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Rapid *in vitro* mass multiplication of orchids *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl. from immature seeds

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The callus embryoid plant regeneration was developed using immature seeds of *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl. Immature capsules of 5-10 wk after pollination (*A. maculosa*) and 10-20 wk after pollination (*R. retusa*) were used for callus induction. In both the species, best response of callus induction was observed on Vacin and Went (VW) medium supplemented with 15% coconut water (CW, v/v). In *A. maculosa*, maximum number of protocorm-like bodies (PLBs; 13.93 per callus) was noted on VW medium supplemented with BA (1 mg L^{-1})+NAA (1 mg L^{-1}) + 15% CW. While in *R. retusa*, maximum number of PLBs (15.83 per callus) was noted in Murashige and skoog (MS) medium supplemented with BA (1 mg L^{-1})+NAA (1 mg L^{-1})+15% CW. *In vitro* regenerated plantlets of both the species were acclimatized to green house conditions. The highest survival rate of plantlets (60%) was noted in the combination of charcoal pieces, brick pieces (1:1) and coconut husk.

Keywords: Callus induction, orchid, plant regeneration, protocorm-like bodies (PLBs)

Introduction

Orchids are one of the most pampered plants and occupy top position among all the flowering plants for cut-flower production and as potted ornamentals. In spite of a very large number of seeds produced, only 0.2-0.3% germinate in nature[1]. Orchid species are under major threat worldwide due to over exploitation by collectors and enthusiasts[2]. The major threat to orchid populations are human activities, such as, land clearing for agriculture purposes, mining and urban development, and collection of plants for medicinal, horticulture and ethno botanical reasons[3].

Plant regeneration from orchid culture usually occurs *via* protocorm. *In vitro* propagation involving a callus phase is considered rather difficult morphogenetic pathway in orchids. So far success with callus culture is restricted only to a few species[4-6]. Orchidaceae consist of high number of threatened species[7]. Rare and threatened orchid species are propagated by seeds rather than by vegetative methods[8]. Regeneration from seeds *via* protocorm-like bodies (PLBs) has become the preferential method for the production of orchids[9]. Mostly horticultural trade depends on wild orchid populations, which are not propagated commercially. Hence, *in vitro* propagation techniques are needed for the continuous survival of the wild populations[10]. In the present study, authors have developed and standardized the protocol for rapid *in vitro* regeneration and mass multiplication of *Aerides maculosa* Lindl. and *Rhynchostylis retusa* (L.) Bl..

Materials and Methods

Callus Induction

Callus cultures were initiated using seeds of immature capsules obtained from controlled pollinated plants grown in shade net condition. In case of *A. maculosa*, immature capsules of 5-10 wk after pollination (AP) (Fig. 1a) and for *R. retusa* 10-20 wk AP (Fig. 2a) were used for *in vitro* propagation.

Capsules were surface cleaned by soaking in detergent Teepol (5% v/v) for 15 min, 70% ethanol for 30 sec., and then by quick flaming. After surface sterilization, the seeds were scooped out and placed on different basal media, such as, Vacin and Went (VW)[11], Murashige and Skoog (MS)[12] and Mitra *et al* (MI)[13]. Callus induction was examined in the media supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). The effect of coconut water (CW) on callus induction was also studied by adding 0 (control), 10, 15 and 20% (v/v) of

