± 0.13	3.65 ± 0.20	3.05 ± 0.17
		1.0	3.71 ± 0.23	2.19 ± 0.12	3.26 ± 0.19	2.32 ± 0.14
		1.5	3.52 ± 0.23	2.41 ± 0.15	3.82 ± 0.23	2.44 ± 0.16
	IBA	0.5	3.24 ± 0.24	2.43 ± 0.16	3.27 ± 0.21	2.84 ± 0.20
		1.0	2.72 ± 0.20	2.14 ± 0.15	2.88 ± 0.20	2.47 ± 0.19
		1.5	1.86 ± 0.13	1.61 ± 0.12	2.12 ± 0.16	2.05 ± 0.14

		0.5	3.73 ± 0.24	3.13 ± 0.22	3.93 ± 0.30	3.25 ± 0.21
	NAA	**1.0**	**4.02 ± 0.23**	**3.32 ± 0.20**	**4.11 ± 0.27**	**3.36 ± 0.22**
		1.5	2.65 ± 0.14	1.86 ± 0.10	3.31 ± 0.20	2.72 ± 0.17

* Based on observations from 50 seedlings/ shoot buds taking five at random from each of ten culture vessels.

1a: Immature seeds turned into larger spherules on agar solidified PM medium

1b: Elongated seedlings on liquid PM + 0.5 mg/l NAA + 1.0 mg/l BAP

1c: An individual seedling elongated on agar solidified PM + 1.0 mg/l IAA + 1.0 mg/l BAP

1d: Multiple shoot buds sprouted from nodal segment on agar solidified MS + 0.5 mg/l NAA + 1.0 mg/l BAP

1e: Development of PLBs from leaf segment on agar solidified MS + 1.0 mg/l Pic + 2.0 mg/l BAP

1f: Development of SPSs at the base of the shoots in liquid MS + 0.5 mg/l Pic + 1.0 mg/l BAP

1g: Elongated seedlings from multiple shoot buds on liquid PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP

1h: Elongated seedlings from multiple shoot buds on agar solidified MS + 0.5 mg/l NAA + 1.0 mg/l BAP

1i: Development of strong and stout root system on agar solidified MS + 1.0 mg/l NAA

Fig 1: *In vitro* seed germination, elongation, SPSs development, Multiple Shoot Buds (MSBs) and seedling development of Acampe rigida.

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