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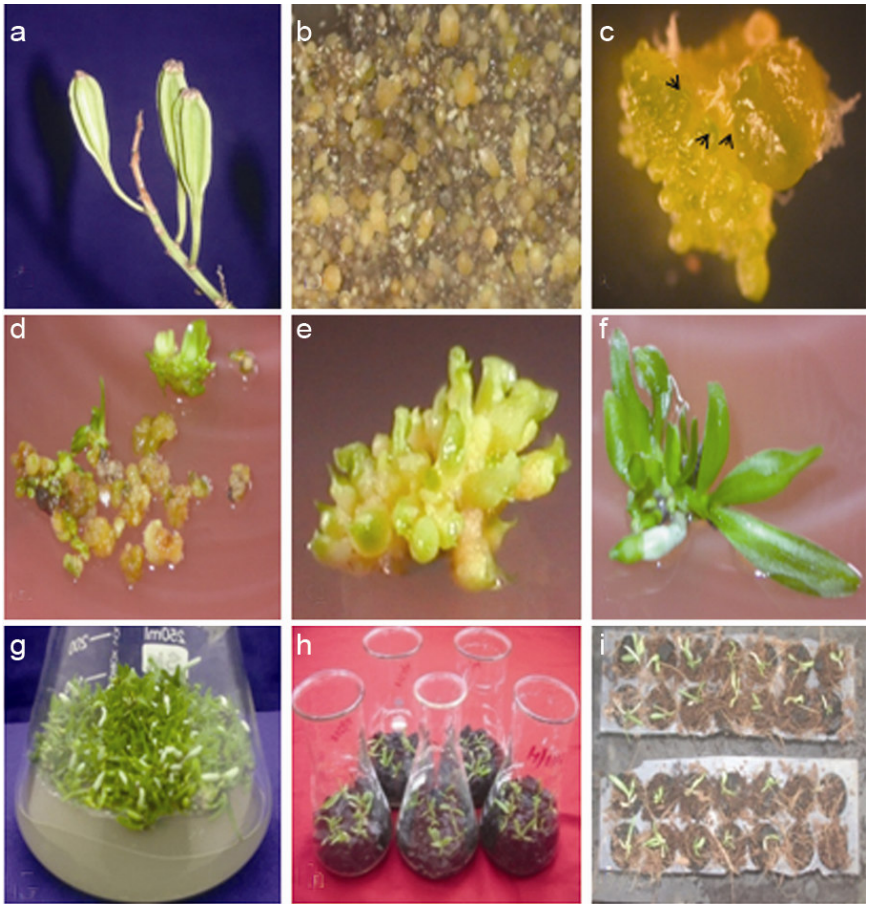

Fig. 1 (a-i)—Micropropagation of *A. maculosa*: a. Immature capsules after 5 wk of pollination; b. Callus developed on VM medium supplemented with 15% CW; c. Callus mass showing PLBs in early stage of development; d-f. Regeneration of PLBs into plantlets; g. Well rooted plantlets; h. Plantlets on charcoal pieces for acclimatization; & i. Plantlets transferred into green house in portray.

CW. Sucrose (3%) was used as organic carbon source. All the media were adjusted to *p*H 5.6±0.2 with 0.1 *N* HCl or NaOH before autoclaving. Media were autoclaved at 121°C for 20 min. Cultures were maintained at 25°C±2 under cool white fluorescent light at 40 µmol m^{-2} s^{-1} for 16 h photoperiod. Experiments were repeated thrice.

Regeneration

To evaluate plant regeneration ability of induced calli, they were cultured on VW and MS medium containing sucrose 3% (w/v) and supplemented with 6-benzyleadenine (BA; 1-5 mg/L) in combination with NAA (1-5 mg/L) and kinetin (KIN; 1-5 mg/L) in conjunction with 15% (v/v) CW.

***Ex Vitro* Establishment**

The regenerated plantlets were transferred to conical flasks containing sterilized charcoal pieces with 10 mL of 1/10th diluted VW medium. The flasks were plugged with cotton and placed in a culture room for 3 wks. Later on, cotton plugs were removed and plantlets were maintained in the culture room for 1 more wk. Then plantlets were treated with bavistin (fungicide) solution at 0.1% concentration for 5 min and transferred to perforated plastic pots. The plastic pots were filled with a mixture of charcoal and brick pieces (1:1) along with coconut husk. Plastic pots containing potting media were again treated with 0.1% solution of bavistin. After transplanting, the plants were sprayed with coconut water 3 times a day for 2 wk. After 2 wk, it was sprayed with 1/10th diluted VW medium once a day up to 1 month. Later, plants were watered regularly twice a day. Care was taken so that roots of the seedling should pass through the space in between charcoal and brick pieces. To prevent fungal and bacterial infections, 0.1% bavistin and 2 ppm of ceftriaxone (antibiotic) were sprayed once in 15 d. The potted plants were kept in a greenhouse (25% light) and mist irrigated. The observation on the establishment of the seedlings was recorded at 2nd wk.

Results

Callus Induction

In *A. maculosa*, callus induction was observed in all the three media supplemented with CW. The callus was friable, opaque and creamish to light yellow in colour. The calli varied in size and were classified into small, medium and large. VW medium was produced higher frequency of callus induction (77-81.8%) compared to MS and MI of media. The callus induction varied from 57.8-60.6% in case of MS medium and 49.4-53.6% in MI medium. It was observed that the VW medium supplemented with 15% CW was found to be the most effective (Table 1; Fig. 1b). The time taken for callus initiation varied amongst the three media tested. In case of VW medium, callus initiation was observed after 27 to 32 d of inoculation, while in MS medium appeared after 33 to 37 d. In MI medium, callus formation was initiated after 38 to 41 d. Thus, earliest callus initiation was noticed on VW medium supplemented with CW (15%) after 27 d of inoculation. However, on MS and MI media callus induction delayed under identical conditions (Table 1).

In *R. retusa*, callus induction was noticed in all the concentrations of CW (10 to 20%). The callus was friable, opaque, creamish to light yellow in colour and varied in size. Among the basal media tried, VW medium was more effective than MS and MI media. In VW medium, 72-80% of callus induction was noted, while considerably lower percentage of callus induction was observed in the case of MS (59-61.4%) and MI (49.6-53.4%) medium. Among the different media tested, VW medium supplemented with CW (15%) was found to be most effective for callus induction (Table 2; Fig. 2b). Early callus initiation was noticed in VW media, *i.e*., 30 to 35 d after inoculation. In comparison to VW medium, slightly more time was taken for callus initiation in MS medium (34-37 d) and MI medium (34-39 d). Compared to all other concentrations, 15% CW was found to be very effective in early initiation of callus in VW medium (30 d).

Regeneration

The regeneration ability of induced callus in *A. maculosa* was evaluated by sub-culturing them on VW medium, while in case of *R. retusa,* it was evaluated on MS medium. The maximum number of PLBs (13.93 per callus) in *A. maculosa* was noted on VW medium supplemented with BA (1 mg L^{-1})+NAA (1 mg L^{-1})+15% CW (Fig. 1c). Among the cytokinin combinations, BA (1 mg L^{-1})+KIN (1 mg L^{-1})+15% CW produced maximum number of PLBs (11.17 per callus). However, the produced PLBs converted into plantlets after 8 to 10 wk of culture in only those cases regenerated on auxin and cytokinin combinations (Fig. 1d-g). The best response of conversion of PLBs into plantets was observed in case of BA (1 mg L^{-1})+NAA (1mg L^{-1})+15% CW (Table 3), while PLBs formed on VW medium with different combinations of cytokinin alone became brown and necrotic upon sub-culturing.

Table 1—Effect of coconut water (CW) on callus induction of immature seeds of *A. maculosa* on Vacin and Went (VW), Murashige and Skoog (MS) and Mitra *et al* (MI) media

Medium supplemented with CW	Responses of immature seeds in culture		
	Morphogenesis*	% callus induction**	No. of days taken
VW (Control)	-	-	-
VW + CW 10%	++	77 ± 0.55	30
VW + CW 15%	+++	81.8 ± 0.82	27
VW + CW 20%	+	78.6 ± 0.54	32
MS (Control)	-	-	-
MS + CW 10%	++	57.8 ± 0.92	33
MS + CW 15%	+	60.6 ± 0.61	35
MS + CW 20%	++	58.4 ± 0.62	37
MI (Control)	-	-	-
MI + CW 10%	+	49.4 ± 0.54	40
MI + CW 15%	+	53.6 ± 0.25	38
MI + CW 20%	+	50.8 ± 0.64	41

*- = No response; + = Small quantity; ++ = Moderate quantity; +++ = Large quantity of calli

**Data showing the mean of 10 replicates±standard error (SE)

Table 2—Effect of coconut water (CW) on callus induction of immature seeds of *R. retusa* on Vacin and Went (VW), Murashige and Skoog (MS) and Mitra *et al* (MI) media

Media supplemented with CW	Responses of immature seeds in culture		
	Morphogenesis*	% callus induction**	No. of days taken
VW (Control)	-	-	-
VW + CW 10%	++	72 ± 0.84	35
VW + CW 15%	+++	80 ± 1.00	30
VW + CW 20%	+	74 ± 0.55	32
MS (Control)	-	-	-
MS + CW 10%	++	59 ± 0.81	37
MS + CW 15%	+	61.4 ± 0.43	34
MS + CW 20%	++	60.6 ± 1.32	35
MI (Control)	-	-	-
MI + CW 10%	+	49.6 ± 0.59	39
MI + CW 15%	+	53.4 ± 0.38	36
MI + CW 20%	+	50 ± 0.98	34

*- = No response; + = Small quantity; ++ = Moderate quantity; +++ = Large quantity of calli

**Data showing the mean of 10 replicates±standard error (SE)

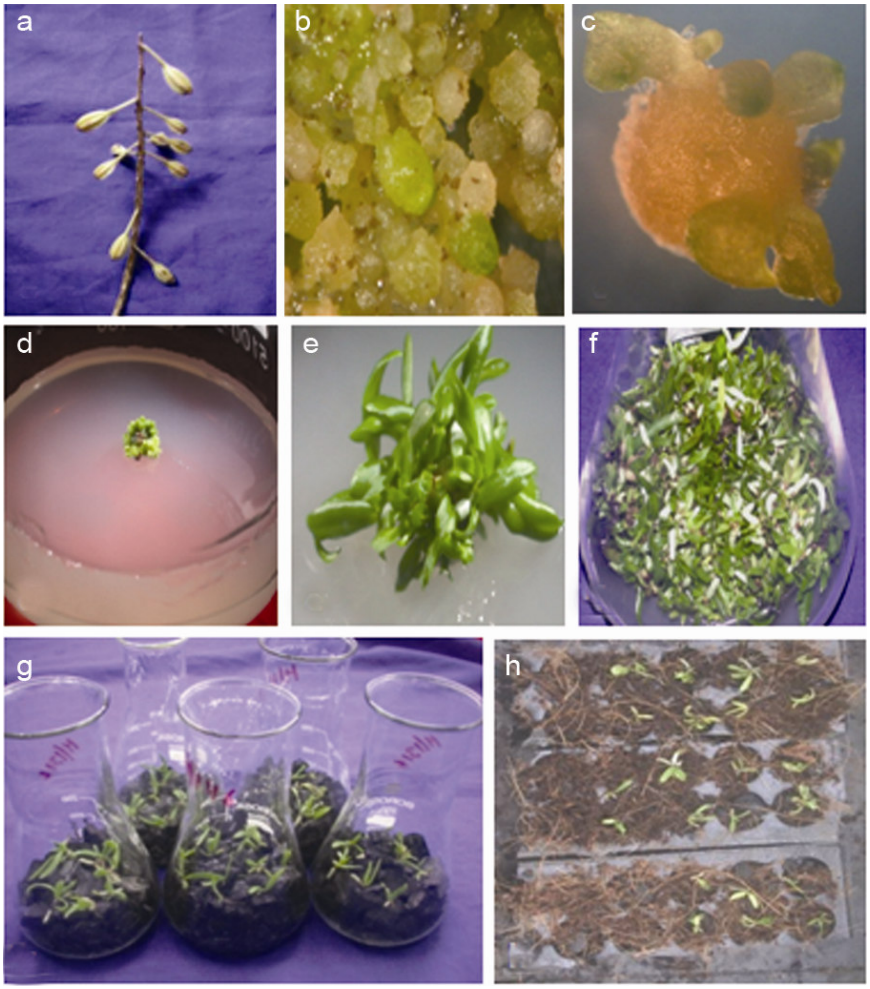

Fig. 2 (a-h)—Micropropagation of *R. retusa*: a. Immature capsules of 10 wk after pollination; b. Callus developed on VW medium supplemented with 15% CW; c. Early stage of PLB formation from the callus; d & e. Conversion of PLBs into plantlets; f. Well rooted plantlets; g. Plantlets on charcoal pieces for acclimatization; & h. Plantlets transferred to greenhouse in portray.

Table 3—Influence of growth regulators (PGRs) on development of protocorm-like bodies (PLBs) from immature seed callus of *A. maculosa* on Vacin and Went (VW) medium supplemented with 15% coconut water (CW)

PGR treatments (mg L^{-1})	No. of PLBs from each callus*	Type of response	Development of shoot buds from PLBs
VW (Control)	-	-	-
BA (5) + NAA (5)	-	-	-
BA (5) + NAA (2)	-	-	-
BA (2) + NAA (2)	4.7 ± 0.36	Greenish PLBs formed	--
BA (2) + NAA (1)	5.33 ± 0.96	Green PLBs formed	Shoot buds & plantlets formation (approx. 2-3 cm)
BA (1) + NAA (1)	13.93 ± 0.64	Healthy and green PLBs formed	Shoot buds & plantlets formation (approx. 2-3 cm)
BA (5) + KIN (5)	-	-	-
BA (5) + KIN (2)	5.17 ± 0.31	Green PLBs formed	--
BA (2) + KIN (2)	9.3 ± 2.72	Green PLBs formed	--
BA (2) + KIN (1)	10.77 ± 0.92	Green PLBs formed	--
BA (1) + KIN (1)	11.17 ± 1.32	Few green PLBs formed	--

*Mean number of PLBs per 0.01 g fresh wt of callus; Data showing mean of 15 replicates±standard error (SE); - = No response; -- = No further growth

Table 4—Influence of growth regulators (PGRs) on development of protocorm-like (PCBs) bodies from immature seed callus of *R. retusa* on MS medium supplemented with 15% coconut water (CW)

PGR treatments (mg L^{-1})	No. of PLBs from each callus *	Type of response	Development of shoot buds from PLBs
MS (Control)	-	-	-
BA (5) + NAA (5)	-	-	-
BA (5) + NAA (2)	-	-	-
BA (2) + NAA (2)	5.33 ± 0.96	Greenish PLBs formed	--
BA (2) + NAA (1)	10.4 ± 1.08	Green PLBs formed	Shoot buds and plantlets formation (approx. 2-3 cm)
BA (1) + NAA (1)	15.83 ± 0.81	Healthy and green PLBs formed	Shoot buds and plantlets formation (approx. 2-3cm)
BA (5) + KIN (5)	4.53 ± 0.65	Few green PLBs formed	--
BA (5) + KIN (2)	10.4 ± 079	Green PLBs formed	--
BA (2) + KIN (2)	11.6 ± 0.46	Green PLBs formed	--
BA (2) + KIN (1)	10.8 ± 1.32	Green PLBs formed	--
BA (1) + KIN (1)	8.13 ± 1.66	Few green PLBs formed	--

*Mean number of PLBs per 0.01 g fresh wt of callus; Data showing the mean of 15 replicates±standard error (SE);
- = No response; -- = No further growth

Similarly, in case of *R. retusa,* the maximum number of PLBs (15.83 per callus) was noted on MS medium supplemented with BA (1 mg L^{-1})+NAA (1 mg L^{-1})+CW (15%). Among the cytokinin combinations, the maximum PLBs (11.6 per callus) were observed in the presence of BA (2 mg L^{-1})+KIN (2 mg L^{-1})+15% CW (Fig. 2c). However, the better response of the conversion of PLBs into plantlets was achieved in auxin and cytokinin combinations (Fig. 2d-f). And combination of BA (1 mg L^{-1})+NAA (1 mg L^{-1})+CW (15%) gave the best results (Table 4). On the contrary, conversion of PLBs into plantlet was not observed in BA and KIN combinations.

***Ex Vitro* Establishment**

For acclimatization, *in vitro* regenerated plantlets of both the species were transferred into conical flasks containing sterilized charcoal pieces with 10 mL of $1/10^{th}$ diluted VW medium and subjected to green house conditions (Figs 1h & 2g). Later, acclimatized plants were transplanted into perforated plastic pots containing a combination of charcoal pieces, brick pieces (1:1) and coconut husk (Figs 1i & 2h). About 60% of the plants survived in the pots.

Discussion

Several workers have reported the advantages of using immature seeds from green capsules for micropropagation[14,15]. These advantages of immature seeds include no loss of seeds by sudden natural dehiscence, easy to surface sterilize, increased rate of seed germination, early start by immature seeds and immature seed culture helps in getting seedlings from wide crosses where embryos in mature seeds often get aborted. Immature seeds (16-18 wk after pollination) of *Arachnis labrosa*, an epiphytic orchid, were used to develop protocol for regeneration and mass multiplication[16]. Other workers have also established protocols for mass multiplication of different orchids using immature seeds[10,17,18].

Plant regeneration using different explants (leaf[19] and immature seeds[18]) has been established in *R. retusa*, while it was only made possible using leaf segments[20] in *A. maculosa*. It has also been reported that regeneration of plantlets in orchids through callus usually occurred through PLB formation, involving somatic embryogenesis[21]. In the present study, calli were induced using immature seeds of two orchids, *A. maculosa* and *R. retusa*, and standard protocols were established for *in vitro* regeneration and mass multiplication *via* PLB formation.

In the present study, CW was found effective in callus induction in both *A. maculosa* and *R. retusa* (Tables 1 & 2). Callus formation was observed in all the three basal media in the presence of CW, whereas better response was noted in VW medium. Percentage callus induction in VW media ranged from 77 to 81% (*A. maculosa*) and 72 to 80% (*R. retusa*), which was much higher as compared to other media tested. Among the CW treatments, VW medium supplemented with CW of 15% (v/v) was found to be most effective for callus induction in both the species (Tables 1 & 2). Large quantities of soft, friable and yellowish green calli were produced on VW medium

supplemented with 15% CW. Similar observations were made in case of *Phalaenopsis* when cultured on VW medium supplemented with 20% CW[22]. Moreover, callus induction in absence of plant growth regulators has also been reported in *Dendrobium fimbriatum*[4] and *D. chrysotoxum*[23].

CW contains considerable amount of auxins and cytokinins[24] and is required for the maintenance of callus[6,25]. Therefore, logical explanation for induction of callus in CW treatments could be presence of auxins and cytokinins. It has been reported that necrosis is a major problem faced during the callus culture of orchids and absence of essentially required plant growth regulators during callus phase usually leads to necrosis[5,22,26]. However, in case of *D. fimbriatum*, complete absence of necrosis was reported[4]. Similarly, in the present study, absence of necrosis was observed in all the treatments used for callus induction.

In orchids, plant regeneration from callus culture *via* PLB formation, which is an intermediary somatic embryo phase, has been reported[6,22,26,27]. It has been observed that differentiation of PLBs from callus in orchids occur in two different ways. In some orchid species, PLB formation requires exogenous plant growth hormones[6,26]; while in others, growth hormones are not required[4,25]. It is believed that cytokinin and auxin ratio plays a vital role in the differentiation of callus into PLBs[28]. In the present study also, callus differentiation into PLBs was found dependent on concentration of cytokinins and auxins used and the best response was observed in the combinations of BA and NAA in both the species. It was observed that PLBs obtained on medium containing BA and NAA combinations developed into well rooted plantlets after 8-10 wk in the same media for both the species. Requirement of auxins and cytokinins for callus differentiation into PLBs and then plantlet formation has also been reported in other orchids[4,5,25,29-31].

The present study shows that the calli of *A. maculosa* and *R. retusa* can be induced from immature seeds on media containing CW. PLB formation from the calli needs the combination of BA and NAA and presence of CW. Further, plantlet developed from PLB on the same medium. Thus, the present protocols provide efficient *in vitro* multiplication method for *A. maculousm* and *R. retusa* from immature seeds. The callus-embryoid-plant regeneration system opens up new avenue for *in vitro* clonal mass multiplication of these potential ornamental orchid species. The development of *in vitro* propagation methods would also contribute to the conservation and commercialization of orchids.

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